Glauco Favot

Production and identification of *Ulva sp.* in multitrophic aquaculture in earth ponds

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Production and identification of *Ulva sp.* in multitrophic aquaculture in earth ponds

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Trabalho efetuado sob a orientação de:

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Glauco Favot

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Resumo

A aquacultura é o setor de produção animal para o consumo humano que mais rapidamente tem crescido no mundo, para além de que é um contribuinte importante para o abastecimento mundial de alimentos e para o crescimento económico. Os efluentes da aquacultura intensiva podem causar eutrofização nas águas costeiras e originar impactos negativos nas comunidades biológicas dessas áreas. É muito importante para o desenvolvimento do sector aquícola que se encontrem soluções adequadas para reduzir o excesso de nutrientes provenientes dos efluentes da aquacultura. A utilização de macroalgas como biofiltros ativos ajuda a reduzir as cargas de nutrientes dissolvidos dos efluentes da aquacultura. As espécies do género *Ulva*, que possuem taxas de crescimento altas e teores de azoto elevadas na composição dos tecidos, são boas candidatas para bio remediar as concentrações de nutrientes na água, além de terem uma função ativa sobre no sequestro de carbono. As espécies de *Ulva* têm sido tradicionalmente utilizadas para nutrição humana e animal pois possuem uma concentração elevada de proteínas. Nos últimos anos desenvolveram-se técnicas que permitem transformá-las numa fonte importante de biocombustível e de ulvano. O enorme potencial comercial deste último produto pode tornar a produção destas algas ainda mais lucrativa. Este trabalho fez a identificação das espécies de *Ulva* que se desenvolvem nos tanques de terra da estação Piloto de Piscicultura de Olhão e que se localizam na Ria Formosa (sul de Portugal), avaliou a taxa de crescimento e a biomassa produzida por uma destas espécies, *Ulva flexuosa*, e determinou o valor da sua produção primária líquida anual (NPP, acrônimo em inglês). Nestes tanques as macroalgas foram cultivadas em dois sistemas multitróficos integrados: um sistema IMTA (acrónimo em inglês para “integrated multitrophic aquaculture”) contendo organismos autotróficos (fitoplâncton, *Ulva flexuosa*), espécies filtradoras (*Crassostrea gigas*) e organismos com alimentação exógena ao sistema (*Argyrosomus regius, Mugil cephalus, Diplodus sargus*); e um sistema constituído apenas por peixes e *Ulva flexuosa*. A espécie de *Ulva* cultivada na estação de aquacultura foi selecionada por se desenvolver naturalmente no canal de descarga dos efluentes da instalação evidenciando uma boa adaptação às variações sazonais de temperatura do local e aos altos níveis de irradiação solar e de amónia. A identificação taxonómica das algas foi feita pela técnica

molecular conhecida como ‘DNA barcoding’. Esta técnica é uma metodologia que utiliza um curto marcador genético presente no DNA do organismo para o identificar como pertencente a uma espécie particular. Neste ensaio foi usado o marcador molecular ITS (acrónimo em inglês para ”internal transcribed spacer”), que permitiu a identificação de seis espécies do gênero *Ulva* presentes nos tanques de terra. Entre eles, a espécie cultivada acabou por ser identificada com *Ulva flexuosa*. Os dados genéticos recolhidos nesta experiência podem levar a concluir que a origem da macroalga cultivada nos tanques de terra da EPPO poderia ser do Pacífico Norte. Esta é a primeira descrição de *Ulva flexuosa* para o sul de Portugal. Contudo, novas questões foram levantadas devido à descoberta de linhagens distintas com o nome desta espécie, usando sequências publicadas Para além disso o morfotipo "folha de alface" foi observado pela primeira vez para as espécies marinhas de *Ulva flexuosa*.

A produção de biomassa e a taxa de crescimento foram testadas comparando: a) os dois sistemas multitróficos utilizados (IMTA (peixe + ostra + *Ulva*) e 'Peixe + *Ulva*'); b) quatro diferentes densidades iniciais (15, 30, 50 e 60 g/m^2) ; c) cinco ciclos de produção e colheita (6, 7, 8, 9 e 15 dias). A taxa de crescimento específico (SGR) de *Ulva flexuosa* resultou ser significativamente diferente entres os dois sistemas multitróficos ($p<0.05$) e maior no sistema de ‘Peixes + *Ulva’*(19.3 ± 0.08% dia^{-1}) do que no sistema IMTA (16.7 ± 0.8% dia^{-1}). A evolução temporal da SGR e da biomassa produzida durante a experiência apresentou um padrão sinusoidal com dois picos. A diminuição no outono parecer ter sido resultante da diminuição sazonal da temperatura e do período de luz enquanto que o decréscimo no mês de Agosto pode ter sido resultante do próprio ciclo de vida da macroalga e da falta de nutrientes. Houve diferenças significativas entre diferentes densidades ($p<0.05$) e diferentes períodos de cultivo($p<0.001$). A densidade de 30g por m^2 foi a que apresentou melhores SGR (23 ± 3.9% dia^{-1}) entre as quatro testadas enquanto que o período de cultivo que produziu melhores SGR foi de sete a nove dias (≈ 21% dia^{-1}). Para obter dados mais pormenorizados sobre os períodos de tempo de cultivo óptimos e a produção de biomassa seca e húmida realizou-se uma experiência de oito dias. Em oito jangadas, de 1 m^2 cada uma, foram colocados 30 gramas de *Ulva sp*.. Nos oitos dias seguintes, uma jangada foi amostrada diariamente, e as algas removidas, pesadas e secas. Para evitar possível perda de biomassa

das algas por distúrbio dos peixes as jangadas foram protegidas por uma rede de plástico. A produção primária e a captura de CO^2 pela *Ulva flexuosa* foi determinada com base numa experiência de incubação realizada em ambiente controlado. A produção primária estimada em condições laboratoriais controladas foi de 1.21 mg C g^{-1} DW h^{-1} resultando numa produção primária anual de 106 g C m^{-2} ano^{-1}. A macroalga *Ulva flexuosa* provou crescer e desenvolver-se bem em condições típicas de aquacultura em tanques de terra. As experiências sobre o ciclo de produção indicaram um período ótimo de cultivo das macroalgas de cerca de 8 dias. Este estudo foi conduzido a uma escala semi-industrial mostrando a viabilidade económica do cultivo desta espécie de macroalga. A presença da *Ulva flexuosa* no Sul de Portugal amplia sua distribuição geográfica e abre a perspetiva de usar esta espécie em sistemas IMTA em diversas partes do país.

Palavras-chave:
Identificação de espécies; DNA-Barcoding; *Ulva flexuosa*; Produção de biomassa; Taxa de crescimento específico (SGR); produção primária líquida (NPP)

Abstract

Waste water from intensive aquaculture can cause eutrophication of coastal waters and subsequent negatively impact downstream biological communities. The use of macroalgae as active biofilter optimizes the reduction of the dissolved nutrient loads in aquaculture effluents. *Ulva* species with their high growth rates and tissue nitrogen contents are very good candidates for bioremediation besides having an active role on carbon sequestration. This study identified the *Ulva sp.* cultivated in earth ponds facing the Ria Formosa lagoon (South Portugal), and assessed the biomass production, the SGR (specific growth rate) and CO_2 uptake performance of this species. Using DNA barcoding with the markers ITS (internal transcribed spacer) I identified six species of the genus *Ulva* growing in the ponds, with *Ulva flexuosa* being the cultivated one. *Ulva flexuosa* was recorded for the first time in South Portugal. However, taxonomic questions were raised because distinct clades were found for this species using published sequences. Moreover, the 'lettuce-leaf' morphotype was observed for the first time for the marine species of *Ulva flexuosa*. The growth and production performance were tested among: a) two different multitrophic systems (IMTA (fish +oyster + *Ulva*) and 'Fish + *Ulva*'); b) four different initial densities (15 ,30, 50 e 60 g/m^2); c) five production and harvest cycles (6, 7, 8, 9 e 15 days). The Specific Growth Rate (SGR) of *Ulva flexuosa* was found to be significantly different between the two multitrophic systems ($p < 0.05$) and higher in the 'Fish + *Ulva*' system (19.3 ± 0.08% day^{-1}) than in the IMTA system (16.7 ± 0.8% day^{-1}). Also, there was significant differences between different densities and varied cultivating periods. Growth of *Ulva flexuosa* was dependent of both densities and time periods. The densities of 30g/m^2 revealed to be the best among the four tested densities (23 ± 3.9 % day^{-1}) whereas the optimal cultivating period was between seven and nine days (≈21 % day^{-1}). The annual NPP of *Ulva flexuosa* was estimated to be of 106 g C m^{-2} year^{-1} a value lower than those reported from different *Ulva* species in other countries. *Ulva flexuosa* showed to grow well under typical conditions of earthen pond aquaculture. The experiments on the production cycle indicated an optimal period of cultivation of about 8 days. The presence of *Ulva flexuosa* in the South Portugal broadens its geographic distribution and

opens the prospect of using this species in IMTA systems in various parts of the country.

Keywords:

Species identification; DNA-Barcoding; *Ulva flexuosa;* Biomass production; Specific Growth Rate (SGR); NPP (Net primary production)

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CHAPTER I.

Introduction

1. INTRODUCTION

1.1 Background

Aquaculture is the fastest growing animal food producing sector in the world and is an increasingly important contributor to global food supply and economic growth (FAO 2016a; Stévant et al., 2017). Aquaculture production by the 28 European Union Member States reached 1.28 million tonnes and 3.96 billion Euros in 2014 according to EUFOMA (EUFOMA, 2016). The greatest contribution to this total comes from finfish farms followed by shellfish (FAO 2016a). Since fish excrete nearly 50 kg N and 7 kg P per ton of finfish produced per year (Troell et al. 2003; Burk et al., 2017) aquaculture industries generate nutrient-rich wastewater streams which can cause environmental problem, mainly in coastal areas (Lawton et al., 2013). To find an appropriate solution to reduce the excess of nutrients coming from aquaculture effluents is very important for the development of the sector both economically and ecologically (FAO, 2016a). A solution could be found by combining extractive and fed aquaculture, an ecological engineering tool known as IMTA system (Abreu et al., 2011; Buck et al., 2017). IMTA represent a practical solution for mitigating the negative effects of fish farming wastes by utilising excess nutrients as a valuable resource for extractive species (Buck et al., 2017; Stévant et al., 2017). This system can prove vital for aquaculture in Portugal, where the activity is developed mainly in land-based farming systems in an extensive or semi-intensive regime (INE, 2016). IMTA system can facilitate the production in land-based aquaculture often limited by strict environmental regulations around water quality of point-source discharges (Lawton et al., 2013). Moreover, IMTA implementation in Portugal could allow the re-use of abandoned saltpens and overcome the problem of finding new spaces for aquaculture facilities (CIGArRA 31.03.05.FEP---0040).

1.1.1 IMTA concept

Integrated multi-trophic aquaculture (IMTA), as the name reveals, is based on the integrated cultivation of aquatic organisms that have different complementary trophic levels. The concept is using the waste products from one food production process (e.g. fin-fish) to feed autotrophs (e.g. phytoplankton, macroalgae) and heterotrophs (i.e. shell-fish) that are co-cultivated with the fed organism and convert in a valuable product (Hughes and Black, 2016) (Figure 1.1).The marine extractive species could be subdivided into three main groups: 1) filter feeders (e.g. oyster),2) deposit feeders (e.g. sea urchins) and 3) dissolved nutrient absorbers (e.g. macroalgae) (Buck et al., 2017). All of them are excellent aquaculture candidates because there are no costs for feeds since they uptake nutrients and particulate matter from the surrounding water column (Paul et al., 2013; Buck et al., 2017). Filter feeders and deposit feeders use mainly particulate organic matter (POM) for their nutrition whereas macroalgae use extract dissolved inorganic nutrients (DIN).

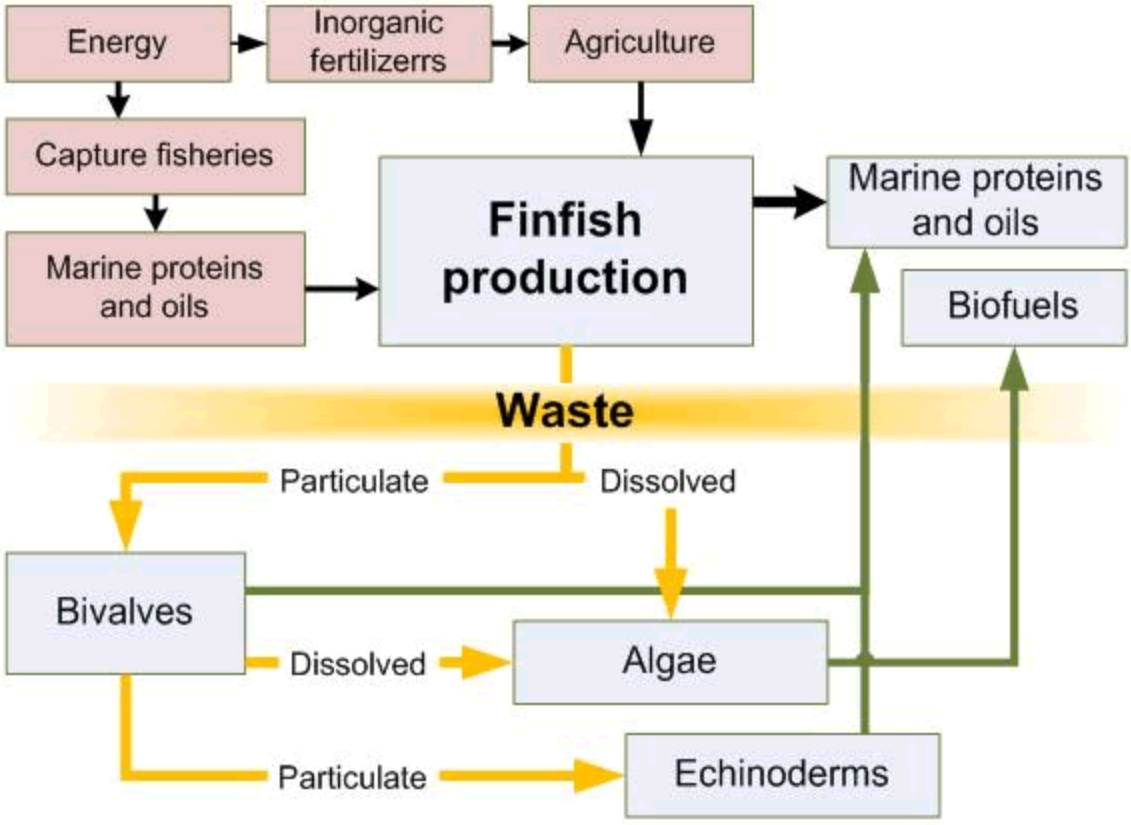

Figure 1.1. Conceptual model of IMTA system (image from http://www.idreem). Yellow arrow indicate the nutrients cycle, green arrows the products obtain from the extractive species and the black arrows the cycle of products used to feed finfish.

1.1.2 *Macroalgae in IMTA*

The ability of macroalgae to be used as excellent biofiltrators has long been demonstrated and their use in the treatment of sewage has proved an acceptable environmental approach, alternative and inexpensive (Troell et al., 2003; Pereira and Correia, 2015; Grote, 2016). Algae act as a biofilter increasing the assimilative capacity of the environment for nutrients (Neori et al., 2004) while simultaneously oxygenating the cultivation medium (Robertson-Andersson, 2003). Macroalgae uptake N, P and C, which they use for growth and production of proteins and carbohydrates. When macroalgae are harvested from IMTA the excess nutrients are also removed from the environment (Burk et al. 2017). Besides reducing the environmental impact of fish aquaculture, macroalgae in IMTA systems add value to the investment in finfish aquaculture by increasing the yield of total biomass produced on a single site (Neori et al. 2004; Stévant et al., 2017). Finally, the macroalgae harvested can be used as low-value commodity energy compounds such as biofuels, biodiesels, biogases and bioalcohols and to produce food, animal feed, bioactive ingredients, pharmaceuticals and cosmetics (Ben-Ari et al., 2014; Burk et al., 2017).

1.1.3 *Macroalgae production in Europe and Portugal*

Despite the growing demand for edible algae in the EU markets, its production is growing slowly with respect to the world's largest producers (EUFOMA, 2016). In 2014 EU macroalgae production amounted to more than 93.000 tonnes, providing approximately 0.3% of the world supply, which represented a decrease in production compared to 2013 (Table 1.1). Traditionally both in Europe and in Portugal the macroalgae industry was based mainly on the harvesting of macroalgae (Pereira and Correia, 2015; EUFOMA, 2016). However, this type of technique is subject to annual fluctuations and poor product quality and raised concerns about the conservation of the marine ecosystem (EUFOMA, 2016). During the years, many different techniques to farm macroalgae have been developed each based on differences in seaweed species, purpose of farming, cultivation techniques, marine environments, scale of operations and coasts (Radulovich et al., 2015) (Figure 1.2).

In Portugal, the production of algae is still developing but the current and

future market prospects (e.g. biofuel) could lead to the development of macroalgae farming in the country.

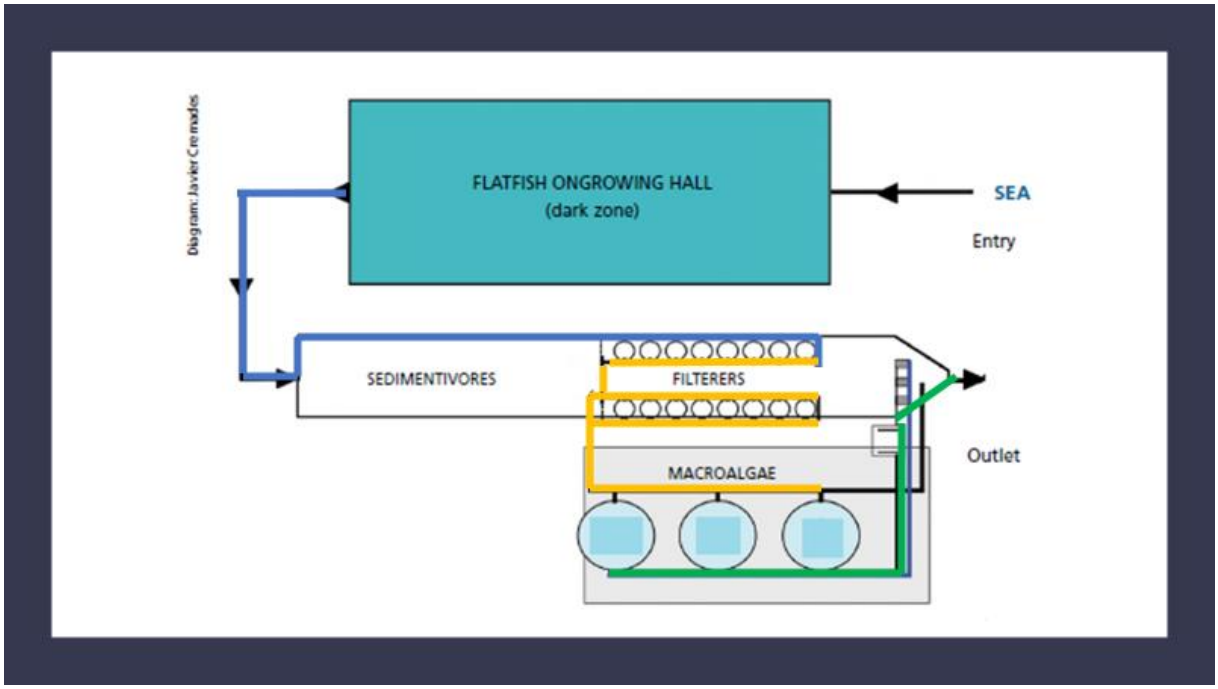

Figure 1.2. IMTA system scheme with species grown separately. The diagram shows the water flow in a turbot farming plant in O Grove (Pontevedra, Spain)*. The blue line is the wastewater coming from fish tank. Yellow line is the water after uptake of POM by shellfish. Green line is the water after the DIN are removed by macroalgae.

* modified from Guerrero and Cremades (2012)

Table 1.1. Production of aquatic plants in Europe*(EUFOMA, 2016)

Country	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
France	23.099	19.192	39.792	39.810	19.032	22.717	47.687	41.579	69.430	58.812
Ireland	29.500	29.500	29.503	29.500	29.500	29.503	29.503	29.509	29.542	29.600
Spain	441	486	134	111	69	125	263	527	1.218	2.154
Italy	1.600	1.400	1.400	1.400	1.400	1.400	1.200	1.200	1.200	1.200
Portugal	624	765	495	198	351	498	461	801	839	786
Estonia	809	394	1.608	1.483	1.032	351	690	430	249	626
Greece	-	-	-	-	-	-	198	174	93	126
Denmark	-	-	-	1.000	1.001	1.000	1.000	1.000	1.800	100
EU total	56.073	51.737	72.932	73.501	52.385	55.594	81.002	75.220	104.370	93.404

*Source: FAO Fishstat (production= harvesting + aquaculture production). No reported production means that data is not available.

1.2 The genus *Ulva*

The cosmopolitan distribution of the genus *Ulva* makes it suitable for cultivation practically everywhere (Ben-ari et al., 2014). Moreover, *Ulva* species possess several factors that make them ideal candidates for bioremediation:

- high growth rate and nitrogen concentration in the tissue;
- efficiency in the removal of inorganic nutrients and ability to resist the high exposure of these elements and compounds derived from them;
- resistance to epiphytes and disease-causing organisms;
- economical value; (Neori et al., 2004; Matos et al., 2006; Lawton et al., 2013; Pereira and Correia, 2015; Grote, 2016).

1.2.1 *Taxonomy*

The genus *Ulva* belongs to the phylum Chlorophyta, family Ulvaceae, class Ulvophyceae and order Ulvales. The species included in this genus are commonly called "green algae" for their distinctive green colour, like that of terrestrial plant leaves (Cormaci et al., 2014). In the past this genus was separated in two distinct genera, *Ulva* and *Enteromorpha,* by Heninrich Friedrich Link in 1820 (Hayden et al., 2003). Link maintained the green algae with distromatic thallus in the *Ulva* genus and moved those with tubular thallus to *Enteromorpha* (Hayden et al., 2003; Cormaci et al., 2014). Only in the 2003 Hayden et al., have proved their congener using nuclear ribosomal internal transcribed spacer DNA (ITS nrDNA) and the chloroplast-encoded *rbc*L gene.

Generally, the thalli can be fixed by a basal disk reinforced by several robust descending filaments produced by all or nearly all near-base cells, or can be freely floating (Cormaci et al., 2014). The cells present a singular chloroplast with a characteristic cup shape and containing a variable number of pyrenoids (Cormaci et al., 2014). Shape and colour of the thallus, number of pyrenoids, shape of cells, type of reproduction, ecology, etc. have been classically used to identify the species of the genus *Ulva* (Marês et al., 2011; Cormaci et al., 2014). Nevertheless, many authors reported that the morphological characters have an

insufficient taxonomic value in several *Ulva* species dues to phenotypic plasticity (Shimada et al. 2003; Hofmann et al. 2010; Comarci, 2014). Studies around the world have shown that only the combination of both molecular and morphological techniques can lead to better characterization of taxa present in different areas of the globe (Loughnane et al., 2008; Heesch et al., 2009; Marês et al., 2011; Wolf et al. 2012; Lawton et al., 2013).

Nowadays, there are 598 species (and infraspecific) names in the Algaebase of which 128 are currently accepted taxonomically (Giury and Giury, 2017). Which makes the *Ulva* genus one of the most numerous of marine and estuarine genera (Kraft et al., 2010).

1.2.2 *Life's cycle*

During their "haplodiplontic" life cycle, species of genus *Ulva* undergo an alternation of two isomorphic generations: the diploid (2n) sporophyte and haploid (n) gametophyte. The gametophyte generation consists of two individual of the opposite sex called zooids. The fusion of the gametes give rise to the second generation, diploid, that will produce haploid zoospores. Finally, these haploid zoospores germinate into male or female haploid gametophyte (Pereira and Correia, 2015) (Figure 1.3). *Ulva* spp. are opportunistic and have a reproductive characteristic comparable to *r* selected species (Castelar et al., 2014). They release a substantial number of small spores (10 μm) with a rapid growth rate, with flagella that allow a rapid dispersion and short life cycle (Castelar et al., 2014). In *Ulva* gametes are released principally from marginal tissue whereas tissue close the holdfast is purely vegetative (Pereira and Correia, 2015). The vegetative thalli have complex glycoproteins that inhibit sporulation, but these substances decreased when thalli age. The variations of environmental factors such as light, temperature, nutrients, tide ranges, etc. are crucial in regulating algae growth, reproduction and sporulation processes. As regards the *Ulva* species, high luminous intensity and high temperatures are among the major factors in increasing zoospore production (Han et al., 2002; Hurd, 2015). Another factor is the fragmentation usually used to enhance the sporulation with the intention of cultivation by inoculation method (Han et al., 2002; Pettett, 2009; Castelar et al., 2014).

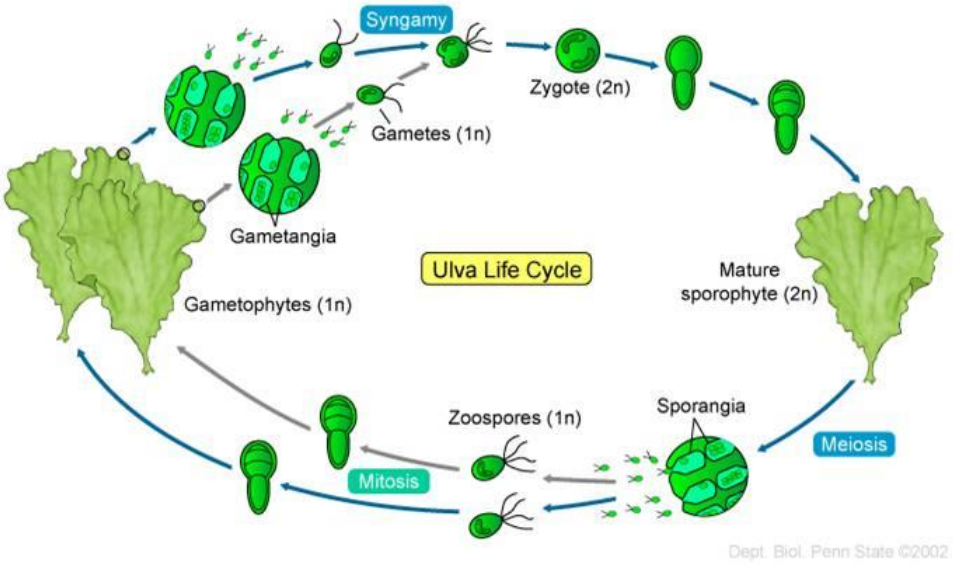

Figure 1.3. Life cycle of *Ulva* (image from http://knowledgeclass.blogspot.pt).

1.2.3 *Taxonomic issue and Barcoding*

The continental coast of Portugal represents the southern limit for several macroalgae species and the combined climatic influences of both Atlantic Ocean and Mediterranean Sea lead to the formation of unique macroalgae communities (Araújo et al., 2009). However, over the years, there was a time gap on the phycological study of the Portuguese coast which has led to poor monitoring of the distribution of macroalgae species (Araújo et al., 2009). The *Ulva* genus is no exception.

Nowadays this genus comprises approximately 17 species in Portugal of which *Ulva rigida* (C. Agardh, 1823), *Ulva clathrata*((Roth) C. Agardh 1811), *Ulva prolifera* (O.F. Müller,1778) and *Ulva mutabilis* (Föyn, 1958) were recorded in the Ria Formosa lagoon (Araújo et al., 2009; Aníbal et al. 2014; Martins, 2014; Alsufyani et al. 2016; Grueneberg et al., 2016) and *Ulva linearis* in the Algarve (South Portugal) (Pereira and Correia, 2015) (Table 1.2).

Nevertheless, the difficulties in the identification of members of genus *Ulva* are known and how many species names have been misapplied along years resulting in artificial ranges for several of them is unknown (Robertson-Andersson, 2003). An accurate assessment of marine macroalgae is important for conservation, monitoring, and management of biological introductions and

invasions (Melton et al., 2016). However, given the growing demand for algae, a proper taxonomic identification has also become necessary in the field of aquaculture (Prasad et al., 2009; Radulovich et al., 2015). Selecting appropriate target species is the first critical step in implementing an algal production programme (Lawton et al., 2013). An example comes from South Africa where it has emerged that the critical matter for South African *Ulva* growers was if the different taxonomic entities had different ecological growth requirements (Bolton et al., 2009). In terms of ecological impact, knowing if a species is broad distributed could permit the translocation of these species between aquaculture facilities without impacting on native biodiversity (Lawton et al., 2013). Another issue arising from improper taxonomic identification is the impossibility to compare results, inhibiting the consolidation of the knowledge about production and other characteristics of the cultivated species (Radulovich et al., 2015).

DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species (Hebert et al., 2003). The main goal is identifying an unknown sample in terms of a pre-existing classification (Kress et al., 2005). The ideal marker should have a highly variable region, useful for species discrimination, flanked by highly conserved region (Saunders and Kucera, 2010). The internal transcribed spacer region of ribosomal cistron (ITS) has been used in several studies concerning the *Ulva* species identification (Marês et al., 2011; Lin et al., 2013 Rybak et al., 2014). It is proving useful for the identification at species level due to its multiple highly variable regions (Shimada et al., 2008; Saunders and Kucera, 2010; Gao et al., 2013). Therefore, the ability of recently developed techniques to analyse more species, more rapidly and in greater detail serves not only to further highlight variability but will act as a platform to optimise their utilisation (Stengel et al., 2011).

Table 1.2. *Ulva* species in the West/North West coast of Portugal and South coast/Ria Formosa lagoon*.

***Ulva* species**	**N.W/W coast**	**S/Ria Formosa**
U. bifrons (Ardré, 1967)	+	-
U. clathrata ((Roth) C. Agardh 1811)	+	+
U. compressa (Linneus, 1753)	+	-
U. curvata ((Kützing) De Toni 1889)	+	-
U. flexuosa (Wulfen, 1803)	+	-
Ulva flexuosa subsp. paradoxa ((C. Agardh) M.J.Wynne 2005)	+	-
U. intestinalis (Linneus 1753)	+	-
U. lactuca (Linneus, 1753)	+	-
U. linearis (P.J.L.Dangeard, 1957)	-	+
U. linza (Linneus, 1753)	+	-
U. mutabilis (Föyn, 1958)	-	+
U. prolifera (O.F.Müller, 1778)	+	+
U. pseudocurvata (Koeman et van den Hoek, 1981)	+	-
U. pseudolinza ((R.P.T.Koeman & Hoek) Hayden et al., 2003	+	-
U. rhacodes ((Holmes, Papenfuss 1960)	+	-
U. rigida (C. Agardh, 1823)	+	+
U. scandinavica (Bliding, 1969)	+	-
U. simplex ((K.L.Vinogradova) Hayden et al., 2003)	+	-

*(+) means presence whereas (-) is for absence.

1.2.4 *Commercial Value*

Macroalgae can be used in a wide range of production processes: production of the hydrocolloids alginate, agar and carrageenan, feed for animals or for the production of green chemicals or bioenergy, are some of the possibilities (van den Burg et al., 2016). In particularly, *Ulva* naturally contain a protein content between 10% and 26% of dry weight of the algae. This characteristic has traditionally been used for human and animal nutrition. *Ulva pertusa*, with a protein level between 20% and 26%, is frequently consumed in Japan. *Ulva reticulata* can be cultured for animal production feeds (Se-Kwon Kim, 2014). Since Growing *Ulva* in effluent media increases its tissue nitrogen and thus protein content (> 40%), it turned out to be a valuable feed for abalone in South Africa abalone farms (Wiencke and Bischof, 2012). Moreover, a consistent relationship between tissue nitrogen and thallus colour was determined and can be used by mariculture farmers to assess the nutrient quality of *Ulva* (Robertson-Andersson et al., 2009). In South Africa, the culture of *Ulva* for abalone feed is more than 1000 t/year (Paul et al., 2013). Usually the weakness of *Ulva* is its low value as a product more than the cost of cultivation, but its use to produce food for the species by the high commercial value can solve this problem. Valente et al., in a study with the aim of evaluate the use of three marine Macroalgae, *Gracilaria bursa-pastoris*, *Ulva rigida* and *Gracilaria cornea*, cultivated in effluents of fish farms, as dietary ingredients in European sea bass (*Dicentrarchus labrax*) juveniles, didn't recorded negative consequences on growth performance, nutrient utilization or body composition (Valente et al., 2006). As already stressed *Ulva* has already naturally good levels of protein (20% dry weight). As well as vitamins, proteins and other rare trace elements, *Ulva* contains arginine, an amino acid used by the animal in function of preventing cardiovascular failure (Pereira and Correia, 2015). A study on *Ulva lactuca* collected in the Tunisian coastline has given a more comprehensive framework about physicochemical, fatty acids and amino acids composition (Yaich et al., 2011). The protein fraction analysis indicated the presence of essential amino acids, which represent 42.0% of the total amino acids. Fat acids represented 7.9% of dry weight and between them palmitic acid was dominant (Yaich et al., 2011). Palmitic acid has anti-microbial activity (Stengel et al., 2011). The most important thing from a commercial point of

view is the high fiber content in in this alga both the insoluble fibre and the soluble dietary fibre (ulvan: sulphated polysaccharide). The problem of high content of heavy metals is solved if *Ulva* is grown in tanks. (Yaich et al., 2011). The biochemical composition of macroalgae depends strongly on the growth conditions and thereby season (Robertson-Andersson et al., 2009). The ratio of protein and carbohydrates can determine which type of species or cultivation system is adequate to obtain one or more of the products listed above, e.g. carbohydrates could be converted into bioenergy, from anaerobic digestion, into biogas (Bruhn et al., 2011).Ulvan is an acidic, sulphated and water-soluble polysaccharide isolated from the proliferative macroalgae of the genus *Ulva*. Give its properties it could be used as an immunostimulator in fish aquaculture, heparin-like drug or as an original biomaterial (Alves et al., 2012a). The main feature of this molecule is the high content in rhamnose, glucuronic acid, xylose, and a small amount of the rare sugar iduronic acid (Coste et al., 2015). The main structural subunit ulvanobiuronic acid type A (A3S) has a glycosaminoglycan-like structure with anticoagulant, antioxidant, immunomodulatory, antihypercholesterolemic, antihyperlipidemic, antiviral, antitumoral and plant defense elicitor activities. It has been used in forming biomaterials such as nanofibers, nanofibrous membrane, microparticles, molecular sponges for cell culture and antiadhesive activity or as ion exchanger hydrogel (Coste et al., 2015; Popa et al., 2014). Nevertheless, this molecule presents a great variability that depends on various factors as the species, life cycle stage and physico-chemical condition.

1.2.5 *Nutrient uptake, specific growth rate (SGR) and biomass yield.*

Ulva is one of the simplest macroalgae to cultivate as it grows vegetatively (Robertson-Andersson, 2003). For instance, in one day *Ulva lactuca* can double its area (Wiencke and Bischof, 2012) achieving a specific growth rate of 35% (Bruhn et al., 2011). Since early 90's, studies conducted in Israel with *Ulva* species has shown that it acts as a bio-filter of waste released by fish in integrated aquaculture stations together with a high growth rate and nutrient uptake capacity. Usually fish assimilate around 20% of N introduced with dry feed, excrete 10% as faeces resulting in 70% of N excreted as dissolved reduced N available to the environment and possibly for macroalgae (Shpigel and Neori,

1996; Neori et al. 2000).In Israel, ammonia-N, as a fraction of total feed-N was reduced from 45% in the fish effluents to 10% in the post-seaweed discharge (Neori et al., 2000) and 1 kg wwt (wet-weight) m^{-2} of *U. lactuca* can remove over 90 % of the ammonium from fish effluents (Robertson-Andersson, 2003). In the course of several experiments, a specific mean growth rate of 18% and a biomass yield of 25 g m^{-2} d^{-1} was estimated when *Ulva* was used as biofilter for marine fishpond effluents (Ben-Ari et al., 2014; Robertson-Andersson, 2003; Bolton et al., 2009). In an experimental integrated system for the intensive land-based culture of abalone, seaweed and fish in Israel *U. lactuca* species grew at a stable rate throughout the year and the nutrients excreted by the fish supported high yields of 78 kg $m^{-2}y^{-1}$ (Coehn and Neori, 1991).

The rapid growth of *U. lactuca* is attributed to its high photosynthetic rates and high ability to uptake dissolved nitrogen (Ben-Ari et al., 2014). A study about a bloom of green algae *Ulva prolifera* in the Yellow sea revealed that the wet weight of *U. prolifera* gradient increased from 11.94% to 25.92% in proportion to contents of dissolved inorganic nitrogen (DIN supply, which indicated DIN content was essentially decisive for the output of *U. prolifera* blooms (Zhou et al., 2015). The cultivation of abalone jointly with *Ulva* in several studies showed a very good performance in terms of removal of nutrients, SGR and biomass growth (Bolton et al., 2009; Robertson-Andersson, 2003; Macchiavello e Bulboa, 2014).

Ulva, besides growing faster and utilise waste nutrients, can out-compete with most species of epiphytic algae. As *Ulva* is often the main epiphyte in monocultures of other seaweed makes it the preferred biofilter seaweed genus. Furthermore, this seaweed suffers from epiphytes only when they get stressed and do not grow at their usual fast rate (Neori et al., 2004).

There is always a certain seasonality in growth capacity and biomass yield of *Ulva* as reported by scientific literature. Seasonality is especially important in the tank cultivation of *Ulva* in temperate zones as all factors, environmental and ecological, vary considerably. A research, presented by Israel et al. (1995), is very comprehensive in this regard: *Ulva lactuca* exhibited high biomass yields correlating with density, photosynthetic photon flux and temperature. During winter when the mean temperature of seawater was 12°C, biomass

increased weekly by an average of 87% while yields in well water at about 18°C averaged 600% per week; biomass increment during spring averaged 215%. A Chilean study recorded that the growth rate and productivity of *U. lactuca* increasing from fall until summer and varying from 0.5 ± 0.2% to 2.6 ± 0.2% d^{-1} and 10 ± 6.1% to 73.6 ± 8.4% g m^{-2} d^{-1} for sustainable growth rate and productivity, respectively (Macchiavello and Bulboa, 2014). Neori et al., 1998 reported a production of *Ulva lactuca* seasonally-dependent lower in winter than in the rest of the year, averaging ≈292 g fresh weight m^{-2} d^{-1} in the summer, and ~83 g fresh weight m^{-2} d^{-1} in winter. The optimal density for the culture of *U. lactuca* was determined to be 1 kg m^{-2} (Ben-Ari et al., 2014) but some authors reported 4 kg m^{-2} as *optimum* (Bruhn et al., 2011).

Microalgae and seaweed have enormous potential for reducing global warming and climate change (Turan and Neori, 2010). Macroalgae lock away atmospheric CO_2 by mean of a process called 'blue carbon' (Chung et al., 2011; Amosu et al., 2013). During photosynthesis they fix CO_2 to create their biomass, releasing oxygen and producing, under anaerobic conditions, CH_4, a clean biofuel (FAO 2009; Turan and Neori, 2010). Macroalgae can also mitigate the effect of finfish uneaten feed that can induces the release of CO_2 into the atmosphere (Fang et al., 2016). Farming macroalgae in combination with fish made IMTA system a sink of CO_2 (Tang et al., 2011). The genus *Ulva* spp. are able to utilise both CO_2 and HCO_3^- as source of carbon. *Ulva lactuca* can be cultivated using flue gas and uses CO_2 from gas as C source increasing it SGR by up to 21% (Bruhn et al., 2011). It was estimated that the removal of 1 million tons of *Ulva prolifera* is equivalent to removing 30000 tons of C (Hurd et al., 2015). This removal has provided a service evaluated around US$100 million (Chopin, 2012). Thus, this mitigative service could be an incentive for the cultivation of *Ulva*, sometimes considered less profitable than other species.

1.2.6 *Ulva sp. in earth ponds*

All the previously IMTA system studies carried out in Portugal used macroalgae native of the Portuguese coast (Abreu et al., 2012). The use of native species is mandatory to avoid the introduction of non-indigenous taxa (Matos et al., 2006; Pereira and Correia, 2015). At IPMA 's Aquaculture Research Station in Olhão (EPPO acronym in Portuguese) the choice of cultivating *Ulva sp.* based

itself on the fact that it grows wild into the settling tank. Furthermore, the genus *Ulva* showed, in previous studies, to withstand the considerable seasonal temperature fluctuations to which the tanks or ponds are subjected (Robertson-Andersson et al., 2003; Guerrero and Cremades, 2012). Others abiotic factors such as high levels of light irradiance and ammonium concentration, commonly elevated in earth ponds, are relevant in the choice of the algae. *Ulva* has shown its ability to grow well under high values of these two variables, reaching high biomass production with high protein content (Floreto et al., 1994; De Casabianca and Posada, 1998; Ben-Ari et al., 2014). Also, the environment of the ponds is improved by this kind of macroalgae. The CO_2 produced by heterotrophs is used by *Ulva* that helps to balance fishpond pH level and oxygen demand (Hurd et al., 2015). Moreover, *Ulva* is able to release spores daily incrementing chlorophyll *a* concentration. That means that in a IMTA systems containing shellfish, as that is carried out at EPPO, *Ulva* species might contribute to the phytoplankton as food for these filter feeders (Robertson-Andersson, 2003). In turn bivalves, acting as bio-filters, remove the phytoplankton that may interfere with the growth of algae and some particulate suspended matter, competing with them for the intake of N, C and P. The mutual benefits that the trophic web of IMTA system may bring to the cultivation of the species that make up it, could result in a boost to both seaweeds cultivation and aquaculture sector in Portugal.

1.3 OBJECTIVES

The present work focused on the feasibility of integrating a land-based production system of *Ulva sp.* on a semi-commercial aquaculture farm, with the objectives of:

- Morphological and genetical characterization (barcoding) of the *Ulva sp.* cultivated and other macroalgae in the ponds.
- Assess Specific Growth Rate (SGR) and Biomass production of *Ulva sp.* in multitrophic aquaculture. Determine the potential for Nutrients and CO_2 uptake

CHAPTER II.

Materials and methods

2. Materials and Methods

2.1 *Ulva sp.* production

The multitrophic aquaculture experiment was conducted at the Aquaculture Research Station in Olhão (EPPO- Estação Piloto de Piscicultura de Olhão). Four rectangular 450 m^2 x 1.5 m deep earthen ponds were used: 2 with fish, oyster and macroalgae (IMTA) and 2 without oysters (Fish + *Ulva*) (Figure 2.1). Autotrophs (phytoplankton, *Ulva sp.*), filter-feeding species (*Crassostrea gigas*) and fed organisms (*Argyrosomus regius, Mugil cephalus, Diplodus sargus*) are grown in the same earthen pond. Stock densities of the organisms cultivated are showed in table 2.1.

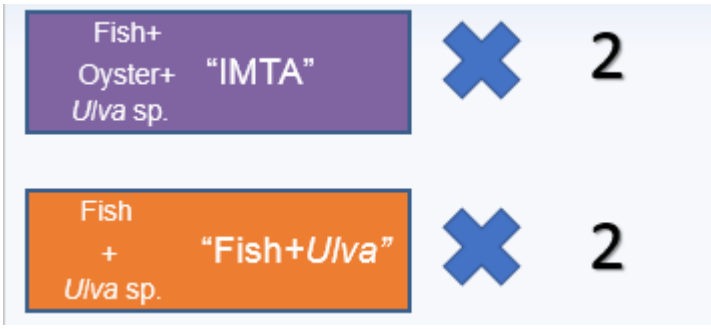

Figure 2.1. Pattern of assay in EPPO earth ponds.

Table 2.1. Stock densities of the organisms present in the pond.

Species	Density
Argyrosomus regius	1500 (N°/pond)
Diplodus sargus	900 (N°/pond)
Mugil cephalus	550(N°/pond)
Crassostrea gigas	18000 (N°/pond)
Ulva sp.	30g/m² x 6 rafts

Growth and biomass production, best cultivation period and CO2 uptake were evaluated for the cultivated macroalgae belong to the genus *Ulva* (Linnaeus, 1753). The time scheduled for the several experiments is shown in Fig 2.2: 1) The first experiment involved the evaluation of the best stock density for *Ulva*'s growth; 2) The best cultivation time to attain the highest growth (best cultivation Period) was determined next in a specific experiment where daily

production of *Ulva sp.* was followed for 8 consecutive days (dry biomass was also measured); 3) After determining this density, the production of *Ulva* in the ponds was assessed by comparing the multitrophic system IMTA and Fish+*Ulva*; 4) The experiment to assess nutrient and CO_2 uptake was the last (August 2017) and was performed in controlled conditions in a laboratory.

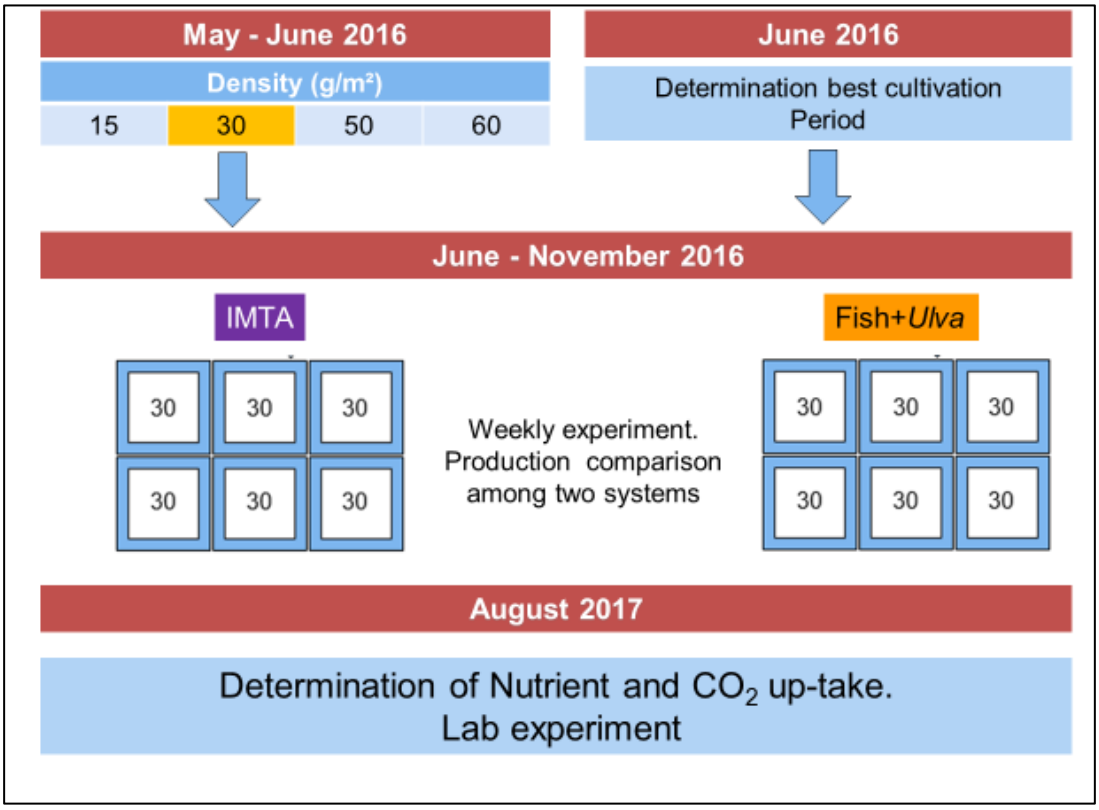

Figure 2.2. Time schedule of experiments ran during the study

Naturally occurring *Ulva* was collected in the main discharge channel and in the settling pond of EPPO (Figure 2.3a). After harvest, the macroalgae were washed with clean saltwater to remove most of the impurities and epibionts and hand-squeezed to eliminate water as much as possible. A portion of the harvest was weighted and individually planted in 6 rafts, each measuring 1 m^2, made of horizontal nets stretched between styrofoam floaters. The individual pieces of macroalgae were attached to the net with brackets (Figure 2.3b and 2.3c).

Figure 2.3. a) Collecting *Ulva* sp. from discharge channel; b) the six floating rafts; c) *Ulva* being fixed with brackets; d) macroalgae draining and weighing.

The stock density that permitted the highest growth of *Ulva* was determined in May-June 2016 in a three-weeks trial to evaluate the growth of the macroalgae (Figure 2.2). Specific growth rate (SGR) and wet biomass production (WBP), was tested using four stock densities: 60, 50, 30 and 15 g/m^2. Each week the growth obtained with different stock densities (60, 50 e 15 g/m^2) were compared with the growth obtained with 30g/m^2 that act as a control for comparison. This was done to prevent the effect of differences in environmental conditions among the three experiments. *Ulva* was distributed among the six rafts in the way shown in Figure 2.4.

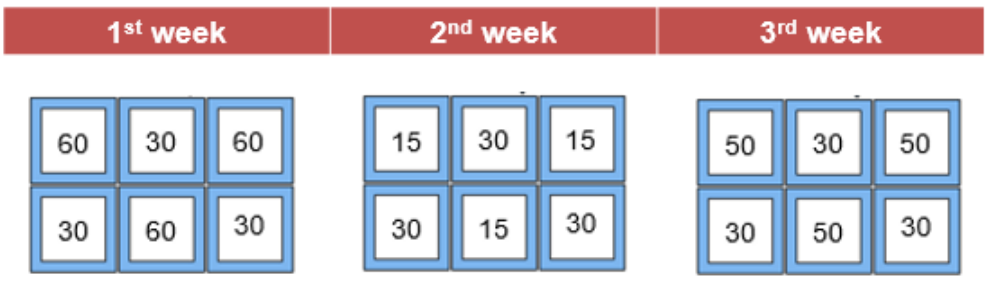

Figure 2.4. Scheme representing the density distribution in the six rafts.

Since the 30g/m^2 showed the best results it was decided to plant the floating structures with this density in all subsequent experiments.

To determine the cultivation time for highest growth the SGR was obtained for 5 different cultivation periods: 6, 7, 8, 9 and 15 days in June 2016. This allowed to draw a growth curve to define the cultivation time that resulted on better growth rates. To accurately determine the daily growth curve another experiment was carry out on an eight-day experiment where the macroalgae biomass was sampled daily. The experiment started on June 2016. Eight floating rafts (each of 1m^2) were placed in a pond containing oysters and fishes (Figure 2.5). The rafts were surrounded by a cage to avoid the detachment and the loss of macroalgae. Moreover, the cages permitted to separate each raft from the others. In the following eight days, a raft was chosen at random and the macroalgae removed, washed, hand drained and weighed. In this experiment the water temperature (°C), pH, turbidity (FNU) and dissolved oxygen (ppm and % saturation) were determined twice a day. *Ulva sp.* were collected, washed and weighed as in previous experiments. 30g of macroalgae was placed on each raft and 3 samples of 30g, were dried up in an oven at 60°C to obtain an average starting dry weight. Obtaining the dry weight allowed to calculate the percentage (17.7%) of dry biomass presents in the wet *Ulva* biomass collected as follow: (DW/WW) *100. The dry weight (DW) was determined by drying the algae at 60°C in a hoven. Dry biomass production (DBP) was calculated by the following equation:

$$DBP=[(DW_f-DW_i)/(A*t)]$$

where DW_f=final dry weight, DW_i=initial dry weight, t=days of culture and A=culture area (Castelar et al., 2014).

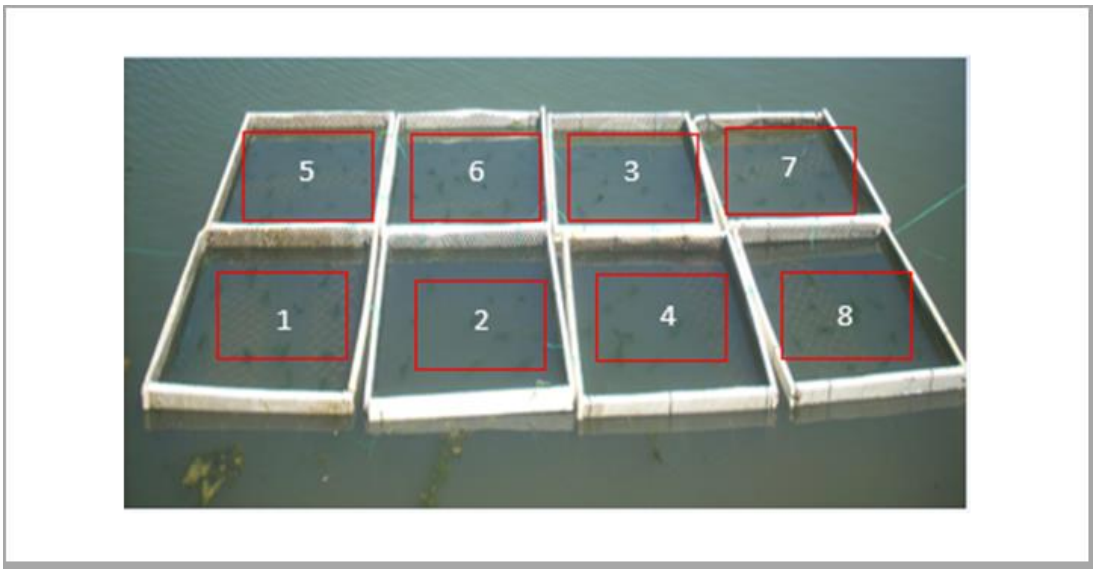

Figure 2.5. Eight-days experiment to determine the growth period. Each raft had 30 g/m2 of initial density. Every number represents after how many days the algae were harvested from that raft.

From June to November 2016 the production of IMTA and Fish+*Ulva* systems was compared. A total of 14 weekly harvests were carried out. During the experiment water temperature (°C), pH, turbidity (FNU, Formazin Nephelometric Units) and dissolved oxygen (ppm and % saturation) were measured with multiparameter probes (Hanna Instruments H9829) twice a day. The irradiance was measured using an Apogee Mark Model SP-214 pyranometer. Furthermore, monthly, samples were taken to determine the concentration of Chlorophyll *a* and nutrients (NH_4, NO_3^-, NO_2^-, HPO_4^-). The nutrients were analysed by colorimetry method (Grasshoff et al., 1983) whereas Chlorophyll *a* was determined by spectrophotometry according to Parsons et al. (1984).

Macroalgae harvesting was done by hand. The floating structures were gently agitated to remove deposited sediments on the surface of the macroalgae before harvest. Prior to weighing *Ulva* was washed with filtered salt water to remove debris and epibionts, squeeze drained and the biomass in each 1 m^2 determined individually in a scale with a 1 mg accuracy (Figure 2.3d).

The daily wet biomass production (WBP) at each 1 m^2 raft composing the floating structure was calculated and expressed in g m^{-2} day^{-1}.

Specific growth rate (SGR, %) of *Ulva* in the rafts was calculated as:

$SGR=\ln (WW_t-WW_i)/t$

where WW_i is the initial wet weight and WW_t is the wet weight after t = time (cultivation days).

To evaluate nutrient (NH_4^+, NO_3^-, NO_2^- and HPO_4^-) uptake, primary production and CO_2 uptake by the cultivated *Ulva sp.* an incubation experiment was carried out in a controlled environment. Primary production was determined by the amount of oxygen production by the macroalgae during a certain time. The experiment was run in lab conditions with constant air temperature (19°C) and light intensity (2 klux). Nine transparent circular plexiglass containers of 5L each, were used as incubation chambers. Three treatments in triplicate with the algae under light (L), three with the algae under no light (D) and three without any algae and under light (C- control). Dark condition was created covering the chambers (D) with black thick plastic sheets. All the chambers were filled with filtered and UV sterilized natural seawater from EPPO reservoir. The C chambers were used to correct for the effect of any eventual planktonic primary production escaping UV sterilization. Before introducing the macroalgae in the chambers water samples were collected to determine the initial concentration of nutrients and dissolved oxygen. Dissolved oxygen was fixed, according to the Winkler method (Grasshoff, 1983).

Ulva sp. samples were collected from the main discharged channel, washed with filtered seawater and cleaned by hand to remove visible epiphytes and organic debris. 10 grams of algae were weighted, with ±1mg accuracy, and placed in 500 ml beakers filled with the sea water like the experimental water, for acclimatization, one hour before the trial. Before sealing the chambers, the water temperature in each was measured with a hand digital thermometer. At the end of experiment and in addition to pH, water temperature was measured again to determine eventual variations.

The incubation period (1 hour) and the macroalgae biomass (10 g of wet weight) were chosen to prevent inhibition of photosynthesis by nutrient depletion and to simultaneously assure that any nutrients and oxygen changes

were detectable (Littler,1979; Serpa, 2005). After the incubation period, water samples for dissolved oxygen were immediately sampled and fixed and, simultaneously, water samples were collected from the incubation bottles in order to determine macroalgal nutrient consumption. Finally, the macroalgae were immediately removed and oven dried (60°C) to obtain the dry weight (DW).

Dissolved oxygen concentration [O_2] in the samples were determined by the Winkler method (Grasshoff, 1983).

The primary production or respiration were determined by the equation:

$$P(R) = \frac{([O2]final - [O2]initial) * V * F * Q}{W * t}$$

P – primary productivity (mg C g^{-1} DW h^{-1}); R – respiration (mg C g^{-1} DW h^{-1}); [O2] final – dissolved oxygen concentration at the end of the incubation time (mg l^{-1}); [O2] initial - dissolved oxygen concentration immediately before the incubation (mg l^{-1}); V – volume of the incubation bottle (l); F – conversion factor of oxygen mass to carbon mass (0.375); Q – photosynthetic quotient; W – macroalgae weight (g DW); t – incubation time (h) (Serpa, 2005; Harrington and Scoggins, 2006).

The net primary production (NPP) was obtained by the equation:

NPP=P(L)-R-P(C)

Where, P(L) = primary production of lighted (L) chambers, R = respiration of dark (D) chambers and P(C)=primary production at the control (C) chambers. The primary productivity, expressed as mg O2 g^{-1} DW h^{-1}, were converted to mg C, assuming a photosynthetic quotient of 1.2 (Valiela, 1995). Values of respiration were converted to carbon equivalents using a respiratory quotient (RQ) of 1.0 (Thomas, 1988). This value is usually used for *Ulva* spp. since they usually metabolize carbohydrates during respiration (Carvalho and Eyre, 2011).

Unfortunately the analysis of nutrients were not performed by the end of this thesis and therefore nutrient uptake results are not presented.

The normality (Shapiro-Wilk's test) and homogeneity of variances (Bartlett's test) within the biotic and abiotic factors were tested before applying parametric test. When these assumptions were not respected, the non - parametric test (Kruskal – Wallis) was used. Statistical test of one-way ANOVA within abiotic factors was performed to identify the possible differences between the two production systems (Altobelli, 2008). One-way ANOVA was also used to test the specific growth rate (SGR) obtained from the two different systems.

The SGR (specific growth rate) of the two systems was used for the following statistical test:

- To determine the correlation (with Spearman variant in case of no normality-homogeneity) between physic-chemical parameters in the pond water and SGR.
- To assess the different densities and periods of cultivation. In this case when statistical difference was found a *pairwise* test was done to know which groups cause the difference ('inhomogeneity') (Altobelli, 2008).

Values for dissolved oxygen, pH, temperature and turbidity used in the correlation analysis (see Figure 3.2 in Results) correspond to the daily mean of a seven days period prior to the sampling for the other parameters.

2.2 Morphological and genetic species identification

2.2.1 *Collection and storage of seaweeds*.

At the beginning of November (3/11/2016), 54 samples of green seaweeds were collected from the 6 earth ponds, among which 17 from the floating structures. The remaining samples were collected from the perimeter of the pond or structures (e.g., ropes). Subsequently, each sample was washed clean with seawater and dried by absorbent paper thoroughly. Of each specimen, a piece of approximately 1 cm^2 was preserved in silica. Each bag was labelled with the date of withdrawal, the tank number, letter "f" or "t" (framework or tank), and sample number. The remainder of each individual collected was preserved as herbarium voucher. This identification system allowed a visual comparison after the species were identified through Barcoding.

2.2.2 *DNA extraction*.

Dried algal biomass was prepared for the DNA extraction through homogenizing the samples by grinding with a tungsten sphere in a mixer mill (Eppendorf A-2-DWP) for 3 minutes at max speed (3,700 rpm). Seaweed DNA was extracted using the NucleoSpin® Plant II Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) following the manufacture's protocol.

The quality of the DNA was verified by running 5µl of the DNA extraction (with 1µl Gel-Red and 2 µl of loading buffer (5X Green GoTaq Flexi Buffer)) of six randomly selected samples on 0.8% agarose gel.

2.2.3 *DNA amplification and sequencing*.

The nuclear primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 3'-CGTATAGTTATTCGCCTCCT-5' were used to amplify nuclear rDNA (ribosomal DNA) fragment (White et al., 1990). This fragment contains, in the 5 '-> 3' order, the ITS1 locus (internal transcribed spacer 1), the 5.8S gene (which encodes the transcription of one of the ribosome components), and the ITS2 locus (internal transcribed spacer 2) (White et al., 1990). Each reaction consisted of 3.95 H2O, 4 µl of 5 X Buffer, 1.6 µl 25mM Mg, 1.25 µl 2mM of each dNTP, 2 µl 1.0 µM of each primer, 0,2µl 5U/µl Go-Taq, 5.0 µL of diluted (1:100 H2O Milli-Q) genomic DNA extract, brought up to a total volume of 20

µL with Milli-Q water.

PCR amplification was run on the Applied Biosystems 2720 Thermal Cycle (Applied Biosystems™, Foster City, CA) and the profile of reaction consisted of an initial denature at 95°C for 5 min followed by 35 cycles of 95°C for 30s, 55°C for 30s min and 72°C for 1 min, a final extension and a final extension at 72°C for 10 min. During the 35 cycles, the extension phase was held for 1 min to assure that both ITS markers were amplified until the end.

The 54 PCR products were visually checked on a stained electrophoreses gel (2% agarose). PCR products consisting of a single band with the right size were sequenced. DNA sequencing was performed on an ABI 3130xl capillary sequencer (Applied Biosystems – CCMAR, Portugal) using the forward primers that were used for PCR.

2.2.4 *Molecular analysis*.

The generated sequences were trimmed and aligned manually using Geneious R7.1.9 (http://www.geneious.com, Kearse et al., 2012). Subsequently identification was based on their DNA sequences by comparing them with sequences present in Genbank. This operation was performed using Nucleotide BLAST web interface (Madden, 2002).

2.2.5 *Phylogenetic analyses - alignment*.

DNA sequence alignment was created using the best quality sequence of each *Ulva* recognized in this study and from respective sequences chosen from BLAST results. Additional sequences for phylogenetic calculation were downloaded from Genbank choosing from other species used in previous papers (Shimada et al., 2003; Mares et al., 2011; Lawton et al., 2013; Rybak et al. 2014) (Annex A, Table 1).

Initial alignment of the nucleotide sets was obtained using Geneious R7.1.9 (http://www.geneious.com, Kearse et al., 2012). Subsequently, the sequences were trimmed to a standard length and the identical sequences removed. The final alignment contained 33 totals taxa (31 ingroup taxa plus one outgroup (*Ulvaria obscura*)), of which 5 were sequences from this study. Since ITS sequences were very variable, the first alignment presented many gaps. Thus,

they were realigned with MAFFT v. 7.310 online application using Q-INS-I algorithm (with default parameters) (Katoh and Toh, 2008). The lasts adjustments of the resulting alignments were carried out using Geneious again.

2.2.6 *Phylogenetic analyses – construction of phylogenetic tree.*

The phylogenetic analyses were performed using the maximum-likelihood (ML) and Bayesian inference (BI) methods (Mareš et al., 2011). The ML tree was obtained using the PhyML online program (Guidon and Gascuel, 2003) and the BI tree was constructed using MrBayes present in Geneious R7.1.9. The program jModelTest version 2.1.10 (Darriba et al., 2012) was used to find the model of sequence evolution that best fit the dataset. ML and Bayesian trees were built using the generalized time reversible (GTR) substitution model with discrete gamma distribution in four categories. One thousand bootstrap replications were performed for both methods using default setting to compare relative support of branches.

The phylogenetic analyses, nucleotide homology (%) and sequence divergence (bp) estimates were based on 520bp, including gaps (Annex A, Table 3).

2.2.7 *Analysis of morphology and anatomy.*

Macroalgae follow the modern nomenclature (Shimada et al., 2003; Cormaci et al., 2014). Morphology of thalli was assessed for fresh algae by Nikon SMZ 1000 Stereomicroscope whereas for anatomy Nikon H550S Microscope (© 2017 Nikon Instruments Europe B.V) was used. All photos were captured and prepared using Nis-Elements Software (© 2017 Nikon Instruments Europe B.V). The fact that not all specimens have obtained a genetic identification and the poor quality of some images has led to the choice to publish only photos of *Ulva flexuosa* taxa.

CHAPTER III.

Results

3. RESULTS

3.1 *Ulva sp.* production

3.1.1 *Abiotic factors* (Table 3.1)

The temperature of the water averaged 25.11±2.92 ºC and 25.08±2.85 ºC at IMTA ponds (Fish + Oysters + *Ulva*) and at ponds without oysters (Fish + *Ulva*) respectively. During the experience, the temperature range between 30.2°C (maximum value found on IMTA ponds on July) and 15.5°C (minimum value found on Fish + *Ulva* ponds on November). Salinity was almost constant (≈ 36 PSU) except on the last day of October when it was raining (minimum value of 32.26 PSU). No significant difference was found between the ponds and systems respecting the temperature and salinity ($p>0.05$).

pH and dissolved oxygen (D.O.) in the water increased on the ponds from morning to afternoon, and this difference was more pronounced during summer (Figures 3.1a and 3.1b). Dissolved oxygen and pH presented higher mean values in the IMTA ponds (pH = 8.47±0.19; D.O.= 5.92±1.03) when compared to Fish + *Ulva* ponds (8.43±0.17; D.O.= 5.67±0.98) and in October when there was a peak at IMTA ponds for both parameters. Either D.O. and pH presented significant difference between the systems ($p<0.01$). Also for the turbidity (FNU) was statistically different among systems but in this case the higher mean corresponded to Fish + *Ulva* system (20.59±8.44). Mean values of nutrients and chlorophyll *a* are presented in Table 3. No significant differences were found between the systems for these factors. Positive correlation was found between specific growth rates (SGR) and temperature and pH, whereas a negative correlation was found between SGR and NH_4^+(p-values< 0.05) (Figure 3.2). Values for dissolved oxygen, pH, temperature and turbidity used in the correlation analysis correspond to the daily mean of a seven days period prior to the sampling for the other parameters.

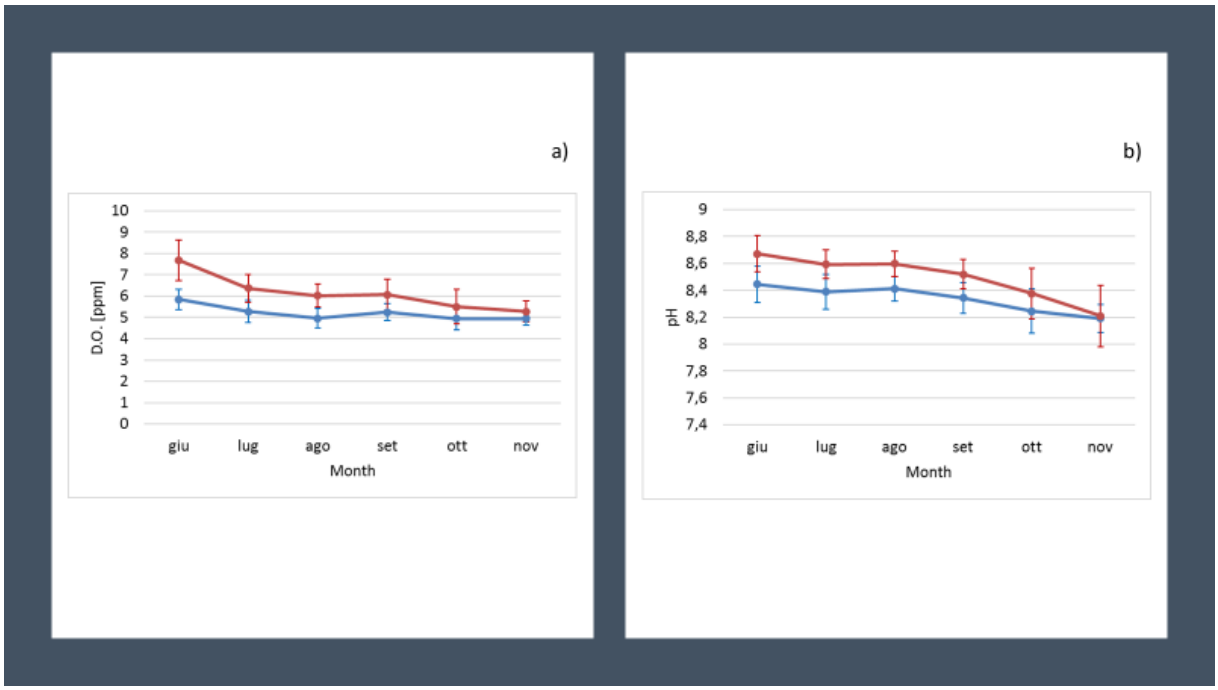

Figure 3.1. Means of daily variation of D.O(a) and pH(b) in the ponds (morning, blue lines; afternoon, red lines) during the 5 months of the experiment (systems are represented together). Vertical bars represent standard deviation.

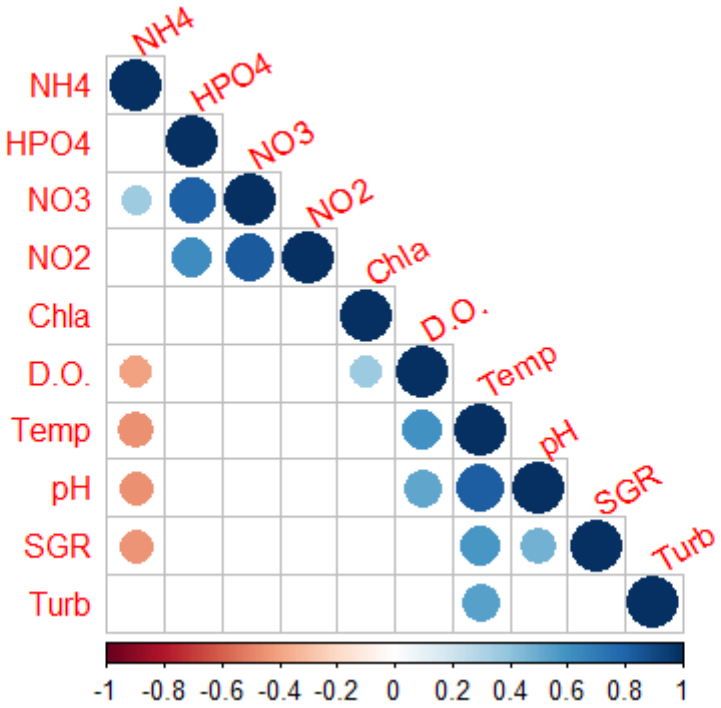

Figure 3.2. Correlation between biotic and abiotic parameters in the ponds. Correlations with p-value > 0.05 were considered as non-significant and leaved blank. Circles represent significant correlations: red - negative correlation, blue - positive correlation. Colour intensity and size of the circles are proportional to the significance of the **correlation coefficient.** (NH_4^+, HPO_4^{-2}, NO_3^-, NO_2^- in µM: Chlorophyll a in µg/l; D.O.: dissolved oxygen in µM; Temp: temperature in °C; SGR: specific growth rate in %, Turb: turbidity in FNU).

Table 3.1. Mean ± standard deviation values of abiotic and biotic factors for the two systems (IMTA and Fish + *Ulva*), and level of significance (p-value) of the comparison between the two using one-way ANOVA.

System	IMTA	Fish + *Ulva*	p-value
Factor			
Temp.(°C)	25.11±2.92	25.08±2.85	p>0.05
pH	**8.47±0.19**	**8.43±0.17**	**p<0.01**
D.O. (ppm)	**5.92±1.03**	**5.67±0.98**	**p<0.01**
Turb. (FNU)	**17.91±7.20**	**20.59±8.44**	**p<0.001**
Irr.[a] (kW m^{-2})	400.47±288.5	400.47±288.5	-
Sal. (psu)	36.08±0.85	36.04±1.76	p>0.05
NH_4^+(µM)	32.20±22.67	36.89±8.63	p>0.05
NO_3^- (µM)	7.84±5.18	6.02±1.73	p>0.05
HPO_4^{-2} (µM)	1.02±0.02	0.93±0.33	p>0.05
NO_2^-(µM)	1.42±1.12	1.37±0.61	p>0.05
Chla (µg/l)	1.07±0.63	0.86±0.66	p>0.05

a. Irradiance equal for both systems because the data came from meteorological station placed on the roof of EPPO building.

3.1.2 *Ulva sp. growth and biomass yield*

Specific growth rate (SGR) of *Ulva sp.* had a mean of 19.3±0.08% at Fish + *Ulva* ponds and 16.7±0.8% at IMTA ponds. Kruskal-Wallis test gave a narrow significant difference between the systems (KW=3.85, p=0.049). The maximum SGR of Fish + *Ulva* systems was achieved on 13 September (36.51%), whereas IMTA registered the higher value on 19 July (31.33%) (Table 3.2).

The mean wet biomass production (WBP) created by the two systems are shown in Table 3.2. The WBP was statistically different (KW=5.84, $p<0.05$) with a maximum value found on Fish + *Ulva* ponds of 65.87 g $m^{-2}d^{-1}$ on 13 of September (Table 3.2).

Table 3.2. Specific growth rate (SGR) and daily wet biomass production (WBP) during the experiment. Kruskal-Wallis (KW) value and significance (p).

System	Min value	Mean ± SD	Max value	KW	p-value
SGR (% d^{-1})					
IMTA	5.6	16.7±0.8	3.,33	3.85	p<0.05
Fish+*Ulva*	3.0	19.3±0.08	36.51		
WBP (g/m^2d)	**Min value**	**Mean ± SD**	**Max value**	**KW**	**p-value**
IMTA	0.25	12.3±9.89	44.85	5.84	p<0.05
Fish+*Ulva*	0.74	17.2±13.60	65.87		

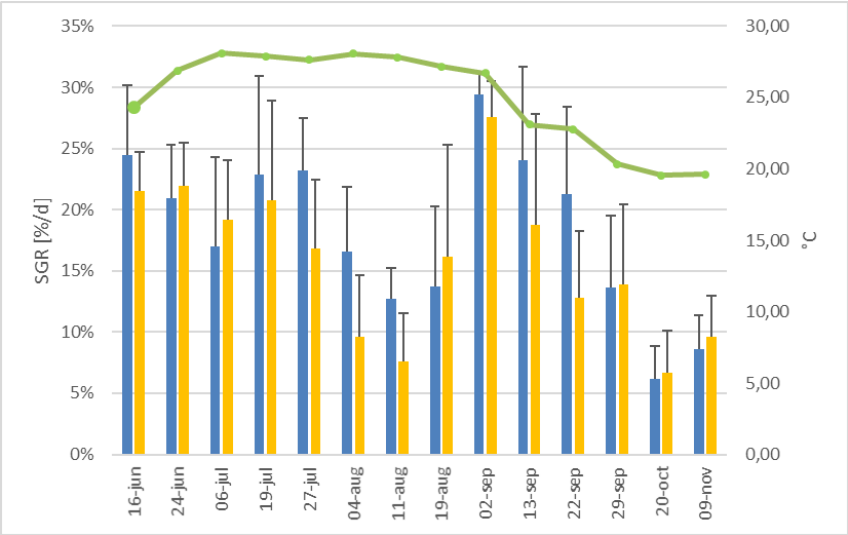

Figure 3.3a. Variation of specific growth rate (SGR) (at right) of *Ulva sp.* along the experiment. XX axis refers to day of harvesting. The green line represents the average water temperature during the 7 days of the cultivation periods (at left). Blue bars: Fish + *Ulva* system; Yellow bars: IMTA system; lines: standard deviation

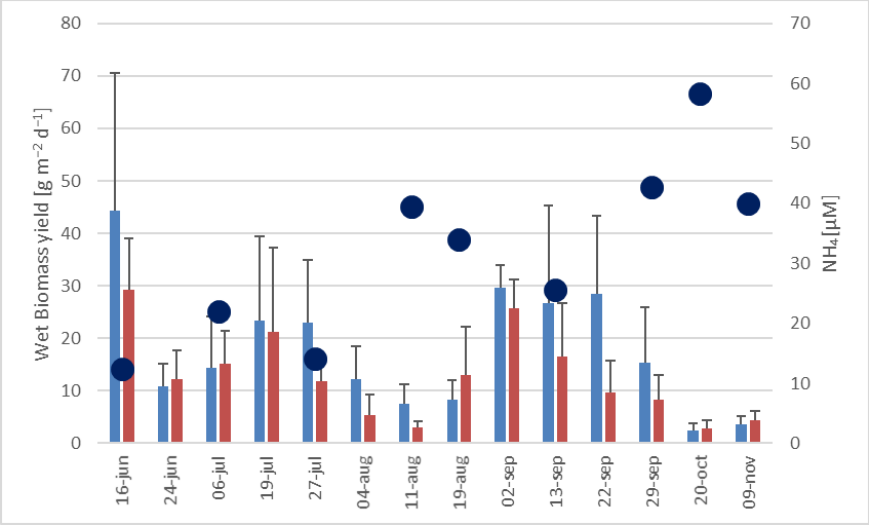

Figure 3.3b. Variation of Wet biomass production (WBP) (at right) of *Ulva sp.* along the experiment. The black dots correspond to the ammonium concentration (at left) in the tanks during the sampling day. Blue bars: Fish + *Ulva* system; red bars: IMTA system; lines: standard deviation

Figs 3.3a and 3.3b show two clear cycles of increase and decrease for both SGR and WBP that corresponds to 6 weeks each. The first increase started in June 24 peaking in 19 July followed by a decrease until August 11 when it reached the minimum value; after this date they started increasing again until September 02. The second decrease reached the minimum value in October 20. The SGR followed the temperature fluctuation only in the last period of the experiment, whereas the ammonium variation is clearly in opposition to the biomass production (Fig. 3.3b).

3.1.3 *Best cultivating periods and stock densities for improved growth*

The Figure 3.4 shows a polynomial trend line of 2nd order (an ascending curve) to illustrate the relationship between the five different cultivation periods and their SGR. The coefficient of determination R^2= 0.9474 represents the fitting of the data to the line. The SGR between the 5 cultivating periods were found to be statistically different (KW = 25.045, p<0.001) and the *pairwise* test stressed that the 6 and 9 days were those that differed significantly from the other three (p=0.0018) (Table 3.3). The SGR of *Ulva sp.* of the 7-8-9 days periods were almost double of the remaining two (Figure 3.4). Abiotic parameters during the experiment to determine the best cultivating period are shown in Table 3.4.

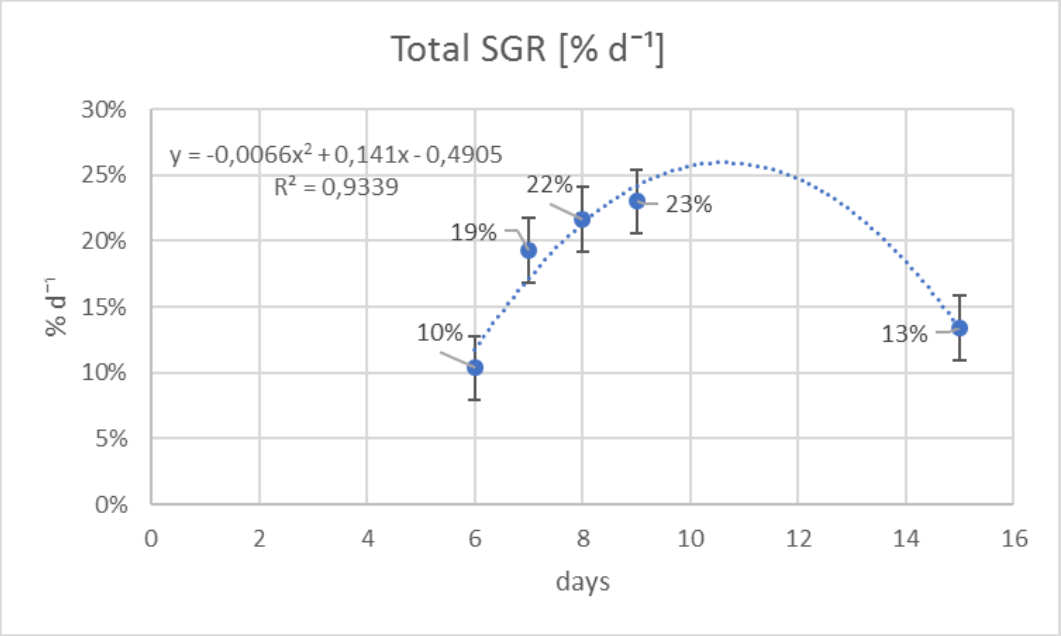

Figure 3.4. Growth curve using SGR recorded from 5 different cultivation periods.

Table 3.3. Numeric matrix containing the p-values of the t- tests calculated for each pair of cultivation period groups. In the output view, the red numbers stressed the periods are significantly different from each other ($p<0.05$).

Cultivation period	6 days	7 days	8 days	9 days	15 days
6 days	—				
7 days	0.018	—			
8 days	0.2109	1.0000	—		
9 days	0.0018	1.0000	1.0000	—	
15 days	1.0000	0.1127	0.7544	0.0058	—

Table 3.4. Mean values (8 days) of abiotic parameters during the experiment to determine the daily growth.

System	Temp.(°C)	pH	D.O. (ppm)	Turb. (FNU)	Sal. (psu)
Morning	25.2±0.81	8.2±0.05	4.6±0.77	15.9±1.71	36.5±0.07
Afternoon	26.9±1.93	8.5±0.06	8.4±2.03	19.2±1.88	36.6±0.07

Different stock densities did show differences for SGR and for WBP (KW= 24.343, $p<0.05$) (Table 3.5). The values for 60 grams were omitted due to a measurement error during weighing. For the densities, the *pairwise* test showed a significant difference in biomass production between 30g/m^2 and the lower value (15 g/m^2) ($p = 0.0004$) but not with 50 g/m^2 (Table 3.6).

Table 3.5. Specific growth rate (SGR) and wet biomass production (WBP) obtained with 3 different initial densities.

	15	30	50
SGR(%/d)	21.1 ± 4.8	23.0 ± 3.9	15.7 ± 7.6
WBP(g/m^{2}d) *	6.9 ± 2.9	22.2 ± 12.6	17.40 ± 13.4

*Significant difference $p<0.05$

Table 3.6. Numeric matrix containing the p-values of the t- tests calculated for each pair of stock densities groups. In the output view, the red numbers stressed the biomass are significantly different from each other ($p<0.01$)

Densities	15g/m^2	30g/m^2	50g/m^2
15g/m^2	—		
30g/m^2	0.0004	—	
50g/m^2	0.004	0.312	—

3.1.4 *Daily growth of Ulva sp.*

Daily growth rates (SGR), obtained during the 8 days experiment, are presented in Figure 3.5. The SGR increased linearly until the third day of cultivation (R^2=0.9969) then entered a stationary phase (R^2=0.0883) with values identical or slightly lower than those reached on the third day (≈ 39 %). The daily increase of dry weight (DW) followed an exponential curve (R^2=0.9756) (Figure 3.6) until the seventh day then slow down sharply. The dry and wet biomass productions on the 8th day was 10.9 g $m^{-2}d^{-1}$ and 60.6 g $m^{-2}d^{-1}$ respectively.

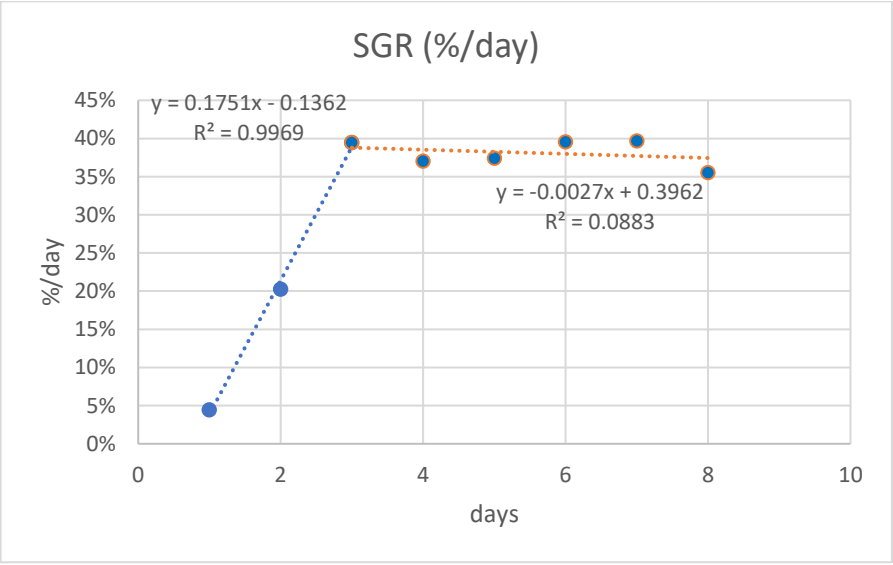

Figure 3.5. Growth curve of *Ulva* sp. SGR grown in eight-days experiment. Blue line represents first 3 days trend. Orange line represents the last 5 days.

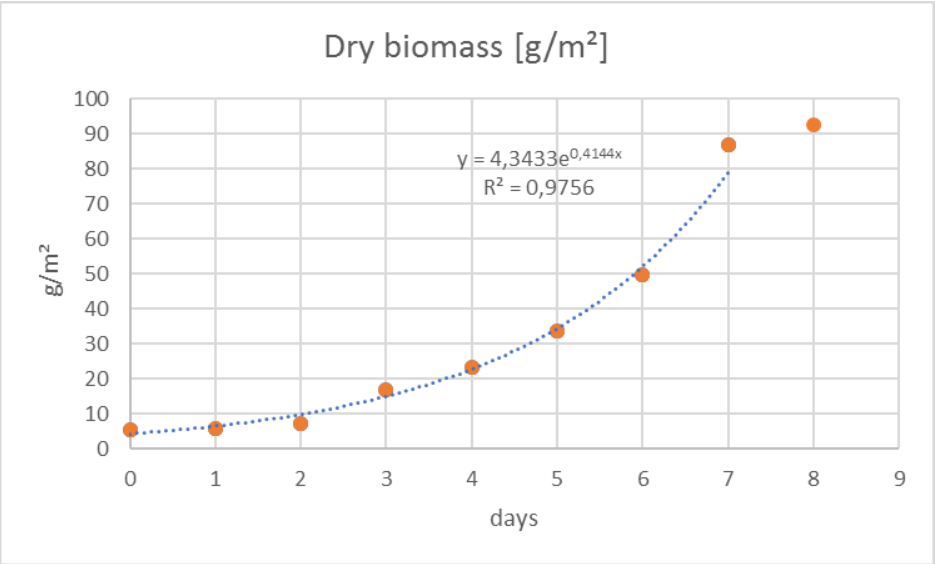

Figure 3.6. Growth curve of *Ulva sp.* dry biomass (DW) grown in eight-days experiment.

3.1.5 *Primary production and Carbon uptake*

Mean values of primary productions expressed in mg O_2 and mg C are shown in Table 3.7. The temperature decreased during the experiment in all the chambers.

Table 3.7. Net Primary production (NPP), temperature and pH (at the end of experiment). pH and temperature are mean values of the 3 chambers for each treatment. To primary production result has already subtracted the respiration and primary production of control (light chamber without algae).

	mg O_2 g^{-1}DW h^{-1}	**mg C g^{-1} DW h^{-1}**	**Initial Temp. (°C)**	**Final Temper (°C)**	**pH**
NPP	1.65	1.21	26.02	23.50±0.15	8.78±0.05

3.2 Morphological and genetic species identification

3.2.1 *Molecular analysis*

The molecular analysis of the macroalgae collected from the EPPO ponds established that the *Ulva* cultivated during the IMTA experiment was an *Ulva flexuosa* (Wulfen, 1803). In addition to the one cultivated, there was found other 5 species belonging to the genus *Ulva* and 2 belonging to *Cladophoora* genus (Annex A, Table 2).

The *Ulva* genus was the well represented and consisted of: *Ulva flexuosa* (Wulfen, 1803), *Ulva clathrata* ((Roth) C. Agardh, 1811), *Ulva intestinalis* (Linnaeus, 1753), *Ulva sapora*[1] (Phillips et al., 2016), *Ulva torta*((Mertens) Trevisan, 1842) and *Ulva prolifera* (O.F.Müller, 1778)

Of the 54 samples used for molecular analysis only 24 had the required quality to be compare with GenBank sequences by BLAST.

3.2.2 *Phylogenetic trees*

The phylogenetic analyses performed with ML (Maximum Likelihood) and BI (Bayesian Inference) methods gave comparable tree topologies with the *Ulva* species coming from the ponds forming four distinct groups (Figure 3.7 and 3.8). These four groups, well supported both in the ML and BI trees, consist of: two monophyletic (C, D) groups, one polyphyletic (A) group and in the group B) *U. torta* is paraphyletic with respect to *U. clathrata*. However, the internal nodes are well supported only in the BI tree, with Bayesian Inference Posterior probability (BP) between 56% and 86%. No support values (nodes with <50% bootstrap support) were reported, for the internal nodes, from ML tree.

Group A showed that *Ulva flexuosa* presents in the EPPO ponds forms a monophyletic clade with *Ulva flexuosa* from Hokkaido, Japan, with a nucleotide homology of 99.47% (2 bp difference) (Table 3.7). According to this phylogram, either *U. flexuosa* are closely related to monophyletic group of *Ulva californica* (internal node value of 69%) and the nucleotide homology showed

[1] *Ulva sapora* is a synonymous name of *Ulva tepida* (Masakiyo, Y. & Shimada, S. (2014)) discovered in Japan for the first time and then reported in Australia (Philipps et al. 2016) as not indigenous species.

between two species (≈ 97%) well supported an evolutionary similarity between these taxa. The *Ulva flexuosa* identified showed a low similarity with other European *U. flexuosa* subspecies with nucleotide homology < 84.3%(Table 3.8).

Table 3.8. Nucleotide homology (in percentage) of ITS region sequences of the four species present in the clade of *Ulva flexuosa* grown within the ponds.

	U. flexuosa_ T11t4ITS	*U. flexuosa* AB097644	*U. californica* AY260560	*U. californica* AY422515
U. flexuosa T11t4ITS	—			
U. flexuosa AB097644	99.47	—		
U. californica AY260560	97.43	96,81	—	
U. californica AY422515	96.80	96,28	99,47	—

Table 3.9. Nucleotide homology (in percentage) of ITS region sequences between *Ulva flexuosa* grown within the ponds and European *Ulva flexuosa* subspp.

	U. flexuosa_ T11t4ITS	*U.flexuosa subsp. flexuosa* HM447564	*U.flexuosa subsp. paradoxa* HM447561	*U.flexuosa subsp. pilifera* HM447579
U. flexuosa T11t4ITS	—			
U.flexuosa subsp. flexuosa HM447564	87.90	—		
U.flexuosa subsp. paradoxa HM447561	84.30	91,71	—	
U.flexuosa subsp. pilifera HM447579	85.75	90.52	85.53	—

Also the groups C, B and D were well supported (BP= 100%, 77%, 100% and ML bootstrap= 96%, 72% and 96% respectively) and showed that all *Ulva*

species sampled were close related with the species sequences from the North Pacific (nucleotide homology between ≈99% to ≈96%) (Annex A, Table 3).

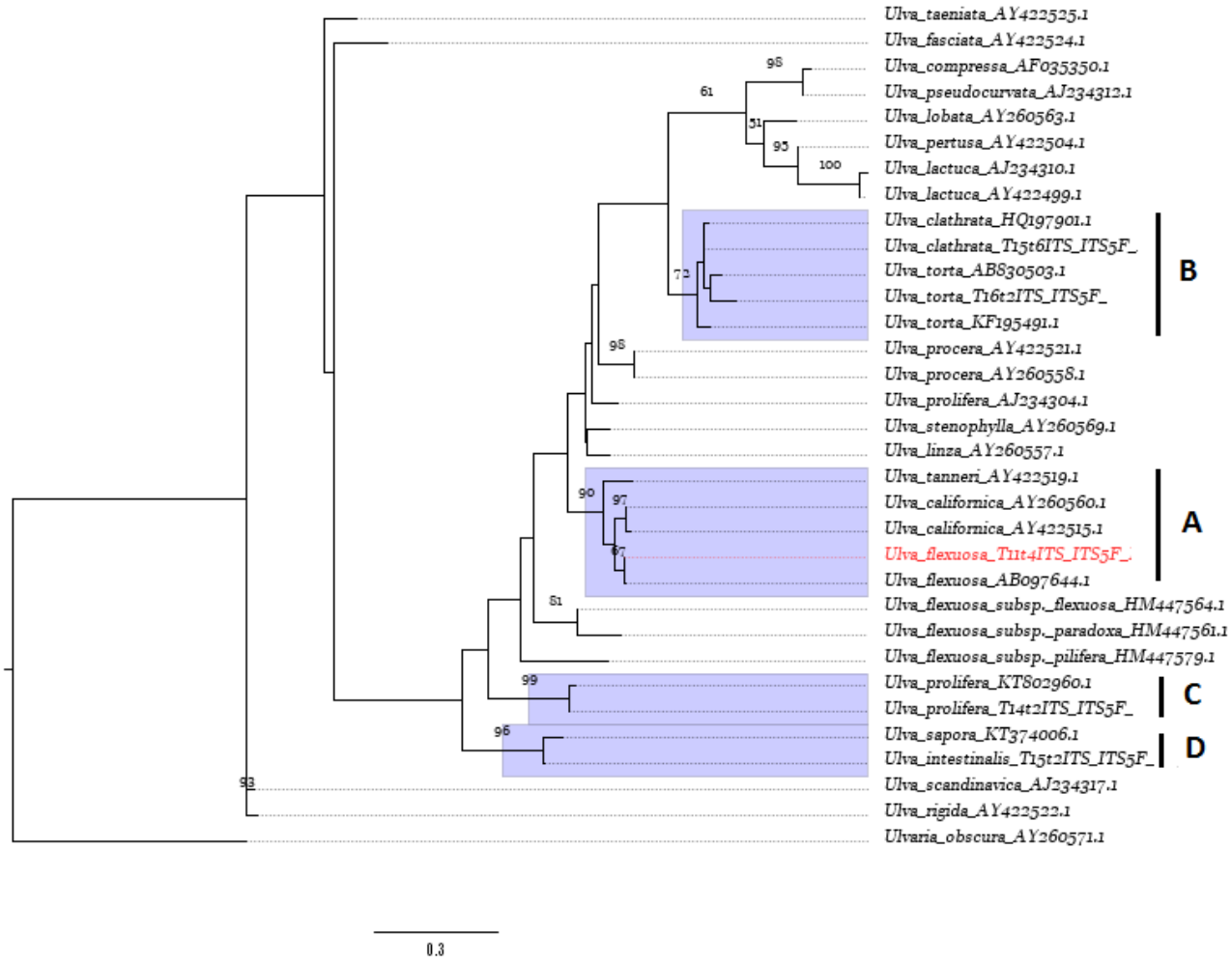

Figure 3.7. Maximum-likelihood (ML) tree of ITS sequences calculated using the evolution model GTR + I + G. ML bootstrap values (1,000 replications) are given on the branches. Values with <50% bootstrap support are not labelled. Sequences are labelled with taxon name and GenBank accession number of ITS sequence (Annex A, Table 1). The tree is rooted using *Ulva*ria *obscura* A, B, C and D refer to Group containing *Ulva* collected from EPPO ponds. In red is stressed the *Ulva flexuosa* identified in this study.

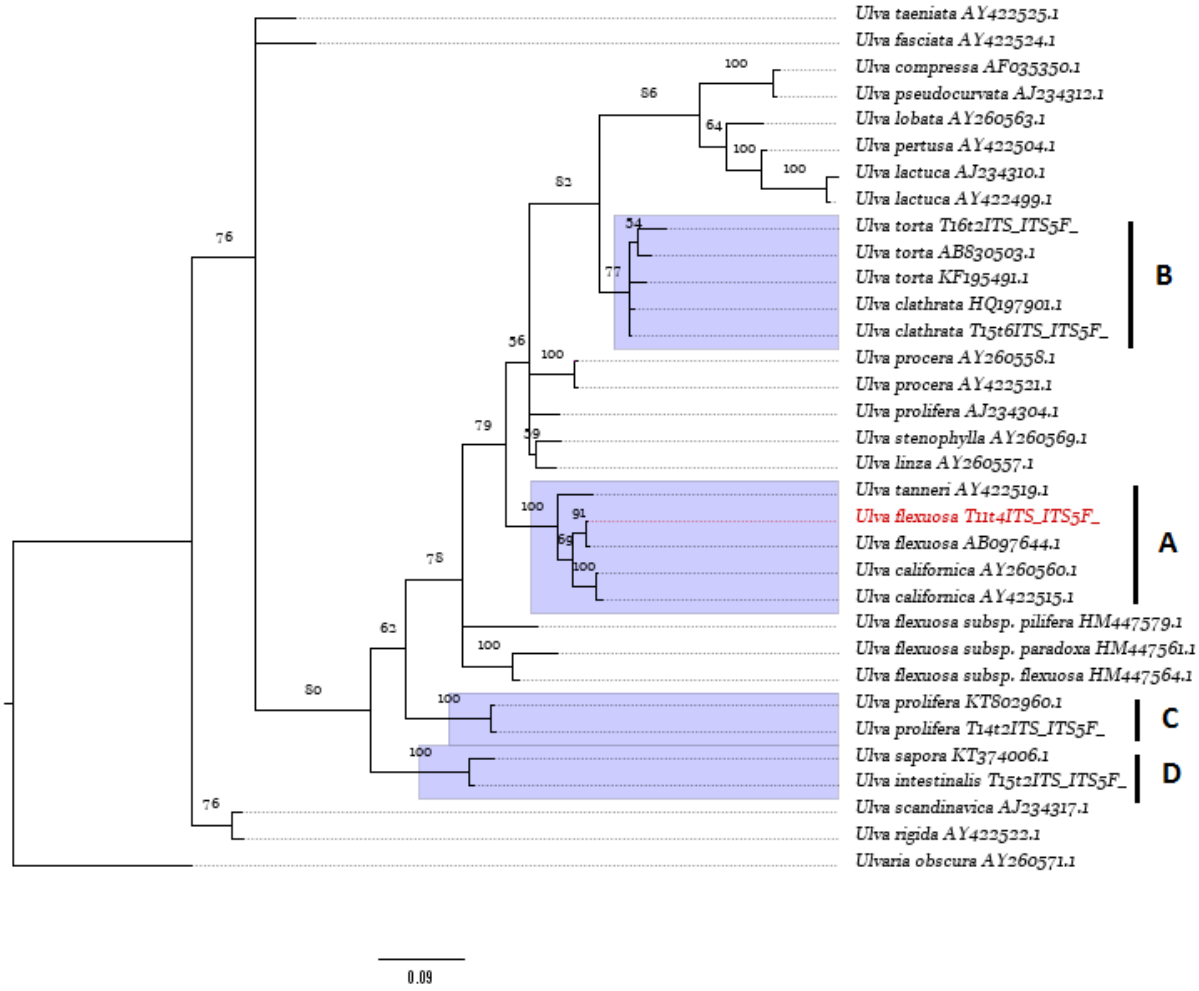

Figure 3.8. Bayesian tree of ITS sequences. Bayesian probabilities (%), BP, are given on the branches. Posterior probabilities < 50% have been omitted. Sequences are labelled with taxon name and GenBank accession number of ITS sequence (Annex A, Table 1). The tree is rooted using *Ulva*ria *obscura*. A, B, C and D refer to Group containing *Ulva* collected from EPPO ponds. In red is stressed the *Ulva flexuosa* identified in this study.

3.2.3 *Morphological observations*

The gross morphological characteristics (Annex A, Table 2) presented a marked homogeneity among the varied species collected, underlining the importance of genetic analysis to identify the different *Ulva* species. The filamentous, herbaceous-like shape was the most common and, with a few exceptions of turf forms (*Ulva sapora* and one *Ulva clathrata*), *Ulva flexuosa* was the only species present with 3 different dominant morphotypes:

a) The lettuce-leaf (Figure 3.9a-3.9b).
b) Narrow and broad gregarious thalli (Figure 3.9c).
c) Filamentous, herbaceous-like shape (Figure 3.10a-e).

The lettuce-like *Ulva flexuosa* was the one that was cultivated. The specimen had a less rigid structure (thin and papery in texture) than those collected in the drainage channel. Moreover, they lost any anchoring structure present in the wild type. Their thallus had medium to light green, broader than long, flat, irregular contoured with undulated margins and is unbranched (Figure 3.9a). Under the microscope the central part of lettuce-like's thallus has showed a disordered cell arrangement with 2-4 pyrenoids per cell. Cells are irregularly arranged, polygonal, usually with rounded corners (Figure 3.9b). Principally measurements are shown in Table 4. The mean number of pyrenoids is three.

Table 3.10. Size of *Ulva flexuosa* cells with wide leaf thalli.

	Length of cells (µm)	**Width of cells(µm)**	**ø of pyrenoids**	**Nº of pyrenoids (in one cells)**
U. flexuosa				
Mean	8.04	5.61	1.84	3.5
Min.	5.19	1.99	0.97	1
Max.	11.27	5.87	2.91	4
SD*	1.20	1.08	0.42	

*SD= Standard Deviation

The two remaining morphotypes belong to *Ulva flexuosa* grown within the ponds or attached to the framework. The first of these was characterized by a narrow and broad gregarious thallus attached to substrate by means of small discoid base and as well as the cultivated morphotype was unbranched, flat with a thin texture and, started from a narrow base, widen towards the top. The second one had a filamentous herbaceous shape and it often presented thalli polyform, slender, tubular compressed or laminar, wide at the top. Observations to the stereoscope revealed the presence of some branches at the base and a stipe that could be hollow. The thalli were fixed by means of a basal disc reinforced by numerous robust rhizoidal filaments. It is worth mentioning the presence of a fourth morphotype, with lanceolate thallus, although it is represented by a single specimen collected around the 13-pond's perimeter.

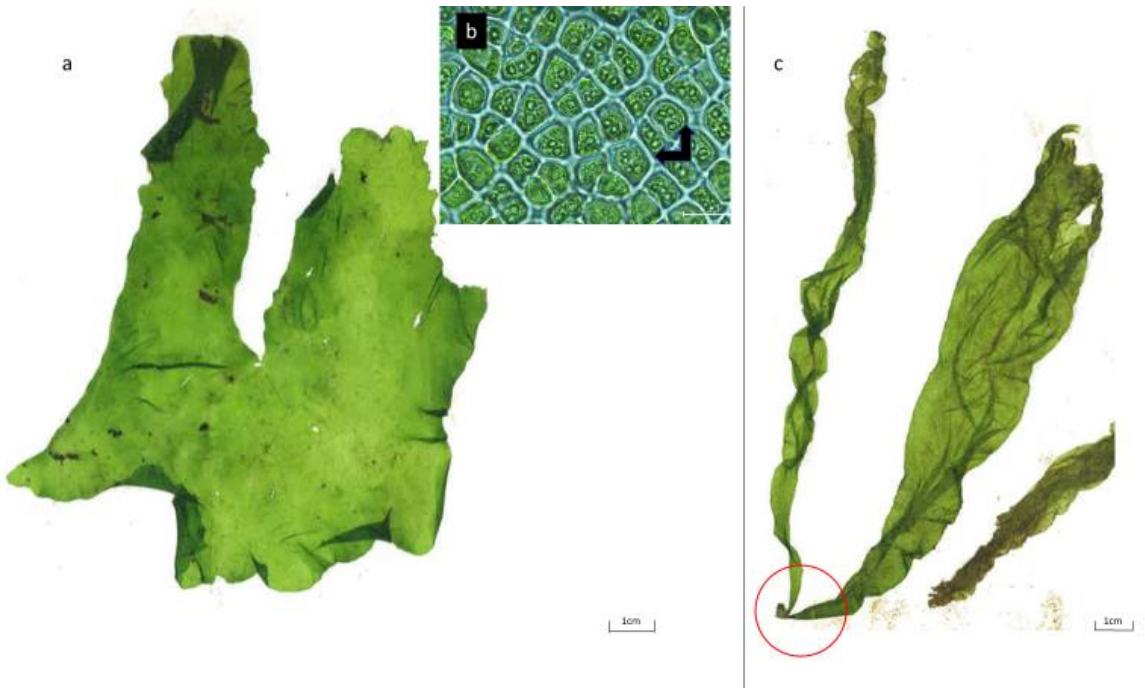

Figure 3.9 a) Lettuce-shape *Ulva flexuosa*; 3b) polygonal cells with pyrenoids (black rows); 3c) Gregarious thalli with discoidal base (red circle). Scale bar a) and c) 1cm. Scale bar for b) is 10µm

Figure 3.10 a) *Ulva flexuosa* filamentous morphotype; b) thallus corrugated; c) laminar; d) branch (red circle); e) hollow stipe. Scale bar a) 1cm; scale bars of b), c), d) and e) are 1mm

CHAPTER IV.

Discussion and Conclusion

4. DISCUSSION

4.1 Morphological and genetic species identification

The identification of *Ulva* spp. present in the EPPO ponds revealed a heterogeneous community. The investigation reported 6 taxa of which three were never reported until now in the Ria Formosa area: *Ulva flexuosa, Ulva torta* and *Ulva intestinalis.* *Ulva flexuosa* was identified as the species cultivated and its lettuce-leaf morphotype is not attributable to any of the subspecies of the marine species.

Despite the ITS had a low amplification success it allowed to differentiate *Ulva* taxa among our samples. The huge morphological plasticity of the kind probably would have leaded to associate the different phenotypes founded with a species already recorded in the Formosa area. The presence of multiple bands sequences between ITS' PCR results has already been reported in the past (Saunders and Kucera, 2010; Couceiro et al., 2011). Therefore, it is commonly associated with *rbc*L (plastid rubisco large subunit) marker to increase the successes of identification (Shimada et al.2003, 2008; Heesch et al. 2009; Kraft et al. 2010, O'Kelly et al.2010; Marês et al., 2011; Rybak et al., 2014).

Ulva flexuosa. *U. flexuosa* was originally described by Wulfen from the Adriatic Sea in the 1803. Currently, *Ulva flexuosa* species includes 5 subspecies and one variety: *E. flexuosa ssp. flexuosa, E. flexuosa ssp. paradoxa* (Dillwyn) Bliding, *E. flexuosa ssp. paradoxa var. profunda* (Bliding), *E. flexuosa ssp. linziformis* (Bliding), *E. flexuosa ssp. biflagellata* (Bliding) and *E. flexuosa ssp. pilifera* (Kützing) Bliding (Shimada et al., 2003; Cormaci et al., 2014).

Among the three morphotype here reported the lettuce-leaf observed is not attributable to any of the marine subspecies belonging to *Ulva flexuosa*. One record of a similar phenotype regarded the subspecies *pilifera* which is a freshwater macroalgae (Marês et al., 2011). This morphotype may have an explanation if is considered that algae grown in IMTA systems tend to develop leaves larger than the wild type (Neori et al., 2004). The remaining two morphologies have a taxonomic response. The filamentous one, based on the polymorphism of the thallus and the presence of a tubular stipe, could be

associated to *Ulva flexuosa ssp. flexuosa* (Cormaci et al., 2014). The one with the gregarious thalli, instead, was similar to the *Ulva flexuosa* morphotype described by Wolf et al. (2102) in the Venice lagoon and *Ulva flexuosa* from Busan and Pohang, Korea (Lee et al., 2014). However, genetic identity discarded the hypothesis of three distinct subspecies confirming instead the enormous plasticity of *Ulva* genus. There are several factors that can explain this phenomenon. *Ulva flexuosa* can 'switch' its thallus morphotype from tubular to foliose along their life and it is more frequent in culture due to stresses unique to artificial systems (Hayden et al., 2003; Rybak et al., 2014). Environmental factors such as salinity and temperature can also affect morphological plasticity (Gao et al., 2016). In our case, the fact of having collected seaweed in November after a week of intense rain may have favoured the finding of different morphotypes due to lowering of the temperature and salinity. Furthermore, in the past has been proved the role of bacterial community on morphology variation of *Ulva* genus (Wichard, 2015; Grueneberg et al. 2016). The capacity of *Crassostrea gigas* to remove a large concentration of bacteria (Jones et al., 2001) could have provoked a change in their community promoting change in *Ulva flexuosa* phenotype. All these assumptions need of further studies to be proven.

Historically the presence of this species in neighbouring countries has been recorded in the coastal zone between Tanger (Morocco) and Melilla (Spain) (Benhissoune et al. 2001) and in the Cadiz Bay (Hernández et al. 2010). Furthermore, *U. flexuosa* has been include in the list of macroalgae of the north coast of Portugal, along Minho, Douro Litoral, and Beira Litoral regions (Araújo et al. 2009) and in Corunna harbour, Spain (Peña and Barbara, 2002).

The *Ulva flexuosa* T11t4 sequence turned out to be almost identical (2bp of difference) to that recorded by Shimada in Hokkaido, Japan (Shimada et al., 2003) forming a well-defined clade in both ML and BI trees. This aspect and the fact that both phylogenetic trees look similar at which encountered in the articles consulted (Shimada et al., 2008; O'Kelly et al., 2010; Heesch et al., 2007; Lawton et al., 2013; Lin et al., 2013; Masakiyo and Shimada, 2013) suggest that the two entities can be conspecific. Some nodes in Bayesian analysis that we have performed have receive high support respect the ML one.

Based on Lewis et al., 2005, these results could reflect the tendency of Bayesian analysis to resolve polytomies with strong support. Nevertheless, I think the feedback with phylogenetic trees of other studies helps to dispel any doubts due to this problem.

These observations may lead to conclude that the origin of these macroalgae could be the North Pacific and other investigations seem to suggest a common origin between the *Ulva flexuosa* of South Europe and the Pacific one. An investigation about cryptic (species with morphologies identical or similar, although genetically different (Wolf et al., 2012)) and new species in North Adriatic reported of an *Ulva flexuosa* quite identical at one reported in the British Columbia (Canada) (Wolf et al., 2012). Moreover, a Greek *Ulva flexuosa spp. linziformis* was found out closer related with a Japanese one (Shimada et al., 2003).

The *Ulva flexuosa* specimens from the EPPO ponds and South Europe did not match genetically with *Ulva flexuosa* subspecies from North Europe (Marês et al., 2011; Rybak et al., 2014). This issue was already detected by Marês and Shimada (Shimada et al., 2008; Marês et al., 2011) and the first one proposed to indicate *U. flexuosa* as indigenous species of the inland waters of the Europe proposing a different nomenclature for the Asians (Marês et al., 2011). However, no mention was made about seawater *Ulva flexuosa* subspecies.

Other taxa. Not only *Ulva flexuosa* was recorded for the first time in the Ria Formosa lagoon, also *Ulva torta* and *Ulva intestinalis* were first reported whereas *Ulva prolifera* and *Ulva clathrata* have been already mentioned in some studies occurred in the lagoon (Aníbal et al. 2014; Alsufyani et al. 2016). Historically all these taxa, with sometimes the exception of *Ulva torta,* have shown a similar geographical distribution, jointly with *U. flexuosa,* in Portugal and closer countries (Benhissoune et al. 2001; Peña et Barbara, 2002; Araújo et al., 2009; Hernández et al. 2010). Moreover, in the port of Corunna they occupied the same environment (Peña et Barbara, 2002). Nevertheless, among the studies listed above only one (Alsufyani et al. 2016) provided a molecular identification by means of molecular techniques. This can lead to some doubts about the real distribution of these species in the Portuguese coast.

Multisource introduction into Ria Formosa lagoon. Since *Ulva spp.* are common components of the hull fouling flora and are known for their rapid, proliferous growth (Couceiro et al. 2011) they are suitable for human-mediated dispersal (Heesch et al., 2008). Several *Ulva* species are considered as cryptogenic due their cosmopolitism and may have been spread over the centuries by sailboats (Heesch et al., 2008). The oysters' culture could a plausible source for the introduction into Ria Formosa lagoon. Shellfishes culture has already considered the cause of introduction of several *Ulva* species in Europe (López et al., 2007; Manghisi et al., 2011), in particular *Crassostrea gigas* transfer from Miyagi prefecture(Japan) after the decline of "Portuguese oyster" *Crassostrea angulata,* until then cultivated (Batista, 2007). Ria Formosa oyster aquaculture was not an exception. Anyway, a recent study revealed *C.angulata* chines origin (Taiwan) and its supposed introduction in Europe during the earliest commercial trade with Asia (Batista et al., 2005). Therefore, *Ulva flexuosa* and the other species here discovered could be present in the Ria since a long time. However, further studies would be required to evidence the precise sources and vectors and if there was a regional spread.

Ulva sapora is not mentioned until now because of the sequence obtained had a bad quality (5.5%), so before making any statement it required a more accurately investigation. However, if these presences will be confirmed could be the first record in Europe.

4. 2 *Ulva flexuosa* production

EPPO pond water and their abiotic factors supported well the *U. flexuosa* growth. The values of specific growth rate (SGR) of both systems gave results similar to other studies (Table 4.1). However, the wet biomass production (WBP) and the dry biomass production (DBP) recorded in this experiment were often lower than the others likely because the use of different tank sizes, techniques or different initial density of *Ulva* (Robertson-Andersson et al., 2008; Castelar et al., 2014).

The optimal cultivation period into EPPO ponds seemed to be positioned between seven to nine days since, after this time, the SGR decreased. Moreover, looking at the growth curve of dry weight (DW) obtained after eight days cultivations, *Ulva flexuosa* seemed to have reached the maximum of biomass around this period. This result and SGR values greater than 10% up to 15 days of cultivation suggest a production cycle of approximately 8 days.

The SGR and WBP during the experience drew a sinusoidal pattern with two spikes and two falls of values. The fall in the autumn can be explained by a decrease in temperatures and a reduction of light period (Ogawa et al., 2013; Amosu, 2016), in addition to a raining week that occurred before the last collection. More complicated is explaining the drop in August. During this period was noted the presence of white spots in the *Ulva* thalli a phenomenon known as "ghost tissue" often indicative of an increase in sporulation. Sporulation can be caused by several factors such as elevated temperatures, irradiance, lack of nutrients and life cycle' stage (Copertino et al., 2008; Chemodanov et al., 2017). However, temperature and irradiance were constant from June to the end of August and the first one was within the optimum range for the species (Castelar et al., 2014; Cui et al., 2015). Even pH values ($7.6<pH<8.8$) were optimal for species growth, since they could be related to a high presence of dissolved bicarbonate (HCO_3^-) in water, the main source of inorganic carbon for the seaweed (Falkowski and Raven, 2007; Raven, 2010; Msuya et al., 2006). Therefore, life cycle could explain the August decreased. The algae could have been harvested at a specific stage of the life cycle and the procedure to weigh it and put it in the structure

could have accelerated these sporulation processes (Pettet, 2009; Chemodanov et al., 2017). In addition to life cycle, another cause of biomass loss in August was probably related to the constant activity of mullet near the rafts. This could have caused the detachment of some algae and the damage of *Ulva* with a consequent increase of sporulation (Pettett, 2009). A confirmation seems to come from the eight-days experiment, where the rafts protected by a cage, gave results of wet biomass production higher than max value of the previous experience. Although the nutrients concentration of EPPO ponds was like if not greater than previous studies (Neori et al., 1991; Nielsen et al., 2012; Ogawa et al., 2013; Macchiavello e Bulboa,2014; Castelar et al., 2014) cannot be ruled out the possibility of a shortage of nutrients, particularly of NH_4^+. The increasing concentration of NH_4^+ during the phases of decline in algal biomass (Figure 3.3b) could represent a phase of renewal of nutrients up to a re-optimal level for algae. Another hypothesis would suggest that this oscillation depicted the *Ulva flexuosa* capacity to remove this nutrient. When macroalgae biomass declined the assimilative capacity of the environment for nutrients declined in turn. However, specific studies will be required for a proper evaluation of both conclusions.

Initial different densities showed better results for 30g/m^2 which led to the decision discussed in the methodology (see Material and methods). Using low initial density has been suggested as a possible optimization of growing space (Castelar et al., 2014). Nevertheless, in macroalgae culture it's usually used an optimum initial density of 1 kg/m^2 but growing macroalgae in tanks equipped with artificial aeration to ensure there is no shading among the algae (Bruhn et al., 2011; Ben-Ari et al., 2014)

Ulva growing in the 'Fish + *Ulva*' system revelled a better performance than in the IMTA. 'Fish + *Ulva*' system presented mean values superior for both SGR and WBP. Since environmental parameters such as temperature, salinity and irradiance were identical for both systems the cause could be attributed to interactions between the different organisms presents into the ponds. It is known that oysters remove suspended particle by filtration (Burk et al., 2017) which explains the turbidity difference between the two systems. However, they contribute to the N pool with their excretions (Jones et al.,

2001) so there might be higher growth of phytoplankton with limitations in the growth of *Ulva* in IMTA system. Nevertheless, the presence of oysters may have also caused a variation in the bacterial community (Jones et al., 2001; Quental-Ferreira et al., 2012). Since the rule of bacteria is important for the growth and the morphogenesis of some species of green algae (Spoerner et al., 2013; Wichard et al., 2015; Grueneberg et al., 2016) the variation in quantity and quality of their community could have affected the growth of algae.

The differences in oxygen concentrations and pH between early morning and afternoon stressed the ability of the primary producers, *Ulva flexuosa* included, to oxygenate the water in both systems. This capacity was also monitored on the primary production experiment were the light chambers after 1 hour gave a higher pH that dark ones.

In order, to compare the results of the net primary production (NPP) with others reports on *Ulva*, the primary production measured in controlled conditions was converted in g C m^{-2} $year^{-1}$ resulting in a value of 106 g C m^{-2} $year^{-1}$. This number is far below than the NPP recorded in Venice (358 g C m^{-2} $year^{-1}$) lagoons or Tel Aviv (838 g C m^{-2} $year^{-1}$) but closer to NPP of *Ulva sp.* found in Ria Formosa lagoon (190 g C m^{-2} $year^{-1}$) (Table 4.2). However, our experiment was carried out under low light intensity (2klux) and, based on a previous study performed in the Ria Formosa, it can be assumed that under natural conditions the performance would be better (Serpa, 2005). In addition to environmental conditions, the differences between the previous studies and our can be attributed to several technical sources of variation (Chemodanov et al., 2017). Anyway, since the experiment has produced 8,052g[2] of dry biomass in total then, along 5 months, they were absorbed 9.7 g of C and produced 13.25 g of O_2.

[2] The overall wet biomass got in 5 months was 45493g. Since was found out dry biomass was in mean 17.7% of wet weight it was obtained the result shown.

Table 4.1. Comparison of averages of specific growth rate(SGR), dry biomass production (DBP), Wet biomass production(WBP) cultured in different systems with different stock density (Table adapted from Neori et al., 2014 and Castelar et al., 2014)

Species	System	Stocking density (kg WW m^{-2})	DBP (g m^{-2} d^{-1})	SGR (%/day)	WBP (g m^{-2} d^{-1})	References
Ulva flexuosa	Earth pond	0.06-0.015	2.6	17	14.75	This study
Ulva lactuca	Tank	1-8	34.5-6	10-1	230-40	Bruhn et al., 2011
Ulva flexuosa	Ropes,sea	0,0005*	0.24	11.95	–	Castelar et al., 2014
Ulva flexuosa	Tank	0,0005*	0.47	22.80	–	Castelar et al., 2014
Ulva clathrata	Tank	0.2-0.5	10.5	7	70	Copertino et al., 2008
Ulva lactuca	Tank	1	16.8 - 56.4	–	112-376	Msuya and Neori, 2008
Ulva lactuca	Tank (continuous aeration)	0.8	47.7	13.3	318	Ben-Ari et al., 2014
Ulva lactuca	Tank (25% aeration)	0.8	26.7	8.1	178	Ben-Ari et al., 2014

Wet biomass values were converted to dry biomass considering that dry/wet *Ulva sp.* biomass is around 15 %(17.7 %in this study); *dry biomass.

Table 4.2 Net primary productivity (NPP) of *Ulva* spp. from different studies (Table adapted from Chemodanov et al., 2017).

Ulva sp.	**NPP** ($g\ C\ m^{-2}\ year^{-1}$)	**References**
Ulva sp. (Ria Formosa Lagoon (estimation))	190	Serpa, 2005
Ulva compressa (Minicoy Atoll)	1460	Kaladharan and Kandan, 1997
Ulva rigida (Venice lagoon)	358	Sfriso et al., 1993
Ulva sp. Reading Power Station, Tel Aviv (grown in a single layer photobioreactor)	838	Chemodanov et al., 2017
Ulva flexuosa (EPPO ponds, Olhao (estimation))	106	This study

4.3 Economic outlook

The quantitative data about the biomass and environmental values obtained in this study together with the identification of *Ulva sp.* cultivated are fundamental to generate hypotheses about its use and possible economic yield. For example, the SGR (≈18%) of algae and the mean NH_4^+µM environmental concentration found in this experiment foreshadow a C: N ratio close to or above 19% (Nielsen et al., 2012). If confirmed, this percentage would be closer to optimal C: N ratio required to convert *Ulva* biomass into bioenergy by anaerobic digestion (Yen and Brune 2007; Bruhn et al., 2011). Going on, the results showed that *Ulva* follows a sinusoidal growth pattern with a high growth rate followed by significant fall (Fig. 3.3a). If further studies will confirm this cyclicity, this observation will allow recommendations to be proposed for an industrial ulvan production objective (Robic et al., 2009). This is important, since in the past *Ulva flexuosa* was proved to contain ≈ 17.7% per DW g of algae without variation yield due to environment condition (Castelar et al., 2014). However, it was proved that the ulvan polysaccharides quality change based on growth period (Robic et al., 2009). Based on required ulvan application is important to know when the highest and lowest growth period occur and then schedule the collection periods. For this reason, it recommends further work considering the impact of *Ulva* gametophyte and sporophyte life phases (Robic et al., 2009). Nevertheless, it is difficult to make a prediction about the possible economic yield of biomass produced without a specific analysis of the dry tissue obtained.

As far as it is concerned the carbon sequestration, the low recorded value can be compensated by promising biomass yield, and anyhow it was higher than land crops (Chemodanov et al., 2017).

5. CONCLUSION

Ulva flexuosa showed to grow well under conditions typical of earth-pond aquaculture. The experiments on the production cycle indicated a period of cultivation of macroalgae of about 8 days. Despite the differences found within the systems, the growing periods and the initial densities of *Ulva flexuosa*, the growth values have always been satisfactory. Moreover, *Ulva flexuosa* shows capacity to oxygenate the pond environment and maintain a pH level recommended for the macroalgae cultivation and release to the sea (Msuya et al., 2006). However, it will be necessary to assess the growth of the species along the year to evaluate better it response at environmental changes. Even higher stock densities should be tested to evaluate a possible cultivation for commercial purposes. The technique used for cultivation has nevertheless proved feasible, in the future we recommend the use of structures that protect *Ulva* from possible contacts with the fish community. The data of NPP obtained are too few to determine the actual potential of this algae in carbon sequestration and more detailed research is required.

The use of the molecular marker ITS was successful on macroalgae cultivated but there was low amplification success. For this reason subsequent investigations of green macroalgae would require the use of markers with a higher success rate such as tufA or associating *rbc*L (plastid rubisco large subunit) with the use of ITS (Saunders and Kucera, 2010). The genetic data collected in this experiment may lead to conclude that the origin of the macroalgae present in EPPO ponds could be the North Pacific. However, the scale of the present study does not allow to state which is the actual distribution area of the *Ulva* spp. identified and their status of native or introduced species.

The importance of the experiment on EPPO station is that it was conducted on a semi-industrial scale providing a base for an economic feasibility of *Ulva flexuosa* cultivation. The presence of *Ulva flexuosa* in the South Portugal broadens its geographic distribution and opens the prospect of using this species in IMTA systems in various parts of the country.

CHAPTER III.

References and annex

6. References

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7. ANNEX A

Table 1. Sources of taxa used to create the phylogenetic trees.

TAXA	COLLECTION SITES	SOURCE	ACCESSION NUMBER ITS
ULVARIA OBSCURA SPP. BLYTII **((ARESCHOUG) BLIDING, 1969)**	Padilla Bay, WA, USA	Hayden et al.,2003	AY260571
ULVA CALIFORNICA **(WILLE IN COLLINS, HOLDEN ET SETCHELL, 1899)**	La Jolla, CA, USA	Hayden et al., 2003	AY260560
ULVA CALIFORNICA **(WILLE IN COLLINS, HOLDEN ET SETCHELL, 1899)**	Northeast Pacific	Lawton et al., 2013	AY422515
ULVA CLATHRATA **((ROTH) C. AGARDH, 1811)**	Yellow Sea, China	Teng et al., 2010	HQ197901
ULVA FLEXUOSA **(WULFEN,1803)**	Oshoro, Hokkaido,Japan	Shimada et al., 2003 Lawton et al., 2013	AB097644
ULVA FLEXUOSA SPP. PILIFERA **(KÜTZING), M.J.WYNNE 2005**	Poland	Marês et al., 2011 Rybak et al., 2014	HM447579
ULVA FLEXUOSA SPP. PARADOXA **((C.AGARDH) M.J.WYNNE, 2005)**	Czech Republic	Marês et al., 2011 Rybak et al., 2014	HM447561
ULVA FLEXUOSA SPP. FLEXUOSA **(WULFEN, 1803)**	Sweden	Marês et al., 2011 Rybak et al., 2014	HM447564
ULVA LACTUCA **(LINNEUS, 1753)**	N.A.*	Marês et al., 2011 Rybak et al., 2014	AJ234310
ULVA LACTUCA **(LINNEUS, 1753)**	Northeast Pacific	Marês et al., 2011 Rybak et al., 2014	AY422499

***ULVA LINZA* (LINNEUS, 1753)**	Humboldt Bay, CA, USA	Hayden et al., 2003	AY260557
***ULVA PROCERA* (K.AHLNER) HAYDE,ET AL., 2003**	N.A.	Hayden et al., 2003	AY260558
ULVA PROCERA	Northeast Pacific	Marês et al., 2011 Rybak et al., 2014	AY422521
ULVA PROLIFERA	Yellow Sea (China)	Zang, 2015	KT802960
***ULVA PSEUDOCURVATA* (KOEMAN ET VAN DEN HOEK, 1981)**	N.A.	Marês et al., 2011 Rybak et al., 2014	AJ234312
ULVA RIGIDA	Northeast Pacific	Marês et al., 2011 Rybak et al., 2014	AY422522
ULVA SAPORA	Shelly Beach, Caloundra Australia	Philips et al., 2016	KT374006
ULVA SCANDINAVICA	N.A.	Marês et al., 2011 Rybak et al., 2014	AJ234317
***ULVA TAENIATA* ((SETCHELL) SETCHELL ET GARDNER, 1920)**	Monterey, CA, USA	Marês et al., 2011 Rybak et al., 2014	AY422525
ULVA TANNERI	Northeast Pacific	Marês et al., 2011 Rybak et al., 2014	AY422519
ULVA TORTA	Fukui (Japan)	Ogawa et al., 2013	AB830503
ULVA TORTA	Clovelly, NSW (Australia)	Lawton et al., 2013	KF195491

*N.A.: Not available

Table 2. *Ulva* taxa identified with short morphological description.

System	Ponds	Sample	Description	Morphological assessment
IMTA	11	11-t3	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
IMTA	11	11-t4	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
IMTA	11	11-f2	*Ulva flexuosa* (Wulfen,1803)	Lettuce-leaf, flat, rounded undulate margins.
IMTA	16	16-t1	*Ulva flexuosa* (Wulfen,1803)	Lettuce-leaf, flat, rounded undulate margins.
IMTA	16	16-t2	*Ulva torta* ((Mertens) Trevisan, 1842)	Narrow small leaf, rounded on top.
IMTA	16	16-t5	*Ulva flexuosa* (Wulfen,1803)	Linear compress thalli, tapering toward the base.
IMTA	16	16-t6	*Ulva sapora* (J.A.Phillips, R.J.Lawton & C.Carl,2016)*	Turf form, Thin-short filamentous
IMTA	16	16-f3	*Ulva flexuosa* (Wulfen,1803)	Filamentous, tubular and linziformis.
IMTA	16	16-f4	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
Fs+Oy	12	12-t2	*Cladophora albida* ((Nees) Kutzing, 1843)	Dark green, musk form
Fs+Oy	12	12-t3	*Cladophora vagabunda* ((Linnaeus) Hoek, 1963)	Narrow liner flat leaf
Fs+Oy	12	12-t5	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
Fs+Oy	14	14-t2	*Ulva prolifera* (O.F.Müller, 1778)	Filamentous, herbaceous shape
Fs+Oy	14	14-t3	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
Fs+Sw	13	13-t2	*Ulva flexuosa* (Wulfen,1803)	Linear compress thalli, herbaceous shape.
Fs+Sw	13	13-t5	*Ulva flexuosa* (Wulfen,1803)	Lettuce-Leaf, flat, rounded edges, undulate margin
Fs+Sw	13	13-t6	*Ulva flexuosa* (Wulfen,1803)	Lanceolate Leaf.
Fs+Sw	13	13-t8	*Ulva clathrata* ((Roth) C.Agardh, 1811)	Filamentous, herbaceous shape
Fs+Sw	13	13-f2	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
Fs+Sw	13	13-f3	*Ulva flexuosa* (Wulfen,1803)	Lettuce-leaf present some perforation
Fs+Sw	15	15-t2	*Ulva intestinalis* (Linnaeus, 1753)	Tubular, herbaceous shape
Fs+Sw	15	15-t3	*Ulva flexuosa* (Wulfen,1803)	Narrow and broad gregarious thalli, small discoid base
Fs+Sw	15	15-t4	*Ulva flexuosa* (Wulfen,1803)	Linear compress thalli, round on top.
Fs+Sw	15	15-t6	*Ulva clathrata* ((Roth) C.Agardh, 1811)	Turf form, Thin-short filamentus

* This name is currently regarded as a synonym of *Ulva tepida* (Masakiyo and S.Shimada, 2014)(Algaedatabased).

Table 3. Nucleotide homology (%) of ITS region sequences of the EPPO samples and other *Ulva* specimens available in GenBank, that grouped in the ITS phylogenetic tree.

CLADE	SPECIES	COLLECTION SITES	ACCESSION NUMBER ITS	HOMOLOGY %	D.B.S (BP)*
A	*Ulva flexuosa* T11t4	EPPO pond			
	Ulva flexuosa	Oshoro, Hokkaido, (Japan)	AB097644	99.47	2
	Ulva californica	La Jolla, California (U.S.A.)	AY260560	97.33	12
	Ulva californica	Northeast Pacific	AY422515	96.80	14
B	*Ulva torta* T16t2	EPPO pond			
	Ulva torta	Fukui (Japan)	AB830503	95.65	17
	Ulva torta	Clovelly, NSW (Australia)	KF195491	94.39	20
	Ulva clathrata T15t6	EPPO pond		95.17	19
	Ulva clathrata	Yellow Sea (China)	HQ197901	94.91	22
B	*Ulva clathrata* T15t6	EPPO pond			
	Ulva clathrata	Yellow Sea, (China)	HQ197901	99.49	2
	Ulva torta	Fukui (Japan)	AB830503	97.71	9
	Ulva torta	Clovelly, NSW (Australia)	KF195491	95.69	17
	Ulva torta T16t2	EPPO pond pond		95.17	19
C	*Ulva prolifera*	EPPO pond			
	Ulva prolifera	Yellow Sea (China)	KT802960	98.60	5
D	*Ulva intestinalis*	EPPO pond			
	Ulva sapora	Shelly Beach, Caloundra (Australia)	KT374006	96.48	14

*Distance between sequences (base-pair)