Universidade de Aveiro Departamento de Biologia
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Bruna Alexandra Martins Marques

Eficiência de poliquetas e halófitas na biorremediação de um efluente de uma piscicultura a operar em regime super intensivo

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Ana Isabel Lillebø, Investigadora principal do Departamento de Biologia da Universidade de Aveiro e CESAM-Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro, e co-orientação científica do Doutor Ricardo Jorge Guerra Calado, Investigador Principal do Departamento de Biologia da Universidade de Aveiro e CESAM-Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro

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“We must plant the sea and herd its animals using the sea as farmers instead of hunters. That is what civilization is all about – farming replacing hunting.”

– Jacques-Yves Cousteau –

o júri

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palavras-chave Aquacultura multi-trófica Integrada; Poliquetas em filtros de areia, Halófitas em Aquaponia, Matéria Orgânica, Nutrientes, Ácidos Gordos

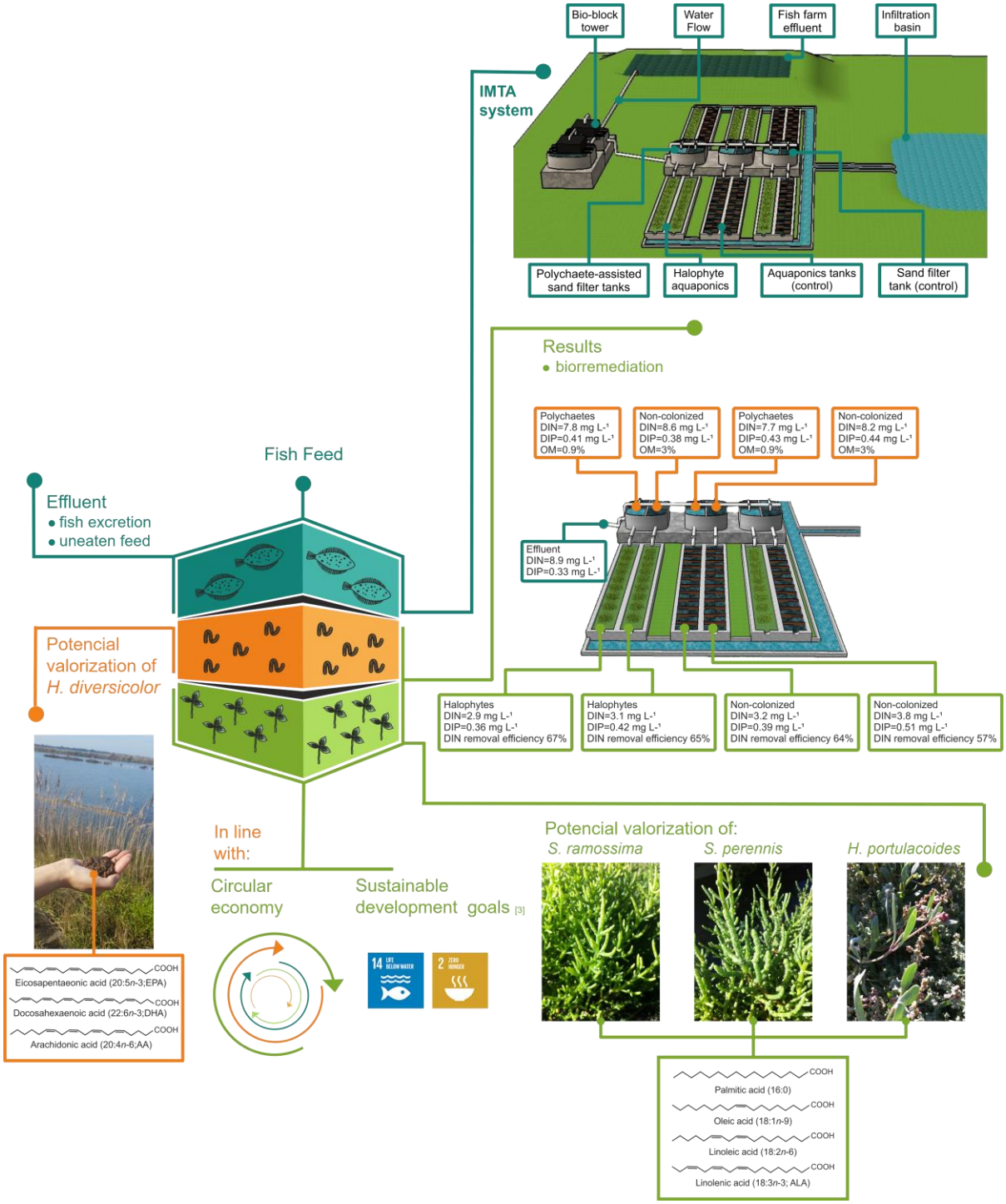
Resumo

Os sistemas de recirculação de aquacultura (RAS) são considerados um dos grandes paradigmas da Revolução Azul, pois permitem "cultivar peixes em qualquer lugar". A expansão destes sistemas RAS, levanta novos desafios face aos custos elevados associados ao tratamento do efluente gerado, existindo assim a necessidade de gestão do efluente orgânico devido ao seu teor de sal (cerca de 5-10% da água circulante). Os sedimentos removidos ricos em matéria orgânica não podem ser utilizados como fertilizantes agrícolas sem tratamento prévio. Deste modo, são encaminhados para estações de tratamento, à semelhança dos resíduos que são rotulados como perigosos para o ambiente de acordo com a legislação Portuguesa do ambiente. Esta imposição legal representa um custo adicional para o modelo produtivo que contemple o uso de RAS para cultivo de peixes marinhos. A aquacultura multi-trófica integrada (IMTA) surge como uma solução sustentável, baseada nos serviços fornecidos pelos ecossistemas. Este conceito envolve a cultura de espécies aquícolas de níveis tróficos diferentes, permitindo assim que os nutrientes presentes no alimento não ingerido e os resíduos produzidos sejam utilizados por outros organismos em cultivo. O presente estudo teve como objetivo testar a eficiência dos poliquetas cultivadas em tanques com filtros de areia e halófitas em aquaponia na remoção da matéria orgânica em suspensão e nutrientes na forma dissolvidos presentes no efluente de uma piscicultura a operar RAS em regime de produção super-intensivo, respetivamente. Pretendeu-se assim avaliar, através do perfil de ácidos gordos, o potencial valor das espécies extrativas escolhidas para este estudo. Este trabalho está dividido em quatro etapas complementares, nomeadamente: 1) testar uma abordagem inovadora de biomitigação com os poliquetas (*Hediste diversicolor*) cultivadas em filtros de areia combinadas com a produção da halófita *Halimione portulacoides* em aquaponia, na remediação de um efluente rico em matéria orgânica em suspensão e nutrientes na forma dissolvida; 2) avaliar o potencial valor acrescentado de *H. diversicolor* cultivado em tanques com filtros de areia abastecidos com o efluente da piscicultura comparando o seu perfil de ácidos gordos com o de conspecíficos selvagens; 3) determinar se o processamento com alta pressão (HPP) altera o teor de ácidos gordos nos poliquetas processados e validar este método para assegurar a biossegurança da biomassa destes organismos para fins comerciais; e 4) comparar os perfis de ácidos gordos de halófitas cultivados em aquaponia com o efluente de piscicultura com espécimes selvagens das áreas doadoras. Este estudo permitiu validar o potencial de *H. diversicolor* em tanques com filtros de areia e *H. portulacoides* em aquaponia na remediação do efluente da piscicultura. Os *H. diversicolor* cultivados em tanques com filtros de areia não só contribuíram para um decréscimo de 70% da matéria orgânica particulada, como também mostraram uma grande capacidade de reter valores elevados de ácidos gordos essenciais, nomeadamente 20:5*n*-3 e 22:6*n*-3 Estes ácidos gordos essenciais, considerados importantes para a nutrição das espécies de aquacultura, não foram encontradas em espécimes selvagens de H. diversicolor. O tratamento com altas pressões induziu uma pequena redução nas quantidades de ácidos gordos altamente insaturados nos poliquetas, no entanto não comprometeu o perfil de ácidos gordos. Desta forma, o tratamento HPP assegura tanto a biossegurança quanto a qualidade nutricional do produto final. As halófitas *H. portulacoides* cultivadas em aquaponia tiveram um crescimento acentuado nos caules e nas folhas, contribuindo para uma diminuição de 65% do azoto inorgânico dissolvido presente nos efluentes, subindo este valor para 67% quando combinadas com *H. diversicolor*. Estudos complementares com *H. portulacoides*, *Salicornia ramosissima* e *Sarcocornia perennis* revelaram que estas halófitas possuem uma grande capacidade para reter nutrientes, apresentando ainda um perfil em ácidos gordos *n*-3 e n-6 que não difere significativamente dos espécimes selvagens. As espécies *H. diversicolor* e *H. portulacoides* apresentam grande capacidade extrativa quando integradas em sistemas IMTA para a biomitigação de efluentes de pisciculturas a operar em regime super-intensivo. As espécies escolhidas representam um potencial valor económico, contribuindo a sua cultura para a redução da dependência da utilização de organismos selvagens, refletindo princípios de economia circular e práticas mais sustentáveis. O sistema IMTA implementado é assim uma ferramenta importante para o tratamento de efluentes, sendo igualmente uma contribuição positiva para a prevenção e redução da poluição marinha, gestão/práticas mais sustentáveis, segurança e crescimento económico, de acordo com o Objetivo de Desenvolvimento Sustentável 14 (ODS14 - “proteger a vida marinha”) proposto pelas Nações Unidas.

Keywords Integrated multi-trophic aquaculture, Polychaete-assisted sand filters, Aquaponics halophytes, Organic matter, Nutrients, Fatty acids

Abstract The concept of recirculating aquaculture systems (RAS) is currently considered one of the paradigms of the Blue Revolution, as it allows to “grow fish anywhere”. One of the main constraints impairing the expansion of RAS, acknowledge to be a more environmentally friendly system, concerns the disposal of the organic rich effluent due to its high content in marine salts (circa 5-10% of the circulating water). The organic rich sediments that result from the settlement of suspended particulate matter (SPM) cannot be further used as fertilizer in traditional agriculture farms, being classified, according to Portuguese environmental legislation, as a dangerous waste. Therefore, it represents an economic burden to the fish farm. Integrated multi-trophic aquaculture (IMTA) has been regarded as a sustainable solution to overcome this constraint, being conceptually framed on an ecosystem-based approach. This concept involves the farming, in proximity, of aquaculture species from different trophic levels with complementary ecosystem functions. In IMTA one species uneaten feed and wastes, including nutrients and by-products, represents a source of energy to the next trophic level, enabling the combination of different extractive species. The present study aimed to test the efficiency of employing polychaete-assisted sand filters and halophytes in aquaponics in the removal of organic-rich SPM and dissolved inorganic nutrients present in a marine RAS effluent. In addition, the potential added value of selected extractive species was evaluated through their fatty acids (FA) profile. To achieve these goals, the present study was divided into four complementary steps: 1) test the capacity of an innovative approach, where *Hediste diversicolor*-assisted sand filters were combined with the production of *Halimione portulacoides* in aquaponics, to bioremediate an organic-rich effluent generated by a super intensive marine fish farm operating a land-based RAS; 2) evaluate the potential added value of RAS cultured *H. diversicolor*, by comparing their fatty acids (FA) profile with that of wild specimens; 3) evaluate, in terms of biosecurity, if high-pressure processing (HPP) of RAS cultured *H. diversicolor*, promoted significant changes on their FA content; 4) assess and compare FA profiles of RAS cultured halophytes, namely *H. portulacoides*, *Salicornia ramosissima* and *Sarcocornia perennis* with that of wild conspecifics from donor sites. The present study revealed that the proposed IMTA system, combining RAS cultured polychaetes *H. diversicolor* and the halophyte plants *H. portulacoides*, contributed to the bioremediation of the marine effluent. In detail, *H. diversicolor*-assisted sand filters promoted a decrease of particulate organic matter (POM) in 70%. The ability of *H. diversicolor* (extractive species) to retain high values of essential FA, namely 20:5*n*-3 e 22:6*n*-3 was also demonstrated. Moreover, 22:6*n*-3, an essential FA paramount for marine aquaculture species’ nutrition, is not found in wild specimens of *H. diversicolor*. The HPP treatment induced a small reduction on polychaetes HUFA levels, but without compromising their FA profile. In this way, HPP treatment ensures both biosecurity and the nutritional quality of polychaetes biomass for high-end products/applications. The halophyte *H. portulacoides* cultured in aquaponics displayed a pronounced growth of stem and leaves biomass, contributing to a decrease of waste water dissolved inorganic Nitrogen (DIN) in 65%. Furthermore, *H. portulacoides* cultured downstream from *H. diversicolor*-assisted sand filters promoted a superior decrease of DIN in effluent water (67%). Although *H. portulacoides*, *S. ramosissima* and *S. perennis* retained high-valued nutrients, their FA profile did not differ significantly from that of wild conspecifics. Both *H. diversicolor* and *H. portulacoides* show a high extractive capacity in IMTA systems for the biomitigation of super-intensive marine fish farms effluents. Selected extractive species display a high potential economic value, with their culture simultaneously contributing for reducing the dependence on wild species and promoting the circular economy agenda and more sustainable practices. The IMTA system implemented represents an important tool for the treatment of marine RAS effluents, as it in holds more sustainable management/practices. Overall, the IMTA system tested contributes to the prevention and reduction of marine pollution and to economic growth, in line with the United Nations Sustainable Development Goal 14 (SDG14 – “life below water”) for 2030.

Graphical Abstract



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Acronym List

AA	Arachidonic acid
AI	Atherogenicity index
ALA	α-linolenic acid
C	Carbon
DHA	Docosahexaenoic acid
DIN	Dissolved inorganic Nitrogen
DIP	Dissolved inorganic phosphorus
DOM	Dissolved organic matter
DON	Dissolved organic nitrogen
DOP	Dissolved organic phosphorus
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acids methyl esters
GC	Gas chromatography
GC-FID	Gas chromatograph system with a flame ionization detector
GLA	γ-linolenic acid
HPP	High-pressure processing
HUFA	Highly unsaturated fatty acid
IMTA	Integrated multi-trophic aquaculture
LA	Linoleic acid
LOI	Loss on ignition
LPC	Large cultured polychaetes

MPC	Medium cultured polychaetes
MUFA	Monounsaturated fatty acids
N	Nitrogen
OM	Organic matter
P	Phosphorus
PI	Polyene index
POM	Particulate organic matter
PUFA	Polyunsaturated fatty acid
RAS	Recirculating aquaculture system
SDG	Sustainable development goal
SFA	Saturated fatty acids
SPC	Small cultured polychaetes
SPM	Suspended particulate matter
TI	Thrombogenicity index
WP	Wild polychaetes

List of Fatty Acid Nomenclature

Class	Shorthand formula	Chain length	Common Name
Saturated fatty acid	**14:0**	14	Myristic acid
	15:0	15	Pentadecylic acid
	16:0	16	Palmitic acid
	17:0	17	Margaric acid
	18:0	18	Stearic acid
	20:0	20	Arachidic acid
	21:0	21	Heneicosanoic acid
	22:0	22	Behenic acid
	24:0	24	Lignoceric acid
M**onounsaturated fatty acid**	**16:1*n*-7**	16	Palmitoleic acid
	16:1*n*-9	16	7-hexadecenoic
	18:1*n*-9	18	Oleic acid
	18:1*n*-7	18	Vaccenic acid
	18:1*n*-5	18	13-octadecenoic
	20:1*n*-7	20	Paullinic acid
	20:1*n*-9	20	Gondoic acid
	22:1*n*-9	22	Erucid acid
Polyunsaturated fatty acid	**18:2*n*-6**	18	Linoleic acid
	18:3*n*-6	18	ɣ-linolenic acid
	18:3*n*-3	18	α-linolenic acid
	20:2*n*-6	20	eicosadienoic acid
Highly unsaturated fatty acid	**20:4*n*-6**	20	Arachidonic acid
	20:5*n*-3	20	Timnodonic acid
	22:4*n*-6	22	Adrenic acid
	22:5*n*-3	22	Clupanodoic acid
	22:6*n*-3	22	Docosahexaenoic acid

Chapter 1

General Introduction

1. General Introduction

1.1 The role of aquaculture

Aquaculture is defined by the Food and Agriculture Organization (FAO, 1988) as: "The *farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated*."

In the last five decades the aquaculture sector has expanded in a context of climate change and depletion of resources. Today, according to FAO (2015), it is recognized as one of the fastest-growing animals-food-producing sectors. With an estimated growth of population reaching the 9 billion by 2050, the increasing need for protein delivery is one of the biggest challenges of the 21st century (Grealis et al., 2017). This boom in aquaculture production, known as the Blue Revolution, emerged as a solution for seafood supply, as the global fisheries capture is becoming exhausted and exceeding its sustainable limit (Neori et al., 2007). In line with the United Nations Sustainable Development Goals and targets for the year 2030, aquaculture might contribute to achieve zero hunger (SDG2) (Ntona and Morgera, 2018), as it is mostly directed for human consumption and already accounts for almost half of the world´s fish consumption (FAO, 2014). In 2016, global aquaculture production reached 110.2 million tonnes, 80.0 million tonnes of aquatic animals and 30.1 million tonnes of aquatic plants (FAO, 2018). In Europe since 2016 the annual aquaculture production has been of 3 million tonnes, including fish, crustaceans and molluscs, and of 2709 tonnes for aquatic plants. In Europe, the average consumption of fisheries and aquaculture products per inhabitant per year is 24.9 Kg (quantity in live weight), 6 Kg more than in the rest of the world. However, consumption varies between European countries, with minimum values in Hungry, (5.3 Kg/person/year) and maximum values in Portugal (56.8 kg/person/year) (European Commission, 2016).

It has been acknowledged that seafood consumption, especially of fish, is highly recommended due to its high-quality protein and nutritional benefits, representing a

valuable source of nutrients. In detail, seafood is an important source of 1) essential amino acids, especially lysine and methionine; 2) essential highly unsaturated fatty acids (HUFA) such as: Arachidonic acid (AA, 20:4*n*-6), Eicosapentaenoic acid (EPA, 20:5*n*-3) and Docosahexaenoic acid (DHA, 22:6*n*-3); and 3) micronutrients (vitamins D, A and B) and minerals (calcium, phosphorus, iodine, zinc iron and selenium) (Sidhu, 2003; Lund, 2013; Venugopal and Gopakumar, 2017).

As aquaculture integrates EU's Blue Growth strategy, EU Member States developed a Multiannual National Strategic plan to improve the sustainability of European marine economies. It is widely known that European aquaculture industries have a great potential for growth, which has not yet been reached. Therefore, a strategic plan was established in order to achieve a sustainable progress in the sector. The strategic guidelines were as follow: 1) "simplifying administrative procedures" to improve the overall competitiveness and development of this sector economics; 2) "securing sustainable development and growth of aquaculture through coordinated spatial planning" through the identification of areas for aquaculture to develop more sustainable practices to aquaculture production, in order to protect landscapes, habitats and biodiversity; 3) "enhancing the competitiveness of EU aquaculture" via identification of business opportunity, marketing strategies (local economies, sustainably produced seafood); and 4) "promoting a level playing field for EU operators by exploring their competitive advantages" through products differentiation e.g., fresh local products and products certification (COM, 2013; Grealis et al., 2017). In addition to the enhancement of aquaculture development, this strategy also provides employment and promotes a socio-economic dynamization in rural areas.

Within the several constrains associated with aquaculture (e.g., excessive exploitation of resources, spread of invasive species, deforestation of coastal areas), effluents have become a topic with increased interest. To ensure environmental responsible actions, waste management regulation and best practice conduction according to the EU directive 2018/851 are being imposed by several organizations (Boyd, 2003). Aquaculture waste is mainly associated with feed and its metabolic end products related factors, namely: feeding composition and regime; biological features related with the cultured species (e.g. feed conversion ratio); and physicochemical parameters of the water (van Rijn, 2013). The paradox of fish carnivorous species requiring feed formulations with fishmeal and fish oil,

has been discussed for quite some time (Nasopoulou and Zabetakis, 2012; Henry et al., 2015; Torrecillas et al., 2017). The replacement of fish by other raw materials, such as plants or insects for reasons of sustainability, can prompt a poorer quality in terms of their omega-3 fatty acids, essential for human consumption (Belghit et al., 2018; Tacon, 2009).

1.2 Aquaculture systems

The aquaculture production comes mostly from extensive e semi-intensive systems. However, due to the concerns about the environment health, as well as the economic and social impacts attached to aquaculture, the proposition of new sustainable practices enhancing and promoting the development of intensive marine aquaculture are daily requested. Briefly, aquaculture systems can be classified into three categories: extensive, semi-intensive and intensive (Welcomme and Bartley, 1998).

Extensive aquaculture systems are part of a natural ecosystem, with limited inputs to maintain animal grown and survive e.g. production of food using natural processes. These systems usually have low density of stock, generally <500 kg/ha/year, and low harvest per unit of area under culture.

Semi-intensive aquaculture systems are used as a mid-level technology, with partial dependence on natural productivity, with dissolved oxygen control, addition of inorganic and organic fertilizers and supplemental feeding. These systems usually have medium input levels and a medium rate of production.

Intensive aquaculture systems are high-tech culture systems employing tanks, raceways or ponds of different sizes and depths suited to different growth stages of fish. These systems display a high-density stock, generally >100000 kg/ha/year, and have very high production costs associated (Welcomme and Bartley, 1998; Ottinger et al., 2016).

Recirculating aquaculture systems (RAS) have become a key solution for large-scale and sustainable fish production. Recirculating aquaculture systems were developed to respond to economic and environmental constraints, such as environmental regulation limiting the land and water access (Mirzoyan et al., 2010), and the dependence of large volumes of

water (Martins et al., 2010). As shown in Figure 1.1, RAS is based on the use of biological and mechanical filters, and the water is recycled back to the system enabling up to 90–99% of the water to be recycled. Therefore, one of RAS advantages is the decrease of waste-water volume.

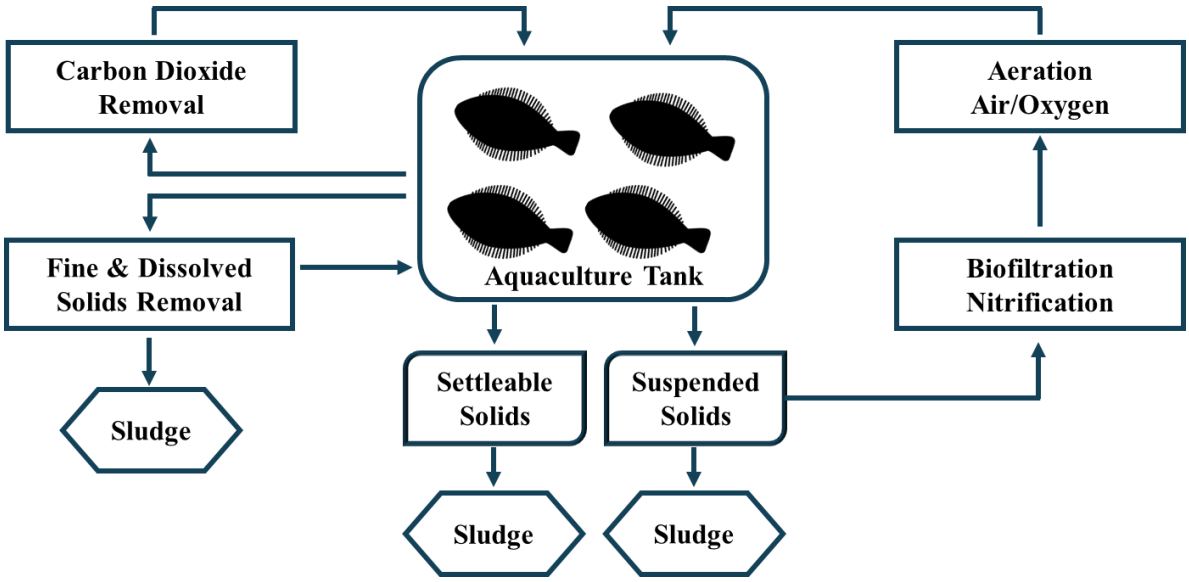

Figure 1.1- Schematic example of recirculated aquaculture system (RAS).

Through denitrification reactors, sludge thickening technologies and ozone treatments, it is possible to promote a decrease in nutrient concentration and a more concentrated waste is achieved (Martins et al., 2010). Suspended solids are removed by sedimentation, ammonia is converted into nitrate (NO_3) through nitrification, oxygen is added to the system through aeration (air/oxygen), and carbon dioxide is removed by degassing. In this way, the operator has greater control over water quality, and several other biotic and abiotic factors, adapting the systems according to the requirements of cultured species. The main constrains of RAS are: 1) the organic-rich suspended particulate matter (POM), since the high concentration of POM might lead to negative impacts on nitrification process; and 2) dissolved organic matter (DOM) and nutrients in dissolved inorganic form (nitrogen, N and phosphorus, P) (Schneider et al. 2005).

1.3 Sustainable Aquaculture

In September 2015, the United Nations adopted the 2030 Agenda with 17 Sustainable Development Goals (SDG) and targets. Each of these goals will be implemented taking into account each country priorities and must fulfil the three dimensions of sustainable development: social, economic and environmental, and for the first-time poverty and sustainable development. Within the topic of this work, two out of the seventeen SDG can be highlighted: SDG2 - End hunger, achieve food security and improved nutrition and promote sustainable agriculture, and SDG - 14 Conserve and sustainably use the oceans, seas and marine resources for sustainable development. This urgent need for food production requests a joint effort and the implementation of new sustainable practices, taking into account the environment and the socioeconomic dimensions. In addition, sustainable aquaculture systems supported by scientific knowledge and new technology approaches can be seen as part of the solution to tackle overfishing and contribute to ocean conservation.

Integrated multi-trophic aquaculture (IMTA) is part of these responsible methods to achieve the desirable green development categories: ecosystem resilience and resource efficiency, while considering the community and their cultural aspects. One of the fundamental concepts of IMTA involves the farming of aquaculture species from different trophic levels that display complementary ecosystem functions (Chopin et al., 2008). By focusing on the synergistic interactions between organisms, this concept allows that uneaten feed, waste, and by-products generated by one species' can be recaptured and converted into fertilizer, feed and energy for other species. The main goal of IMTA, in marine and coastal waters, is the combined culture of fed aquaculture species, with particulate organic matter (POM) extractive species and dissolved inorganic matter (DIM) extractive species, to create a balanced ecosystem and minimize environmental impacts (e.g. Chopin et al., 2008; Barrington et al., 2009; Wu et al., 2015). In this way, IMTA reduces the costs associated with the treatment of organic rich-effluents by replacing or complementing water treatment technological solutions commonly employed. The multiple variation concept of IMTA has been applied throughout human history (Figure

1.2).

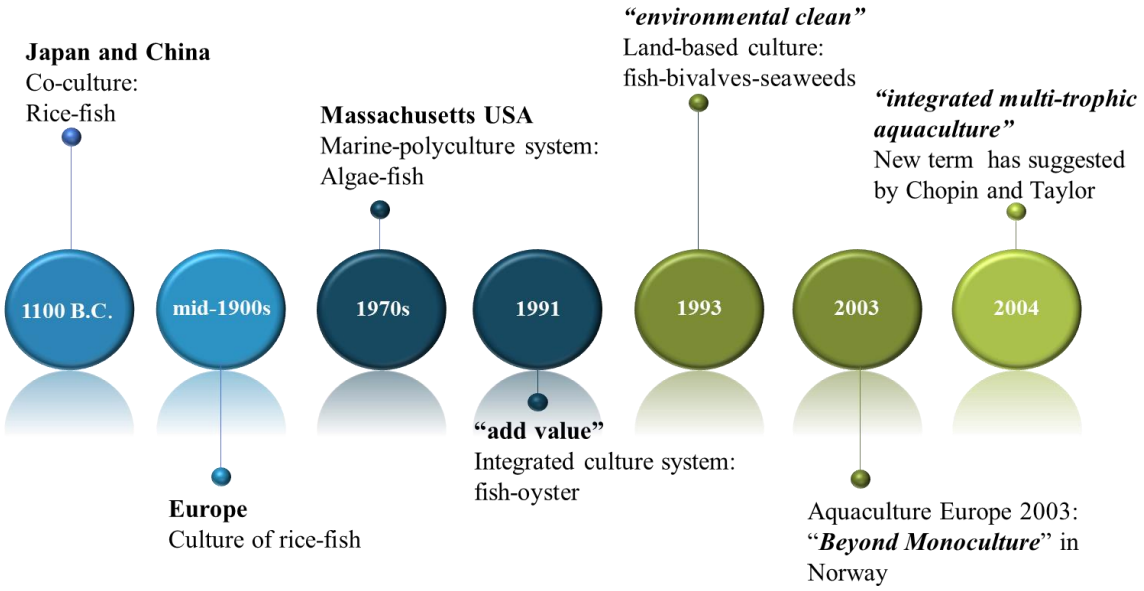

Figure 1.2 - Timeline of IMTA implementation: From past to present

In China and Japan, where aquaculture had its early beginnings, the co-culture of rice and fish, known as aquatic culture, has been practiced for millennia (Neori et al., 2004). The global interest in rice-fish farming was renewed by the mid-1900 in Africa, Asia, Australia, Europe, North America and South America (FAO, 2004). In the 1970s a waste-recycling marine-polyculture system was developed with red algae (e.g., *Gracilaria* sp. and *Neoagardhiella baileyi*) to remove the inorganic nutrients from fish culture effluents (Deboer and Ryther, 1978). By then, IMTA systems emerged as a possible solution, based on ecosystems management, for the reutilization of effluents. For example, Shpigel and Blaylock (1991) showed the potential of integrated fish-oyster culture system to add additional value to commercial product through algae production, with a reduction of 50% in clean water input. In line with the previous study, Shpigel et al., (1993), constructed a model system "environmental clean" to remove particulate and dissolved metabolites from the fish culture effluents. The integration of fish, bivalves and seaweeds resulted in a reduction of 96% in the nitrogen budget (Shpigel et al., 1993). In 2003 the European Aquaculture Society (EAS) organized the first international conference "Aquaculture Europe 2003: Beyond Monoculture" focused in research and industrial applications of "integrated" or "multispecies" aquaculture technologies (Aquaculture Europe, 2003). This technology appeared to be a new step in aquaculture evolution. In 2004, to standardize

IMTA terminology (e.g., integrated culture, polyculture, multispecies culture), Chopin and Taylor combined the multiple expressions into the term integrated multi-trophic aquaculture. However, it is important to point out the differences between polyculture and IMTA. In contrast to IMTA, polyculture practices the co-culture of species in the same site, without considering different trophic levels (Barrington et al., 2009). For example, the co-culture of two or more species of finfish is characterized as polyculture without environmental benefits, opposed to IMTA systems. According to Chopin, the IMTA concept is resumed by the principle of conservation of mass, formulated by Antoine-Laurent de Lavoisier in 1789: "*Nothing is created, nothing is lost, everything is transformed*". In other words, "*The solution to nitrification is not dilution but conversion*" (Barrington et al., 2009). From this perspective, IMTA is pointed as a sustainable solution when compared to monocultures, once that monocultures depend of supplementation with an exogenous source of food without mitigation solutions (Chopin et al., 2001). The strengths, weaknesses, opportunities, and threats (SWOT) analysis can be briefly resumed in Table 1.1.

Table 1.1 - Swot analysis of the IMTA system

Strengths	**Weaknesses**
Effluent management Nutrient recycling Wide site applicability Reduce the "ecological footprint" and coastal impact Exploitation of waste and production of a marketable products with little/without associated costs Economic diversification and increased profit Replacement or reduction of fish meal or fish oil	Needs the best business plan and management to achieve an effective balanced system Insufficient scientific knowledge Production cost-efficiency assessment is still in progress Spread of diseases Non-acceptance by consumers of cultured species Lack of acceptance from aquaculture farmers and investors
Opportunities	**Threats**
Providence of jobs in a sustainable development Supply local food and nutrition Coastal and rural community development New sale products opportunities and eco-food concept Technology improvement Green approach to economy development Possibility to increase aquaculture and crop productivity	Food safety concerns Climate change Global Economy status Absence of polices and regulations

1.4 IMTA species cultivation

Ecosystem-based management is a modern approach to more mainstream management methods. It considers all interactions within the ecosystem, balancing the ecosystems health and the human's need for the ecosystems goods and services, in a long-term perspective (Foley et al., 2010; Ansong et al., 2017). Considering this approach, it is imperative to have an equilibrium between IMTA compartments at the ecosystem level (see Figure 1.3). With the appropriate proportions, the cultivation of fed aquaculture species (e.g. finfish/shrimp) with organic extractive aquaculture species (e.g. shellfish and sea worm) and inorganic extractive aquaculture species (e.g. seaweed and plants), can play a crucial role in the sequestration of nutrients (Barrington et al., 2009).

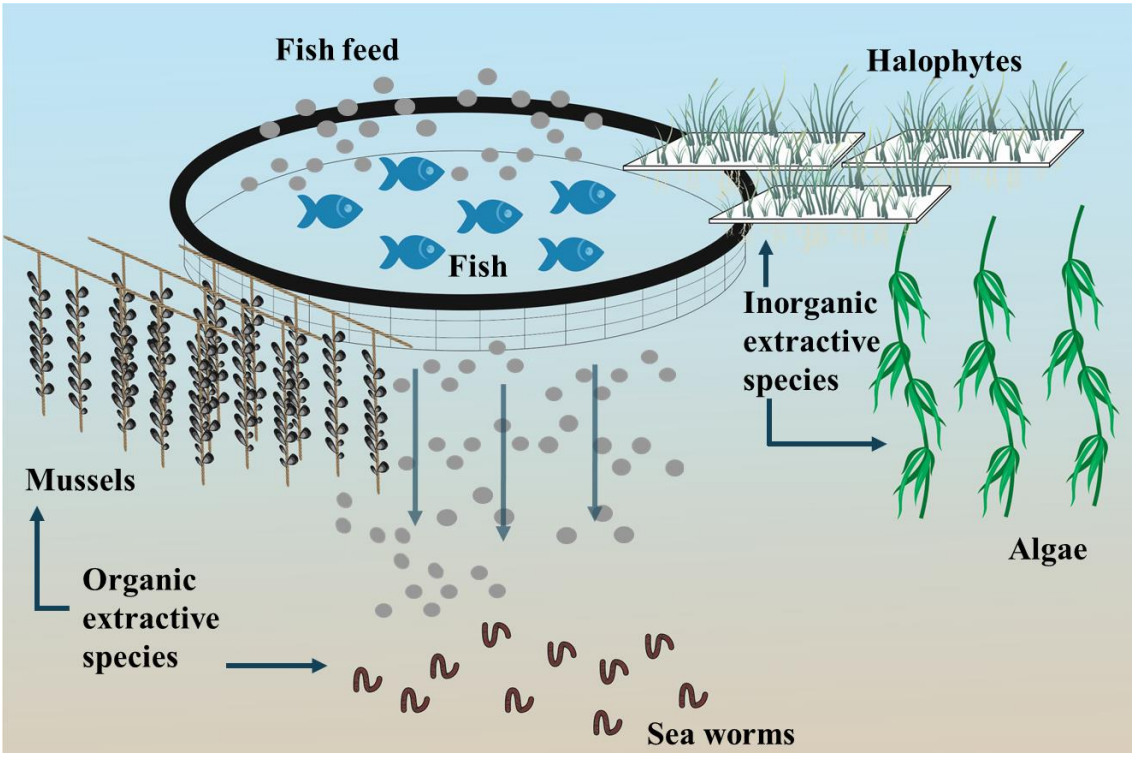

Figure 1.3 - Conceptual model for an Integrated Multi-Trophic Aquaculture (IMTA).

The selected organisms to integrate IMTA are the key factor for the effectiveness of the system itself. There are several fed culture species in aquacultures where IMTA has been applied with variable degrees of success: gilthead seabream (*Sparus aurata*) (Sarà et al., 2012; Shpigel et al., 2016; 2017; 2018); black seabream (*Sparus macrocephalus*) (Wu et

al., 2015); turbot (*Scophthalmus rhombus*) (Abreu et al., 2011); European sea bass (*Dicentrarchus labrax*) (Abreu et al., 2011; Sarà et al., 2012; Waller et al., 2015); among others.

There are several extractive species showing great potential for the consumption of particulate organic matter from the fish farm effluent, namely: flathead grey mullet (*Mugil cephalus*) (Shpigel et al., 2016); sea urchin (*Paracentrotus lividus*) (Shpigel et al., 2018); snail (*Viviparus bengalensis*) (Kibria, 2018), Pacific oyster (*Crassostrea gigas*) (Sarà et al., 2012); Mediterranean mussel (*Mytilus galloprovincialis*) (Sarà et al., 2012). Polychaetes are within these group of extractive species, representing an added value for IMTA systems. Polychaetes are detritivores and filter-feeders, reducing the bacterial concentrations in water wastes (Stabili et al., 2010). In addition, polychaetes can be used has bait in the sports fishing industry (Olive and Cowin, 1994), and furthermore for broodstock maturation diets (Marhematizadeh et al., 2015, Palmer et al., 2014). These organisms represent an expanding global business, being already cultured in several countries, including the USA and China (Fidalgo e Costa et al., 2006). According to Olive, in 1999 the European bait worm market had already been evaluated in ~€200 million. In Portugal, the collection (bait digging) of worms from their natural habitat has been insufficient to keep-up with market demand, and the solution has often been the import of these organisms (Fidalgo e Costa et al., 2006). The annual average of imports has been declining in the recent years, i.e., between 2002-2003 49.52 ton of live worms were imported and in 2012-2015 this number decreased to 14.24 ton. This decrease can be associated with an increased collection of polychaetes or reduction in market demand (Sá et al., 2017). The introduction of non-indigenous species, through bait import, can originate several ecological problems, such as: i) competition with native species, ii) transmission of pathogenic agents, and iii) negative impacts on biological diversity (Sá et al., 2017). The IMTA systems might be a good alternative to produce native species of polychaetes to fulfill the national demand on bait for sports-fishing with no additional cost.

Regarding nutrient sequestration and dissolved inorganic matter extraction, there are several important species pointed out as suitable for IMTA systems, namely: sea lettuce (*Ulva lactuca*) (Shpigel et al., 2017); lettuce (*Lactuca sativa*) (Bakhsh and Chopin, 2012). Seaweeds application is a wise choice for IMTA (Abreu et al., 2011; Wu et al., 2015), as

besides being effective in the extraction of dissolved inorganic nutrients, they can be used for food, as fertilisers, pharmaceutics and energy conversion (Lucas and Southgate, 2012). Halophytes, such as *Salicornia dolichostachya* (Waller et al., 2015) and *Salicornia* spp. (Webb et al., 2013), were already proven to be effective in the bioremediation of aquaculture effluents. These vascular plants have been chosen due to their capacity to tolerate fluctuations of salt concentrations, to balance oxygen, pH, and CO_2 concentrations (Neori et al., 2004), as well as to mitigate nutrients concentration present in the water through their incorporation (Webb et al., 2013; Waller et al., 2015). Also, halophytes have great economic value, related to functional foods, oil seed and nutraceutical industry (Boestfleisch et al., 2014; Singh et al., 2014).

Aim and outline of the thesis

Coastal wetlands provide important ecosystem services that are underpinned by functions and processes mediated by living organisms. Both salt marsh halophytes (salt-tolerant plants) and ragworms are recognized as ecosystem engineers due to their ability to alter the surrounding physical environment. Namely, halophytes promote water flow attenuation and enhance the settling of organic-rich suspended matter, whilst ragworms promote sediment reworking through bioturbation and bioirrigation. In addition, ragworms are omnivorous and their scavenging feeding habits include organic-rich particulate matter and detritus.

This study aimed to mimic the processes supporting coastal wetlands ecosystem services for bioremediation, and is supported by the only super-intensive marine fish farm operating in Portugal employing RAS technology - Aquacria Piscícolas S.A.. The final aim of this work was to evaluate the performance of polychaete-assisted sand filters employing ragworms (*Hediste diversicolor* (O.F. Müller, 1776)) and aquaponic systems using the halophyte sea purslane *Halimione portulacoides* (L.) Aellen in the reduction of organic-rich SPM present in the effluent of a super-intensive fish farm culturing Senegalese sole (*Solea senegalensis*) with RAS technology. These species were chosen due to their common distribution in coastal systems at temperate latitudes and their abundance in Ria de Aveiro coastal lagoon, Portugal.

To accomplish the proposed objectives, the research work plan was divided into 4 complementary tasks, which correspond to Chapters 2 to 5.

Chapter 2 presents an innovative biomitigation approach, where polychaete-assisted (*H. diversicolor*) sand filters were combined with the production of the halophyte sea purslane *H. portulacoides* in aquaponics, to bioremediate an organic-rich effluent generated by a super intensive fish farm. The main objectives of this chapter were to: 1) test the potential of polychaete-assisted sand filters using the polychaete *H. diversicolor* to remediate the particulate organic matter (POM) of a farm effluent; and 2) test the potential of *H. portulacoides* aquaponics to remediate the dissolved inorganic matter (DOM) of a farm effluent. To achieve the objectives set out in this chapter, an IMTA system was constructed and tested. The system included four different experimental combinations to evaluate the potential of polychaete-assisted sand filters and halophyte aquaponics to bioremediate the effluent of super-intensive marine fish farm. **Chapter 3** explores the potential added value of *H. diversicolor* cultured in sand bed tanks supplied with effluent water from a super-intensive marine fish farm given their fatty acid profile. The main objectives of this chapter were: 1) to test if the ragworm fatty acids profiles depend on food source, by comparing cultured and wild specimens, and 2) to test if the fatty acids profiles of ragworms cultured in sand bed tanks supplied with RAS effluent are size-dependent, by comparing small, medium and large ragworms.

Chapter 4 addresses the use of a non-thermal preservation technology on cultured ragworms *H. diversicolor* in order to validate this approach to safeguard their biosecurity. The main objective of this chapter was to test the differences in the fatty acid profiles and lipid quality indexes of fresh whole depurated small, medium and large-sized *H. diversicolor* and conspecifics exposed to high pressure processing (HPP).

Chapter 5 evaluates the added value of halophytes cultured in aquaponics using the effluent from a super intensive fish farm considering their fatty acids profile. The main objective of this chapter was to compare the fatty acid signatures of cultured and wild specimens of *H. portulacoides*, *Salicornia ramosissima* (J. Woods) and *Sarcocornia perennis* (Mill.) A.J. Scott. to determine if and/or how fatty acid profiles can be affected by the effluent from a super-intensive marine fish farm.

Lastly, in Chapter 6, a summary, integration and overall discussion of results from previous chapters is provided, along with future guidelines and suggestions for further research.

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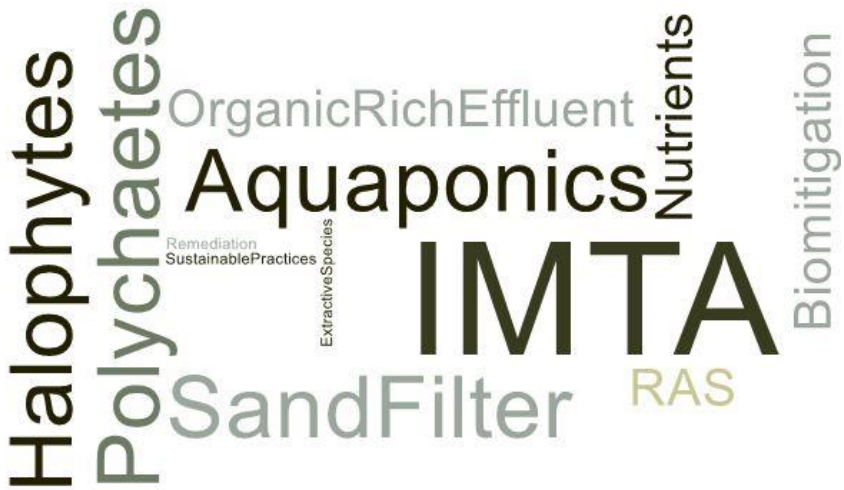
Chapter 2

New species for the biomitigation of a super-intensive marine fish farm effluent: combined use of polychaete-assisted sand filters and halophyte aquaponics



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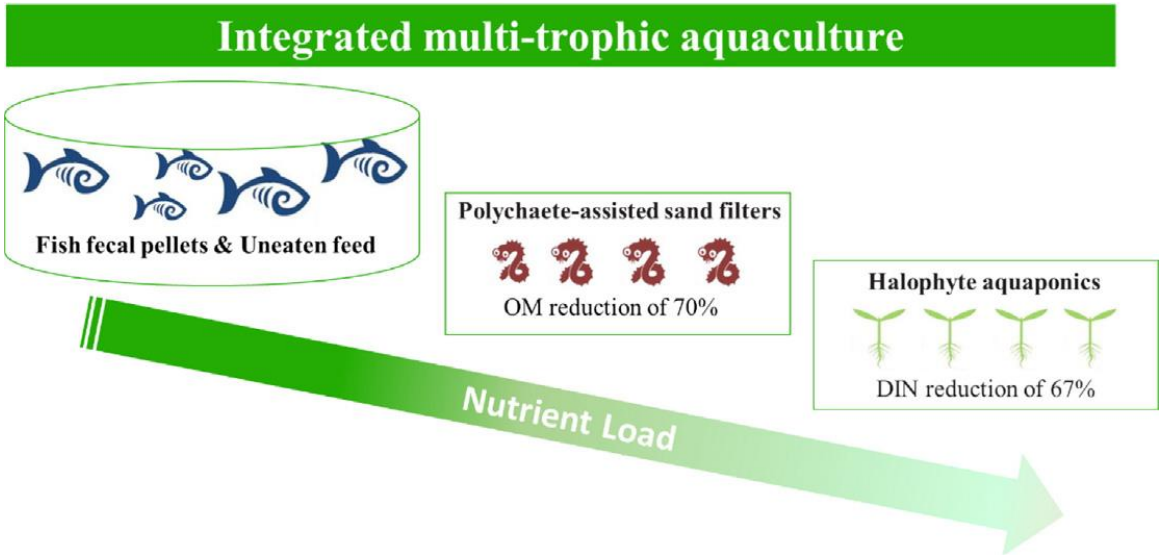
2. New species for the biomitigation of a super-intensive marine fish farm effluent: combined use of polychaete-assisted sand filters and halophyte aquaponics

Abstract

The main objective of this study was to test an innovative biomitigation approach, where polychaete-assisted (*Hediste diversicolor*) sand filters were combined with the production of *Halimione portulacoides* in aquaponics, to remediate an organic-rich effluent generated by a super intensive fish farm operating a land-based RAS (Recirculating aquaculture system). The set up included four different experimental combinations that were periodically monitored for 5 months. After this period, polychaete-assisted sand filters reduced in 70% the percentage of OM and the average densities increased from ≈400 ind. m^{-2} to 7000 ind. m^{-2}. *H. portulacoides* in aquaponics contributed to an average DIN (Dissolved inorganic Nitrogen) decrease of 65%, which increased to 67% when preceded by filter tanks stocked with polychaetes. From May until October (5 months) halophytes biomass increased from 1.4 kg m^{-2} ± 0.7 (initial wet weight) to 18.6 kg m^{-2} ± 4.0. Bearing in mind that the uptake of carbon is mostly via photosynthesis and not though the uptake of dissolved inorganic carbon, this represents an approximate incorporation of ≈1.3 kg m^{-2} carbon (C), ≈15 g m^{-2} nitrogen (N) and ≈8 g m^{-2} phosphorus (P) in the aerial part (76% of total biomass), and an approximate incorporation of ≈0.5 kg m^{-2} carbon (C), ≈3 g m^{-2} nitrogen (N) and ≈2 g m^{-2} phosphorus (P) in the roots (24% of total biomass). In the present study, the potential of the two-extractive species for biomitigation of a super-intensive marine fish farm effluent could be clearly demonstrated, contributing in this way to potentiate the implementation of more sustainable practices.

Keywords: Organic matter; Nutrients; Recirculating aquaculture system (RAS); sand filter; aquaponics; Integrated multi-trophic aquaculture (IMTA)


Integrated multi-trophic aquaculture
Fish fecal pellets & Uneaten feed
Polychaete-assisted sand filters
OM reduction of 70%
Halophyte aquaponics
DIN reduction of 67%
Nutrient Load

2.1 Introduction

The water column of any aquatic ecosystem is characterized by the presence of particulate organic matter (POM), dissolved organic matter (DOM) including dissolved organic nutrients (nitrogen DON and phosphorus DOP), and dissolved inorganic nutrients (nitrogen DIN=NO_x-N+NH_4-N and phosphorus DIP=PO_4-P) (Worsfold et al., 2008), which represent the nutrient forms taken up by primary producers. The proportion of these components allows to classify these ecosystems from oligotrophic to hypereutrophic, as is the case of organic-rich effluents originated by super-intensive fish farms. The treatment of these effluents before they can be discarded into the aquatic environment, within legal regulatory limits, might represent an economic burden to fish farms, namely those operating super-intensive recirculating aquaculture systems (RAS). The main goal of integrated multi-trophic aquaculture (IMTA), in marine and coastal waters, is the combination of fed aquaculture species (e.g. super intensive shrimp or fish farms) with particulate (POM) or dissolved (DOM) organic matter extractive cultured species (e.g. detritivorous fish or invertebrates for POM or filter feeding invertebrates for DOM) and dissolved inorganic nutrients extractive cultured species (e.g. micro or macroalgae, as well as halophytes) to create balanced systems and minimize environmental impacts (e.g. Chopin et al. 2008, Barrington et al. 2009, Wu et al. 2015). In this way, IMTA reduces the costs associated with the treatment of organic rich-effluents by replacing or complementing water treatment technological solutions commonly employed to treat organic-rich aquaculture effluents. Some of the technological solutions available are: woodchip bioreactors, which are recommended to be used downstream of a filtration device (Christianson et al., 2016); single-stage activated sludge reactor with citrate-rich liquid wastes, a common by-product of the food industry (Fox et al., 2015); and up flow anaerobic sludge blanket reactors for digestion of hypereutrophic effluents combined with a biogas production unit (Mirzoyan and Gross 2013). All these reactors seem effective for the treatment of OM and nitrogen sources but require regular maintenance and/or high running costs. The inclusion of extractive cultured species (marine invertebrates and/or primary producers) in IMTA systems will target the energy available in the different water compartments aiming to culture ‘environmentally friendly’ products that in addition might have a market value. The incorporation of specific extractive species in IMTA systems has the potential to generate new added

commercial value to aquaculture operations with relatively little or no additional costs (Alexander et al., 2015).

The main objective of the present study was to test a biomitigation approach with new extractive species to remediate an organic-rich effluent generated by a super intensive fish farm operating a land-based RAS to produce the flatfish *Solea senegalensis* Kaup, (1858). This approach combines the use of polychaete-assisted sand filters using the polychaete *Hediste diversicolor* (O.F. Müller, 1776) with the production of the halophyte *Halimione portulacoides* (L.) Aellen in aquaponics. The results achieved are also discussed taking into account the already existing market value for these two species.

2.2 Material and methods

2.2.1 Selected extractive species

The polychaete *H. diversicolor* (Figure 2.1a), also known as ragworm, was selected due to its wide distribution along the shallow marine and brackish waters of European and North American estuaries and by being an infaunal species that makes U or Y-shaped burrows in sandy-mud bottoms; moreover its "bentho-pelagic" life cycle is characterized by females brooding their embryos in the maternal burrow, the same location where its short pelagic larval life also takes place; this species is an active predator that displays omnivorous feeding habits, being ranked within the deposit-feeders polychaetes functional group (Scaps, 2002). The halophyte *H. portulacoides* (Figure 2.1b), also known as sea purslane, was chosen due to its distribution along the Atlantic coast of Europe and to the fact that it is one of the most abundant and productive plant species in European salt marshes (Bouchard et al., 1998). In addition, it is an evergreen halophyte flowering from July to September in the study area (in Aveiro lagoon, see below).

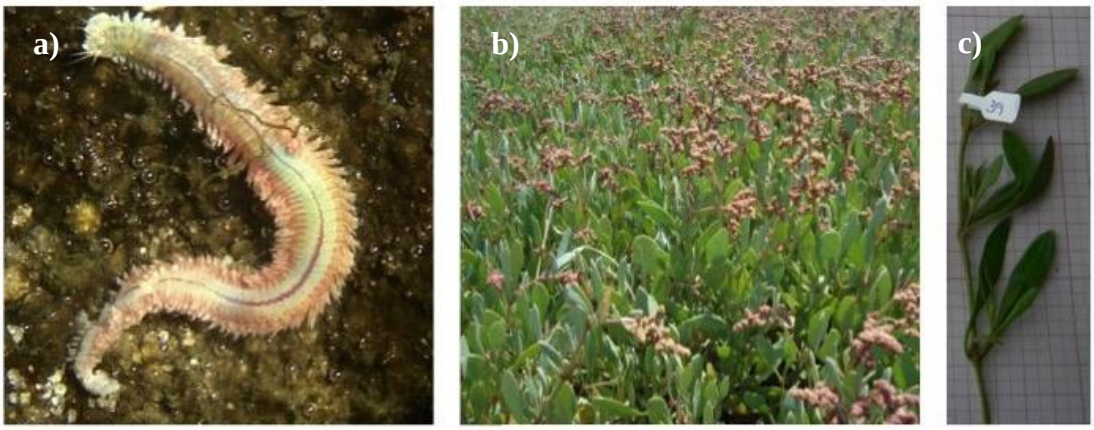


Figure 2.1 - Species employed to validate the IMTA concept used in the present study: a) *Hediste diversicolor* (ragworm) used as organic matter extractive cultured species; b) *Halimione portulacoides* (sea purslane) used as the dissolved inorganic nutrients extractive cultured species and detail of graft used to stock aquaponics tanks and c) grafts of *Halimione portulacoides*.

2.2.2 IMTA experimental design

The effluent originating from the super intensive fish farm was pumped from a nearby settling basin and allowed to trickle flow through a bio-block tower, in order to aerate and increase oxygen levels (from less than 2 mg L^{-1} to 8-9 mg L^{-1}). Following this procedure, the effluent was collected in a 4 m^3 header tank where it was further aerated through a flexible air diffusor hose secured to the bottom of the tank; this procedure also prevented the unwanted settling of any POM in the header tank. The aerated effluent was then allowed to gravity flow from the header tank to the twelve sand filter tanks, which were set-up in parallel. The outflow from each sand filter tank was connected to an aquaponics tank. Each sand filter tank had an approximate volume of 1 m^3 and a surface area of 1 m^2, with the bottom of the tank being covered with 200 mm of sand (1-2 mm grain size). Each tank was also equipped with a draining pipe in the bottom, to allow water recirculation through the sand filter, and two outlets, a mechanical spillway for regular outflow and a spillway to prevent eventual tank overflow. Sand filter tanks were placed in parallel and supplied with the RAS aerated effluent by gravity at a flow of 180 L h^{-1}. Each aquaponics tank, with and without halophytes, was 6 m long by 2 m wide and 0.3 m deep, and in order to maximize the effluent retention time within each tank, 11 alternating wooden barriers were placed transversally to the flow; a full water renewal was achieved ≈ every 12 h. The treated effluent was sent to a collector channel for subsequent

infiltration in the soil, meaning that it was an IMTA open system. Six of the twelve independent experimental sand filter tanks were stocked with wild polychaetes, with the remaining six tanks being used as control. Six aquaponics tanks were stocked with halophytes, three of them being connected to three sand filter tanks stocked with polychaetes and the other three to control tanks (no polychaetes). The schematic representation of the experimental set up is shown in Figure 2.2. The experiment run for five months (from May to October) and during this period no additional feed was provided to the system apart from the organic rich effluent pumped from settling basin.

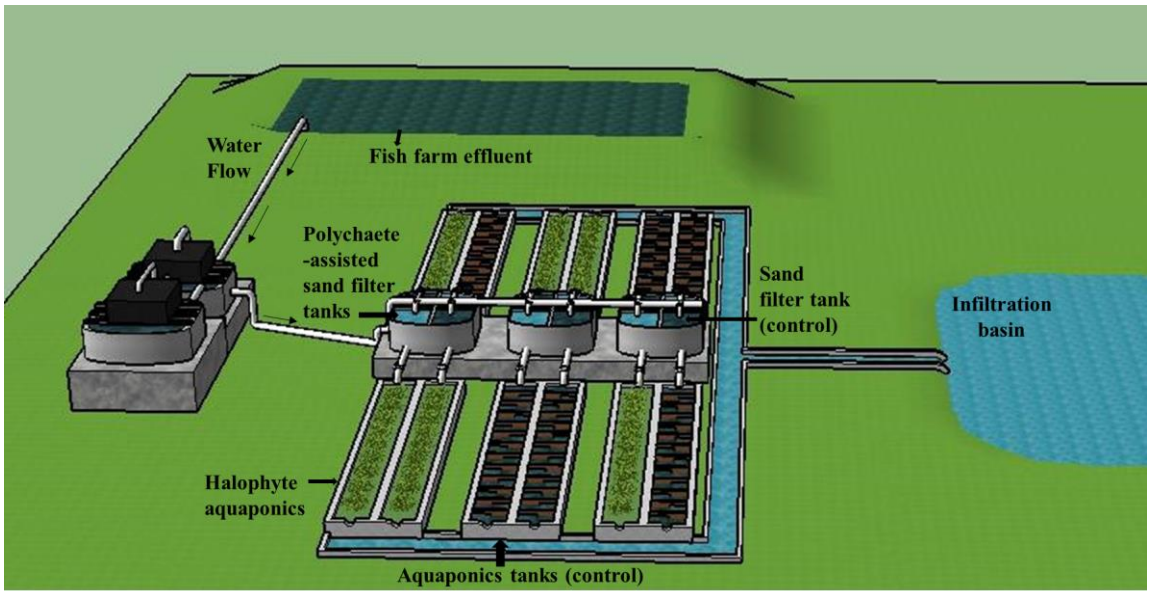


Figure 2.2 - Experimental design employed in the present study with indication of all experimental combinations employed using the polychaete *Hediste diversicolor* (ragworm) cultivated in sand filters and the halophyte *Halimione portulacoides* (sea purslane) cultivated in aquaponics tanks.

2.2.3 IMTA extractive species cultivation

Wild specimens of polychaetes *Hediste diversicolor* (average total length of 80-100 mm) were collected in Ria de Aveiro coastal lagoon by local fishermen, and 400 individuals were stocked in each of the six sand filter tanks. After five months polychaetes were sampled using a hand corer (Ø 110 mm, 150 mm depth). Three corers were taken from each colonized sand filter tank and sorted *in situ* to determine polychaetes densities and biomass. Sample specimens were sorted into three size classes (small <30 mm, medium 30-50 mm and large >50 mm). Substratum samples were taken in triplicate at the beginning of the experiment from all sand filter tanks (reference condition), as well as

five months after the beginning of the experiment from each of the six control tanks (no polychaetes) and the six tanks stocked with polychaetes). Samples were characterized for particle size through sequential sieving (granulometry) and for organic matter (OM) content through loss on ignition (LOI%; 5 h combustion at 450 ºC of substratum previously dried at 105 ºC, until constant weight).

Wild specimens of the halophyte *H. portulacoides* were collected in Ria de Aveiro coastal lagoon salt marsh to make grafts. These grafts were kept in Hoagland's nutrient solution until the growth of new root biomass (Sousa et al., 2011). Afterwards, halophytes were transplanted to aquaponics tanks. Halophytes were marked, measured and weighed at the beginning of the experiment. A selected number of rooted grafts was used to establish the initial weight for above and belowground biomass. At the end of the experiment halophytes were randomly sampled and their above and belowground biomass was determined. For mass balance calculations carbon and nitrogen contents (total C and N) were quantified in a CHNS/O analyser (Fisons Instruments Model EA 1108, Beverly, Massachusetts, USA), whilst phosphorus content (total P) was inferred using data from wild specimens of *H. portulacoides* from the Tagus estuary (≈ 200 km south from Ria de Aveiro) (Sousa et al., 2010).

2.2.4 IMTA monitoring

During the experimental period, effluent pH, temperature, concentration of dissolved oxygen and salinity were measured monthly *in situ*, using a WTW – pH 330i/set equipped with SenTix® 41; a WTW – cond 3110/set 1 equipped with TetraCon® 325 and a WTW – Oxi 3210/set 2 equipped with CellOx® 325-3. Effluent samples were also collected monthly during the experimental period to monitor the concentrations of suspended particulate matter (SPM) between the head tank and the outlets of the sand filter tanks; dissolved inorganic nutrients (NO_x-N, NH_4-N and PO_4-P) were also determined between the head tank and the outlets of the aquaponics tanks. As the composition of the effluent was considered to be relatively constant and stable due to the fine-tuning of the fish farm production, with emphasis on the amount of feed being provided and the fish load per grow-out system, a monthly characterization was considered as suitable for the present study. Effluent aliquots were transported to the lab under dark and refrigerated conditions

and immediately filtered (Whatman GF/C, Ø47 mm dehydrated (105 ºC) and pre-weighed filters). Afterwards effluent aliquots were frozen (-20 ºC) until analysis, whilst filters containing suspended particulate matter (SPM) were processed following the Environmental Protection Agency (EPA) Method 160.2. Effluent aliquots were analyzed for oxidized forms of dissolved inorganic nitrogen (NO_x-N) using a flow injection system (FIAstar 5000 Analyzer, Höganäs, Sweden), ammonium (NH_4-N) and phosphates (PO_4-P) following the standard methods in (Limnologisk Metodik 1992). To ensure analytical quality control, calibration curves, using a standard solution, were run at the beginning of the analysis and in parallel with blanks and samples.

2.2.5 Statistical analysis

Statistical analyses were performed using the Software STATISTICA. The experimental set up with the spatial distribution of experimental conditions is available as supplementary information (Figure S 2.1). Monthly measurements of environmental parameters and nutrient concentrations in the fish farm effluent were considered as independent variables; as the effluent water was never re-used in the biomitigation system (see Figure S 2.1), no repeated measurement statistical test was employed. Following the described objectives, the existence of significant differences (at $p<0.05$) were investigated in environmental parameters, concentration of suspended particulate matter (SPM) and organic matter (OM) between sand filter tanks with polychaetes and without polychaetes at the end of the experimental period (5 months) using a one factor pairwise comparison (Student´s t-test). Additionally, the existence of significant differences (at $p<0.05$) in DIP and DIN concentrations between the 4 different experimental conditions (aquaponics tanks with halophytes preceded by sand filter tanks with polychaetes, aquaponics tanks with halophytes preceded by sand filter tanks without polychaetes, aquaponics tanks without halophytes preceded by sand filter tanks with polychaetes and aquaponics tanks without halophytes preceded by sand filter tanks without polychaetes (control)) was also evaluated using a non-parametric test, Kruskal-Wallis, on a 2x2 crossed design.

2.3 Results

All the results regarding environmental measured monthly are now organized into two tables made available as supplementary information. Results show that the environmental parameters were not significantly different between sand filter tanks, i.e., the average pH in the polychaete-assisted sand filter tanks was 7.9 ± 0.2, while in the non-colonized sand filter tanks was 8.0 ± 0.1 (t-test, $p < 0.055$, n=6); the average water temperature was 19.7 ± 1.8 °C and 19.6 ± 2.0 °C, respectively (t-test, $p < 0.586$, n=6); and the concentration of dissolved oxygen in the water was 8.8 ± 0.7 mg L^{-1} and 9.5 ± 0.8 mg L^{-1}, respectively (t-test, $p < 0.020$, n=6). Average salinity was 20 throughout the IMTA system. The average concentration of suspended particulate matter (SPM) in the head tank was 50.2 ± 5.2 mg L^{-1} (maximum 59.1 mg L^{-1} and minimum 40.9 mg L^{-1}). The average SPM concentrations at the outlet of the sand filter tanks with and without polychaetes was 43.5 ± 8.6 mg L^{-1} and 55.0 ± 15.2 mg L^{-1}, respectively. While these values reveal an average decrease of 21% of SPM concentration when polychaetes were present in the sand filter tanks, these differences were not statistically significant (t-test; $p = 0.129$, n=6). It must be highlighted that small drifting filamentous green algae were often retained in the glass fiber filters employed to determine SPM. The initial substratum was characterized by 0.09 ± 0.01% of MO (%LOI, n=3). Results show significant differences between experimental conditions in the sand filter tanks concerning the percentage of OM at the end of the experiment (after 5 month) (t-test, $p < 0.0491$, n=6). The average percentage of OM was 0.9 ± 0.2% and 3.0 ± 0.8% in sand filter tanks with and without polychaetes (respectively), which reveals a 70% decrease in OM due to the presence of polychaetes. During this period polychaetes average densities increased from ≈400 ind. m^{-2} to 7000 ind. m^{-2}. Solely taking into account specimens from the larger size class (> 50 mm), the average biomass increased from 129 g m^{-2} to 2298 ± 664 g m^{-2}.

Dissolved inorganic nitrogen average concentration (DIN = NO_x-N + NH_4-N) in the head tank was 8.9 ± 1.3 mg L^{-1} (maximum 10.0 mg L^{-1} and a minimum 6.8 mg L^{-1}). The average concentration of DIN in the outlet of the aquaponics tanks with halophytes preceded by sand filter tanks without polychaetes was 3.1 ± 2.6 mg L^{-1} (n=3), representing an average decrease of 65%. This value increased to 67% for sand filter tanks stocked with polychaetes (n=3). DIN removal efficiency decreased to 57% when neither polychaetes or halophytes were present in the tanks. Despite the differences in

DIN removal efficiency between experimental conditions: there was no interaction between the presence of polychaetes and halophytes; DIN concentrations within each experimental condition exhibited a high variability; and concentrations in the aquaponics tanks with and without halophytes did not differ significantly ($H_{3,60}$ =1,887; p =0,596).

Figure 2.3 shows a schematic representation of the experimental set-up with the average percentage of OM, the average concentrations of DIN and DIP in the different steps of the IMTA, and the mass balance of DIN (average percentage of removal efficiency).

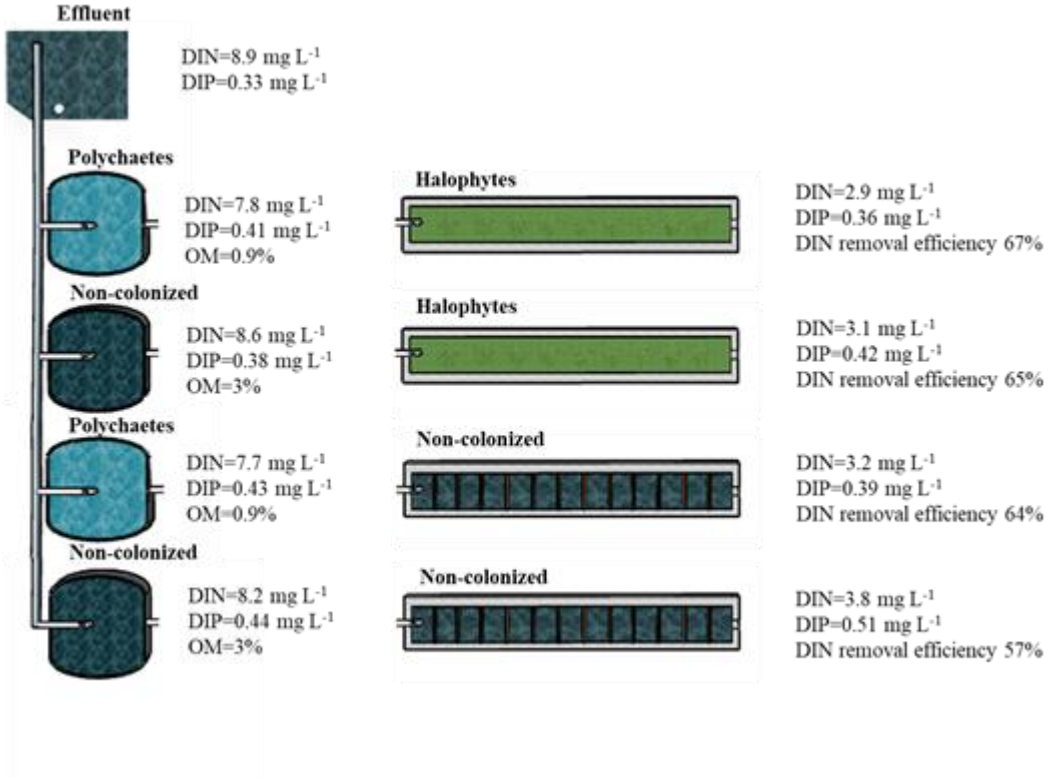


Figure 2.3 - Schematic representation of the experimental set-up with the average percentage of organic matter (OM), the average concentrations of dissolved inorganic Nitrogen (DIN) and dissolved inorganic Phosphorous (DIP) along the different compartments of the IMTA system and the mass balance of DIN (average percentage of removal efficiency).

Dissolved inorganic phosphorus average concentration (DIP) in the head tank was 0.32 ± 0.11 mg L^{-1} (maximum 0.50 mg L^{-1} and minimum 0.21 mg L^{-1}). The average concentration in the outlet of aquaponics tanks with halophytes preceded by sand filter tanks without polychaetes was 0.42 ± 0.13 mg L^{-1} (n=3), representing an increase in the concentration of DIP by 27%. However, the average concentration of DIP increased in sand filter tanks with or without polychaetes, being higher in the presence of polychaetes

(0.41 ± 0.07 mg L^{-1}). The highest average increase in DIP was recorded when neither polychaetes (0.44 ± 0.25 mg L^{-1}) or halophytes (0.51 ± 0.40 mg L^{-1}) where present, while the lowest values were those recorded when polychaete assisted sand filters where employed (0.42 ± 0.13 mg L^{-1}) followed by aquaponics tanks with halophytes (0.36 ± 0.10 mg L^{-1}); this last combination accounted to an average increase in the concentration of DIP by 55% and 9%, respectively (n=3). As for DIN, these results reveal a high variability, with no significant differences being recorded for DIP among the different experimental conditions tested ($H_{3,60}$=2,488; p =0,478).At the end of the experimental period halophytes biomass increased from 1.4 ± 0.7 kg m^{-2} (initial wet weight) to 18.6 ± 4.0 kg m^{-2} (after five months), with 76% corresponding to stems and leaves biomass (14.2 ± 3.1 kg m^{-2}) and 24% to roots biomass (4.5 ± 1.4 m^{-2}). This increase in halophytes biomass (in dry weight) can be converted into C, N and P units. In dry weight (dwt) *H. portulacoides* biomass increased from 0.4 kg m^{-2} to 5.6 kg m^{-2}, which gives an approximate increase of 5.2 kg dwt m^{-2} during the five months of the experiment. Taking into account C, N and P values specifically for *H. portulacoides*: C=34% and C=39%; N=3.7% and N=2.3%; P=0.18% ±0.07 and P=0.35 ±0.08% (for above and belowground biomass, respectively) (Sousa et al., 2010). This increase in biomass represents an approximate incorporation of ≈1.3 kg m^{-2} carbon (C), ≈15 g m^{-2} nitrogen (N) and ≈8 g m^{-2} phosphorus (P) in the aerial part, and an approximate incorporation of ≈0.5 kg m^{-2} carbon (C), ≈3 g m^{-2} nitrogen (N) and ≈2 g m^{-2} phosphorus (P) in the roots system, during the five months of the experiment.

2.4 Discussion

The integration of additional extractive species from different trophic levels, in marine and brackish aquaculture systems is summarized in Table 2.1. The examples provided clearly show how IMTA enables the transformation of organic rich wastes produced by one species into a food source to another; when extractive species are produced in an integrated way with the main species being targeted by aquaculture they are able to significantly reduce the percentage of OM in the effluents generated by such operations (between 20% and 56%) (Lefebvre et al., 2000, Palmer, 2010, Fang et al., 2017). A similar trend is recorded for the percentage of DIN (between 47% and 98%) (e.g. Lymbery et al. 2006, Graber and Junge, 2009, Webb et al., 2012, 2013) and the percentage of DIP (between neg< and 88%) (Lymbery et al. 2006, Graber and Junge,

2009, Webb et al., 2012). In the IMTA set up used in the present study polychaete-assisted sand filters reduced the percentage of OM in 70%, which is within the above mentioned range. In the combined set up employing polychaete-assisted sand filters and aquaponics tanks with halophytes the percentage of DIN was reduced in 67%, which is also within the above mentioned range.

Table 2.1 - Summary table with relevant examples of IMTA combining fed aquaculture species with other extractive cultured species (only studies presenting relevant data on removal efficiencies and published in international peer-reviewed publications (in English) are summarized, being listed following chronological order of publication).

Fed aquaculture species (system)	Country	Extractive cultured species		Removal efficiency in %			Reference
		POM	DOM, DIN, DIP	MO	P	N	
Dicentrarchus labrax (European sea bass) (land-based fish-farm)	France	*Crassostrea gigas* (bivalve) (chambers; indoor experiment)	-	56%	-	-	Lefebvre et al., 2000
Litopenaeus vannamei (Pacific white shrimp) (outdoor RAS)	Taiwan	-	*Phragmites australis* (macrophyte) (free water surface (FWS) and subsurface flow (SF) constructed wetlands)	-	5.40% (PO4–P) 80 days	57% TAN 80 days	Lin et al., 2003
Penaeus monodon (giant tiger shrimp) (earth ponds)	Australia	*Perinereis nuntia and Perinereis helleri* (polychaetes) (sand filters)	*Perinereis nuntia and Perinereis helleri* (polychaetes) (sand filters)	from 4–5% to 1–2% > 50%	Non-significant	Non-significant	Palmer, 2010
Shrimp, sole and turbot (Intensive marine farm)	UK	-	*Salicornia europaea* (halophyte) (wetland filter beds)		70% DIP 88 days	98% DIN 88 days	Webb et al., 2012
Litopenaeus vannamei (Pacific white shrimp) (land based RAS)	UK	-	*Salicornia europaea* (halophyte) (constructed wetland)	-	47%	67%	Webb et al., 2013
Raft bivalve-macroalgae polyculture (experimental set up with bivalve biodeposits and macroalgae detritus)*	China	*Apostichopus japonicus* (sea cucumber) (deposit-feeding)	-	54% increase in biomass (from ≈35 g to 65 g) at 15 ºC. From the OM pool available for feeding 5% of the C pool and 14% of the N pool were allocated to growth (28 days)			Yuan et al., 2013
Paralichthys olivaceus (olive flounder) (fish farm cages)	*China*	*Perinereis aibuhitensis* (polychaete) (fiberglass tank; indoor experiment)	*Perinereis aibuhitensis* (polychaete) (fiberglass tank; indoor experiment)	20-50%	-	30-65%	Fang et al., 2017
Solea senegalensis (Senegalese sole) (super intensive land-based RAS)	Portugal	*Hediste diversicolor* (polychaete) (sand filters)	*Halimione portulacoides* (halophyte) (aquaponics)	70%	-	67% 150 days	Present study

The average DIN concentrations outflowing from the system into the infiltration basin (≈3-4 mg L^{-1} DIN) are also within the concentrations recorded by Webb et al., (2013) using *Salicornia europaea* to treat a shrimp farm effluent from a land-based RAS system (≈2-5 mg L^{-1} DIN). The phosphorus mass balance evidences the mineralization of OM (Coelho et al., 2004, Lillebø et al., 2004), probably enhanced by the bioturbation and bio-irrigation activity of polychaetes present in the sand filters, which is able to promote an increase in the concentration of the dissolved form (DIP) (e.g. Mortimer et al., 1999). The fact that total N and total P was not quantified limits the complete mass balance calculations, namely regarding the P cycle. However, the increase in DIP does not mean an increase in the pool of P, but the mineralization of OM and release of DIP to the water column. The increase in the percentage of DIP, due to mineralization of OM, has also been recorded in woodchip bioreactors systems and in activated sludge reactors with citrate-rich liquid wastes, both applied for the treatment of RAS effluents (Fox et al., 2015, Christianson et al., 2016), as well as in other IMTA systems (Graber and Junge, 2009). Nevertheless, in the IMTA set up tested in the present work the average DIP concentration outflowing from the system into the infiltration basin (≈0.4-0.5 mg L^{-1} DIP) is one order of magnitude lower than those recorded by Webb et al., (2013) using *Salicornia europaea* to treat a shrimp farm effluent from a land-based RAS (1.1-2.8 mg L^{-1} DIP).

Overall, the present results show the potential of the two extractive species employed in our study (*H. diversicolor* and *H. portulacoides*) for biomitigation of a super-intensive marine fish farm effluent. Besides mitigating the concentration of DIN in the effluent generated by the RAS, C, N and P present in the DOM and POM fractions, as well as dissolved in the inorganic pool, are also incorporated into polychaetes and halophytes biomass. Furthermore, both cultured species already have well-established value chains and markets. Wild ragworms collected in Aveiro coastal lagoon are sold as sports fishing bait at €2 per pack of ≈ 60 adults; at the internet site http://www.baitsrus.com/ these polychaetes are sold at £8.00 ≈ €9 for ≈220 g (1/2 pound (lb)); at the internet site http://www.finefoodspecialist.co.uk/ sea purslane is sold at £18.50 ≈ €21per 500 g; at the internet site https://www.farmdrop.com/ sea purslane is sold at £1.40 ≈ €2 for 50 g (1 Punnet) (Note: internet prices and monetary conversions refer to November 2016). Table 2.2 summarizes the potential market value of *H. diversicolor* and *H. portulacoides* per unit area of production (m^2) using the IMTA system tested in the present study.

Table 2.2 - Summary of the potential market value of the extractive cultured species per unit area of production (m^2) in the IMTA system employed in the present work.

***Hediste diversicolor* (Sand filters)**			***Halimione portulacoides* (Aquaponics)**		
Initial set up	**After 5 months**	**Potential market value**	**Initial set up**	**After 5 months**	**Potential market value**
≈ 400 ind. m^{-2} 129 g	7000 ind. m^{-2} (>5mm) 2298 g	≈ €90 per m^{-2} production (bait)	1.4 kg dwt m^{-2}	18.6 kg wwt m^{-2} (76% = stems & leaves) 14.2 kg wwt m^2	≈ €280 per m^{-2} production (human consumption)
					

This combined production under IMTA conditions falls within the category of 'environmentally friendly' production methods with new added commercial value and with little or no additional costs. Through the implementation of a strategy based on the concept "less technology, more biology", the IMTA system employed clearly contributed to the decrease of OM present in the effluents generated by the land-based RAS using autochthonous species from the production site. The system employed also enabled to reduce the pool of dissolved inorganic nutrients, particularly DIN present in the effluent through its incorporation into halophytes biomass. This result is particularly relevant, as nitrogen is usually the limiting nutrient in marine and coastal ecosystems and therefore a nutrient of concern in case of eutrophication (Lillebø et al., 2007). It is worth highlighting that *H. portulacoides* is an evergreen halophyte and that new grafts can be regularly added to the aquaponics system to optimize production and the uptake of nutrients present

in the effluent, thus compensating for the harvest of tender shoots for biomass valorization (e.g. human consumption, pharmaceuticals and technical implementations) (Ksouri et al., 2011). The principles of 'Blue Economy' aim for the optimization of the benefits received from the sustainable development of marine environments, including aquaculture, which is one of the economic activities considered in the Blue Growth agenda (UNEP 2015). Overall, the IMTA approach employed in our work is in line with these principles, as it allows to transfer the concept of a 'Green Economy' into the 'Blue World', thus actively contributing to the "Greening of the Blue Revolution".

2.5 Conclusions

The integrated use of polychaete-assisted sand filters and halophyte aquaponics for the biomitigation of a super-intensive marine fish farm effluent revealed a considerable potential for the mitigation of DIN (67% decrease efficiency). Furthermore, both extractive cultured species employed are 'environmentally friendly' solutions that already have well-established value chains and whose production value may reach up to ≈ €90 per m^{-2} for *H. diversicolor* (if sold as bait) and ≈ €280 per m^{-2} for *H. portulacoides* (if sold for human consumption) with little additional production costs. In this way, the IMTA concept presented and validated in the present study clearly falls into the 'Blue Growth' and the circular economy agendas and contributes to the implementation of more sustainable practices in marine aquaculture.

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2.7 Supporting Information

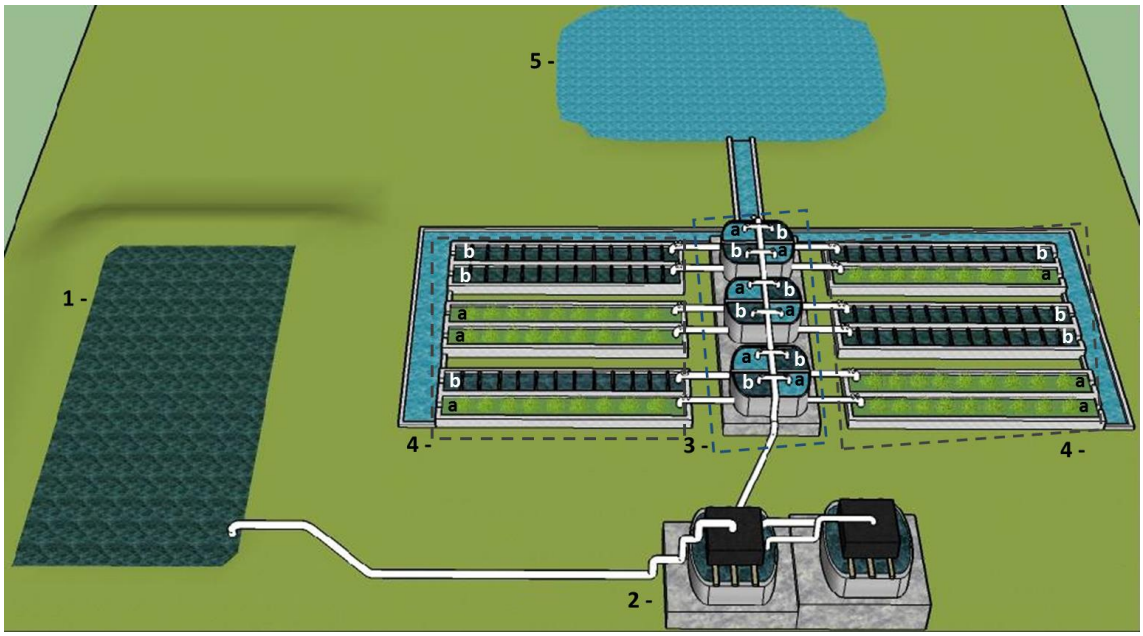

Figure S 2.1 - Top view of IMTA design with indication of all experimental combinations employed using the polychaete *Hediste diversicolor* cultivated in sand filters and the halophyte *Halimione portulacoides* cultivated in aquaponics tanks. 1 – effluent; 2 - head tank; 3 - sand filter tank: 3a – polychaete-assisted sand filter tanks, 3b – sand filter tanks/control; 4 – aquaponic tanks: 4a - aquaponics tank with halophytes, 4b - aquaponics tank without halophytes; 5 – Infiltration basin

Table S 2.1 - Summary table with physicochemical parameters (pH, temperature, dissolved oxygen (DO)), suspended particulate matter (SPM) and organic matter (OM) of polychaete-assisted sand filter tanks and non-colonized sand filter tanks (average values ± standard deviation)

	Polychaete-assisted sand filter tanks (n=6)					**Non-colonized sand filter tanks (n=6)**				
	pH	**Temperature (ºC)**	**DO (mg L^{-1})**	**SPM (mg L^{-1})**	**OM (%)**	**pH**	**Temperature (ºC)**	**DO (mg L^{-1})**	**SPM (mg L^{-1})**	**OM (%)**
Month 1	-	20.5 ± 0.3	9.5 ± 0.3	47.34 ± 5.07	0.1	-	20.8 ± 0.5	9.8 ± 0.4	52.11 ± 10.38	0.1
Month 2	-	21.8 ± 0.2	8.0 ± 0.3	31.22 ± 2.05	-	-	21.7 ± 0.2	9.0 ± 0.7	64.33 ± 65.35	-
Month 3	8.0 ± 0.1	20.3 ± 0.2	8.4 ± 0.2	51.22 ± 5.40	-	8.0 ± 0.2	20.4 ± 0.4	8.6 ± 0.2	65.35 ± 13.07	-
Month 4	7.9 ± 0.2	19.1 ± 0.2	9.2 ± 0.3	45.72 ± 8.84	-	8.0 ± 0.1	19.0 ± 0.2	10.3 ± 0.5	43.28 ± 9.31	-
Month 5	7.8 ± 0.1	17.0 ± 1.3	9.0 ± 0.5-	41.93 ± 3.83	0.9 ± 0.2	7.9 ± 0.1	16.2 ± 0.5	9.6 ± 0.6	49.80 ± 18.19	3.0 ± 0.8

Table S 2.2 - Summary table with the average concentrations of DIN and DIP in the different steps of the IMTA system (average values ± standard deviation).

	Month 1		Month 2		Month 3		Month 4		Month 5	
	DIP (mg L^{-1})	**DIN (mg L^{-1})**	**DIP (mg L^{-1})**	**DIN (mg L^{-1})**	**DIP (mg L^{-1})**	**DIN (mg L^{-1})**	**DIN (mg L^{-1})**	**DIN (mg L^{-1})**	**DIP (mg L^{-1})**	**DIN (mg L^{-1})**
Head tank	0.29	9.51	0.21	9.99	0.37	9.77	0.28	6.77	0.50	8.37
Polychaete-assisted sand filter tanks (n=3)	0.47 ± 0.02	12.46 ± 0.36	0.37 ± 0.01	7.25 ± 0.43	0.47 ± .08	5.85 ± 2.06	0.34 ± 0.02	5.95 ± 0.42	0.40 ± 0.05	7.41 ± 0.95
Aquaponics tanks with halophytes preceded by sand filter tanks with polychaetes (n=3)	0.23 ± 0.06	6.36 ± 2.44	0.34 ± 0.07	0.16 ± 0.26	0.47 ± 0.10	0.87 ± 0.49	0.34 ± 0.02	1.96 ± 0.27	0.44 ± 0.02	5.34 ± 0.98
Non-colonized sand filter tanks (n=3)	0.37 ± 0.04	12.67 ± 0.94	0.31 ± 0.05	7.81 ± 0.97	0.45 ± 0.09	8.69 ± 0.37	0.32 ± 0.02	6.11 ± 1.66	0.44 ± 0.05	7.49 ± 0.33
Aquaponics tanks with halophytes preceded by sand filter tanks without polychaetes (n=3)	0.25 ± 0.07	6.54 ± 3.55	0.48 ± 0.19	0.42 ± 0.07	0.51 ± 0.06	2.88 ± 1.26	0.38 ± 0.02	1.49 ± 0.58	0.49 ± 0.00	4.08 ± 0.35
Non-colonized sand filter tanks (n=3)	0.38 ± 0.04	12.35 ± 0.34	0.37 ± 0.08	8.42 ± 0.72	0.73 ± 0.50	6.63 ± 2.44	0.29 ± 0.02	5.29 ± 0.54	0.41 ± 0.04	8.19 ± 0.51
Aquaponics tanks without halophytes preceded by sand filter tanks without polychaetes (control) (n=3)	0.18 ± 0.08	6.36 ± 2.59	0.62 ± 0.39	2.01 ± 0.72	0.96 ± 0.67	3.70 ± 2.95	0.40 ± 0.10	2.06 ± 0.66	0.40 ± 0.02	4.89 ± 0.97

Chapter 3

Adding value to ragworms (*Hediste diversicolor*) through the bioremediation of a super-intensive marine fish farm

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3. Adding value to ragworms *(Hediste diversicolor)* through the bioremediation of a super-intensive marine fish farm

Abstract

The aim of this study was to evaluate the potential added value of *Hediste diversicolor*, cultured for 5 months in sand bed tanks supplied with effluent water from a super-intensive marine fish farm, by comparing their fatty acid (FA) profile with that of wild specimens. The polychaetes showed an approximately 35-fold increase in biomass during the experimental period and their FA profile was significantly different from that of wild specimens. In cultivated specimens, the most abundant FA class was that of highly unsaturated FA (HUFA), with eicosapentaenoic acid (EPA, 20:5*n*-3) being the best represented. Similar percentage (SIMPER) analysis showed an average 20.2% dissimilarity between the FA profile of wild and cultivated specimens, supporting the view that the culture system employed enables the recovery of high value nutrients (e.g. EPA and docosahexaenoic acid [DHA, 22:6*n*-3]) from fish feeds into the tissues of *H. diversicolor* that would otherwise be lost from the production environment. While the nutritional value of wild ragworms is well established in marine aquaculture (namely for broodstock maturation diets), the higher level of DHA displayed by the specimens produced under the proposed culture system may grant them a premium market value.

Keywords: Integrated multi-trophic aquaculture (IMTA); Polychaete-assisted sand filters; Fatty acids.

Figure 3.1 - Ragworms (*Hediste diversicolor*) on sand filters of a super-intensive brackish-water fish farm, cultured using the farm's organic-rich effluent and displaying a greater content of docosahexaenoic acid (DHA, 22:6*n*-3) than wild conspecifics.

3.1 Introduction

Recirculating aquaculture systems (RAS) are currently considered one of the paradigms of the Blue Revolution, as they allow people to 'grow fish anywhere' (Martins et al. 2010). The principles behind RAS promote the treatment and reuse of culture water, with 10% or less of the total water volumes having to be replaced per day (Hutchinson et al. 2004, van Rijn et al. 2006). However, one of the constraints impairing the expansion of these production systems (in a closed or semi-closed operation) is the challenge associated with the load of organic-rich suspended particulate matter (POM), dissolved organic matter (DOM) and nutrients in dissolved inorganic form (nitrogen, N, and phosphorus, P) present in its effluent (Schneider et al. 2005, Marques et al. 2017).

A number of integrated multi-trophic aquaculture (IMTA) systems have been developed in order to reduce the nutrient load present in the effluents produced when growing finfish or shrimp. In other words, IMTA combines the integrated culture of fed target species (e.g. finfish/shrimp) with that of extractive species that use particulate or dissolved organic matter (e.g. shellfish/herbivorous fish) or dissolved inorganic nutrients (e.g. seaweed/halophytes) generated through the excretion products of fed species and uneaten feed (Schneider et al. 2005, Chopin et al. 2008, Alexander et al. 2015). Overall, this environmentally friendly approach aims to address the impacts commonly associated with conventional aquaculture, such as nutrient loading and sedimentation (Schneider et al. 2005, Barrington et al. 2009). In this way, promoting IMTA practices may help to overcome the current bottlenecks faced by enterprises operating RAS, as the nutrient-rich effluents that they generate may be used to culture additional species. While on one side this approach allows the nutrient load issue to be addressed from a biomitigation perspective, through the incorporation of nutrients in extractive species biomass, it also opens up the opportunity of adding new cash-crops to the production model through the valorization of those extractive species (Chopin et al. 2008, Alexander et al. 2015).

The polychaete *Hediste diversicolor* (O.F. Müller, 1776), popularly known as ragworm, is a candidate species for land-based IMTA systems, as it can efficiently recycle particulate organic nutrients present in fish farm effluents (Scaps 2002, Bischoff et al. 2009, Santos et

al. 2016). This polychaete is a burrowing species that inhabits the soft bottoms of shallow marine and brackish waters environments, generally in sediments with high organic contents, in the North temperate zone of European and North American Atlantic coasts (Scaps 2002, Lillebø et al. 2012). *H. diversicolor* exhibits a 'bentho-pelagic life cycle' (Scaps 2002, Nesto et al. 2012). It is one of the rare Nereid species which remain atokous throughout their lifespan, contrasting with some other Nereids which undergo a metamorphosis to a typical epitokous heteronereid form (Scaps 2002, Breton et al. 2003, Aberson et al. 2011). During reproduction, the female incubates the eggs for a period of 10 to 14 d inside the gallery and dies soon afterwards. In this way, the direct or brooded larval development permits a greater flexibility in the level of development of the released offspring (Aberson et al. 2011). The feeding modes of this polychaete are diversified, ranging from surface deposit to suspension feeding, and they are also able to scavenge and actively prey on other organisms (including conspecifics) (Luis & Passos 1995, Fidalgo e Costa et al. 2000, Bischoff et al. 2009). The interactions with its environment show an efficient adaptation to a variation of environmental parameters such as salinity, temperature and dissolved oxygen (Smith 1964, Fritzsche & von Oertzen 1995, Murray et al. 2017). Polychaetes also play an important ecological role in the marine environment, as they are major contributors to the resuspension of organic matter via bioturbation (Würzberg et al. 2011), a feature of great relevance for their potential use in IMTA. Indeed, some studies have already highlighted how polychaetes can be successfully employed in the bioremediation of aquaculture effluents under an IMTA framework (Palmer 2010, Fang et al. 2017). The nutritional value of ragworms is well established in marine aquaculture, with these organisms being a highly valued item in marine finfish and shrimp maturation diets (Olive 1999, Techaprempreecha et al. 2011, Santos et al. 2016). One of the main reasons for their popularity is their fatty acid (FA) profile, namely the levels they display of important polyunsaturated FA (PUFA) (Brown et al. 2011, Santos et al. 2016). Some polychaete species, including *H. diversicolor*, are able to biosynthesize PUFA, such as 20:5*n*-3 eicosapentaenoic acid (EPA) and 22:6*n*-3 docosahexaenoic acid (DHA), which are known to be essential for marine finfish and shrimp (Olive 1999, Fidalgo e Costa et al. 2000).

In order to evaluate the potential valorization of *H. diversicolor* cultured in sand bed tanks supplied with effluent water from a super-intensive marine fish farm, their fatty acid

profile was determined and compared with that of wild specimens. The experimental procedure aimed to test (1) whether ragworm FA profiles depend on food source, by comparing cultured and wild specimens, and (2) whether the FA profiles of ragworms cultured in sand bed tanks supplied with RAS effluent are size-dependent, by comparing small, medium and large ragworms.

3.2 Material and methods

3.2.1 Experimental set-up

Effluent originating from a super intensive RAS system farming Senegalese sole *Solea senegalensis* was pumped from a settling basin to a bio-block tower system, in order to allow the effluent water to trickle and increase its oxygen levels before reaching the header tank reservoir. In this header tank, the effluent was strongly aerated and set to flow in parallel into 6 experimental sand bed tanks. Each of these tanks had an approximate volume of 1 m^3 and a surface area of 1 m^2. The bottom of each tank was covered by 200 mm of sand (1 to 2 mm grain size substratum) beneath which a draining pipe allowed the effluent water to percolate through the sand bed. Each tank was equipped with 2 outlets, one that regulated the water level inside the tank and allowed the water to drain and another one that was set to prevent the tank from overflowing in case the sand bed became clogged and impaired water percolation. Sand bed tanks were placed in parallel and supplied with aerated RAS effluent water by gravity at a flow of 180 l h^{-1}. All tanks were equipped with a 0.8 m diameter ring of aeration hose supplying compressed air (see Marques et al. 2017). Three of the 6 experimental sand bed tanks were stocked with wild polychaetes (WP), while the remaining 3 were set as controls. The experiment was run for 5 months (from May to October) and no additional feed was provided to the polychaetes during this period to supplement the organic-rich effluent containing uneaten/undigested feed and fish feces. During the experimental period, temperature, salinity, pH and dissolved oxygen concentration of the effluent water being supplied to the tanks was monitored twice a month using a WTW Cond 3110/SET 1 equipped with TetraCon® 325,

a WTW pH 330i/SET equipped with SenTix® 41, and a WTW Oxi 3210/SET 2 equipped with CellOx® 325-3, respectively.

3.2.2 Polychaete stocking and sampling

Wild specimens of *Hediste diversicolor* (average total length between 80 and 100 mm, the commercial size of this species when traded as bait for sports fishing) were collected in the Ria de Aveiro coastal lagoon (Portugal 40° 38' 04.8" N, 8° 39' 52.4" W) by local fishermen, and 200 individuals (with a combined weight of 130 g) were stocked in each of the 3 sand bed tanks randomly selected to act as polychaete-assisted sand filters. At 5 months post-stocking, polychaetes were sampled using a hand corer (110 mm diameter and 150 mm long), with 3 cores being taken from each colonized sand bed tank. Polychaete specimens were sorted in situ and transported to the laboratory in sterilized sand and clean seawater. At the laboratory, all specimens were left to depurate overnight in pre-combusted sand and artificial seawater prepared to match salinity in situ (20). Subsequently, all sampled specimens were separated into 3 pre-established size classes (small <30 mm, medium 30 to 50 mm and large >50 mm). At the beginning of the experiment, triplicate samples of WP (all from the large class) that were used to stock the tanks, were left to depurate overnight under identical conditions as those previously described. After depuration, wild polychaetes and those originating from the sand bed tanks were freeze-dried and stored at -80°C for subsequent FA analysis.

3.2.3 Sampling of potential nutrient sources of POM for FA analysis

To perform the FA characterization of all potential nutrient sources available to the ragworms stocked in the sand bed tanks, triplicate samples of the 2 fish feed types (FEED A and B) used during the grow-out of *S. senegalensis* in the RAS system, as well as uneaten/undigested feed and fish feces accumulated in the cyclone filters (POM), were collected, freeze dried and stored at -80°C for posterior FA analysis. The top 10 mm of the sand bed surface of each tank stocked with ragworms, all organic-rich settled particles (OM), were also collected in triplicate from each tank and were also processed as described above for FA analysis.

3.2.4 FA extraction and analysis

The derivation of FAs for gas chromatography (GC) analysis was performed following the methodology described by Aued-Pimentel et al. (2004), adjusting the weight, as this method has the advantage of being performed at room temperature and thus reducing the risks of FA decomposition. All freeze-dried samples were powdered and homogenized, weighed accurately in a Sovirel Pyrex glass tube (40 mg of ragworm biomass, 20 mg of FEED, ~40 mg of POM and 150 mg of OM) and dissolved in 1 ml of the internal standard solution of a methyl ester fatty acid C21:0 in n-hexane (0.35 g L^{-1}). In the same tube, 0.2 ml of a methalonic KOH solution (2 mol L^{-1}) was added, and the tube was sealed and mixed vigorously in a vortex shaker for 30 s. Following this procedure, 2 ml of a saturated NaCl solution was added to the tube, and the mixture was centrifuged for 5 min at 1409 × g. The separated organic phase (1 ml) was transferred into another tube and the excess solvent was removed under vacuum. The oil obtained was dissolved in n-hexane (200 µl) and analyzed by gas chromatography with a flame ionization detector (GC-FID), using a Perkin Elmer 400 instrument (PerkinElmer). The detector and injector were kept at 250°C, with hydrogen as carrier gas. FAs were separated in a fused-silica capillary column, DB-FFAP (30 m length, 0.32 mm internal diameter, 0.25 µm film thickness, J & W Scientifics) with the following temperature program: 50°C for 3 min, 40°C min^{-1} to 160°C, 2°C min^{-1} to 210°C, 20°C min^{-1} to 250°C (for 1 min). The identification of FAs was done by matching with a previously injected standards mixture (Supelco® 37 component FAME mix, Sigma-Aldrich). The FA content (µg mg^{-1} dry weight, DW) in the samples analyzed was calculated considering the relationship between mass, the area of FAs and the internal standard (C21:0). PUFA are defined as all FA with ≥2 double bonds; in the present study, highly unsaturated fatty acids (HUFA, FAs with ≥4 double bonds) are considered separately.

3.2.5 Statistical analysis

Statistical analysis was performed using PRIMER v6 with the PERMANOVA+ add-on. A resemblance matrix using the content (µg mg^{-1} DW) of each FA in each sample was prepared using the Bray-Curtis similarity coefficient, after performing a log(x + 1)

transformation to emphasize compositional rather than quantitative differences (Anderson 2008). Hypotheses were tested by performing 2 independent 1-way analyses of similarities (ANOSIM). To assess the differences between FA profiles of cultured polychaetes versus WP and those of small, medium and large cultured polychaetes (SPC, MPC and LPC, respectively) in sand bed tanks, a global R statistic was calculated where values close to 1 indicate maximum differences between groups and values near 0 indicate a complete group overlay. Similarity percentage (SIMPER) analysis was also performed to evaluate the percentage that each FA contributed to the dissimilarity recorded between samples, with those contributing 50% of cumulative dissimilarities being highlighted. Hierarchical cluster analysis was performed to group the samples according to their similarity. A dendrogram was used to highlight the hierarchical similarity among the samples. Similarity among the samples was calculated using the Bray-Curtis similarity measure and the group average algorithm was used to group the samples successively in a hierarchical way. A canonical analysis of principal coordinates (CAP) was performed to evaluate the strongest correlation of LPC in the predefined groups (OM, POM, FEED A and B). For a detailed description of all the statistical analysis described above please refer to Clarke & Gorley (2006).

3.3 Results

3.3.1 Biomass production of ragworms

During the 5 months experimental period, water parameters (average ± standard deviation, SD) within the sand bed tanks remained stable (see Table S 3.1 in the supporting information), with an average water temperature of 19.6 ± 1.3°C, salinity 21.2 ± 0.2, pH 7.8 ± 0.2 and dissolved oxygen 8.4 ± 0.7 mg l−1. The final average (±SD) weight of cultured polychaetes biomass was 2622 ± 869 g, corresponding to 104 ± 68, 226 ± 137 and 2292 ± 664 g for SPC, MPC and LPC, respectively. During this period, polychaetes density increased from 200 individuals (ind.) m^{-2} to up to 7094 ind. m^{-2}, which represented an approximately35-fold increase in density, solely considering LPC (initial biomass of 130 g increasing to 2292 g 5 mo later) and approximately 18-fold considering the whole biomass of cultured polychaetes (initial biomass of 130 g increasing to 2622 g 5 mo later).

On average, the total numbers of polychaetes per tank were 9608 ± 5922, 3374 ± 1464 and 7094 ± 1375 ind. M^{-2}, for SPC, MPC and LPC, respectively. The average number of LPC recorded represented approximately 100 bait packages similar to those originally introduced into the system (each pack of bait holds ~70 individuals).

3.3.2 FA profile analysis

The FA profiles of WP, SPC, MPC and LPC (these displaying a similar size to that of wild specimens), POM, OM, and FEED A and B are summarized in Table 3.1. Considering FA classes, WP and LPC displayed similar profiles; however, the ANOSIM test revealed the existence of significant differences (R = 1, p = 0.002) between the FA profiles of WP and LPC. HUFA was the most abundant FA class (8.78 ± 0.70 and 14.23 ± 1.08 μg mg^{-1} DW for WP and LPC, respectively), with EPA (20:5*n*-3) being the best represented FA (5.51 ± 0.29 and 8.34 ± 0.36 μg mg^{-1} DW for WP and LPC, respectively). PUFA averaged 2.34 ± 0.38 and 3.99 ± 0.46 μg mg^{-1} DW for WP and LPC, respectively, while monounsaturated fatty acids (MUFA) averaged 6.69 ± 1.28 and 10.05 ± 1.08 μg mg^{-1} DW for WP and LPC, respectively. Concerning saturated fatty acids (SFA), WP presented 6.52 ± 1.62 μg mg^{-1} DW, while LPC displayed 8.99 ± 1.16 μg mg^{-1} DW, with the most representative SFA being palmitic acid (16:0) (4.22 ± 1.1 and 5.63 ± 0.85 μg mg^{-1} DW for WP and LPC, respectively). The identification and quantification of the FA profiles of FEED A and B, POM and OM revealed that the most representative FA in each of these samples was: (MUFA) vaccenic acid (18:1*n*-7) (25.13 ± 3.17 μg mg^{-1} DW) for FEED A; (SFA) palmitic acid (16:0) for FEED B (23.44 ± 1.22 μg mg^{-1} DW) and POM (8.16 ± 1.16 μg mg^{-1} DW), and (PUFA) eicosadienoic acid (C20:2*n*-6) (2.29 ± 0.20 μg mg^{-1} DW) for OM. SIMPER analysis showed that the FA profiles of WP and LPC displayed an average dissimilarity of 20.2%, with more than 50% of that dissimilarity being explained by the following FAs: 18:3*n*-3 (*α*-linolenic acid, ALA), 22:4*n*-6 (docosatetraenoic acid, DTA), 18:2*n*-6 (linoleic acid), 22:6*n*-3 (DHA), 18:1*n*-9 (oleic acid), 18:3*n*-6 (*γ*-linolenic acid, GLA) and 22:1*n*-9 (erucic acid) (Table 3.2). The ANOSIM test revealed the existence of significant differences between the FA profiles of SPC and LPC (R = 0.474, *p* = 0.011), and MPC and LPC (R = 0.319, *p* = 0.017).

Table 3.1 - Fatty acid (FA) profiles (µg g–1 DW) of wild (WP), and small, medium and large *Hediste diversicolor* cultured in the sand bed tanks (SPC, MPC and LPC, respectively), organic matter (OM), particulate organic matter (POM) and fish feed (FEED A and FEED B). Values are average of 9, 6 or 3 replicates± standard deviation. ND: fatty acid not detected. SFA: saturated fatty acids (14:0; 15:0; 16:0; 17:0; 18:0; 20:0); MUFA: monounsaturated fatty acids (16:1*n*-7; 16:1*n*-9; 18:1*n*-9; 18:1*n*-7; 18:1*n*-5; 20:1*n*-9; 22:1*n*-9); PUFA: polyunsaturated fatty acids (16:2*n*-6; 18:2*n*-6; 18:2*n*-3; 18:3*n*-6; 18:3*n*-3; 20:2*n*-6; 20:2*n*-9; 20:3*n*-6); HUFA: highly unsaturated fatty acids (20:4*n*-6; 20:5*n*-3; 22:4*n*-6; 22:5*n*-3; 22:6*n*-3). PUFA are defined as all FA with ≥2 double bonds; in the present study, HUFA (FA with ≥4 double bonds) are not considered within ≥PUFA. Others refers to the FAs 14:0 (iso), 15:0 (iso) and 16:0 (iso)

FA	WP (n = 9)	SPC (n = 9)	MPC (n = 9)	LPC (n = 9)	OM (n = 9)	POM (n = 6)	FEED A (n = 3)	FEED B (n = 3)
14:0	0.10 ± 0.03	0.22 ± 0.03	0.25 ± 0.04	0.33 ± 0.05	0.36 ± 0.03	2.39 ± 0.37	4.09 ± 0.44	8.18 ± 0.63
15:0	0.27 ± 0.05	0.23 ± 0.03	0.21 ± 0.03	0.28 ± 0.03	0.06 ± 0.01	0.22 ± 0.03	ND	ND
16:0	4.22 ± 1.10	3.53 ± 0.28	3.66 ± 0.57	5.63 ± 0.85	1.31 ± 0.22	8.16 ± 1.16	13.53 ± 1.33	23.44 ± 1.22
17:0	0.27 ± 0.06	0.34 ± 0.03	0.34 ± 0.02	0.46 ± 0.07	ND	0.35 ± 0.09	0.60 ± 0.06	1.48 ± 0.21
18:0	1.65 ± 0.37	1.56 ± 0.19	1.64 ± 0.13	2.28 ± 0.16	0.34 ± 0.06	1.87 ± 0.23	0.61 ± 0.03	3.39 ± 1.21
∑SFA	6.52 ± 1.62	5.89 ± 0.55	6.10 ± 0.79	8.99 ± 1.16	2.14 ± 0.36	13.16 ± 1.89	18.83 ± 1.86	36.50 ± 3.27
16:1*n*-7	0.27 ± 0.06	0.57 ± 0.08	0.63 ± 0.07	0.90 ± 0.13	0.14 ± 0.20	2.38 ± 0.40	ND	ND
16:1*n*-9	ND	ND	ND	ND	0.86 ± 0.16	ND	4.66 ± 0.44	9.09 ± 0.76
18:1*n*-9	1.96 ± 0.43	1.83 ± 0.22	1.96 ± 0.06	2.39 ± 0.19	0.48 ± 0.08	7.77 ± 1.95	2.97 ± 0.31	9.64 ± 5.07
18:1*n*-7	0.31 ± 0.05	0.77 ± 0.06	0.89 ± 0.20	1.39 ± 0.20	0.58 ± 0.10	1.53 ± 0.63	25.13 ± 3.17	5.98 ± 4.13
18:1*n*-5	1.74 ± 0.44	1.49 ± 0.20	1.43 ± 0.11	1.87 ± 0.22	ND	ND	ND	ND
20:1*n*-9	1.86 ± 0.22	2.14 ± 0.24	2.27 ± 0.17	2.80 ± 0.20	0.14 ± 0.18	0.86 ± 0.17	2.71 ± 0.25	1.99 ± 0.10
22:1*n*-9	0.54 ± 0.08	0.48 ± 0.09	1.00 ± 0.12	0.71 ± 0.11	0.05 ± 0.02	1.64 ± 0.60	2.00 ± 0.21	2.11 ± 0.19
∑MUFA	6.69 ± 1.28	7.28 ± 0.90	8.18 ± 0.73	10.05 ± 1.08	2.26 ± 0.74	14.17 ± 3.74	37.47 ± 4.38	28.81 ± 10.25
18:2*n*-6	0.22 ± 0.03	0.56 ± 0.08	0.72 ± 0.16	1.13 ± 0.09	0.16 ± 0.03	4.31 ± 1.10	9.68 ± 1.13	5.31 ± 0.40
18:3*n*-6	1.12 ± 0.15	0.14 ± 0.05	0.17 ± 0.01	0.32 ± 0.04	0.09 ± 0.02	0.65 ± 0.20	2.75 ± 0.26	1.62 ± 0.12
18:3*n*-3	0.49 ± 0.10	ND	ND	ND	ND	0.29 ± 0.10	ND	ND
20:2*n*-6	0.25 ± 0.05	0.49 ± 0.05	0.56 ± 0.09	1.08 ± 0.24	2.29 ± 0.20	ND	0.18 ± 0.31	0.11 ± 0.08
∑PUFA	2.34 ± 0.38	2.08 ± 0.33	1.89 ± 0.33	3.99 ± 0.46	2.55 ± 0.25	5.26 ± 1.40	14.53 ± 1.86	10.79 ± 1.16
20:4*n*-6 (AA)	0.84 ± 0.10	0.99 ± 0.33	1.25 ± 0.15	1.61 ± 0.17	0.12 ± 0.02	0.15 ± 0.04	0.74 ± 0.13	1.10 ± 0.04
20:5*n*-3 (EPA)	5.51 ± 0.29	5.65 ± 0.62	6.43 ± 0.51	8.34 ± 0.36	0.36 ± 0.12	0.11 ± 0.04	7.06 ± 0.71	16.20 ± 2.15
22:4*n*-6	0.39 ± 0.19	1.13 ± 0.26	1.24 ± 0.04	1.75 ± 0.23	ND	0.31 ± 0.07	0.17 ± 0.02	0.31 ± 0.02
22:5*n*-3	2.03 ± 0.12	1.25 ± 0.16	1.37 ± 0.17	1.72 ± 0.18	0.04 ± 0.01	0.22 ± 0.09	1.25 ± 0.16	1.32 ± 0.21
22:6*n*-3 (DHA)	ND	0.60 ± 0.19	0.49 ± 0.07	0.82 ± 0.14	0.37 ± 0.06	1.73 ± 0.58	8.25 ± 0.75	10.61 ± 1.29
∑HUFA	8.78 ± 0.70	9.62 ± 1.56	10.79 ± 0.92	14.23 ± 1.08	0.89 ± 0.21	2.52 ± 0.83	17.48 ± 1.77	29.54 ± 3.70
∑Others	0.09 ± 0.00	0.51 ± 0.10	0.43 ± 0.08	0.38 ± 0.08	0.21 ± 0.11	0.22 ± 0.02	ND	ND

Table 3.2 - SIMPER overall average dissimilarities (%) between the mean fatty acid (FA) profiles of wild (WP), and small, medium and large (SPC, MPC and LPC, respectively) *Hediste diversicolor* cultured in the sand bed tanks, and organic matter (OM), particulate organic matter (POM) and fish feed (FEED A and FEED B)

W & LPC			SPC & LPC			MPC & LPC			SPC & MPC		
FA	**Contrib. %**	**Cum. %**	**FA**	**Contrib. %**	**Cum. %**	**FA**	**Contrib. %**	**Cum. %**	**FA**	**Contrib. %**	**Cum. %**
18:3*n*-3	8.98	8.98	18:2*n*-6	9.53	9.53	16:0	8.87	8.87	20:4*n*-6	10.76	10.76
22:4*n*-6	8.54	17.53	22:5*n*-3	8.52	18.05	18:2*n*-6	8.24	17.11	20:5*n*-3	8.22	18.98
18:2*n*-6	8.30	25.83	20:4*n*-6	7.73	25.78	20:5*n*-3	7.67	24.79	16:0	7.31	26.29
22:6*n*-3	8.08	33.91	22:1*n*-9	7.41	33.19	22:1*n*-9	6.79	31.57	22:4*n*-6	6.93	33.23
18:1*n*-9	7.70	41.61	16:0	7.30	40.49	20:2*n*-6	6.76	38.34	22:6*n*-3	6.78	40.01
18:3*n*-6	6.85	48.46	20:2*n*-6	6.58	47.07	18:1*n*-9	6.63	44.97	18:2*n*-6	6.61	46.62
22:1*n*-9	6.51	54.97	18:1*n*-9	6.18	53.25	18:0	5.68	50.65	18:1*n*-9	5.90	52.53

FEED A & LPC			FEED B & LPC			POM & LPC			OM & LPC		
FA	**Contrib. %**	**Cum. %**	**FA**	**Contrib. %**	**Cum. %**	**FA**	**Contrib. %**	**Cum. %**	**FA**	**Contrib. %**	**Cum. %**
18:1*n*-9	14.48	14.48	16:1*n*-9	11.87	11.87	20:5*n*-3	15.74	15.74	20:5*n*-3	13.72	13.72
22:6*n*-3	10.01	24.50	14:0	10.21	2208	18:1*n*-11	8.25	23.99	20:1*n*-9	8.39	22.10
16:1*n*-9	9.86	34.36	20:2*n*-6	10.18	32.27	14:0	7.91	31.90	18:1*n*-7	6.77	28.87
18:2*n*-6	9.62	43.98	16:0	7.99	40.25	18:2*n*-6	7.76	39.66	16:0	6.32	35.19
14:0	7.95	51.93	18:2*n*-6	6.00	46.25	18:1*n*-7	6.95	46.62	22:4*n*-6	6.31	41.50
			18:1*n*-11	5.90	52.15	16:1*n*-7	5.71	52.52	22:5*n*-3	6.23	47.74
									22:1*n*-9	5.89	53.63

However, the low R-values displayed in both comparisons suggest that these differences were more likely due to natural variability than promoted by the different size of the polychaetes. No significant differences were recorded in the FA profiles of SPC vs. MPC ($R = 0.131$, $p = 0.121$). SIMPER analysis (Table 3.2) revealed that the average dissimilarities recorded between the FA profiles of SPC, MPC and LPC in sand bed tanks were as follows: 6.1% for LPC vs. MPC; 8.2% for LPC vs. SPC; and 5.7% for MPC vs. SPC. The single FAs contributing the most to the recorded dissimilarities were: 16:0 (explaining 8.9% of the dissimilarity between LPC and MPC); 18:2*n*-6 (explaining 9.5% of the dissimilarity between LPC and SPC); and 20:4*n*-6 (explaining 10.8% of the dissimilarity between MPC and SPC). Regarding the FA profiles of LPC and potential sources of food (POM, OM, FEED A and B), SIMPER revealed a dissimilarity of 40.9% between LPC and POM, 60.8% between LPC and OM, and 43.7 and 44.3% between LPC and FEED A and B, respectively (Table 3.2). A first hierarchical cluster analysis (Figure 3.2) revealed 2 distinct clusters (with a 90% similarity) separating the FA profiles of WP *Hediste diversicolor* from those of conspecifics cultured in sand bed tanks. A second hierarchical cluster analysis (Figure 3.3) showed that the FA profiles displayed by OM samples were clearly separated (similarity <60%) from those exhibited by cultured, large *H. diversicolor* and POM, as well as FEED A and B.

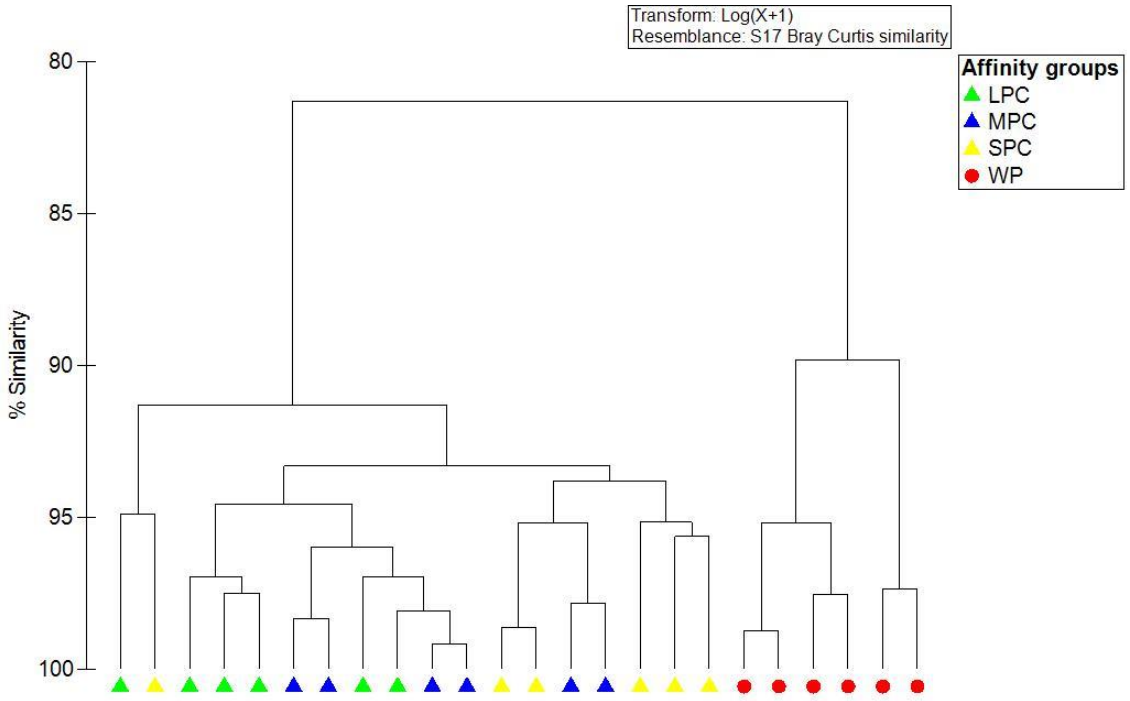

Figure 3.2 - Hierarchical cluster analysis groups of the fatty acid profiles of wild (WP), and small, medium and large cultured *Hediste diversicolor* (SPC, MPC and LPC, respectively)

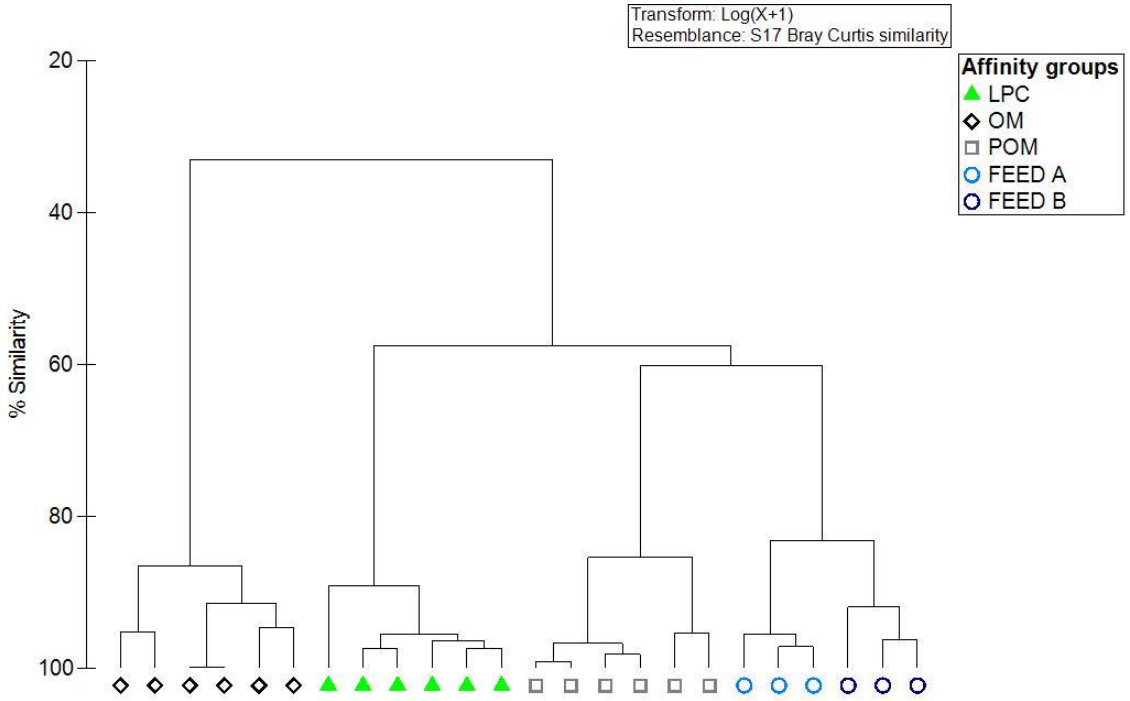

Figure 3.3 - Hierarchical cluster analysis groups of the fatty acid profiles of large *Hediste diversicolor* (LPC) cultured in the sand bed tanks, organic matter (OM), particulate organic matter (POM) and fish feed (FEED A and FEED B)

The CAP analysis revealed that the FA profile most closely resembling that of LPC was that of POM, as 100% of all LPC profiles were allocated to POM when LPC was selected for the 'leave-one-out' allocation routine (Table 3.3).

Table 3.3 - Cross validation success of large *Hediste diversicolor* (LPC) FA profile based on FA profiles of organic matter (OM), particulate organic matter (POM) and fish feed (FEED A and FEED B)

	Allocation of observations to groups				**Total per group**	**% correct**
	OM	POM	FEED A	FEED B		
OM	6	0	0	0	6	100
POM	0	6	0	0	6	100
FEED A	0	0	3	0	3	100
FEED B	0	0	0	3	3	100
New sample (LPC)	0	6	0	0	6	100

3.4 Discussion

The reproduction success of wild *Hediste diversicolor* under cultivated conditions (i.e. using RAS effluents displaying a high level of uneaten/ undigested feed and fish feces) confirms the ability of this species to switch its feeding behavior according to trophic/environmental conditions (Bischoff et al. 2009). In fact, it has been demonstrated that under nutrient enrichment conditions, the surface deposit-feeding behavior of *H. diversicolor* is enhanced over suspension feeding and/or predation (Aberson et al. 2016). The increment recorded in biomass and density during the experimental period corroborates that the available food was sufficient to secure growth and reproduction. In addition, the bentho-pelagic life cycle of *H. diversicolor* and its direct development in the sand bed tank environment, without loss of offspring, allows reproductive success and a significant increase in polychaete density. The FA profiles of cultivated specimens (SPC, MPC and LPC) and wild polychaetes (WP) are in agreement with previous studies (Table 3.4) reporting that the most abundant FA recorded in cultured and wild polychaetes are palmitic acid, stearic acid, oleic acid and EPA (García-Alonso et al. 2008, Bischoff et al. 2009, Techaprempreecha et al. 2011, Lillebø et al. 2012). Of these, EPA (8.34 ± 0.36 μg mg^{-1} DW in LPC and 5.51 ± 0.29 μg mg^{-1} DW in WP) was the most abundant HUFA

present in polychaete biomass. It can be seen that cultivated specimens are able to incorporate almost all available EPA in their food sources into their tissues, even when this FA is present in low levels (e.g. 0.11 ± 0.04 μg mg^{-1} DW for POM, 0.36 ± 0.12 μg mg^{-1} DW for OM, 7.06 ± 0.71 μg mg^{-1} DW for FEED A and 16.20 ± 2.15 μg mg^{-1} DW for FEED B). EPA levels present in food sources can be complemented by the polychaetes through *de novo* synthesis (Santos et al. 2016). This FA is one of the major components of fish oil, a precursor of prostaglandins and thromboxane. It cannot be synthesized *de novo* in humans (García-Alonso et al. 2008) and is therefore classified as an essential FA (Olive 1999). DHA is also an essential FA of great importance in marine fish nutrition. The significant increase in DHA concentrations in cultured specimens may be due to a selective retention in tissues and/or their ability to elongate FAs through a pathway that involves chain elongation of EPA and its desaturation to obtain DHA (Olive et al. 2009). The absence of DHA in the tissues of wild specimens might reflect a deficiency of this FA in natural intertidal mudflat food sources. Arachidonic acid (AA, 20:4*n*-6) is another n-6 long-chain FA essential to fish diets. AA was detected in all samples of polychaetes (WP, SPC, MPC and LPC with 0.84 ± 0.10, 0.99 ± 0.33, 1.25 ± 0.15 and 1.61 ± 0.17 μg mg^{-1} DW, respectively) and takes part in several metabolic pathways in invertebrates, e.g. *Perinereis nuntia* (Techaprempreecha et al. 2011) and *Nereis virens* (Brown et al. 2011). This FA can also be biosynthesized from linoleic acid (Bischoff et al. 2009).

Table 3.4 - Summary table with the most abundant fatty acids in polychaete worms with different food sources

Polychaete worm	Food source	Top 5 of the most abundant fatty acids					Reference
Hediste diversicolor (O.F. Müller, 1776)	Mud flats (Cardiff Bay)	20:5*n*-3	16:0	18:1*n*-9	18:0	15:0	García-Alonso et al. (2008)
	Fish food	16:0	20:5*n*-3	18:1*n*-9	18:2*n*-6	18:0	
	Eel sludge	16:0	20:5*n*-3	18:1*n*-9	20:1	17:0	
Hediste diversicolor (O.F. Müller, 1776)	Sedimentation tank (OM, feces and uneaten food of *Sparus. aurata*)	20:5*n*-3	16:0	18:1*n*-9/*n*-12	18:0	20:1*n*-9	Bischoff et al. (2009)
	Wild (French Atlantic coast)	20:5*n*-3	16:0	18:1*n*-7	16:1	18:0	
Perinereis nuntia	Wild (Bangphra beach. Chonburi providence, Thailand)	16:0	18:1	18:0	16:1	20:5	Techaprempreecha et al. (2011)
	Commercial shrimp diet	16:0	18:2	18:1	18:0	20:2	
	Commercial worm diet	16:0	18:2*n*-6	20:5*n*-3	18:1*n*-9	16:1	
Nereis virens	Fecal waste	16:0	22:1*n*-11	18:1*n*-9	20:5*n*-3	20:1*n*-9	Brown et al. (2011)
	Mixed waste	16:0	22:1*n*-11	20:5*n*-3	18:1*n*-9	20:1*n*-9	
	Pellet waste	16:0	20:5*n*-3	22:1*n*11	18:1*n*-9	20:1*n*-9	
Hediste diversicolor (O.F. Müller, 1776)	Mud flats colonized by *Juncus maritimus* (Laranjo basin)	20:5*n*-3	16:0	18:0	20:4*n*-6	18:1*n*-7	Lillebø et al. (2012)
	Mud flats colonized by *Bolboschoenus* (Laranjo basin)	20:5*n*-3	16:0	18:0	20:4*n*-6	C18:1*n*-7	
	Without vegetation (Laranjo basin)	20:5*n*-3	16:0	20:4*n*-6	18:0	C22:6*n*-3	
Hediste diversicolor (O.F. Müller, 1776)	Commercial diet (seabream dry feed)	16:0	20:5*n*-3	18:1*n*-9	18:0	C22:6*n*-3	Santos et al. (2016)
	Commercial diet (semi-wet pellets for cultured sole)	16:0	20:5*n*-3	18:1*n*-9	18:2*n*-6	C22:6*n*-3	
	Non-processed diet of mackerel fillets	16:0	20:5*n*-3	22:6*n*-3	18:0	C18:1*n*-9	

It has been shown that, to some extent, polychaetes (*Arenicola marina*) have the ability to elongate 18:2*n*-6 (linoleic acid) to produce 20:2*n*-6, which through a desaturation pathway can yield *de novo* 20:4*n*-6 (Olive et al. 2009).The absence of DHA in the tissues of wild specimens might reflect a deficiency of this FA in natural intertidal mudflat food sources. Arachidonic acid (AA, 20:4*n*-6) is another n-6 long-chain FA essential to fish diets. AA was detected in all samples of polychaetes (WP, SPC, MPC and LPC with 0.84 ± 0.10, 0.99 ± 0.33, 1.25 ± 0.15 and 1.61 ± 0.17 μg mg^{-1} DW, respectively) and takes part in several metabolic pathways in invertebrates, e.g. *Perinereis nuntia* (Techaprempreecha et al. 2011) and *Nereis virens* (Brown et al. 2011). This FA can also be biosynthesized from linoleic acid (Bischoff et al. 2009). In addition, polychaetes can also retain most 20:4*n*-6 (AA) present in their diet (OM = 0.12 ± 0.02 μg mg^{-1} DW). AA is an essential FA in fish nutrition, particularly during early life phases (e.g. larval stages), thus being paramount when selecting ingredients to fulfil the nutritional needs of cultured marine fish (Bell & Sargent 2003). According to other studies, palmitic acid (16:0) represents one of the most abundant saturated FAs present in polychaetes (García-Alonso et al. 2008, Brown et al. 2011, Techaprempreecha et al. 2011, Santos et al. 2016). Palmitic acid is the first FA to be biosynthesized and is a precursor of longer-chain saturated FA (Nelson & Cox 2004). Palmitic acid is also a precursor of many types of molecules with physiological relevance, such as membrane lipids, fats and waxes (García-Alonso et al. 2008). Results show that the proposed system for culturing *H. diversicolor* enables the recovery of HUFAs (e.g. EPA, DHA and AA) and palmitic acid into the tissues of these polychaetes. These FA can be reintroduced into productive systems through the potential of *H. diversicolor* to recycle these key ingredients available in different food sources originating from cultivated fish (POM, OM, FEED A and B). Without the action of *H. diversicolor*, these essential FA would likely be lost into the environment. In its natural habitat, *H. diversicolor* is prey for higher trophic levels, either small fish such as the common goby *Pomatoschistus microps* and the sand goby *P. minutus* (Scaps 2002), or larger fish also used for human consumption, such as the Senegalese sole *Solea senegalensis* (Rosa et al. 2008). This may somehow explain the high level of acceptability displayed by this prey when offered to farmed fish species. In the present study, it was shown that cultivated *H. diversicolor* displays a FA profile that holds great potential to marine fish aquaculture as: (1) it is able to reduce the loss of essential FA from the productive system to the environment; and (2) it

displays an enhanced nutritional value when compared to wild specimens (e.g. the presence of DHA in its FA profile). Moreover, the average number of LPC recorded in the present study after 5 months is the equivalent of approximately 100 bait packages identical to those initially used to stock each tank (with circa 70 specimens per package).

3.5 Conclusions

Overall, the present study confirms the potential of *Hediste diversicolor* for the bioremediation of super-intensive marine fish farm effluents and highlights its ability to retain high value nutrients (e.g. HUFA in general and EPA in particular, and to a lesser extent DHA) from fish feeds that would otherwise be lost from the production environment. Ragworm biomass of large specimens may be valued selectively if traded live for sport fish bait, as these are traded at a unitary level (pack of live bait) and not per kg. LPC can also be frozen and traded for maturation diets for fish and/or shrimp broodstock, with their higher level of DHA being used as a feature that may grant them a premium market value over wild conspecifics. The biomass of small, medium or large cultured polychaetes (SPC, MPC and LPC, respectively) may be valued as a whole through their use as a premium ingredient for finishing diets of farmed marine organisms, thus overcoming the need to grade cultured ragworms.

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3.7 Supporting Information

Table S 3.1 - Physicochemical parameters (means ± SD) of non-colonized (N-C) and colonized (C) sand bed tanks (SBT) containing *Hediste diversicolor*.

Parameter	**Month 1**		**Month 2**		**Month 3**		**Month 4**		**Month 5**	
	N-C SBT	C SBT	N-C SBT	C SBT	N-C SBT	C SBT	N-C SBT	C SBT	N-C SBT	C SBT
Temperature (°C)	20.5 ± 0.6	20.6 ± 0.6	18.8 ± 0.1	18.8 ± 0.0	19.4 ± 0.1	19.4 ± 0.2	20.4 ± 0.1	20.4 ± 0.1	18.5 ± 0.1	18.9 ± 0.2
Salinity	21.7 ± 0.0	21.7 ± 0.0	21.0 ± 0.0	21.0 ± 0.0	21.2 ± 0.0	21.2 ± 0.0	21.2 ± 0.0	21.2 ± 0.0	21.1 ± 0.0	21.1 ± 0.0
pH	-	8.95 ± 0.12	-	-	8.04 ± 0.18	7.90 ± 0.08	7.71 ± 0.08	7.56 ± 0.03	7.93 ± 0.04	7.95 ± 0.03
Dissolved oxygen (mg l^{-1})	9.69 ± 0.34	8.90 ± 0.31	8.80 ± 0.04	7.96 ± 0.15	8.40 ± 0.49	7.77 ± 0.25	8.45 ± 0.30	7.08 ± 0.04	9.00 ± 0.31	9.09 ± 0.14

Chapter 4

Effect of high-pressure processing (HPP) on the fatty acid profile of different sized ragworms *Hediste diversicolor* cultured in an integrated multi-trophic aquaculture (IMTA)

4. Effect of high-pressure processing (HPP) on the fatty acid profile of different sized ragworms *Hediste diversicolor* cultured in an integrated multi-trophic aquaculture (IMTA)

Abstract

Ragworms *Hediste diversicolor* cultured in integrated multi-trophic aquaculture (IMTA) display higher levels of important fatty acids (FA) than conspecifics collected from the wild. Thus, these ragworms hold a high potential to be used in maturation diets for several marine fish and shrimp species. Nonetheless, their use may represent a pathway for pathogens. The objective of the present study was to determine if high-pressure processing (HPP) of whole ragworms, as an approach to safeguard their microbiological safety, would promote any significant change on their FA content and validate this approach to safeguard biosecurity. The FA profiles and lipid quality indexes (atherogenicity, thrombogenicity and polyene) of different sized ragworms (small, total length (TL) <30 mm; medium, TL between 30 and 50 mm; and large, TL >50 mm) cultured in an IMTA was compared before and after being subjected to HPP treatment. The ANOSIM test revealed the existence of significant differences between FA content of control and HPP treatment considering each size class. The lipid quality indexes suggest that nutritional quality of FA treated with HPP does not seem to be affected, however, these results indicate that HPP does not damage FA, enabling its application for polychaetae meal pasteurization without compromising its nutritional value and supporting the principles of circular economy.

Keywords: Integrated multi-trophic aquaculture; Polychaete-assisted sand filters; Highly unsaturated fatty acids (HUFA); Biosecurity.

4.1 Introduction

In line with United Nations sustainable development goal 14 (SDG 14 – "*life below water*"; specifically, "*conserve and sustainably use the oceans, seas and marine resources*") the greatest global aquaculture challenges consist in harmonizing the environmental, social and economic perspectives (FAO, 2016). Integrated multi-trophic aquaculture (IMTA) systems are seen as green technology solutions in aquaculture (Chopin et al., 2008), which enable a more sustainable aquaculture and reduce the dependency on wild stocks. Therefore, by being more socially accepted, IMTA has the potential to foster environmental sustainability and economic growth (Alexander et al., 2016; Stevens et al., 2018).

The inclusion of the polychaete *Hediste diversicolor* O.F. Müller, 1776, popularly known as ragworms, in IMTA systems has shown a high potential for the bioremediation of organic-rich waste supply from super-intensive fish farms, due to its ability to feed on particulate organic matter (Bischoff et al., 2009; Palmer, 2010; Fang et al., 2017; Marques et al., 2017). Plus, it has been shown that this extractive species has the ability to selectively retain and/or biosynthesize essential fatty acids (EFA), namely highly unsaturated fatty acids (HUFAs) (García-Alonso et al., 2008; Bischoff et al., 2009; Marques et al., 2018). In general, HUFAs are essential cell membranes constituents, playing a key role in membrane fluidity, in the modulation of enzyme activity, in neural development, and regulation of stress resistance, as shown for marine finfish and shrimp (e.g. Soudant et al., 1996; Suloma and Ogata, 2011). When cultured in IMTA systems, ragworms can be regarded as a potential added value product for marine fish and shrimp broodstock, as they are commonly employed whole in maturation diets for a number of species e.g., *Penaeus monodon* (Fabricius) (Meunpol et al., 2005), *Solea solea* (Linnaeus, 1758) (Cardinaletti et al., 2009). In broodstock management, the lipid and fatty acid profile of maturation diets are paramount for a high-quality development of gonads, enhanced fecundity and fertility (Watanabe et al., 1984; Soudant et al., 1996; Izquierdo et al., 2001). The lack of essential fatty acids can compromise fecundity and hatching rate, and induce anomalies in larvae (Soudant et al., 1996). Specifically concerning marine fish and shrimp, docosahexaenoic acid (DHA - 22:6*n*-3), arachidonic acid (AA - 20:4*n*-6) and

eicosapentaenoic acid (EPA - 20:5*n*-3) have already been identified as key essential fatty acids for a normal growth and survival of marine fish and shrimp that can be provided by polychaetes (Sargent et al., 1999; Bell and Sargent, 2003). In more detail, DHA has a major role in the structural and functional assets of cell membranes, being involved in oogenesis and embryogenesis, and impairing larval mortality and malformations (Soudant et al., 1996). Arachidonic acid (AA) are the most important precursor of prostaglandins and also play an important role in the metabolism of lipid membrane during gametogenesis in females (Khajeh et al., 2017). Eicosapentaenoic acid (EPA) is also a precursor of some of another type of prostaglandins and a strong inhibitor of AA-derived eicosanoid production (Bell et al., 1997). Therefore, it is essential to have adequate DHA/EPA and AA/EPA ratios to ensure reproductive success and improve eggs quality (Bell et al., 1997). The profile of *H. diversicolor* in these specific fatty acids somehow explains why this species stimulates gonad maturation and spawning in marine fish and shrimp (Luis and Passos, 1995). If one considers the way how polychaete meal is prepared (Barba et al., 2015; Moreirinha et al., 2016; Rastogi et al., 2007), the use of IMTA-cultured ragworms to produce a premium polychaete meal may not be of concern from a biosecurity point of view. However, the use of whole *H. diversicolor*, either fresh or frozen, even if depurated and flash frozen, may represent a vector of diseases for broodstock fish or shrimp. Therefore, the present study aims to determine the effects of high-pressure processing (HPP) on *H. diversicolor* FA profile, verifying if this technology can be used for whole ragworms preservation without compromising its nutritional value.

High-pressure processing is a non-thermal preservation technology that has rapidly become highly relevant in the food industry, as it represents a physical additive-free food preservation technology (Heinz and Buckow, 2010). The most important advantages of HPP is the ability to process food at ambient or a lower temperature, while simultaneously inactivate microorganisms and spoilage catalyzing enzymes with a minimal change of the food taste and nutrient content; moreover, it also improves the recovery and bioavailability of bioactive compounds and reduces food allergenicity (Barba et al., 2015; Moreirinha et al., 2016; Rastogi et al., 2007).

As the inactivation of microorganisms through HPP has already been thoroughly demonstrated, including for the most common pathogens in marine aquaculture (see Table

4.1), this aspect was not specifically addressed in the present manuscript. The following null hypothesis was tested: H_0 There are no significant differences in the FA profile and lipid quality indexes of fresh whole depurated small, medium and large-sized *H. diversicolor* and conspecifics exposed to HPP.

4.2 Material and methods

4.2.1 Sampling and processing of ragworms *H. diversicolor* cultured in IMTA system

Ragworms were cultured under the IMTA set-up described by Marques et al. (2017). At the end of experimental period (5 months), each sand filter tank stocked with *H. diversicolor* was sampled (5 replicates per tank) using a hand corer (Ø 110 mm, 150 mm depth). In the laboratory, all sampled specimens were sorted into three pre-established size classes (small, total length (TL) <30 mm; medium, TL between 30 and 50 mm; and large, TL >50 mm) and left to depurate for 24 h in aerated containers with pre-combusted sand (at 450 ºC for 5 h) and artificial seawater (prepared by mixing Tropic Marin Pro Reef salt (Tropic Marine, Germany) and freshwater purified by a reverse osmosis unit and matching the salinity of 21 at the IMTA facility). In order to test the effect of HPP in *H. diversicolor*, six samples of each polychaete size class were weighted to obtain a similar biomass (5 g), with half of those samples (three per size class) being used as a control group (fresh specimens) and the other half being stored in heat sealed hermetic plastic bags and exposed to HPP.

4.2.2 High-pressure processing treatments

High-pressure processing treatment was performing using a hydrostatic press (high-pressure system U33, Unipress Equipment Division, Poland) in a pressure vessel of 35 mm diameter and 100 mm height, at room temperature (21 ºC) using as pressurizing fluid a mixture of water and propylene glycol. Hermetic plastic bags with *H. diversicolor* samples

were exposed to 300 MPa (3000 bar) during 15 min. Moreirinha et al., (2016) reported that such HPP conditions were sufficient to inactivate pathogenic organisms (see Table 4.1). Following HPP treatment, all samples, including those from the control, were stored at -80 ºC and then dehydrated in a freeze dryer during 24 h. Freeze dried sub-samples were mechanically homogenized and stored at -80 ºC for posterior biochemical analysis.

Table 4.1 - High Pressure Processing (HPP) conditions applied for inactivation of common pathogens in aquaculture.

Pathogens	**Isolated from:**	**Pressure applied and duration of HPP**	**References**
Photobacterium damselae *Vibrio anguillarum* *Aeromonas* *Salmonella* sp. *Escherichia coli*	Hake (*Merluccius merluccius*) and dried salted cod (*Gadus morhua*)	300 MPa (15 min)	Moreirinha et al., (2016)
Listeria monocytogenes *Escherichia coli*	Smoked rainbow trout fillets (*Oncorhynchus mykiss*) and fresh catfish fillets (*Silurus glanis*)	400/600 MPa (1/5 min)	Mengden et al., (2015)
Vibrio parahaemolyticus	Oysters (*Crassostrea gigas*)	293 MPa (2 min)	Ma and Su, (2011)
Pseudomonas spp. *Shewanella* spp.	Coho salmon (*Oncorhynchus kisutch*)	135/170/200 MPa (30 s)	Aubourg et al., (2010)
ND*	Atlantic salmon (*Salmo salar*)	150/300 MPa (15 min)	Yagiz et al., (2009)
ND*	Rainbow trout (*Oncorhynchus mykiss*) and Mahi Mahi (*Coryphaena hippurus*)	150/300/450/600 MPa (15 min)	Yagiz et al., (2007)
Psycrophiles	albacore tuna (*Thunnus alalunga*)	310 MPa (6 min)	Ramirez-Suarez & Morrissey, (2006)

4.2.3 Fatty acids analysis

The separation of fatty acids methyl esters (FAMEs) was performed using a 7890B gas chromatograph system with a flame ionization detector (GC-FID) following the methodology described by Aued-pimentel et al. (2004). The main advantage of this method is that it can be done at room temperature, thereby it reduces the risks of FA decomposition. Previous to analysis all freeze-dried samples were powdered and homogenized, being weighted accurately in a sovirel/pyrex glass tube (~50 mg of *H. diversicolor* biomass) and dissolved in 1 ml of the internal standard solution of a fatty acid 21:0 in n-hexane (0.021 g L^{-1}). Afterwards, 0.2 mL of methalonic KOH solution (2 mol L^{-1}) was added, the tube was sealed and mixed vigorously in a vortex shaker for 2 min. Following this procedure, 2 mL of a saturated NaCl solution was added, and centrifugation of the mixture took place for 5 min at 3000 rpm to separate the organic phase. Then 1 mL of organic phase was transferred into a vial and the excess of solvent was left to evaporated with nitrogen gas. The dried sample obtained was dissolved in n-hexane (1 mL) and analysed using a GC-FID. The detector and injector were kept at 250 ºC, with hydrogen as carrier gas. Fatty acid methyl ester (FAME) were separated in a fused-silica capillary column, DB-FFAP column (30 m x 320 µm x 0.25 µm) (Agilent 123-3232) with the following temperature programme: 75 ºC (initial), 20 ºC min^{-1} to 155 ºC (4 min), 2 ºC min^{-1} to 180 ºC (16.5 min), 4 ºC min^{-1} to 250 ºC (44 min). The identification of the fatty acids was done by matching the peaks with previously inject internal standards. The FA content (µg.mg^{-1}DW) in the analysed samples was calculated considering the relation between mass the area of fatty acids and the internal standard (21:0). In the present study PUFA are defined as all FA with ≥ 2 double bonds and highly unsaturated fatty acids (HUFA) (FA with ≥ 4 double bonds) are considered separately and not within PUFA.

4.2.4 Lipid quality indexes

Lipid quality indexes, namely the atherogenicity index (AI) and thrombogenicity index (TI) were determined according to Ulbricht and Southgate (1991), while the polyene index (PI) was calculated according to Lubis and Buckle (1990).

AI and TI can be used to assess the nutritional quality of polychaetes, being calculated through the following equations:

$$AI = (12:0 + 4 \times 14:0 + 16:0)/[\sum MUFAs + PUFA\ n\text{-}6 + PUFA\ n\text{-}3]$$

$$TI = (14:0 + 16:0 + 18:0)/[\ 0.5 \times \sum MUFAs + 0.5 \times PUFA\ n\text{-}6 + 3 \times PUFA\ n\text{-}3 + PUFA\ n\text{-}3/PUFA\ n\text{-}6]$$

Concerning PI, this index can be used as a measure of PUFA damage, being a good proxy for lipid oxidation, and can be calculated according to the following equation:

$$PI = (EPA+DHA)/16:0$$

4.3 Statistical analysis

A resemblance matrix using the content of each FA on each polychaete sample was prepared using the Bray-Curtis similarity coefficient, after performing a log (x + 1) transformation to emphasize compositional similarity rather than on quantitative differences (Anderson, 2008). A two-way analyses of similarities (ANOSIM) was performed to test the null hypotheses. A global R statistic was calculated to determine the differences between FA content of small, medium and large *H. diversicolor* (S, M and L, respectively) in the different treatments (control and HPP), with R values close to one indicating maximum differences between groups and values near zero indicating a complete group overlay. An identical analysis was used to identify if there were any significant differences in lipid quality indexes. To evaluate the percentage that each FA contributed to the dissimilarities recorded between treatments a SIMPER (similarity percentage) analysis was also performed, with those contributing with 50% of cumulative dissimilarities being highlighted. For a detailed description of all the statistical analysis referred above please see Clarke and Gorley, (2006). All Statistical analyses were performed using PRIMER v6 with the PERMANOVA+ add-on.

4.4 Results

4.4.1 Fatty acid profiles

Table 4.2 summarizes the results of the fatty acid content for *H. diversicolor* in the control group and those exposed to HPP. Both in control and HPP samples, a total of 23 fatty acids were recorded for the different *H. diversicolor* size classes (S, M and L). In control samples of *H. diversicolor*, EPA was the dominant FA in all size classes contributing to 25%, 24% and 22% of the total FA content presented by S, M and L, respectively. The average content of this FA was 7.53 ± 0.11 µg mg^{-1} DW, 6.32 ± 0.02 µg mg^{-1} DW and 6.68 ± 0.17 µg mg^{-1} DW for S, M and L, respectively. Palmitic acid was the most dominant SFA, with 4.09 ± 0.06 µg mg^{-1} DW, 3.46 ± 0.18 µg mg^{-1} DW and 4.57 ± 0.11 µg mg^{-1} DW for S, M and L, respectively. Concerning MUFA, the most representative fatty acid was 20:1*n*-9, with 2.20 ± 0.03 µg mg^{-1} DW, 1.91 ± 0.07 µg mg^{-1} DW and 2.06 ± 0.02 µg mg^{-1} DW being recorded for S, M and L, respectively.

In the *H. diversicolor* HPP treatment group, EPA was the most representative FA with 6.77 ± 0.11 µg mg^{-1} DW, 5.07 ± 0.05 µg mg^{-1} DW and 5.33 ± 0.17 µg mg^{-1} DW to S, M and L, respectively. Palmitic acid content was 4.58 ± 0.06 µg mg^{-1} DW, 4.77 ± 0.07 µg mg^{-1} DW and 6.57 ± 0.18 µg mg^{-1} DW in S, M and L, respectively. The class MUFA was characterized by the fatty acids 18:1*n*-5 (2.35 ± 0.05 µg mg^{-1} DW, 1.99 ± 0.02 µg mg^{-1} DW and 2.86 ± 0.08 µg mg^{-1} DW to S, M and L, respectively) and the 20:1*n*-9 groups (2.34 ± 0.05 µg mg^{-1} DW, 1.94 ± 0.02 µg mg^{-1} DW and 2.18 ± 0.04 µg mg^{-1} DW to S, M and L, respectively).

Table 4.2 - Fatty acid profiles (µg mg^{-1} DW) of different sized (small, total length (TL) <30 mm; medium, TL between 30 and 50 mm; and large, TL >50 mm) cultured *Hediste diversicolor* fresh (Control) or exposed to high-pressure processing (HPP). Values are average of three replicates ± standard deviation.

FA	Control Small	Control Medium	Control Large	HPP Small	HPP Medium	HPP Large
14:0	0.29 ± 0.01	0.24 ± 0.01	0.35 ± 0.00	0.35 ± 0.00	0.41 ± 0.02	0.54 ± 0.02
15:0	0.25 ± 0.00	0.21 ± 0.01	0.25 ± 0.00	0.27 ± 0.00	0.28 ± 0.00	0.33 ± 0.02
16:0	4.09 ± 0.06	3.46 ± 0.18	4.57 ± 0.11	4.58 ± 0.06	4.77 ± 0.07	6.57 ± 0.18
17:0	0.45 ± 0.01	0.35 ± 0.02	0.42 ± 0.01	0.51 ± 0.01	0.44 ± 0.01	0.55 ± 0.02
18:0	1.92 ± 0.02	1.63 ± 0.08	1.98 ± 0.03	2.09 ± 0.03	1.95 ± 0.02	2.34 ± 0.05
∑ SFA[1]	**7.01 ± 0.10**	**5.89 ± 0.30**	**7.58 ± 0.16**	**7.81 ± 0.11**	**7.85 ± 0.12**	**10.32 ± 0.29**
16:1*n*-7	0.78 ± 0.01	0.72 ± 0.02	0.98 ± 0.04	0.93 ± 0.01	1.00 ± 0.01	1.44 ± 0.03
18:1*n*-9	1.93 ± 0.03	1.65 ± 0.04	1.84 ± 0.02	2.04 ± 0.05	1.85 ± 0.02	1.88 ± 0.07
18:1*n*-7	1.00 ± 0.01	0.91 ± 0.03	0.99 ± 0.01	1.04 ± 0.04	1.04 ± 0.01	1.23 ± 0.06
18:1*n*-5	2.08 ± 0.02	1.69 ± 0.03	1.98 ± 0.06	2.35 ± 0.05	1.99 ± 0.02	2.86 ± 0.08
20:1*n*-9	2.20 ± 0.03	1.91 ± 0.07	2.06 ± 0.02	2.34 ± 0.05	1.94 ± 0.02	2.18 ± 0.04
22:1*n*-9	0.84 ± 0.01	1.05 ± 0.04	1.07 ± 0.01	0.76 ± 0.02	0.85 ± 0.01	0.82 ± 0.02
∑ MUFA[2]	**8.83 ± 0.11**	**7.92 ± 0.23**	**8.92 ± 0.15**	**9.46 ± 0.23**	**8.67 ± 0.08**	**10.55 ± 0.32**
18:2*n*-6	0.85 ± 0.01	0.73 ± 0.03	0.78 ± 0.01	0.81 ± 0.01	0.64 ± 0.01	0.69 ± 0.03
18:3*n*-6	0.25 ± 0.00	0.24 ± 0.01	0.32 ± 0.02	0.25 ± 0.00	0.22 ± 0.00	0.30 ± 0.02
20:2*n*-6	0.63 ± 0.01	0.61 ± 0.02	0.44 ± 0.00	0.64 ± 0.01	0.56 ± 0.00	0.82 ± 0.02
∑ PUFA[3]	**2.11 ± 0.02**	**1.99 ± 0.07**	**2.32 ± 0.03**	**2.04 ± 0.03**	**1.73 ± 0.10**	**2.24 ± 0.10**
20:4*n*-6 (AA)	0.79 ± 0.01	0.83 ± 0.04	0.82 ± 0.00	0.68 ± 0.01	0.63 ± 0.01	0.67 ± 0.03
20:5*n*-3 (EPA)	7.53 ± 0.11	6.32 ± 0.02	6.68 ± 0.17	6.77 ± 0.11	5.07 ± 0.05	5.33 ± 0.17
22:4*n*-6	0.90 ± 0.01	1.08 ± 0.05	1.01 ± 0.01	0.78 ± 0.01	0.77 ± 0.01	0.85 ± 0.04
22:5*n*-3	1.49 ± 0.02	1.40 ± 0.06	1.27 ± 0.04	1.38 ± 0.03	1.04 ± 0.01	1.09 ± 0.04
22:6*n*-3 (DHA)	0.76 ± 0.01	0.71 ± 0.02	0.59 ± 0.01	0.63 ± 0.01	0.44 ± 0.02	0.50 ± 0.03
∑ HUFA[4]	**11.47 ± 0.16**	**10.35 ± 0.16**	**10.37 ± 0.23**	**10.25 ± 0.18**	**7.97 ± 0.08**	**8.44 ± 0.30**
∑ Others[5]	**0.78 ± 0.01**	**0.62 ± 0.03**	**0.65 ± 0.02**	**0.93 ± 0.01**	**0.82 ± 0.1**	**1.05 ± 0.05**
∑ Total	**30.20 ± 0.04**	**26.77 ± 0.79**	**29.84 ± 0.59**	**30.49 ± 0.56**	**27.03 ± 0.39**	**32.60 ± 1.05**

The ANOSIM analysis performed on FA content of polychaete samples from the control group and those exposed to HPP revealed the existence of significant differences. The statistical analysis showed significant differences (R = 1, p = 0.003) between the control group and HPP treatment. Considering size class (S, M and L) the ANOSIM analysis showed significant differences (R=1; p= 0.001) between the size group with a strong

difference within each group. In fact, in HPP treated *H. diversicolor* SFA increased from 7.01; 5.89, and 7.58 µg mg^{-1} DW to 7.81; 7.85, and 10.32 µg mg^{-1} DW for S, M and L, respectively. Regarding HUFA, there was a decrease in FA content from 11.47; 10.35, and 10.37 µg mg^{-1} DW to 10.25; 7.97, and 8.44 µg mg^{-1} DW for S, M and L, respectively, with EPA content being mostly responsible for this decrease (Figure 4.1).

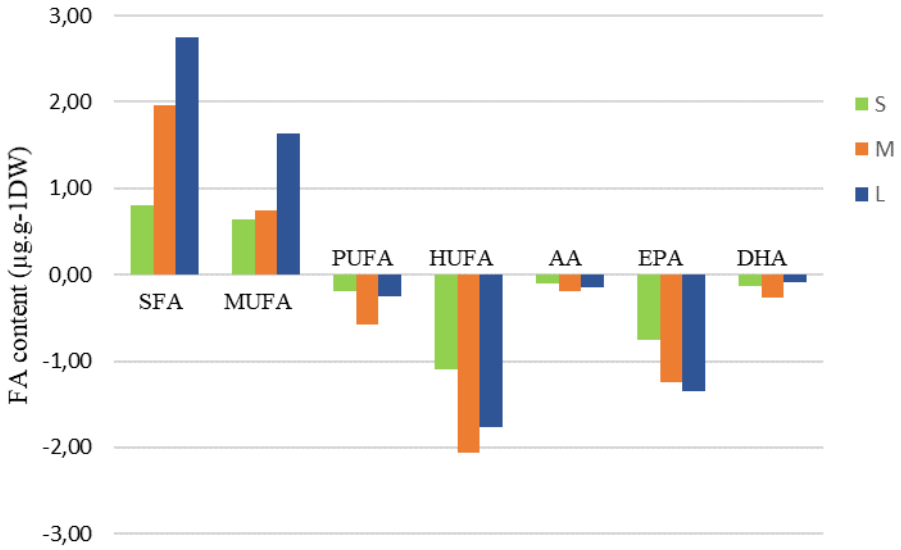

Figure 4.1 - Shifts in the fatty acid content of different sized (small (S), total length (TL) <30 mm; medium (M), TL between 30 and 50 mm; and large (L), TL >50 mm) cultured *Hediste diversicolor* exposed to high-pressure processing (HPP) when compared to fresh specimens. SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - Polyunsaturated fatty acids; HUFA - Highly unsaturated fatty acids, PUFA are defined as all FA with ≥ 2 double bonds; in the present study HUFA (FA with ≥ 4 double bonds) are not considered within ∑PUFA; AA - arachidonic acid, 20:4*n*-6; EPA - eicosapentaenoic acid, 20:5*n*-3; DHA - docosahexaenoic acid, 22:6*n*-3).

Indeed, the SIMPER analysis (Table 4.3) confirmed these trends, with average dissimilarities recorded between the FA content of *H. diversicolor* in the control and HPP group being as follows: 3.3% for S; 7.5% for M; and 6.9% for L. Up to 8.7% of the dissimilarities recorded between the S in the control and HPP group was explained by EPA alone. Palmitic acid explained 11.4% and 13.4% of the dissimilarities recorded between the control and HPP group for M and L, respectively.

Table 4.3 - SIMPER overall average dissimilarities (%) between the mean fatty acid (FA) of different sized (small, total length (TL) <30 mm; medium, TL between 30 and 50 mm; and large, TL >50 mm) cultured *Hediste diversicolor* fresh (Control) or exposed to high-pressure processing (HPP).

Control *vs.* HPP			**Control *vs.* HPP**			**Control *vs.* HPP**		
Small			**Medium**			**Large**		
FA	**Contr. %**	**Cum. %**	**FA**	**Contr. %**	**Cum. %**	**FA**	**Contr. %**	**Cum. %**
20:5*n*-3	8.7	8.7	**16:0**	11.43	11.43	**16:0**	13.4	13.4
16:0	8.53	17.23	**20:5*n*-3**	8.27	19.7	**18:1*n*-7**	11.37	24.77
18:1*n*-7	8.09	25.32	**22:6*n*-3**	7.65	27.35	**16:1*n*-7**	9.2	33.98
16:1*n*-7	7.6	32.92	**22:5*n*-3**	7.13	34.48	**20:5*n*-3**	8.49	42.47
22:6*n*-3	7.14	40.06	**22:2*n*-6**	7.12	41.6	***iso* 16:0**	7.07	49.53
22:2*n*-6	6.12	46.18	**16:1*n*-7**	6.58	48.18			
20:4*n*-6	5.83	52.01	**14:0**	5.57	53.75			

4.4.2 Lipid quality indexes

Table 4.4 summarizes the lipid quality indexes (AI, TI and PI) recorded for the different sized classes of *H. diversicolor* in the control and exposed to HPP. The ANOSIM test revealed significant differences (R=1; p=0.003) between the FA content of polychaetes in control versus HPP treatment, and between the indexes (R=0.926; p=0.001) (see Figure 4.2)

Table 4.4 - Lipid quality indexes of different sized (small, total length (TL) <30 mm; medium, TL between 30 and 50 mm; and large, TL >50 mm) cultured *Hediste diversicolor* fresh (Control) or exposed to high-pressure processing (HPP). AI - Atherogenicity index; TI - Thrombogenicity index and PI - Polyene index.

Lipid quality index	Control			HPP		
	Small	Medium	Large	Small	Medium	Large
AI	0.30 ± 0.00	0.29 ± 0.02	0.37 ± 0.00	0.35 ± 0.00	0.46 ± 0.01	0.55 ± 0.00
TI	0.21 ± 0.00	0.21 ± 0.01	0.26 ± 0.00	0.26 ± 0.00	0.35 ± 0.00	0.44 ± 0.00
PI	2.03 ± 0.01	2.04 ± 0.12	1.59 ± 0.01	1.62 ± 0.02	1.16 ± 0.01	0.89 ± 0.01

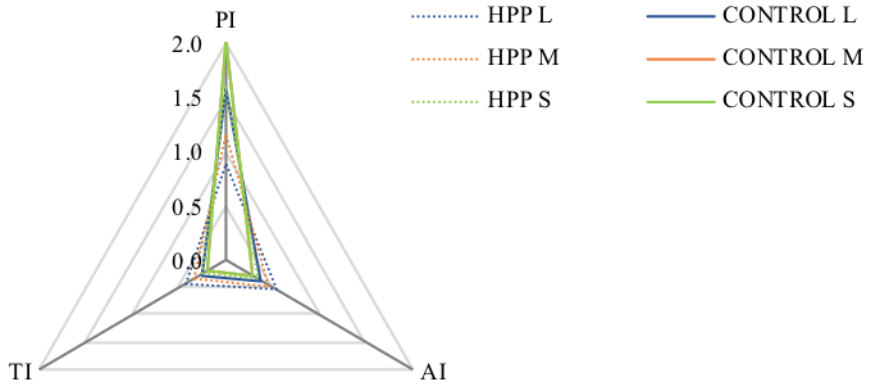

Figure 4.2 - Nutritional quality indices of different sized (small (S), total length (TL) <30 mm; medium (M), TL between 30 and 50 mm; and large (L), TL >50 mm) cultured *Hediste diversicolor* fresh (CONTROL) or exposed to high-pressure processing (HPP). AI - Atherogenicity index; TI - Thrombogenicity index and PI - Polyene index.

4.5 Discussion

High-pressure processing has been used with success as a food preservation process since the 1990´s (Ohshima et al., 1993). This food treatment has the potential to inactivate microorganisms and reduce microbial growth (Cruz-Romero et al., 2008; Ohshima et al., 1993). Moreirinha et al. (2016) showed that a group of important pathogenic bacteria (e.g., *Acinetobacter*, *Aeromonas*, *Escherichia coli*, *Enterobacter*, *Klebsiella*, *Photobacterium*, *Pseudomonas aeruginosa*, *Salmonella* and *Vibrio anguillarum*), some of which infect fish, decreased to undetectable levels when treated at 300 MPa during 15 min (see Table 4.1).

Microorganisms differ in their response to HPP treatments depending on their physiological state, temperature, time and magnitude of the induced pressure (Thakur and Nelson, 1998; Moreirinha et al., 2016). Several authors showed with success the reduction of the microbial growth in fish and fish food products through the use of HPP. Relevant examples of HHP treatments are albacore tuna (*Thunnus alalunga*) treated at 310 MPa during 6 min (Ramirez-Suarez and Morrissey, 2006); smoked rainbow trout fillets (*Oncorhynchus mykiss*) and fresh catfish fillets (*Silurus glanis*) treated at 400, or 600 MPa during 1 and 5 min (Mengden et al., 2015); oysters (*Crassostrea gigas*) treated at 293 MPa during 2 min (Ma and Su, 2011); coho salmon (*Oncorhynchus kisutch*) treated at 135, 170 and 200 MPa during 30 s (Aubourg et al., 2010); rainbow trout (*Oncorhynchus mykiss*) and Mahi Mahi (*Coryphaena hippurus*) treated at 150, 300, 450, and 600 MPa during 15 min (Yagiz et al., 2007); Atlantic salmon (*Salmo salar*) treated at 150 MPa and 300 MPa during 15 min (Yagiz et al., 2009) (see Table 4.1).

Concerning the effects of HPP on the FA content, the present study shows that HPP treatment at 300 MPa during 15 min, had a significant effect on *H. diversicolor* content. Even though these results differ from Yagiz et al. (2009), where Atlantic salmon (*S. salar*) was exposed to the same HPP treatment, this may be explained due to the antioxidation capacity of astaxanthin present in the salmon, recognized to be higher than that of other antioxidants (Shimidzu et al., 1996).

However, changes in SFA content with HPP treatment are in accordance with previous results recorded for: rainbow trout (*Oncorhynchus mykiss*); mahi mahi (*C. hippurus*) (Yagiz et al., 2007); Atlantic salmon (*S. salar*) (Yagiz et al., 2009); and Rongchang pig (He et al., 2012). SFA are not prone to oxidation in opposite to HUFA. Actually, HUFA are more reactive and more easily oxidized due to the double bonds that these molecules display in their carbon chain (Barba et al., 2015; Vázquez et al., 2013; Wang et al., 2013). As a consequence of lipid oxidation, FA content may display more or less pronounced shifts (Canto et al., 2015; Yagiz et al., 2009). These shifts can also be related to the action of heme proteins which act as catalysts and, under HPP, can become denatured and more pro-oxidative (Yagiz et al., 2009). The potential destruction of lipid membranes may also explain the shifts recorded (Barba et al., 2015).

In the present study it was expected that the loss of HUFA would be reflected in quality indices. Our results demonstrated that HPP treatment induced a reduction of HUFA, including AA, EPA and DHA in *H. diversicolor*. However, the nutritional quality of the polychaetes treated with HPP does not seem to be affected, as revealed by the analyses of lipid quality indexes. These results are in agreement with previous findings. Aubourg et al. (2010) showed that at 170 MPa during 30 s, and 200 MPa during 30 s, the PI values of farmed coho salmon (*O. kisutch*) had no significant differences, contributing in this way for the preservation of lipid's nutritional value. Another study by Vázquez et al., (2013) revealed that only minor differences in PI were recorded on frozen mackerel (*Scomber scombrus*) exposed to 150, 300, 450 MPa with a holding time of 0, 2.5 and 5 min. According to Telahigue et al., (2013) the decrease in PI values after HPP indicated that the oxidation process was in progress in control samples and was stooped. In the present study, there was a decrease in PI following HPP and an increase in AI and TI values. These results are in agreement with previous works on hake (*M. merluccius*) and sardinella (*Sardinella aurita*) (Telahigue et al., 2013).

According to Bischoff et al., (2009) and Marques et al., (2018), *H. diversicolor* has a high potential for bioremediation of IMTA systems, as this species is capable of retaining highly-valued FA, such as HUFAS (e.g. EPA, DHA and AA). The amount of DHA in polychaetes exposed to HPP decreased 25% (from 0.20 to 0.15 mg in 100 g of total dry weight for LP; from 0.26 to 0.16 mg in 100 g of total dry weight for MP and from 0.25 to 0.21 mg in 100g of total dry weight for SP) when compared with the control group. However, polychaetes farmed in the sand filters and exposed to HPP still display a higher content of DHA than wild conspecifics (see Marques et al., 2018). Therefore, the present study highlights the suitability of employing HPP for the inactivation of microorganisms and reduction of microbial growth without compromising the nutritional value of ragworms.

4.6 Conclusions

From a biosecurity perspective, HPP is a suitable approach to treat ragworms and safeguard that these do not act as a pathway for pathogens when fed to valuable fish and shrimp broodstock. When such ragworms are produced under an IMTA framework and display a higher percentage of valuable HUFAs than conspecifics from the wild, there is still a positive trade-off between using this premium polychaetes and sacrifice part of their HUFA content (including EPA, DHA and AA) due to HPP to secure microbiological safety. Overall, the basis for a circular economy is supported using the present approach and contributes to SDG 14 targets concerning aquaculture, as it integrates environmental sustainability, safety and economic growth.

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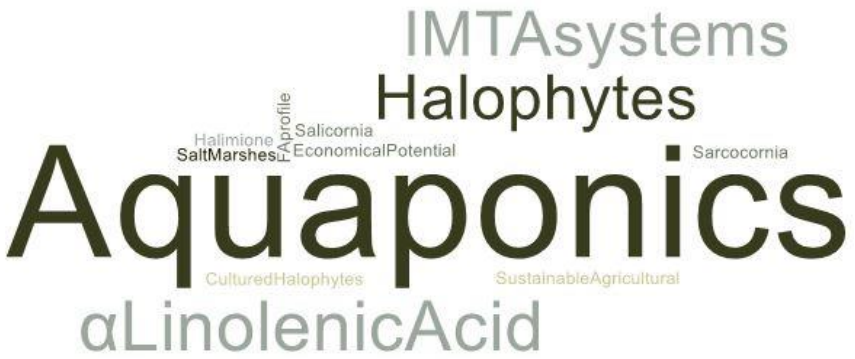
Chapter 5

Halophyte plants farmed in aquaponics display fatty acid profiles similar to conspecifics in the wild



5. Halophyte plants farmed in aquaponics display fatty acid profiles similar to conspecifics in the wild

Abstract

Halophytes have gradually been introduced in marine integrated multi-trophic aquaculture (IMTA) systems due to their capacity to bioremediate nutrient-rich marine effluents and their potential use for human consumption due to their content in omega-3 and omega-6 fatty acids (FA). To foster the valorization of halophytes produced using an IMTA framework, it is important that culture conditions do not promote significant shifts in their FA profile, when compared to that displayed by conspecifics in the wild. The main objective of the present study was to compare the FA profiles of three halophyte species (*Halimione portulacoides*, *Salicornia ramosissima* and *Sarcocornia perennis*) cultured in aquaponics coupled to an IMTA system with that of wild conspecifics retrieved from donor sites. The FA profiles were compared considering different plant organs (edible parts and roots) and sampling dates (spring, summer and autumn). Results show that the FA profiles of specimens cultured in aquaponics were not significantly different from that of wild conspecifics, displaying a high content of omega-3 FAs in edible parts, particularly during summer, and mostly in the form of α-linolenic acid (ALA, 18:3*n*-3). In more detail, for the specimens cultured in aquaponics, ALA concentration in the edible parts of each species ranged from 5.10 to 7.11 μg mg^{-1} DW in *H. portulacoides*, from 5.66 to 9.19 μg mg^{-1} DW in *S. ramosissima* and from 5.49 to 7.20 μg mg^{-1} DW in *S. perennis*. Concerning omega-6, in the form of linoleic acid (LA, 18:2*n*-6) present in edible parts, concentrations ranged from 2.25 to 2.46 μg mg^{-1} DW in *H. portulacoides*, from 3.26 to 4.84 μg mg^{-1} DW in *S. ramosissima* and from 2.17 to 3.06 μg mg^{-1} DW in *S. perennis*. The nutritional quality assessed through the ratio of PUFA/SFA, for both wild and cultured plants, revealed values well above the threshold (0.45). Overall, the culture conditions tested in the present work reinforce the potential of aquaponics coupled to marine IMTA to produce high-quality halophytes suitable for human consumption.

Keywords: α-linolenic acid; cultured halophytes; aquaponics; economical potential

5.1 Introduction

Building upon the Goals for the Millennium, the United Nations (UN) elaborated further and proposed Sustainable Development Goals (SDG) and targets for the year 2030, with 17 areas of critical importance for humanity and the planet having been identified (United Nations, 2015). From these, SDG 14 - "*life below water*" aiming to "*conserve and sustainably use the oceans, seas and marine resources for sustainable development*"; and SDG 2 - "*zero hunger*" aiming to "*end hunger, achieve food security and improved nutrition and promote sustainable agriculture*" (United Nations, 2017) are aligned with the UN/FAO's blue growth initiative, targeting responsible and sustainable fisheries and aquaculture (FAO, 2014). These SDG are also in line with EU blue growth strategy targeting sectors that have a high potential for sustainable jobs and growth (European Commission, 2012). Under this strategy, aquaculture is seen an opportunity to ensure a better use of marine resources and improve nutrition, therefore holding the potential to support SDG14 and SDG2 aims and targets. In this context, an increase in science based-knowledge might enhance the link between the development of environmental and economic sustainable aquaculture, namely by reducing the dependency from wild specimens, ensure the nutritional value of cultured specimens and enhancing their overall added value.

Aquaponics, a variation of integrated multitrophic aquaculture (IMTA) concept, combines two technologies: recirculation aquaculture systems (RAS) (e.g., fish-farm) and hydroponics plant production (soilless cultivation of crops) (König et al., 2018). In a similar way as for agriculture (part of SDG 2), where aquaponics appears to be a solution to achieve a sustainable agricultural production system (Lehman et al., 1993), aquaponics holds the potential to play a significant role in aquaculture production (Goddek et al., 2015; Junge et al., 2017). In an aquaculture environment, namely in marine aquaculture, these systems use nutrient rich effluents from fish production to culture a range of extractive species, namely salt tolerant plants, i.e., halophytes. In aquaponics, the excess of organic matter in the fish farm effluent becomes a source of energy for plant growth, stimulating the recycling of dissolved inorganic nutrients (Goddek et al., 2015).

Halophytes have been gradually introduced in marine aquaculture systems to enhance the implementation of more sustainable practices, due to their extractive capacity and suitability for bioremediation of nutrient rich marine effluents, as well as their potential for human consumption (Alexander et al., 2015; Custódio et al., 2017). Relevant examples regarding the use of halophytes as a primary driver for the mitigation of nutrient rich effluents from super-intensive marine fish farm are the works by Brown et al. (1999) (using *Suaeda esteroa* Ferren & S.A. Whitmore (1983), *Salicornia bigelovii* Torrey, 1858 and *Atriplex barclayana* D.Dietr.), Webb et al. (2012) (using *Salicornia europaea* Linnaeus), Shpigel et al. (2013) (using *Salicornia persica* Akhani) and Marques et al. (2017) (using *Halimione portulacoides* (L.) Aellen). In addition, a growing number of studies have highlighted the potential use of halophyte plants as food for human consumption, namely *Salicornia* spp. (Isca et al., 2014; Webb et al., 2012), *Sarcocornia perennis* (Mill.) A.J. Scott, *Salicornia ramosissima* J. Woods and *Arthrocnemum macrostachyum* (Moric.) Moris (Barreira et al., 2017). Wild specimens of the above-mentioned halophyte species are recognized to be rich in omega-3 and omega-6 fatty acids (FA) (Maciel et. al., 2018), specifically *α*-linolenic and linoleic acid (ALA, 18:3*n*-3 and LA, 18:2*n*-6, respectively) (Amjad Khan et al., 2017). These essential FA are the precursors of some of the most important polyunsaturated fatty acids (PUFAs) for human nutrition, such as 20:4*n*-6 arachidonic acid (ARA), 20:5*n*-3 eicosapentaenoic acid (EPA) and 22:6*n*-3 docosahexaenoic acid (DHA) (e.g., Simopoulos, 1999; 2004; Singh et al., 2005). Therefore, from a valorization perspective, it is of paramount importance that the production of halophytes in aquaponics associated to marine fish production do not promote any major shift in their FA profile, that may render cultured specimens less appealing to consumers than conspecifics collected from the wild.

This study aims to compare the FA profiles of halophytes cultured under aquaponics conditions with that of wild specimens harvested from donor sites. The present study addresses three halophyte species from family Chenopodiaceae (*Halimione portulacoides* (L.) Aellen, previously known as *Atriplex portulacoides* (L.); *Salicornia ramosissima* (J.) Woods; and *Sarcocornia perennis* (Miller) A. J. Scott) whose potential for aquaponics production as part of IMTA systems has already been documented (e.g. Marques et al., 2017; Webb et al., 2013, 2012), as well as their nutritional properties for human consumption (Barreira et al., 2017; Glenn et al., 2013; Maciel et al., 2016; Ventura et al.,

2011). In brief, *H. portulacoides* is an evergreen halophyte present in salt marshes along the Atlantic coast of Europe (Bouchard et al., 1998; Waisel, 1972); *S. ramosissima* is a pioneer annual halophyte, commonly distributed in the salt marshes of the Iberian Peninsula (Davy et al., 2006); and *S. perennis* has a perennial life cycle being one of the most common halophytes in low-middle elevations of salt marshes in European (Davy et al., 2006). The FA profile of each selected halophyte was studied in spring, summer and autumn, as these are the three most relevant periods of the year for their annual life-cycle. To evaluate whether the nutrient rich effluent from a super-intensive marine fish farm affected the FA profiles of halophytes cultured in aquaponics, their profile in these valuable biomolecules was analyzed and compared with that from wild specimens. The following null hypothesis was tested: H_o1: there are no significant differences in FA profiles in the edible parts and roots, at spring, summer and autumn of *H. portulacoides*, *S. ramosissima* and *S. perennis* from the wild and cultured in aquaponics.

5.2 Material and methods

5.2.1 Short description of the donor site and selected halophyte species

The halophyte species used in the present study were collected in Ria de Aveiro coastal lagoon salt marshes (40º38´N 08º44´W). This coastal system is approximately 45 km long and 10 km wide with one single connection with the Atlantic Ocean. The lagoon geomorphology is characterized by four main channels, each of them with small channels and islands, large areas of intertidal sand and mudflats, seagrasses meadows and one of the largest continuous saltmarshes in Europe (Sousa et al., 2017). The region is characterized by a temperate maritime climate with an average temperature of 14°C and an average precipitation of 1000 mm (Stefanova et al., 2015). Figure 5.1 features the three selected species, which can be briefly described, according to Flora Iberica (Castroviejo, 1986), as follows:

Halimione portulacoides – shrubby perennial up to 150 mm tall, with woody stems at the base and succulent leaves at the top. Stems can grow prostrate, ascending or erect. Leaves (spatulate or lanceolate to linear-lanceolate, exceptionally deltoid) are opposite in lower part and alternate in upper part of the plants, with a light petiole. The inflorescences consist of inconspicuous flowers.

Salicornia ramosissima – annual erect, rarely decumbent subshrub up to 400 mm tall. Stems are generally quite branched, terminating in spike-like apparently jointed inflorescences, with two opposite three-flowered cymes partly hidden in the internode tissue. Each cyme holds one large central flower and two smaller lateral flowers. Central flower has its base generally covered by the scarious margin of the lower segment.

Sarcocornia perennis – perennial subshrub up to 700 mm tall, with woody steams at the base, prostrate to procumbent, and above with fleshy-articulated, erect-ascending, simple or sparingly branched stems. Leaves are reduced to a sharp scale, with a hyaline border. It has a spiciform inflorescence, lateral or terminal, formed by opposing triflora crests - at the base of each fertile core - and decussate. Central flower slightly larger than the lateral ones.

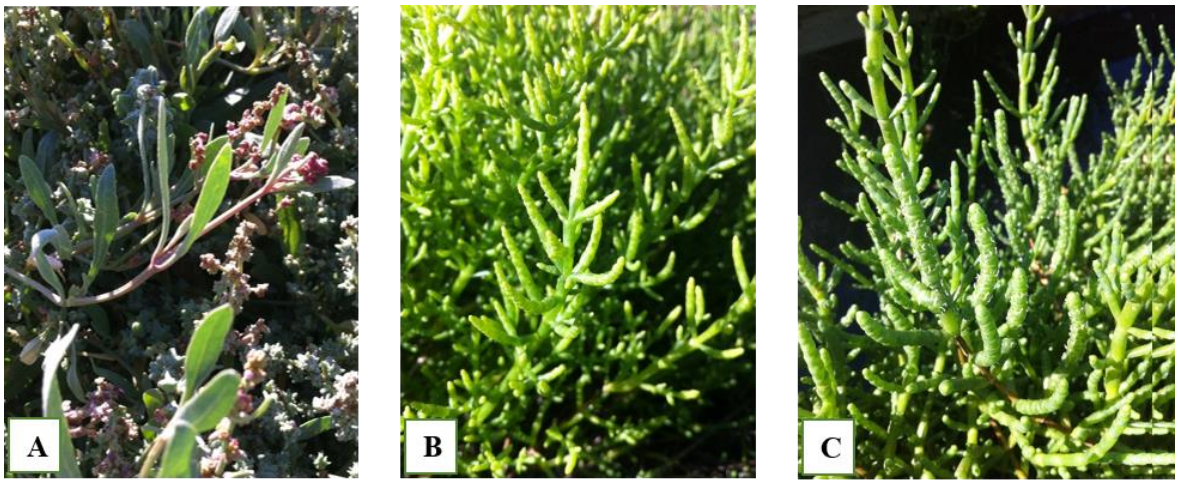


Figure 5.1 - Halophytes cultured in aquaponics tanks supplied by the effluent from a super intensive fish farm: A) *Halimione portulacoides*; B) *Salicornia ramosissima* and C) *Sarcocornia perennis*.

5.2.2 Aquaponics system

Figure 5.2 details schematically the experimental set-up employed, showing the water flow of the organic rich effluent originating from a super-intensive marine RAS system to produce Senegalese sole (*Solea senegalensis*) to aquaponics tanks. In brief, the organic rich effluent was pumped from a settling basin to a 4 m^3 header tank coupled to a bio-block tower (to aerate and increase oxygen levels, from ~2 mg L^{-1} to 8-9 mg L^{-1}). From there the aerated effluent was allowed to flow by gravity at 180 L h^{-1} to sand filter tanks (1-2 mm grain size), each with a volume of 1 m^3 and a surface area of 1 m^2. Sand filter tanks were set-up in parallel, with each one of them being connected to an aquaponics tank. Each aquaponics tank was 6 m long by 1 m wide and 0.3 m deep. To maximize effluent retention time within each tank, 11 alternating wooden barriers were placed transversally to the flow. A full water renewal was achieved ≈ every 12 h. For this study, due to the tanks inter-variability regarding nutrient dynamics (Marques et al., 2017) one tank stocked with each of the selected halophytes was selected in order to have composite samples of the halophyte plants covering the 6 m long x 1 m wide tank (see below for further details).

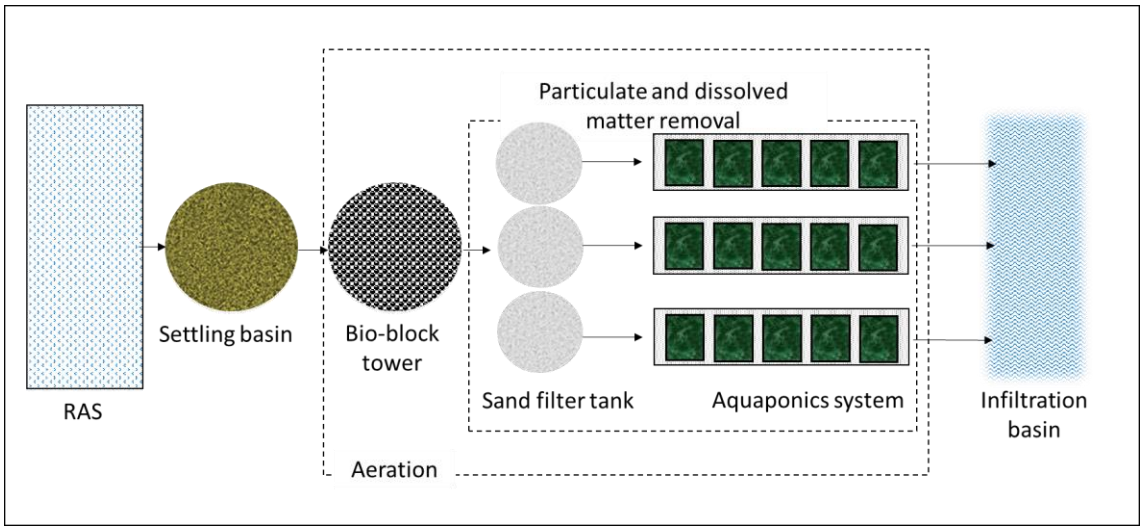


Figure 5.2 - Configuration and design of aquaponics system coupled to sand filters tanks.

5.2.3 Stocking and sampling of halophytes

The aquaponics tanks were stocked with selected halophyte species as follows: *H. portulacoides* grafts were kept in Hoagland's nutrient solution and transplanted to the aquaponics tank after new roots were recorded (Marques et al., 2017); *S. ramosissima* new shoots (circa 50 mm) were washed to remove the sediment from the rhizosphere and transplanted to the aquaponics tank; and *S. perennis* grafts were transplanted directly to the aquaponics tank. Each aquaponics tank was stocked with circa 800 plants equally distributed over 9 Styrofoam trays floating in the tank water. The aquaponics experiment was started in March, with halophytes being randomly sampled in Ria de Aveiro donor sites and each aquaponics tank during spring (May), summer (July) and autumn (October). Sampling from each aquaponics tank was performed by haphazardly selecting 2 plants from each of the 9 floating Styrofoam trays (thus assembling a composite sample of 18 specimens), with an identical number of specimens per halophyte species also being in each donor site. Each halophyte plant was separated into plant organs (edible parts and roots) and subsequently freeze-dried and stored at -80 ºC for posterior FA analysis. During the experimental period temperature, salinity and pH of the effluent was monitored in situ, using a WTW – cond 3110/set 1 equipped with TetraCon® 325 and WTW – pH 330i/set equipped with SenTix® 41. Effluent aliquots were filtered (Whatman GF/C) and analyzed for dissolved inorganic nitrogen (NOx-N) using a flow injection system (FIAstar 5000 Analyzer, Höganäs, Sweden), ammonium (NH4-N) and phosphates (PO4-P) following the standard methods in Limnologisk Metodik, 1992.

5.2.4 Fatty acids extraction and analysis

The derivatization of FA for gas chromatography (GC) analysis was performed following the methodology described by Aued-Pimentel et al. (2004) with some adaptations. Briefly, all freeze-dried samples were powdered and homogenized, being weighted accurately in a sovirel/pyrex glass tube (~50 mg of plant organs) and dissolved in 1 ml of *n*-hexane containing a FAME internal standard from FA 21:0 (heneicosanoic acid) (0.021 g L^{-1}) was added. In the same tube was added 0.2 mL of a methalonic KOH solution (2 mol L^{-1}), with the tube being sealed and mixed vigorously in a vortex shaker for 2 minutes. Following

this procedure, 2 mL of a saturated NaCl solution was added to the tube, with the mixture being centrifuged during 5 min at 3000 rpm and the organic phase separated. Afterwards, 1 mL of organic phase was transferred into a vial and the excess of solvent was evaporated with gas nitrogen. The oil obtained was dissolved in *n*-hexane (1 mL) and analyzed using a GC-FID. Separation of FA was performed using a 7890B gas chromatograph (GC) system with a flame ionization detector (FID). The detector and injector were kept at 250 ºC, with the carrier gas used being hydrogen. FA were separated in a fused-silica capillary column, DB-FFAP column (30 m x 320 µm x 0.25 µm) (Agilent 123-3232) with the following temperature programme: 75 ºC (initial), 20 ºC min^{-1} to 155 ºC (4 min), 2 ºC min^{-1} to 180 ºC (16.5 min), 4 ºC min^{-1} to 250 ºC (44 min). The advantage of this method is that it can be performed at room temperature, which reduce the risks of FA decomposition. The identification of the FA was done by matching with previously inject internal standards. The FA content (µg.mg^{-1}DW) in the samples analyzed was calculated considering the relation between mass, the area of fatty acids and the internal standard (21:0).

5.2.5 Statistical analysis

Statistical analysis regarding each halophyte species was performed using PRIMER v6 with the PERMANOVA+ add-on. A resemblance matrix using the content (µg g^{-1} DW) of each FA in each halophyte was performed using the Bray-Curtis similarity coefficient, following a log (x + 1) transformation in order to empathize the compositional differences rather than on quantitative differences (Anderson, 2008). Permutational multivariate analysis of variance (PERMANOVA) was used to assess the differences between FA profiles of wild versus cultured halophytes, considering edible parts and roots and the three sampling dates (spring, summer and autumn). Three factors were included in test design: 1) condition was introduced as a fixed factor, with cultured and wild being used as levels; 2) sampling dates were introduced as a fixed factor, with spring, summer and autumn being used as levels; 3) plant organs were introduced as a random factor nested in conditions, with edible parts and roots being used as levels. The statistical significance of multivariate variance components was tested using 9999 permutations of residuals under a reduced model, with significance level of 0.05. Therefore, a permutation analysis of multivariate dispersions (PERMDISP) was used if the PERMANOVA result showed a significant

difference (Anderson, 2017). The PERMDISP measures the distance between each individual and the group median (centroid) and evaluates the difference in the centroid distances between the groups (Anderson, 2017). A Principal Coordinates Analysis (PCO) was performed to calculate the variability in FA content considering the factors. This analysis enables to plot the inter-individual differences in FA content along the first two axes into the multidimensional space. For a detailed description of the statistical analysis described above please refer to Clarke & Gorley, 2006.

5.3 Results

During the experimental period, water temperature displayed the same trend among the three aquaponics tanks, following expected seasonal variations. Temperature ranged between 18.2 and 21.4 ºC in the tank with *H. portulacoides*, between 19.1 and 23.1ºC in the tank with *S. ramosissima* and between 18.1 and 22.3 ºC in the tank with *S. perennis*. Concerning pH, this parameter showed a higher variability between systems, ranging from 7.47 to 7.76 in the tank with *H. portulacoides*, 7.45 to 8.73 in the tank with *S. ramosissima* and 7.24 to 7.57 in the tank with *S. perennis*.

Table S 5.1, presented as supplementary material, displays the average concentration of dissolved inorganic phosphorous (DIP) and dissolved inorganic nitrogen (DIN) during the experimental period. In the head tank the average concentration of DIP was 0.32 ± 0.11 mg L^{-1} (maximum 0.50 mg L^{-1} and minimum 0.21 mg L^{-1}), while the average concentration of DIN (DIN=NOx-N+NH4-N) was 8.9 ± 1.3 mg L^{-1} (maximum 10.0 mg L^{-1} and a minimum 6.8 mg L^{-1}). The average concentration of dissolved inorganic nutrients (DIP +DIN) in both the inlets and outlets of each halophytes aquaponics tank increased over time, indicating that the concentration of dissolved inorganic nutrients from the RAS system increased during the experimental period.

The removal capacity of DIP displayed by each halophyte species was negligible, as in average DIP concentrations were higher in the outlet of the aquaponics tank than in the inlet. Concerning DIN, the removal capacity of each halophyte species was higher in the summer (circa 90%). In detail, DIN removal capacity in spring, summer and autumn, was 13%, 91% and 51% in the tank with *H. portulacoides*, 12%, 89% and 21% in the tank with *S. ramosissima*, and 52%, 98% and 60% in the tank with *S. perennis*, respectively.

The FA profiles of cultured and wild halophytes are summarized in Table S 5.2-5.4, presented as supplementary material. The most representative saturated fatty acid (SFA) in each of the three selected halophyte species, solely considering the edible parts and roots, was palmitic acid (16:0) in both cultured and wild halophytes. The average content of 16:0 in *H. portulacoides* ranged between 15-18% in the edible parts and 19-26% in the roots. Concerning *S. ramosissima*, the average content of 16:0 ranged between 17-20% in the edible parts and 22-33% in the roots. In *S. perennis*, the average content of 16:0 ranged between 18-21% in the edible parts and 16-29% in the roots. The most representative monounsaturated fatty acid (MUFA) in each of the three selected halophytes was oleic acid (18:1*n*-9) with similar concentrations between edible parts and roots. Considering the whole plant biomass, the average content of 18:1*n*-9 ranged between 6-13% for *H. portulacoides*, 1-5% for *S. ramosissima* and 2-10% for *S. perennis*. Regarding polyunsaturated fatty acids (PUFA), α-linolenic acid (ALA, 18:3*n*-3) was the most representative in the edible parts of the three-halophytes, considering cultured and wild halophytes. In detail, ALA ranged between 38-49% in *H. portulacoides*, 38-56% in *S. ramosissima* and 20-53% in *S. perennis*. On the other hand, the roots of cultured and wild halophytes exhibited linoleic acid (LA, 18:2*n*-6) as the most abundant PUFA, ranging between 36-41% for *H. portulacoides*, 27-52% for *S. ramosissima* and 34-54% for *S. perennis*. Figure 5.3 represents the relative percentage (A) and the absolute value (expressed as µg of FA per mg of dry weight) (B) of each FA class (SFA, MUFA and PUFA) in the edible parts and roots of the three-halophyte species evaluated in the present study, both cultured and wild. The PUFA/SFA ratio of edible parts ranged between 1.7-2.7, 2.9-3.2 and 2.5-2.8 for wild *H. portulacoides*, *S. ramosissima* and *S. perennis* (respectively). Concerning specimens cultured in aquaponics, the PUFA/SFA ratios displayed where commonly slightly higher, ranging between 2.7-3.3, 2.8-3.4 and 3.2-3.3 for *H. portulacoides*, *S. ramosissima* and *S. perennis*. All species, regardless of being wild or cultured in aquaponics, displayed a PUFA/SFA ratio > 0.45, the threshold value indicative of good nutritional quality.

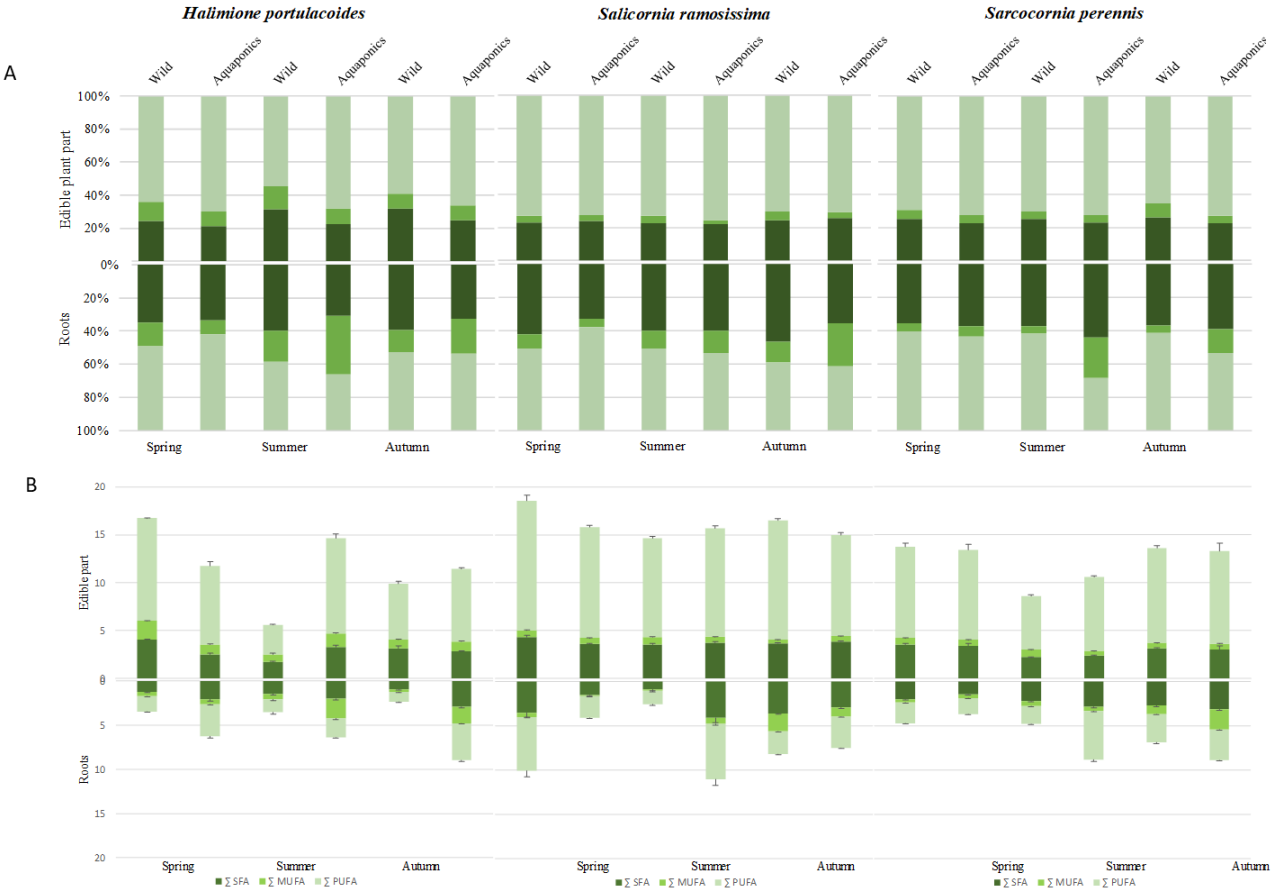


Figure 5.3 - Fatty acids (FA) class relative content (% of the total pool of fatty acids) (A) and absolute values (µg of FA per mg of dry weight) (B) in the edible parts and roots, at spring (beginning of the experiment), summer (middle of the experiment) and autumn (end of the experiment) of *H. portulacoides*, *S. ramosissima* and *S. perennis* from the wild (W) and cultured in aquaponics (A). ∑SFA – sum of saturated fatty acids; ∑MUFA – sum of monounsaturated fatty acids; ∑PUFA – sum of polyunsaturated fatty acids.

PERMANOVA performed revealed that there were no significant differences among the FA profiles for cultured and wild halophyte species (*H. portulacoides*; Pseudo-$F_{1,35}$ = 0.091; p = 0.6712; *S. ramosissima*; Pseudo-$F_{1,35}$ = 0.157; p = 1 and *S. perennis*; Pseudo-$F_{1,35}$ = 0.149; p = 1), neither between spring, summer and autumn (*H. portulacoides*; Pseudo-$F_{2,35}$ = 0.607; p = 0.6116; *S. ramosissima*; Pseudo-$F_{2,35}$ =1.374; p = 0.3459 and *S. perennis*; Pseudo-$F_{2,35}$ = 1.1176; p = 0.4249). Significant differences were recorded in the FA profiles of plant edible parts and roots of each halophyte species (*H. portulacoides*; Pseudo-$F_{2,35}$ = 9353,3; p = 0,0001; *S. ramosissima*; Pseudo-$F_{2,35}$ = 5008,3; p = 0,0001 and

S. perennis; Pseudo-$F_{2,35}$ = 4021,1; p = 0,0001). The PERMDISP test performed revealed that there was also a significant difference in the inter-individual variability displayed by plant organs (*H. portulacoides*; $F_{1,34}$ = 5.85; p = 0.017; *S. ramosissima* $F_{1,34}$ = 91.41; p = 0.001 and *S. perennis*; $F_{1,34}$ = 107.67; p = 0.001). The major part of this variability can be explained by the first two axes of the PCO performed for each halophyte species (Figure 5.4), with 98.6% of total variance being explained for *H. portulacoides*, with PCO1 axis explaining 90.6% of total variance and clearly separating edible parts from and roots without discriminating cultured from wild specimens. The same trend was displayed for *S. ramosissima*, where the two first axis of the PCO analysis explained 97.7% of total variance, with PCO1 axis explaining 82%. Regarding *S. perennis*, the first two axis of the PCO analysis explained 97% of total variance, with PCO1 axis explaining 82.4%.

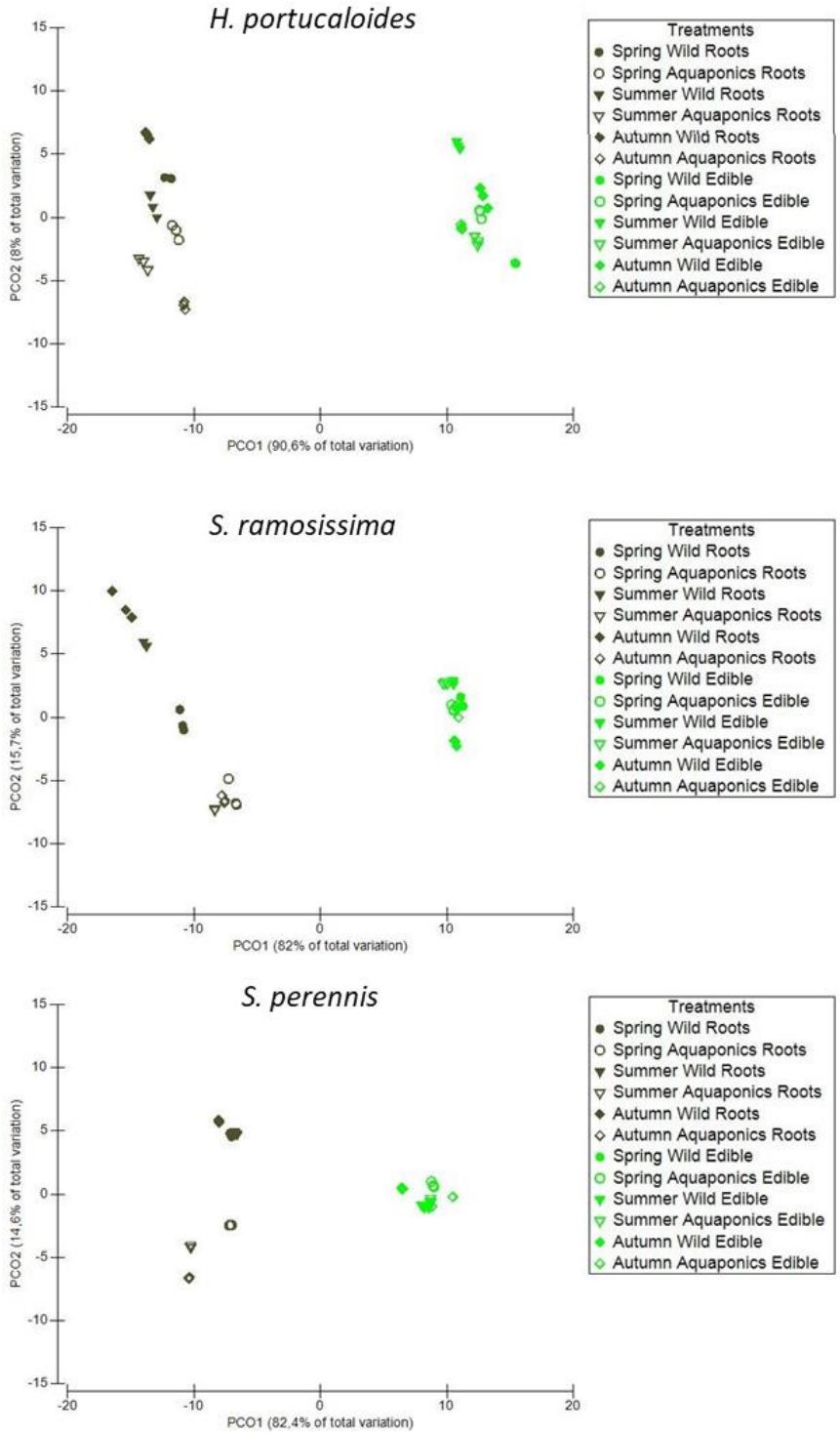


Figure 5.4 - Principal coordinates analysis (PCO) of fatty acid profiles in the edible parts and roots, at spring (beginning of the experiment), summer (middle of the experiment) and autumn (end of the experiment) of *H. portulacoides*, *S. ramosissima* and *S. perennis* from the wild and cultured in aquaponics.

5.4 Discussion

Halophytes are widely recognized to be well-adapted and thrive under saline environments, developing different morphological, anatomical, and physiological strategies (Flowers and Colmer, 2015). The added value of culturing halophytes using aquaculture effluents has already been demonstrated through irrigation (Singh et al., 2015), constructed wetlands (Buhmann and Papenbrock, 2013) and IMTA systems (Marques et al., 2017; Webb et al., 2013, 2012). While the use of halophytes in IMTA systems for nutrient uptake and remediation of aquaculture effluents has been studied by several authors (Webb et al., 2013; Waller et al., 2015, Marques et al., 2017), there is still room to enhance the economic potential of this practice if the nutritional properties of cultured specimens is shown to be at least comparable to that of conspecifics in the wild. In other words, it is paramount to show that halophytes cultured under an IMTA framework do not display a shift in their nutritional properties, namely on their FA profile (e.g., omega-3 and omega-6 FA content). Under the studied conditions, i.e., halophytes were cultured under aquaponics conditions receiving the nutrient rich from a marine RAS system, their FA profile did not differ significantly from that of wild specimens from donor sites. The FA profile of the three species, *H. portulacoides*, *S. ramosissima* and *S. perennis*, considering both wild plants and specimens cultured in aquaponics were dominated by three main FA: palmitic (16:0), LA and ALA. Both the omega-3 ALA and the omega-6 LA are known to be essential for humans (Burdge and Calder, 2005; Simopoulos, 2004), as they cannot be synthetized *de novo* and play a key role for human health (Isca et al., 2014; Ksouri et al., 2012). It is known that ALA is the main precursor of several longer chain and more unsaturated omega-3 FAs, such as EPA and DHA (Simopoulos, 2004, 1999), which are paramount for a number of role vital organ functions and intracellular activities (Ksouri et al., 2012). Linoleic acid and ARA are the most useful form of omega-6 FA for human nutrition (Amjad Khan et al., 2017; Harwood, 1996; Singh et al., 2005). According to previous studies, C18 are the most representative FAs in halophytes, with *S. ramosissima* with 39.60 % (of total FA) of ALA and 20.02 % (of total FA) of LA and *H. portulacoides* with 43.53 % (of total FA) of ALA and 14.21 % (of total FA) of LA (Maciel et al., 2018);

Salicornia persica with 48.28 % (of total FA) of ALA and 1.72% (of total FA) of LA and *Sarcocornia fructicosa* with 44.17 % (of total FA) of ALA and 1.46 % (of total FA) of LA (Ventura et al., 2011).

It was not surprising to verify that for each halophyte species studied, the FA profile of the edible parts were significantly different from that displayed by the roots, regardless of plants being wild or cultured in aquaponics. Such differences are inherent to the contrasting functional and physiological roles played by the different organs of these plants. To our best knowledge, only the study by Kaur et al. (2013) addressed FA composition in different plant organs, revealing that leaves (edible parts) of the perennial pepperweed *Lepidium latifolium* L. displayed a higher concentration of omega-3 (ALA), followed by omega-6 (LA) and palmitic acid than the plant roots. The high content of ALA in chloroplast membranes acts as protection for plants against damage during cold spells, as a process for acclimation (Sinclair et al., 2002).

Results suggests that under the cultured conditions, i.e., in aquaponics receiving a nutrient rich effluent from a marine RAS system, halophytes were not nutrient limited. When calculating the PUFA/SFA ratio, an indicator of good nutritional quality and good health status (Bertin et al., 2014), our data show that both wild and cultured *H. portulacoides*, *S. ramosissima* and *S. perennis* displayed this ratio above the threshold (0.45). Moreover, the PUFA/SFA ratio was often higher in halophytes cultured in aquaponics than in wild conspecifics, evidencing a higher level of FA unsaturation. These findings support the suitability for human consumption of *H. portulacoides*, *S. ramosissima* and *S. perennis*, cultured in aquaponics, namely in IMTA systems coupled to marine RAS system. A similar finding had also been previously reported by Bertin et al. (2014), with the halophyte *Sarcocornia ambigua* (Amaranthaceae) also exhibiting a PUFA/SFA ratio averaging 3.4 (therefore higher than the threshold value of 0.45), hence being considered suitable for human consumption.

One major advantage of aquaponic systems is their resistance and resilience against threats from soil-borne pests and diseases (Goddek et al., 2015). Beyond this advantage, the following additional benefits from halophytes cultured coupled to marine IMTA systems for human consumption, can be highlighted: i) in opposition to freshwater, saltwater is not a limited resource (Gunning et al., 2016); ii) it enables to reduce dependency on wild

specimens of halophytes, which is particularly relevant as most of them are classified under environmental regulations (e.g., EU Habitat Directive; Bern Convention on the Conservation of European Wildlife and Natural Habitats; RAMSAR convention on wetlands); iii) it is a sustainable way to improve food production (Junge et al., 2017; König et al., 2018) in line with SDG2; iv) RAS provides a continuous source of high quality nutrient-rich effluents for aquaponics (Marques et al., 2017) so no additional input is required (Singh et al., 2015); v) it promotes the recycling of nutrients, minimizing losses and environmental impacts on water bodies receiving the effluent; vi) it promotes water filtration, thus reducing the costs of wastewater treatment (Hu et al., 2015; Junge et al., 2017; and Marques et al., 2017), being in line with SDG14; and vii) it is in line with the framework for circular economy (European Commission, 2018). The production of halophytes in aquaponics coupled to a marine IMTA system can be regarded as an important way to create added value, through the retention of nutrients within the productive system and avoidance of their loss to the environment, thus achieving the objectives of Agenda 2030 for Sustainable Development (European Commission, 2018).

5.5 Conclusions

Under the culture conditions tested, i.e., aquaponics culture using the effluent water from a super-intensive marine fish farm, the FA profile of *H. portulacoides*, *S. ramosissima* and *S. perennis* did not change significantly from that displayed by conspecifics in the wild. The present study shows that halophytes cultured under IMTA conditions display a FA profile rich in omega-3 and omega-6 FA, thus holding the same potential for valorization as wild conspecifics from donor sites. The cultivation of these species for human consumption through aquaponics is therefore technically viable and can be applied to enhance food production, in line with SDG2 aims and targets, while fostering the implementation of more sustainable practices in aquaculture as advocated in SDG14 aims and targets.

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5.7 Supporting Information

Table S 5.1 - Concentration of dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorous (DIP) along the experimental period in *H. portulacoides* aquaponics tank system, *S. ramosissima* aquaponics tank system and *S. perennis* aquaponics tank system.

Halophytes	Aquaponics tank	Parameter	Spring	Summer	Autumn
H. portulacoides	Effluent inlet	DIP (mg L^{-1})	0.078 ± 0.002	0.131 ± 0.002	0.433 ± 0.002
	Effluent outlet		0.054 ± 0.007	0.461 ± 0.008	0.495 ± 0.008
	Effluent inlet	DIN (mg L^{-1})	0.481 ± 0.002	4.902 ± 0.001	8.315 ± 0.002
	Effluent outlet		0.739 ± 0.002	0.461 ± 0.001	3.455 ± 0.001
S. ramosissima	Effluent inlet	DIP (mg L^{-1})	0.090 ± 0.003	0.445 ± 0.001	0.443 ± 0.027
	Effluent outlet		0.096 ± 0.005	0.520 ± 0.006	0.513 ± 0.010
	Effluent inlet	DIN (mg L^{-1})	0.869 ± 0.002	4.736 ± 0.001	8.096 ± 0.003
	Effluent outlet		0.766 ± 0.004	0.514 ± 0.001	6.371 ± 0.003
S. perennis	Effluent inlet	DIP (mg L^{-1})	0.084 ± 0.005	0.312 ± 0.002	0.462 ± 0.015
	Effluent outlet		0.042 ± 0.001	0.380 ± 0.011	0.474 ± 0.004
	Effluent inlet	DIN (mg L^{-1})	0.754 ± 0.001	4.543 ± 0.003	8.225 ± 0.002
	Effluent outlet		0.359 ± 0.017	0.085 ± 0.001	3.250 ± 0.001

Table S 5.2 - Fatty acid profile (μg mg^{-1} DW) of wild and aquaponics *Halimione portulacoides* in edible plant part and roots biomass in spring, summer and autumn. Values are averages 3 replicates ± standard deviation. ND: fatty acid not detected. Saturated fatty acids (SFA): 14:0; 16:0; 17:0; 18:0; 20:0; 22:0; 24:0. Monounsaturated fatty acids (MUFA): 16:1*n*-7; 16:1*n*-9; 18:1*n*-9; 18:1*n*-7; 20:1*n*-7. Polyunsaturated fatty acids (PUFA): 16:3*n*-3; 18:2*n*-6; 18:3*n*-3; 20:2*n*-11.

H. portulacoides	**Wild**						**Aquaponics**					
	Edible plant part			**Root**			**Edible plant part**			**Root**		
	Spring	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**
16:0	2.94 ± 0.01	0.97 ± 0.02	1.52 ± 0.08	0.71 ± 0.00	0.69 ± 0.10	0.46 ± 0.01	1.82 ± 0.11	2.35 ± 0.10	1.81 ± 0.04	1.64 ± 0.12	1.49 ± 0.10	2.01 ± 0.07
17:0	0.04 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:0	0.21 ± 0.01	0.07 ± 0.00	0.10 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.10 ± 0.01	0.15 ± 0.01	0.12 ± 0.00	0.08 ± 0.00	0.08 ± 0.01	0.12 ± 0.01
20:0	0.11 ± 0.00	0.06 ± 0.00	0.09 ± 0.01	0.10 ± 0.00	0.10 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.12 ± 0.01
22:0	0.21 ± 0.01	0.16 ± 0.00	0.28 ± 0.03	0.22 ± 0.01	0.39 ± 0.02	0.25 ± 0.01	0.12 ± 0.00	0.20 ± 0.01	0.26 ± 0.01	0.17 ± 0.02	0.21 ± 0.02	0.38 ± 0.02
24:0	0.57 ± 0.03	0.50 ± 0.04	1.16 ± 0.10	0.13 ± 0.02	0.19 ± 0.01	0.11 ± 0.01	0.39 ± 0.02	0.51 ± 0.09	0.57 ± 0.02	0.17 ± 0.01	0.14 ± 0.01	0.28 ± 0.01
∑ SFA	**4.07 ± 0.06**	**1.77 ± 0.07**	**3.17 ± 0.22**	**1.21 ± 0.03**	**1.42 ± 0.14**	**0.94 ± 0.03**	**2.50 ± 0.14**	**3.28 ± 0.21**	**2.83 ± 0.07**	**2.I10 ± 0.15**	**1.97 ± 0.14**	**2.91 ± 0.11**
16:1*n*-7	ND	ND	ND	0.03 ± 0.00	0.08 ± 0.02	0.01 ± 0.00	ND	ND	ND	0.09 ± 0.01	1.29 ± 0.11	0.77 ± 0.02
16:1*n*-9	0.23 ± 0.00	0.05 ± 0.00	0.12 ± 0.01	ND	ND	ND	0.17 ± 0.01	0.20 ± 0.01	0.12 ± 0.00	ND	ND	ND
18:1*n*-9	1.57 ± 0.01	0.64 ± 0.13	0.67 ± 0.03	0.43 ± 0.01	0.46 ± 0.06	0.27 ± 0.01	0.78 ± 0.03	1.10 ± 0.04	0.81 ± 0.01	0.34 ± 0.02	0.45 ± 0.02	0.79 ± 0.03
18:1*n*-7	0.10 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.14 ± 0.03	0.03 ± 0.00	0.03 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.08 ± 0.01	0.50 ± 0.03	0.34 ± 0.02
20:1*n*-7	0.07 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	ND	ND	ND	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	ND	ND	ND
∑ MUFA	**1.97 ± 0.01**	**0.77 ± 0.14**	**0.88 ± 0.04**	**0.50 ± 0.01**	**0.67 ± 0.11**	**0.31 ± 0.01**	**1.04 ± 0.04**	**1.41 ± 0.06**	**1.04 ± 0.02**	**0.51 ± 0.04**	**2.25 ± 0.17**	**1.90 ± 0.07**
16:3*n*-3	0.34 ± 0.00	0.05 ± 0.00	0.12 ± 0.01	ND	ND	ND	0.40 ± 0.02	0.25 ± 0.01	0.14 ± 0.00	ND	ND	ND
18:2*n*-6	2.91 ± 0.02	0.80 ± 0.01	1.52 ± 0.07	1.43 ± 0.03	1.27 ± 0.14	0.96 ± 0.01	2.41 ± 0.13	2.46 ± 0.12	2.25 ± 0.04	3.18 ± 0.20	1.91 ± 0.09	3.42 ± 0.10
18:3*n*-3	7.32 ± 0.02	2.05 ± 0.05	4.03 ± 0.19	0.34 ± 0.01	0.21 ± 0.03	0.16 ± 0.00	5.29 ± 0.29	7.11 ± 0.33	5.10 ± 0.08	0.45 ± 0.04	0.25 ± 0.01	0.71 ± 0.02
20:2*n*-6	0.15 ± 0.00	0.15 ± 0.01	0.16 ± 0.02	ND	ND	ND	0.13 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	ND	ND	ND
∑ PUFA	**10.71 ± 0.04**	**3.06 ± 0.06**	**5.82 ± 0.29**	**1.78 ± 0.04**	**1.48 ± 0.17**	**1.12 ± 0.02**	**8.23 ± 0.44**	**9.93 ± 0.46**	**7.59 ± 0.12**	**3.63 ± 0.23**	**2.17 ± 0.10**	**4.14 ± 0.12**

Table S 5.3 - Fatty acid profile (μg mg^{-1} DW) of wild and aquaponics *Salicornia ramosissima* in edible plant part and roots biomass in spring, summer and autumn. Values are averages 3 replicates ± standard deviation. ND: fatty acid not detected. Saturated fatty acids (SFA): 14:0; 16:0; 17:0; 18:0; 20:0; 22:0; 24:0. Monounsaturated fatty acids (MUFA): 16:1*n*-7; 16:1*n*-9; 18:1*n*-9; 18:1*n*-7; 20:1*n*-7. Polyunsaturated fatty acids (PUFA): 16:3*n*-3; 18:2*n*-6; 18:3*n*-3; 20:2*n*-11.

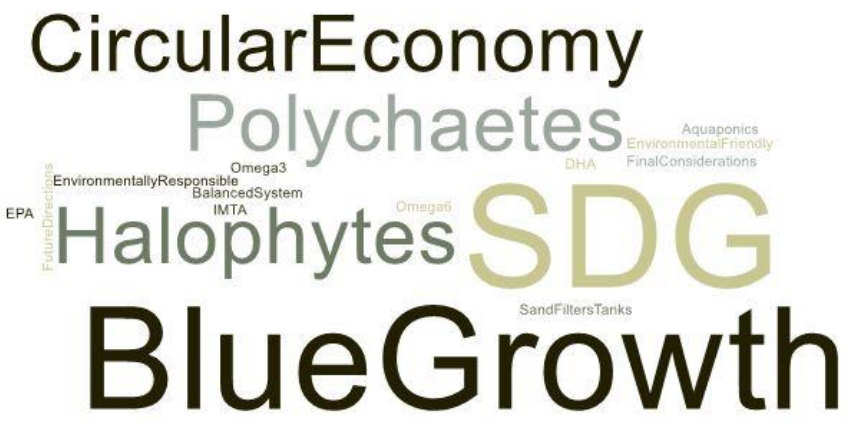
S. ramosissima	**Wild**						**Aquaponics**					
	Edible plant part			**Root**			**Edible plant part**			**Root**		
	Spring	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**
16:0	3.49 ± 0.16	2.86 ± 0.05	2.74 ± 0.07	2.68 ± 0.33	1.03 ± 0.03	0.57 ± 0.04	2.84 ± 0.09	2.77 ± 0.06	2.93 ± 0.07	2.98 ± 0.36	2.71 ± 0.00	1.94 ± 0.08
17:0	ND	0.06 ± 0.00	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:0	0.32 ± 0.01	0.26 ± 0.00	0.16 ± 0.01	0.10 ± 0.01	0.05 ± 0.00	0.03 ± 0.00	0.31 ± 0.01	0.36 ± 0.01	0.26 ± 0.01	0.13 ± 0.02	0.16 ± 0.00	0.14 ± 0.01
20:0	0.11 ± 0.01	0.06 ± 0.00	0.08 ± 0.00	ND	ND	ND	0.11 ± 0.00	0.11 ± 0.00	0.11 ± 0.00	0.16 ± 0.02	0.13 ± 0.00	0.16 ± 0.01
22:0	0.12 ± 0.01	0.16 ± 0.00	0.26 ± 0.00	0.32 ± 0.05	0.16 ± 0.00	0.13 ± 0.02	0.17 ± 0.00	0.14 ± 0.00	0.22 ± 0.01	0.30 ± 0.04	0.26 ± 0.01	0.34 ± 0.01
24:0	0.25 ± 0.01	0.26 ± 0.01	0.32 ± 0.01	0.48 ± 0.08	0.29 ± 0.01	0.23 ± 0.04	0.29 ± 0.01	0.25 ± 0.00	0.33 ± 0.01	0.56 ± 0.10	0.37 ± 0.01	0.35 ± 0.01
∑ SFA	4.28 ± 0.19	3.66 ± 0.06	3.55 ± 0.09	3.58 ± 0.47	1.53 ± 0.04	0.96 ± 0.11	3.73 ± 0.11	3.64 ± 0.07	3.84 ± 0.09	4.12 ± 0.55	3.63 ± 0.02	2.92 ± 0.12
16:1*n*-7	ND	ND	ND	0.06 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	ND	ND	ND	0.22 ± 0.03	1.11 ± 0.03	0.34 ± 0.02
16:1*n*-9	0.29 ± 0.01	0.36 ± 0.01	0.21 ± 0.00	ND	ND	ND	0.29 ± 0.01	0.12 ± 0.00	0.16 ± 0.01	ND	ND	ND
18:1*n*-9	0.38 ± 0.02	0.26 ± 0.00	0.39 ± 0.01	0.32 ± 0.04	0.12 ± 0.00	0.08 ± 0.01	0.24 ± 0.00	0.25 ± 0.00	0.33 ± 0.01	0.19 ± 0.03	0.27 ± 0.00	0.43 ± 0.02
18:1*n*-7	0.05 ± 0.00	0.06 ± 0.00	0.11 ± 0.00	0.08 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.06 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.26 ± 0.04	0.63 ± 0.01	0.29 ± 0.01
20:1*n*-7	0.03 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	ND	ND	ND	0.02 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	ND	ND	ND
∑ **MUFA**	0.76 ± 0.03	0.66 ± 0.01	0.78 ± 0.01	0.47 ± 0.06	0.18 ± 0.00	0.13 ± 0.02	0.61 ± 0.01	0.43 ± 0.01	0.56 ± 0.02	0.67 ± 0.10	2.01 ± 0.04	1.06 ± 0.04
16:3*n*-3	ND	0.06 ± 0.00	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:2*n*-6	4.31 ± 0.21	3.66 ± 0.06	4.82 ± 0.11	5.28 ± 0.65	2.09 ± 0.04	1.28 ± 0.09	4.28 ± 0.10	3.26 ± 0.05	4.84 ± 0.10	5.24 ± 0.58	2.21 ± 0.03	2.85 ± 0.10
18:3*n*-3	9.18 ± 0.38	7.96 ± 0.13	5.41 ± 0.12	0.75 ± 0.09	0.30 ± 0.01	0.25 ± 0.02	7.02 ± 0.16	9.19 ± 0.15	5.66 ± 0.18	1.03 ± 0.12	0.39 ± 0.01	0.66 ± 0.02
20:2*n*-6	0.03 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	ND	ND	ND	0.04 ± 0.00	0.03 ± 0.00	0.07 ± 0.00	ND	ND	ND
∑ **PUFA**	13.53 ± 0.59	11.6 ± 0.19	10.31 ± 0.22	6.02 ± 0.74	2.39 ± 0.05	1.54 ± 0.11	11.34 ± 0.26	12.47 ± 0.20	10.57 ± 0.28	6.27 ± 0.70	2.60 ± 0.03	3.51 ± 0.12

Table S 5.4 - Fatty acid profile (μg mg^{-1} DW) of wild and aquaponics *Sarcocornia perennis* in edible plant part and roots biomass in spring, summer and autumn. Values are averages 3 replicates ± standard deviation. ND: fatty acid not detected. Saturated fatty acids (SFA): 14:0; 16:0; 17:0; 18:0; 20:0; 22:0; 24:0. Monounsaturated fatty acids (MUFA): 16:1*n*-7; 16:1*n*-9; 18:1*n*-9; 18:1*n*-7; 20:1*n*-7. Polyunsaturated fatty acids (PUFA): 16:3*n*-3; 18:2*n*-6; 18:3*n*-3; 20:2*n*-11.

S. perennis	**Wild**						**Aquaponics**					
	Edible plant part			**Root**			**Edible plant part**			**Root**		
	Spring	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**
16:0	2.83 ± 0.13	2.75 ± 0.17	1.54 ± 0.02	0.90 ± 0.01	0.72 ± 0.01	0.76 ± 0.03	1.97 ± 0.05	2.60 ± 0.07	2.53 ± 0.26	2.15 ± 0.07	2.02 ± 0.05	2.40 ± 0.06
18:0	0.18 ± 0.02	0.19 ± 0.02	0.18 ± 0.00	0.07 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.14 ± 0.00	0.15 ± 0.01	0.14 ± 0.02	0.10 ± 0.00	0.11 ± 0.00	0.19 ± 0.01
20:0	0.08 ± 0.01	0.09 ± 0.01	0.07 ± 0.00	0.19 ± 0.00	0.14 ± 0.01	0.20 ± 0.01	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.02	0.13 ± 0.01	0.13 ± 0.00	0.15 ± 0.00
22:0	0.15 ± 0.01	0.15 ± 0.02	0.20 ± 0.00	0.36 ± 0.01	0.26 ± 0.01	0.47 ± 0.02	0.08 ± 0.00	0.13 ± 0.00	0.12 ± 0.04	0.19 ± 0.01	0.23 ± 0.01	0.23 ± 0.01
24:0	0.24 ± 0.02	0.23 ± 0.03	0.23 ± 0.01	0.49 ± 0.05	0.32 ± 0.02	0.74 ± 0.04	0.13 ± 0.00	0.16 ± 0.00	0.15 ± 0.05	0.31 ± 0.02	0.29 ± 0.01	0.22 ± 0.01
∑ SFA	3.48 ± 0.18	3.41 ± 0.25	2.22 ± 0.04	2.02 ± 0.08	1.48 ± 0.05	2.23 ± 0.11	2.37 ± 0.06	3.10 ± 0.09	2.99 ± 0.39	2.88 ± 0.11	2.77 ± 0.08	3.18 ± 0.09
16:1*n*-7	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.04 ± 0.00	0.34 ± 0.01	1.28 ± 0.03
16:1*n*-9	0.24 ± 0.01	0.26 ± 0.01	0.10 ± 0.00	ND	ND	ND	0.09 ± 0.00	0.15 ± 0.00	0.22 ± 0.02	ND	ND	ND
18:1*n*-9	0.48 ± 0.02	0.34 ± 0.02	0.66 ± 0.03	0.34 ± 0.01	0.36 ± 0.00	0.51 ± 0.02	0.43 ± 0.01	0.46 ± 0.01	0.37 ± 0.05	0.35 ± 0.01	0.34 ± 0.01	0.49 ± 0.01
18:1*n*-7	ND	ND	ND	0.06 ± 0.00	0.04 ± 0.00	0.08 ± 0.00	ND	ND	ND	0.05 ± 0.00	0.26 ± 0.01	0.52 ± 0.01
∑ MUFA	0.73 ± 0.03	0.60 ± 0.04	0.76 ± 0.03	0.40 ± 0.01	0.40 ± 0.01	0.59 ± 0.02	0.52 ± 0.01	0.61 ± 0.02	0.59 ± 0.07	0.45 ± 0.02	0.93 ± 0.03	2.29 ± 0.05
18:2*n*-6	3.32 ± 0.13	3.21 ± 0.21	3.61 ± 0.11	1.97 ± 0.04	1.49 ± 0.02	1.62 ± 0.06	2.17 ± 0.05	2.69 ± 0.08	3.06 ± 0.31	4.75 ± 0.17	2.81 ± 0.09	3.03 ± 0.07
18:3*n*-3	6.17 ± 0.27	6.19 ± 0.33	1.96 ± 0.02	0.37 ± 0.01	0.34 ± 0.00	0.34 ± 0.01	5.49 ± 0.09	7.20 ± 0.18	6.64 ± 0.50	0.75 ± 0.03	0.40 ± 0.01	0.42 ± 0.01
∑ PUFA	9.49 ± 0.40	9.40 ± 0.53	5.57 ± 0.13	2.34 ± 0.05	1.82 ± 0.02	1.96 ± 0.07	7.66 ± 0.13	9.89 ± 0.26	9.70 ± 0.81	5.50 ± 0.20	3.22 ± 0.10	3.45 ± 0.08

Chapter 6

Final Considerations and Future Perspectives



6. Final considerations and future perspectives

Coastal wetlands provide important ecosystem services that are underpinned by functions and processes mediated by living organisms. Both ragworms and salt marsh halophytes (salt-tolerant plants) are recognized as *ecosystem engineers* due to their ability to alter the surrounding physical environment. Namely, ragworms are omnivorous scavengers that are able to include in their diet organic-rich particulate matter and detritus. In addition, ragworms promote sediment reworking through bioturbation and bioirrigation, whilst halophytes promote water flow attenuation and enhanced the settling of organic-rich suspended matter.

The Integrated Multi-Trophic Aquaculture (IMTA) system developed in the scope of this study represents an innovative valuable approach towards the development of a sustainable aquaculture industry. The system has shown to be environmentally responsible by using organisms from different trophic levels. From an economic point of view, it can be considered as having the potential for being a profitable system and likewise to support the creation of new jobs in coastal regions through smart specialization.

To perform a successful IMTA systems it is necessary to optimize the flow rates of effluents, as well as the density of *H. diversicolor* in sand filters and the growth performance of *H. portulacoides* cultured in aquaponics tanks. The implementation of this "*environmental friendly*" system combining polychaetes and halophytes proved to be efficient by reducing particulate organic matter and dissolved inorganic matter of the effluent generated by a super intensive fish farm operating a land-based RAS. This balanced system minimizes environmental impacts, reduces the "*ecological footprint*" and coastal impacts. Moreover, the exploitation of the marine aquaculture effluent can be a profitable practice through the production of marketable products with low or negligible associated costs. For example, polychaetes displayed (unlike wild conspecifics) a significant content of docosahexaenoic acid (DHA, 22:6*n*-3), showing the ability to retain high value nutrients (e.g. HUFA in general, particularly EPA, and DHA) from fish feeds that would otherwise be lost from the production environment. Additionally, the polychaetes cultured in sand filters and subjected to HPP treatment can be used to replace or reduce fish meal or fish oil in formulated feeds without compromising their nutritional

value. The halophytes cultured in aquaponics systems show a remarkable ability to retain high value nutrients such as omega-3 and omega-6 FA. Indicators of good nutritional quality, i.e. PUFA/SFA ratios, proved that the halophytes fulfill the prerequisites to be used for food consumption.

The IMTA concept developed in this study is an evident contribution for a development of responsible aquaculture practices, integrating economics and environmental issues. It tackles overexploitation of natural resources, by reducing the dependency on wild specimens, together with a promotion of circular economy, through the reutilization of nutrients. Approaches like this enable industries to increase their profitability without exhausting natural resources. This circular economy activity allows cost-effectiveness by reusing capital and enlarging the life cycle of otherwise finished products, while contributing for the United Nations Sustainable Development Goal 14 (SDG14 – "*life below water*") for 2030.

The blue growth is an initiative with a sustainable basis, balancing economic growth, social development and sustainable exploitation of aquatic resources through low ecological imprint. This work is a contribution for this sustainable growth, but further questions and challenges still need to be addressed, namely:

- Development of tools and methods to help aquaculture industry overcome, environmental, socio-economic and legislative constraints to achieve more efficient environmental and economical practices.
- Development of protocols to ensure the quality and safety of IMTA products to be included in local markets and avoid the spread of diseases.
- In a more socio-economical approach, perform a cost-efficiency evaluation on the production of IMTA products, in partnership with fish farmers, local markets and consumers.
- To increase the scientific knowledge, it is important to: 1) include more extractive species such as: mussels (filter feeders), sea urchins (deposit feeders), and kelps (seaweeds) in IMTA systems; 2) perform lab-scale experiments to study the life cycle of extractive species when supplied with effluent water from a marine farm; and 3) test multiple configurations of the system to improve IMTA systems

efficiency, for example, testing vertical configurations to minimize the implementation footprint.