Universidade de Aveiro
2022

Daniel Baptista Jerónimo

RECUPERAÇÃO DE NUTRIENTES DE EFLUENTES DE AQUACULTURA ATRAVÉS DO USO INTEGRADO DE FILTROS DE POLIQUETAS E HALÓFITAS EM AQUAPONIA

RECOVERING NUTRIENTS FROM AQUACULTURE EFFLUENTS THROUGH THE INTEGRATED USE OF POLYCHAETE FILTERS AND HALOPHYTES IN AQUAPONICS

Universidade de Aveiro
2022

Daniel Baptista Jerónimo

RECUPERAÇÃO DE NUTRIENTES DE EFLUENTES DE AQUACULTURA ATRAVÉS DO USO INTEGRADO DE FILTROS DE POLIQUETAS E HALÓFITAS EM AQUAPONIA

RECOVERING NUTRIENTS FROM AQUACULTURE EFFLUENTS THROUGH THE INTEGRATED USE OF POLYCHAETE FILTERS AND HALOPHYTES IN AQUAPONICS

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

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através de uma bolsa de doutoramento atribuída a Daniel Baptista Jerónimo (PD/BD/127989/2016)

“This aquaculture practice (IMTA) is based on a very simple principle: ‘the solution to nutrification is not dilution, but extraction and conversion through diversification”

Thierry Chopin and co-authors (2012)

o júri

Presidente

Prof. Doutor Carlos Manuel Martins da Costa
Professor Catedrático da Universidade de Aveiro

Vogais

Prof. Doutora Luísa Maria Pinheiro Valente
Professora Associada com Agregação da Universidade do Porto

Prof. Doutor Henrique José de Barros Brito Queiroga
Professor Associado com Agregação da Universidade de Aveiro

Doutora Luísa Margarida Batista Custódio
Investigadora Principal da Universidade do Algarve

Doutor Tiago Filipe Baptista da Rosa Repolho
Investigador Júnior da Universidade de Lisboa

Doutor Ricardo Jorge Guerra Calado
Investigador Principal com Agregação da Universidade de Aveiro

Acknowledgement

À minha esposa e filha que são a minha essência e foram o meu grande apoio durante estes 5 anos. Muito obrigado pela vossa paciência e desculpem todas as horas que estive ausente.

Aos meus pais e irmãos que são as minhas raízes e onde vou sempre carregar baterias.

Aos meus orientadores, Ricardo Calado, Ana Lillebø e Javier Cremades pela orientação científica, disponibilidade e confiança.

Ao Ricardo Calado e Ana Lillebø pela amizade, pela força e motivação que sempre me transmitiram nos momentos mais difíceis e pela enorme ajuda na análise de dados e correção científica.

Ao Javier Cremades por ter-me acolhido de braços abertos no seu laboratório e me ter dado a conhecer uma cidade fantástica, como A CORUÑA. À Erika Garcia e Eduardo Casais por toda a amizade e ajuda durante este período.

À Professora Rosário Domingues, Beta Maciel e Felisa Rey pela amizade e por me terem orientado no Departamento de Química da Universidade de Aveiro.

Ao Marco por ter sido um grande companheiro de estudo, de viagem e principalmente de amizade.

À Daniela Rodrigues, Luísa Marques e Bruna Marques pela amizade, ajuda e pelas palavras de incentivo e força durante todo este percurso.

A todos os amigos da grande família MBA.

Ao Departamento de Biologia, Departamento de Química e ao ECOMARE.

A todos os amigos que fiz ao longo desta jornada académica (um enorme abraço apertadinho😊).

palavras-chave **Filtros de areia com poliquetas; Halófitas em aquaponia; Aquacultura multi-trófica integrada; Biorremediação; Biotecnologia marinha**

Resumo

A indústria da aquacultura desempenha um papel fundamental na segurança alimentar mundial, sendo os efluentes derivados destes sistemas ricos em nutrientes que não são incorporados na biomassa das espécies em produção (ex., alimento não ingerido, fezes e outros produtos de excreção). A aquacultura multi-trófica integrada (IMTA) conceptualiza a recuperação destes nutrientes em biomassa valiosa de espécies extrativas, sendo os poliquetas e as plantas halófitas, respetivamente, espécies chave para incorporarem os nutrientes presentes na matéria orgânica particulada (POM) e inorgânica dissolvida (DIM - incluindo azoto e fosforo inorgânico dissolvido, DIN e DIP, respetivamente). O presente estudo teve como objetivo avaliar a eficiência na capacidade de filtração de efluentes aquícolas, resultante da integração de poliquetas cultivadas em filtros de areia (PASFs) e de plantas halófitas produzidas em aquaponia, encontrando-se dividido em 5 estudos complementares. O primeiro trabalho teve como alvo avaliar a eficiência alcançada com PASFs (recorrendo ao poliqueta *Hediste diversicolor*) quando sujeitos a diferentes concentrações de nutrientes presentes no efluente de um sistema de produção de peixe (*Sparus aurata*) em regime semi-intensivo em tanque de terra. Neste estudo concluiu-se que o sucesso reprodutivo de *H. diversicolor* foi alcançado apenas nos PASFs que revelaram uma maior concentração de POM presente no efluente (1.5-1.8 mg L^{-1}), tendo estes contribuído para reter ≈70% desta matéria orgânica. Foi ainda possível identificar a colonização natural dos PASFs pelas espécies de poliquetas *Diopatra neapolitana*, *Sabella* cf. *pavonina* e *Terebela lapidaria*, que se adaptaram às condições de cultivo de IMTA. O segundo trabalho teve como objetivo avaliar a valorização do perfil em ácidos gordos essenciais (EFA) das quatro espécies de poliquetas referidas anteriormente, tendo estas revelado um perfil com elevada concentração de EFA *n*-3, tais como o ácido eicosapentaenóico e docosahexaenóico (≈ 1.5 – 4.8 and 1.0 – 1.1 µg mg^{-1} DW, respetivamente). O perfil de ácidos gordos (FA) de *D. neapolitana*, *S.* cf. *pavonina* e *T. lapidaria* foi descrito pela primeira vez no presente estudo. *Hediste diversicolor* e *T. lapidaria* revelaram a maior similaridade em termos de perfil de FA, exibindo ainda a maior similaridade com o alimento fornecido ao peixe produzido no sistema de IMTA. O terceiro estudo teve como intuito otimizar a janela temporal necessária para produzir uma biomassa *premium* de *H. diversicolor* enriquecida com EFA quando sujeita a dieta comercial de aquacultura (durante 10, 20 e 40 dias) e quando cultivada sob diferentes condições de temperatura (20 e 25 ºC) e salinidade (15, 20 e 25). As diferentes condições de cultivo testadas não contribuíram para alterações significativas no perfil de FA. Foi possível assistir a um aumento progressivo da concentração total de FA (70 - 90 µg mg^{-1} DW), assim como da concentração de FA *n*-3 e *n*-6 (17-19 e 13-17 µg mg^{-1} DW, respetivamente), não tendo sido atingido um *plateau*. No final do estudo, os poliquetas cultivados exibiram uma maior similaridade para com o perfil de FA da dieta comercial utilizadas do que com o perfil de FA dos espécimes iniciais/selvagens. Foi ainda possível reportar evidências da biossíntese *de novo* de alguns FA (ex., 20:3 *n*-6, 20:3 *n*-3, 20:4 *n*-3). O quarto estudo apresentado teve como finalidade avaliar a eficiência de diferentes configurações (1 único tanque de policultura com 0.3 m^2 [1T] ou 2 tanques independentes para separar níveis tróficos com 0.6 m^2 [2T]) de cultivo combinado de PASFs (recorrendo ao poliqueta *H. diversicolor* ou *Arenicola marina*) e halófitas em aquaponia (*Salicornia ramosissima*) na filtração do efluente de uma unidade de testes zootécnicos de produção de camarão (*Litopenaeus vannamei*). Pode concluir-se que as espécies extrativas quando cultivadas numa configuração 1T exibiram valores de biorremediação similares ao apresentado numa configuração 2T (redução de 74-87% POM, 56-64% de DIN e 60-65% de DIP). O poliqueta *H. diversicolor* exibiu uma produtividade considerável (≈5.000 ind. m^{-2}; 78-98 g m^{-2}), enquanto a espécie *A. marina* falhou na adaptação às condições de cultivo e exibiu sobrevivência residual (<10%). A produtividade de *S. ramosissima* obtida na configuração 1T foi aproximadamente o dobro daquela verificada para a configuração 2T (≈ 150-170 e 60-90 g FW m^{-2} biomassa edível, respetivamente). A coloração amarelada

registada nas plantas halófitas, terá muito provavelmente sido devida ao tratamento de água aplicado no sistema de recirculação (oxidação química e consequente filtração) que removeu ferro (e provavelmente outros oligoelementos), destacando assim limitações que devem ser consideradas em estudos futuros. O quinto estudo avaliou o crescimento, a biorremediação e a composição elementar da halófita *S. ramosissima* quando cultivada sob diferentes salinidades dentro da gama tolerável pela espécie (Sal.15, Sal.20 and Sal.25 ≈257, 342 and 428 mM NaCl, respetivamente) e sob diferentes concentrações de ferro (Fe) (FeDeficiency, FeNormal and FeEnriched ≈5-10, 10-30 and 250-500 µg Fe^{2+} L^{-1}, respetivamente). No ensaio de salinidade, as plantas que cresceram a Sal.20 exibiram valores de produtividade ligeiramente superiores, mas sem diferenças significativas para as outras condições de salinidade testadas (biomassa aérea edível fresca entre 23 – 30 g $plant^{-1}$). Durante os 60 dias de estudo, as plantas sob as diferentes condições de salinidade incorporaram na sua biomassa edível 50 - 63 mg de azoto (N), 4.2 - 5.5 mg de fósforo (P) e 296 - 368 mg de carbono (C). Relativamente ao ensaio para avaliar o efeito do ferro, foi possível reportar uma correlação positiva entre o crescimento e o aumento da concentração deste elemento no meio hidropónico. Foi registada uma geração de biomassa significativamente inferior para as plantas FeDeficiency relativamente àquela registada nas restantes condições testadas. O tratamento FeDeficiency afetou o perfil de pigmentos e a eficiência dos fotossistemas das plantas (com valores significativamente inferiores de clorofila e carotenoides a serem registados, assim como valores inferiores da máxima eficiência quântica do fotossistema II [Fv/Fm]). Foi verificada uma correlação positiva entre o aumento da concentração de Fe no meio hidropónico e o aumento da concentração de C, hidrogénio (H), manganês (Mn), Fe, cobre (Cu), zinco (Zn) e molibdénio (Mo) na biomassa edível da planta. Durante os 60 dias de estudo, as plantas FeEnriched incorporaram uma quantidade significativamente superior de N, P e C na sua biomassa edível (≈63, 5.5 e 369 mg $plant^{-1}$, respetivamente) do que as plantas FeDeficiency (≈28, 3.7 e 161 mg $plant^{-1}$, respetivamente). Este último estudo chama a atenção para os possíveis efeitos que podem resultar da integração destas plantas como espécies extrativas em sistemas RAS que utilizam tratamentos de água que promovem, a precipitação e eliminação oxidativa de Fe (entre outros micro e macronutrientes essenciais) (ex., ozonificação e oxidação química). Demonstrou ainda que sob condições controladas, é possível produzir vegetais marinhos enriquecidos em Fe através de uma abordagem amiga do ambiente, destacando assim o potencial das plantas halófitas para IMTA. Os resultados aqui reportados constituem informação relevante para o desenvolvimento de uma indústria aquícola sustentável, sendo uma contribuição positiva para a circularidade, produção e valorização de biomassa marinha, estando alinhada com os Objetivos de Desenvolvimento Sustentável propostos pelas Nações Unidas.

keywords **Polychaete assisted sand filters; Halophytes in aquaponics; Integrated multi-trophic aquaculture; Bioremediation; Marine biotechnology**

abstract

The aquaculture industry plays a key role in world food security and the effluents from these production systems are rich in nutrients that have not been incorporated into biomass of fed species being farmed (these nutrients are present in the form of uneaten feed, faeces and other excretion products). Integrated multi-trophic aquaculture (IMTA) conceptualizes the recovery of these nutrients into valuable biomass of extractive species, with polychaetes and halophyte plants, respectively, being key species to recover nutrients present in the form of particulate organic matter (POM) and dissolved inorganic matter (DIM - includes dissolved inorganic nitrogen and phosphorus, DIN and DIP, respectively). This study aimed to evaluate the efficiency from integrating polychaete assisted sand filters (PASFs) and halophyte plants produced in aquaponics in the filtration of aquaculture effluents, being divided into 5 complementary studies. The first study aimed to evaluate the efficiency achieved with PASFs (using *Hediste diversicolor*) when under contrasting concentrations of unused nutrients present in the effluent water of a semi-intensive fish farming system (*Sparus aurata*) using earthen ponds. It was possible to conclude that the reproductive success of *H. diversicolor* was achieved only in the two PASFs receiving effluent water with a higher concentration of POM (1.5-1.8 mg L^{-1}), with PASFs contributing to retain ≈ 70% of available POM. The larvae of polychaetes *Diopatra neapolitana*, *Sabella* cf. *pavonina* and *Terebella lapidaria* naturally colonized the PASFs employed in this study and adapted to IMTA culture conditions. The second study evaluated the valorisation, in terms of essential fatty acids (EFA), of the biomass of the four above-mentioned polychaete species. Their fatty acid (FA) profile revealed to be enriched with *n*-3 EFA, such as eicosapentaenoic and docosahexaenoic acids (≈ 1.5 - 4.8 and 1.0 - 1.1 µg mg^{-1} DW, respectively). The FA profile of *D. neapolitana*, *S.* cf. *pavonina* and *T. lapidaria* was described for the first time in this study. The FA profiles of *H. diversicolor* and *T. lapidaria* revealed the highest level of similarity to that of aquafeed provided to fish farmed in this IMTA design. The third study aimed to optimize the timeframe to produce a premium biomass of *H. diversicolor* enriched with EFA when supplied a commercial aquafeed (during 10, 20 and 40 days) and grown under different combinations of temperature (20 and 25 ºC) and salinity (15, 20 and 25). Here, the different culture conditions did not contribute to significantly modify the FA profile of *H. diversicolor*. Total FA concentration (70 - 90 µg mg^{-1} DW) and *n*-3 and *n*-6 FA concentration (17-19 and 13-17 µg mg^{-1} DW, respectively) incremented progressively, and no plateau was achieved. At the end of the study period, polychaetes exhibited a FA profile with greatest similarity to the one displayed by the aquafeeds than to the ones exhibited by initially stocked/wild polychaetes. It was also possible to report evidence of *de novo* FA biosynthesis (e.g., 20:3 *n*-6, 20:3 *n*-3, 20:4 *n*-3). The fourth study evaluated the efficiency of different IMTA configurations (1 single polyculture tank with 0.3 m^2 [1T] or 2 tanks to separate trophic levels with 0.6 m^2 [2T]) of combined culture of PASFs (using *H. diversicolor* or *Arenicola marina*) and halophyte plants produced in aquaponics (*Salicornia ramosissima*) using effluent water of a facility performing zootechnical trials using shrimp (*Litopenaeus vannamei*).It was concluded that extractive species when cultured in 1T design exhibited similar bioremediation values than those achieved under 2T design (reduction of 74-87% POM, 56-64% DIN and 60-65% of DIP). Considerable productivities were obtained for the polychaete *H. diversicolor* (≈5,000 ind. m^{-2}; 78-98 g m^{-2}), while *A. marina* failed to adapt to culture conditions and exhibited a low survival (<10%). The productivity of *S. ramosissima* obtained in 1T design was approximately twice the one achieved under 2T design (≈ 150-170 and 60-90 g FW m^{-2} edible biomass, respectively). The yellowish coloration exhibited by halophyte plants, was most likely due to the water treatment applied in the RAS system (chemical oxidation and consequent filtration) which removed iron (and likely other oligoelements), thus highlighting limitations that should be considered in future studies. The fifth study evaluated the growth, bioremediation and elemental composition of the halophyte *S. ramosissima* when cultured under different salinities within the species tolerance range (Sal.15, Sal.20 and Sal.25

≈257, 342 and 428 mM NaCl, respectively) and under different concentrations of iron (Fe) (FeDeficiency, FeNormal and FeEnriched ≈5 - 10, 10 - 30 and 250 - 500 µg Fe^{2+} L^{-1}, respectively). In the salinity experiment, plants grown under Sal.20 exhibited slightly higher productivities, but with no significant differences for the other salinity conditions tested (edible aboveground biomass between 23 – 30 g FW $plant^{-1}$). During the 60-days trial, plants from the different salinity treatments incorporated in its edible biomass 50 - 63 mg of nitrogen (N), 4.2 - 5.5 mg of phosphorus (P) and 296 - 368 mg of carbon (C). Concerning the iron effect experiment, a positive correlation was recorded between the growth and the increment of the concentration of this element in hydroponic media. FeDeficiency plants generated a significantly lower biomass than the plants cultured under the other conditions tested. FeDeficiency treatment affected the pigment profile and photosystems efficiency of plants (with significantly lower values of chlorophyll and carotenoids being recorded, as well as lower maximum quantum efficiency of photosystem II [Fv/Fm]). A positive correlation between the increment of Fe concentration in the hydroponic media and the increment of C, hydrogen (H), manganese (Mn), Fe, cooper (Cu), zinc (Zn) and molybdenum (Mo) in plant edible biomass was recorded. During the 60 days trial, FeEnriched plants incorporated a significantly higher amount of N, P and C into edible biomass (≈63, 5.5 and 369 mg $plant^{-1}$, respectively) than FeDeficiency plants (≈28, 3.7 and 161 mg $plant^{-1}$, respectively). This last study draws attention to the possible effects that may result from the integration of these plants as extractive species in RAS that use water treatments that promote the precipitation and oxidative elimination of Fe (among other essential micro and macronutrients) (e.g., ozonation and chemical oxidation). It further demonstrates that under controlled conditions, it is possible to produce Fe enriched salty vegetables using an environmentally friendly approach, and therefore highlighting the potential of halophyte plants for integrated multi-trophic aquaculture (IMTA). Results here reported are a relevant contribution to foster the development of a more sustainable aquaculture industry, with emphasis on circularity, production, and valorisation of marine biomass, in line with United Nations Sustainable Development Goals.

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Acronym List

AdA	Adrenic Acid
AFDW	Ash Free Dry Weight
ALA	Alpha-Linolenic Acid
ARA	Arachidonic Acid
DGLA	Dihomo-Gamma-Linoleic Acid
DGR	Daily Growth Rate
DHA	Docosahexaenoic Acid
DIN	Dissolved Inorganic Nitrogen
DIP	Dissolved Inorganic Phosphorus
DO	Dissolved Oxygen
DOM	Dissolved Organic Matter
DON	Dissolved Organic Nitrogen
DOP	Dissolved Organic Phosphorus
DPA	Docosapentaenic Acid
EFA	Essential Fatty Acids
EPA	Eicosapentaenoic Acid
ETA	Eicosatetraenoic Acid
ETE	Eicosatrienoic Acid
FA	Fatty Acid
FAME	Fatty Acids Methyl Esters
FR	Feeding Rate
GC-MS	Gas Chromatography-Mass Spectromet
HUFA	Highly Unsaturated Fatty Acids
IMTA	Integrated Multi-Trophic Aquacultur
LA	Linoleic Acid
LOI	Loss On Ignition
MUFA	Monounsaturated Fatty Acids
N	Nitrogen
OM	Organic Matter
P	Phosphorus
PASFs	Polychaete Assisted Sand Filters
POM	Particulate Organic Matter

PUFA	Polyunsaturated Fatty Acid
RAS	Recirculating Aquaculture System
SDG´s	Sustainable Development Goal´s
SFA	Saturated Fatty Acids
SGR	Specific Growth Rate
SPM	Suspended Particulate Matter
TP	Total Phosphorus
TN	Total Nitrogen

List of Fatty Acid Nomenclature

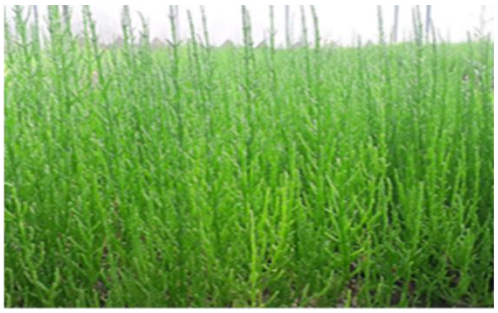
Class	Shorthand formula	Chain length	Name referred throughout the work
Saturated fatty acid	**14:0**	14	Myristic acid
	15:0	15	Pentadecylic acid
	16:0	16	Palmitic acid
	17:0	17	Margaric acid
	18:0	18	Stearic acid
	20:0	20	Arachidic acid
	21:0	21	Heneicosanoic acid
	22:0	22	Behenic acid
M**onounsaturated fatty acid**	**16:1 *n*-9**	16	7-hexadecenoate
	16:1 *n*-7	16	Palmitoleic acid
	16:1 *n*-5	16	11-hexadecenoate
	18:1 *n*-14	18	l 4-octadecenoate
	18:1 *n*-9	18	Oleic acid
	18:1 *n*-7	18	Vaccenic acid
	20:1 *n*-13	20	7-eicosenoate
	20:1 *n*-11	20	9-eicosenoate
	20:1 *n*-9	20	Gondoic acid
	20:1 *n*-7	20	13-eicosenoate
	22:1 *n*-11	22	11 – docosenoate acid
Polyunsaturated fatty acid	**16:3 *n*-3**	16	7,10,13 – hexadecatrienoic acid
	18:2 *n*-6 (LA)	18	Linoleic acid
	18:3 *n*-6	18	Gamma-linolenic acid
	18:3 *n*-3 (ALA)	18	Alpha-linolenic acid
	$^{\Delta 5,11}$ 20:2	20	5,11 - eicosadienoate
	$^{\Delta 5,13}$ 20:2	20	5,13-eicosadienoate
	20:2 *n*-6	20	eicosadienoic acid
	$^{\Delta 8,11}$ 20:2	20	8,11-eicosadienoate
	20:3 *n*-6 (DGLA)	20	Dihoma-gamma-linoleic acid
	20:3 *n*-3 (ETE)	20	Eicosatrienoic acid
	$^{\Delta 7,13}$ 22:2	22	7,13 – docosadienoate acid
	$^{\Delta 5,13}$ 22:2	22	5,13 – docosadienoate acid
	$^{\Delta 7,13,16}$ 22:3	22	7,13,16 – docosatrienoate acid

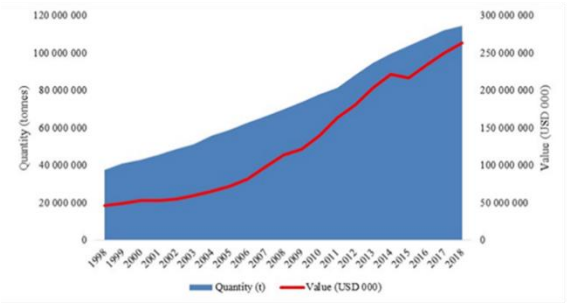
Continued…

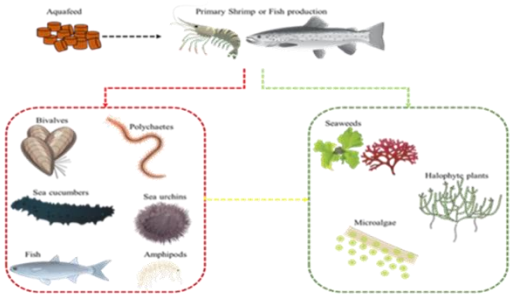
Highly unsaturated fatty acid	**18:4 *n*-3**	18	Stearidonic acid
	20:4 *n*-6 (ARA)	20	Arachidonic acid
	20:4 *n*-3 (ETA)	20	Eicosatetraenoic acid
	20:5 *n*-3 (EPA)	20	Eicosapentaenoic acid
	22:4 *n*-6 (AdA)	22	Adrenic acid
	22:5 *n*-3 (DPA)	22	Docosapentaenoic acid
	22:6 *n*-3 (DHA)	22	Docosahexaenoic acid

Chapter 1

1.1. General Introduction







1.1. General Introduction

1.1.1. The Role of Aquaculture for world food security

According to the latest statistics on aquaculture compiled by the Food and Agriculture Organization (FAO), world aquaculture production attained another all-time record high at 114.5 million tonnes in live weight being produced in 2018 (USD 263.6 billion)[1]. In the last 20 years, aquaculture production has increased by about 3 and 5 times in terms of quantity and value produced (Fig. 1.1).

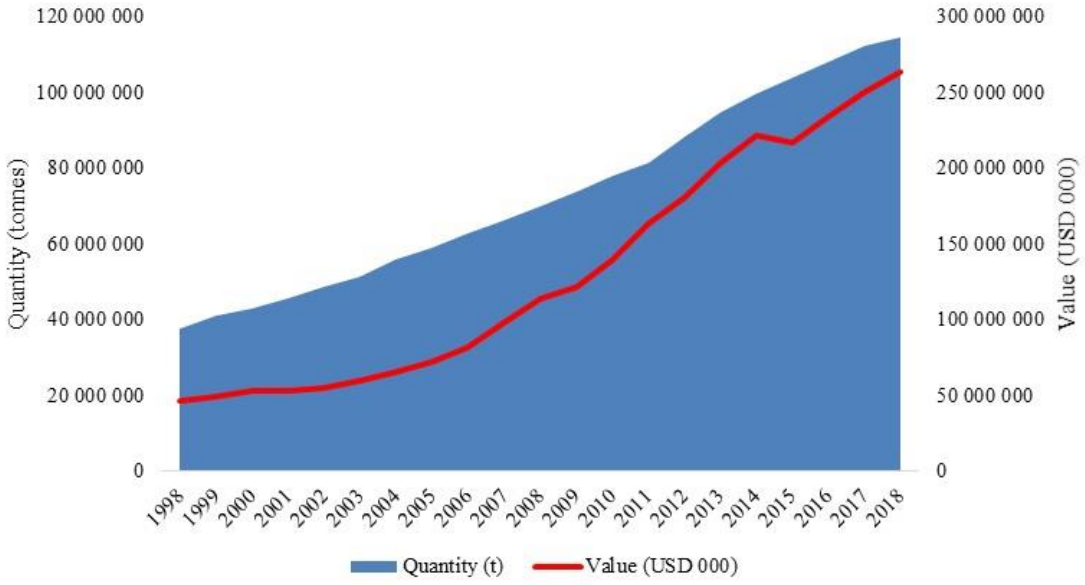


Figure 1.1. Evolution of biomass (tonnes) and value (USD 000) generated by aquaculture industry in the last 20 years (https://www.fao.org/fishery/statistics/global-aquaculture-production/query/en)[2].

Historically, aquaculture was defined in the eighties by the FAO (FAO, 1988)[3] 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*."

Total production in 2018 consisted of 82.1 million tonnes of aquatic animals (USD 250.1 billion), 32.4 million tonnes of aquatic plants (mostly macroalgae or seaweeds) (USD 13.3

billion) and 26 000 tonnes of ornamental seashells and pearls (USD 179 000)[1,2]. For total aquatic plants production, accounted mostly the red and brown seaweeds (54.9 and 44.8% of total production, respectively) with green seaweeds and other miscellaneous plants accounted to less than 1%[2]. Here it is also important to elucidate that 99.8% of this production was achieved under the saline environment (brackish and marine waters), which has considerable expression at world level but residual at European level (51% and 0.2% of marine aquaculture production, respectively)[2]. In Europe there is a predominance of production of carnivorous species (mainly finfish) and for the growth of the aquaculture sector in this continent following the SDGs, it becomes quite clear to us that it will be imperative to work on the development of production models for primary/autotrophic species (e.g., seaweeds, microalgae, halophyte plants) which form the basis of food chain and are very important to supplement human and animal nutrition.

For aquatic animals', production accounted mostly for finfish, followed by molluscs, crustaceans, and other organisms (such as several marine invertebrates, turtles and frogs) (Fig. 1.2). Here it is also important to consider that approximately 13% of the total production of finfish and 98% of molluscs were produced under a saline environment (brackish and marine waters), while crustaceans and other organisms accounted for 61 and 42%, respectively[1,2].

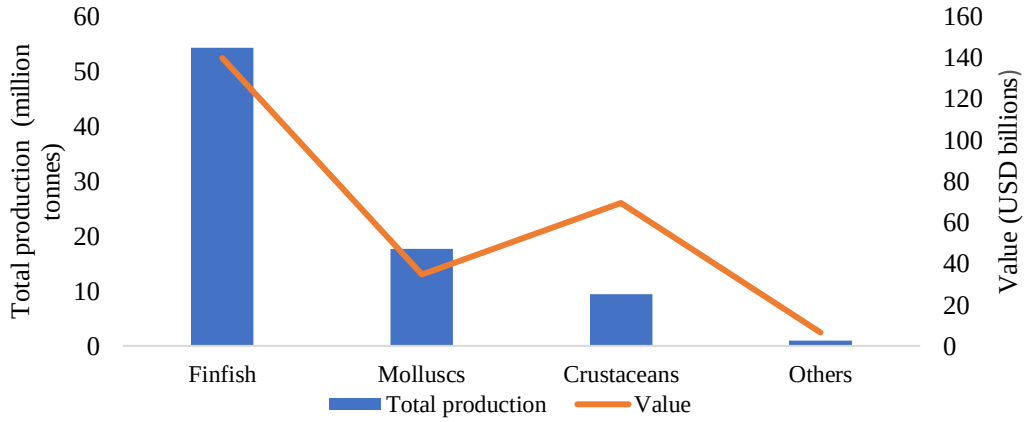


Figure 1.2. Characterization of aquatic animals' production in 2018 (https://www.fao.org/fishery/statistics/global-aquaculture-production/query/en)[2].

The contribution of world aquaculture to global fish production (including finfish, crustaceans, molluscs, and others) reached 46.0% in 2018 (total of about 179 million tonnes;

USD 401 billion)[1,2]. The production rates of this sector have been growing at 7.5% since 1970 (farmed aquatic animals grew by an average of 5.3% year^{-1} between 2001 - 2018), while captures through fisheries have remained stable, as shown by the annual values reported in the last 20 years that varied between 80.0 – 84.4 million tonnes (includes finfish, crustaceans, molluscs and other animals)[1]. It is also important to note that a growing percentage of fish stocks are being captured at biologically unsustainable levels (34.2% of assessed stocks)[1]. It is under this scenario that aquaculture will increasingly be the main driver to supply aquatic animals to a growing population. The current world population of more than 7.4 billion is projected to reach 9.7 billion by 2050[4]. Under this scenario and considering that nowadays 820 million people are undernourished globally, hunger and malnutrition are already considered the world´s most devastating concerns[5,6]. The dietary contribution of fish and fish co-products is paramount in terms of high-quality animal proteins, polyunsaturated and highly unsaturated fatty acids (PUFA and HUFA, respectively) and micronutrients for diversified and healthy diets. Our needs in essential fatty acids (EFA) are due to limitations that vertebrate species (including humans and most marine teleost species) exhibit in the *de novo* synthesis of these molecules due to the lack of desaturases (Δ12 and Δ15) that allow to produce PUFA from oleic acid (18:1 *n*−9); consequently, their inclusion in aquafeeds is essential[7-10]. In that way, a balanced profile of EFA must be included in formulated aquafeeds to satisfy the needs of cultured species, but especially so that these at the end of a productive cycle exhibit an optimal profile for human nutrition[7,9,10]. The growth and development of the aquaculture industry allowed to continue to fulfil a relevant part of human needs in these EFA (e.g., *n*-3 HUFA such as eicosapentaenoic [20:5 *n*-3; EPA] and docosahexaenoic [22:6 *n*-3; DHA] among others). Nowadays it is acknowledged that a dose of 500 mg/day per person of the above-mentioned *n*-3 HUFA is recommended to reduce the risk of cardiovascular disease[7,11-13], and that based on this dose alone there is a global need of approximately 0.4 million metric tonnes of these EFA per year[7]. Besides EFA, fish are also a valuable source of essential amino acids, vitamins (particularly A, B and D) and minerals, such as iron, calcium, zinc and selenium. Global food fish consumption increased at an average annual rate of 3.1% from 1961 to 2017, a rate almost twice that of annual world population growth (1.6 %) for the same period. Food fish consumption per capita grew from 9.0 kg (live weight equivalent) in 1961 to 20.5 kg in 2018, at about 1.5 % per year[12].

1.1.2. The evolution of fed aquaculture and the need to produce alternative raw materials to those traditionally used

Fed aquaculture production has outpaced that of the non-fed subsector in world aquaculture (57 vs 25 million tonnes in 2018, respectively)[1]. This trend is largely due to the production of carnivorous fish (e.g., Atlantic salmon, European seabass, and gilthead seabream) and several crustacean species (e.g., whiteleg shrimp and giant tiger prawn). The total use of aquafeeds estimated for 2016 alone was approximately 49.6 million tonnes, being expected to rise to 76.2 million tonnes by 2025[14]. As already mentioned above, the aquaculture of marine carnivorous species depends on diets rich in *n*-3 HUFA, which are secured through well-balanced aquafeed formulations that commonly contain fishmeal and fish oil, two increasingly scarcer and costly ingredients for aquafeeds[1,15-18]. Figure 1.3 displays the production of fishmeal and fish oil over the last twenty years (from 2001 and 2020), being unlikely that these production trends will increase in the future[1]. While in recent years, a growing percentage of fish meal is derived from fish processing co-products (estimated at 25–35%), it is important to have in mind that these raw materials (e.g., muscle, fishbone, gills, guts, head, liver, skin) display different nutritional composition than that achieved from the whole fish[1,19].

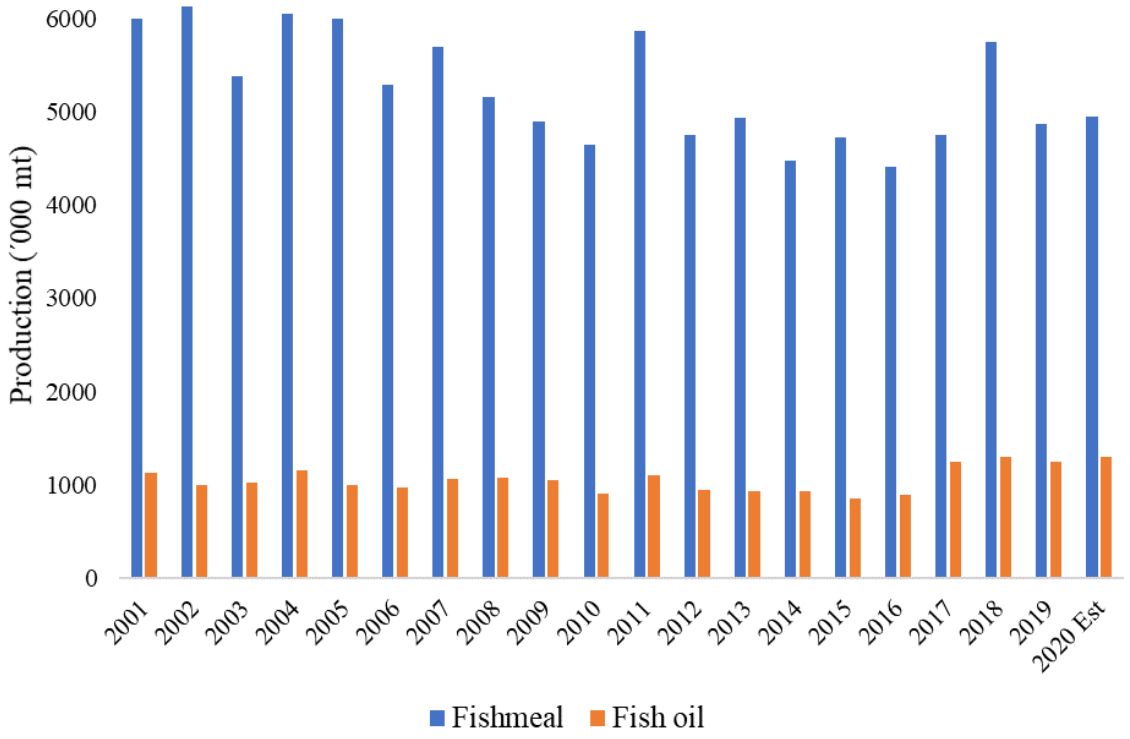


Figure 1.3. Production (´000 mt) of fishmeal and fish oil in the period 2001-2020. Values for 2020 were estimated. Adapted from EUMOFA (2021)[20].

In 2018, the Marine Ingredients Organization (IFFO) estimated that about 75% of all fish oil and fishmeal produced globally is used for aquafeeds formulation[21]. Their inclusion rates in aquafeeds have shown a clear downward trend, largely because of supply and price variation, coupled with a continuously increasing demand from the aquaculture industry[1]. These key ingredients are increasingly used selectively at specific stages of production, such as during hatchery production, broodstock maturation and finishing diets during grow-out; their share in grow-out diets is significantly decreasing (e.g., for Atlantic salmon, these ingredients accounted for 90% of the whole composition in 1990, 30% in 2010 and less than 10% nowadays[22,23]). Currently is still settled that to guarantee a high growth efficiency of carnivorous species it will always be necessary to include in aquafeeds composition some percentage of this marine limited resources (fishmeal and fish oil) or other equivalent raw material[1,15-17]. The importance of this inclusion is linked to the fact that these ingredients continue to be the most nutritious and most digestible ones for farmed fish, as well as their major source of *n*-3 HUFA (e.g., EPA and DHA)[1,17]. The level of inclusion in aquafeed

formulas has been optimized to provide these nutrients to cultivated species, but also so that at the end of the production cycle they present a nutritional profile rich in *n*-3 HUFA to supplement human diets[7]. The expected growth of aquaculture production worldwide will require efficient aquafeeds formulation and, therefore, a huge demand for both traditional and alternative marine origin raw materials is anticipated. It is also important to bear in mind that currently there is an increasing trend to address marine origin raw materials for the development of products to directly suppress issues related with human nutrition (e.g., nowadays values above 20% of fish oil production is processed for direct human consumption[24]). Nowadays it is estimated that an additional amount of >100 million tonnes protein and oils will be needed to serve the expected growth in aquaculture sector; these are predicted to come from currently unused species: Krill and mesopelagic organisms (20 million tonnes, but on a longer timescale), algae including seaweeds (>50 million tonnes), and a better use of discards and processing waste (30 million tonnes)[25]. Regarding alternative raw materials, in recent years, animal by-product meals (e.g., meat, bone and blood meals), cultured organism's meals (e.g., insect and worm meal), oilseed meals (e.g., soybean, rapeseed and cottonseed), cereal meals (e.g., maize and wheat) and seaweed meals (mainly from green and red seaweeds) have all been considered to replace fish meal, while microalgae, Antarctic krill and oilseed crops have been considered as substitutes of fish oil[24, 26-28]. However, it is important to acknowledge that the production of biomass for animal nutrition using resources that are already depleted for direct human nutrition should be avoided. The production of alternative raw materials must be therefore performed, whenever possible, using rather unexplored resources (e.g., soils affected by salinity and effluents from aquaculture industry). It is under this scenario that the adoption of more sustainable practices, such as integrated multi-trophic aquaculture (IMTA), may allow to recycle nutrients from farmed species productive environment that would otherwise be wasted via effluents. These may be incorporated into valuable extractive species biomass and acquire a primordial relevance on the pathway towards sustainability. This approach, supports a more efficient use of aquafeed and its ingredients (with emphasis on fish meal and fish oil), thus contributing to alleviate the growing pressure that the aquafeed industry continues to exert on marine based ingredients originating from fisheries. If one focus on the extractive species considered in the present thesis, the culture of polychaetes such as *Hediste diversicolor* and halophyte plants such as *Salicornia ramosissima* under IMTA conditions should be

prioritized, as both allow to successfully recover unused nutrients into valuable biomass. This biomass can subsequently be used to supplement human and/or animal nutrition.

1.1.3. Why Integrated Multi -Trophic Aquaculture (IMTA) should be a priority?

Integrated multi-trophic aquaculture consists in the farming, in proximity, of aquaculture species from different trophic levels and with complementary ecosystem functions, in such a way that allows one species uneaten feed and wastes, nutrients and by products to be reused and converted in fertilizer, feed and energy for other crops, and as such take advantage of synergistic interspecific interactions[29-32]. Figure 1.4 displays some examples of organisms from different trophic levels already employed in IMTA designs to recover particulate and dissolved nutrients resulting from the production of farmed marine fish/crustaceans. By adopting IMTA one can contribute to increase the economic (i.e., increase the productivity per unit input) and environmental performances of the aquaculture industry[30,33]. This concept aims to mimic the natural interactions that occur between different species for an industrial production. Despite this concept being already studied for over 30 years, its adoption at a commercial scale has fallen short of expectations. At the origin of this failure to transfer IMTA to an industrial/commercial scale may be some the following limitations: 1) complex licensing processes; 2) limited areas for aquaculture activity; 3) producers decision to use the available area for the production of fed species rather than extractive species that sometimes present lower commercial value; 4) increasing the complexity of the production system; 5) the need to have work teams specialized in the production of different species being used in IMTA; and 6) contrasting growing conditions required by the different species being produced[33]. Integrated multi-trophic aquaculture allows to put the aquaculture industry on a path of sustainability and in line with the principles of circularity advocated by the modern paradigms of a blue bioeconomy; overall IMTA can contribute to achieve some of the Sustainable Development Goals (SDG´s) defined for 2030 by the United Nations. Table 1.1 summarizes the contributions that IMTA can provide to achieve some of these SDG´s. The adoption of IMTA is, above all, a social responsibility that should be considered by all decision makers, stakeholders and community.

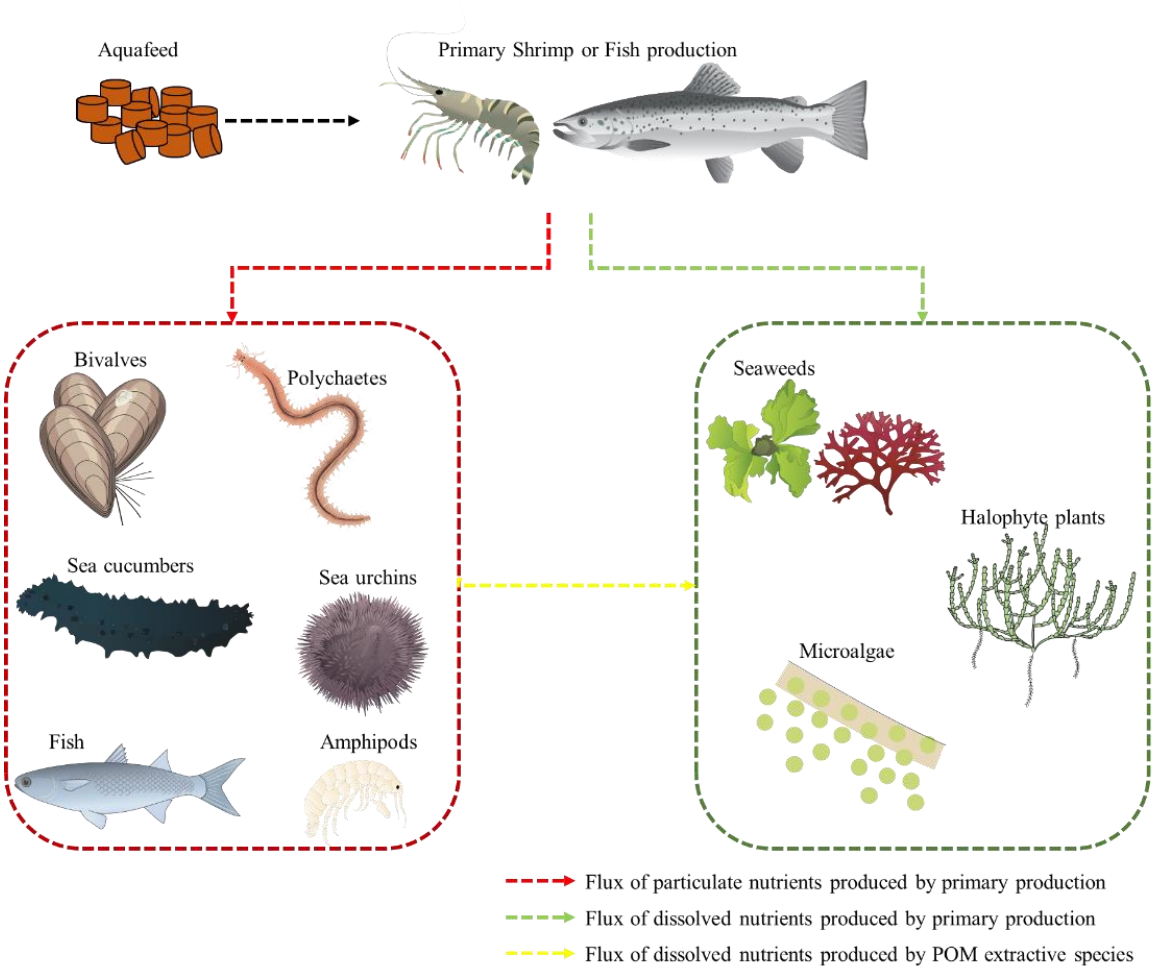


Figure 1.4. Species from different trophic levels already included in integrated multi-trophic aquaculture (IMTA) designs to recover particulate and dissolved nutrients. Image credits: shrimp, fish – salmon (Jane Hawkey); bivalves, polychaetes, fish - Flathead Mullet, halophyte plant (Dieter Tracey); sea cucumbers, sea urchins, seaweeds (Tracey Saxby); Microalgae (Diana Kleine) (Integration and Application Network, University of Maryland Center for Environmental Science [ian.umces.edu/imagelibrary/])

Table 1.1. Summary of the contribution of integrated multi-trophic aquaculture (IMTA) to achieve specific targets within Sustainable Development Goals (SDG´s) defined by the United Nations for the current decade.

SDG´s	Contribution of IMTA to accomplish SDG´s specific targets
	**2.4:** Ensure sustainable food production systems and implement resilient agricultural practices that increase productivity and production, that help maintain ecosystems (2030). **2.5:** Maintenance of the genetic diversity of seeds, cultivated plants and farmed and domesticated animals (through hatcheries and nursery systems) (2020).
	**6.6:** Protection and restoration of water-related ecosystems including mountains, forests, wetlands, rivers, aquifers and lakes (2020).
	**12.2:** Sustainable management and efficient use of natural (marine) resources (2030). **12.5:** Substantial reduction in waste generation through prevention, reduction, recycling and reuse (2030).
	**14.1:** Prevent and significantly reduce marine pollution of all kinds, in particular from land-based activities, including marine debris and nutrient pollution (aquaculture industry) (2025). **14.2:** Sustainable management and protection of marine and coastal ecosystems to avoid significant adverse impacts (2020). **14.A:** Enhance scientific knowledge, develop research capacity and transfer marine technology. **14.C:** Enhance the conservation and sustainable use of oceans and their resources.

1.1.4. The role of polychaetes and halophyte plants to foster marine land based IMTA

To better frame the state of the art concerning the use of polychaetes and halophyte plants in marine land-based IMTA designs, a systematic review was performed. In this review all studies which aimed to evaluate the efficiencies of marine extractive species integrated in commercial or experimental land based IMTA designs were considered (see research procedure and criteria used to select studies in the schematic representations in Fig. S1.1 and Table S1.1, respectively). In supplementary tables S1.2-S1.7, all works selected in this review are organized in chronological order and by group of extractive species. Polychaetes were included in 11% of all studies analysed after applying the selection criteria (total of 128 studies selected). The value of the global harvesting of polychaetes in 2016 (approx. 121,000 tonnes) is comparable to that of several most important fisheries at world level[34]. Besides polychaetes, bivalves and echinoderms (included in 14 and 5% of studies, respectively) and fish and crustaceans (included in 3 and 1% of studies, respectively) were the other taxonomic groups considered as most interesting to recover unused nutrients from POM into biomass of extractive species (Fig. 1.5). It was also possible to verify that halophyte plants were also present in 11% of all studies performed out on the recovery of dissolved inorganic nutrients, with seaweeds and microalgae being the other groups of extractive species also being explored (present in 46% and 9% of the studies performed, respectively) (Fig. 1.5). The analysis of all studies surveyed allowed to conclude that only 14% of them employed in the same IMTA design species belonging to different trophic levels, thus aiming to simultaneously recover particulate and dissolved nutrients that remain in the productive environment and will otherwise be wasted to the surrounding environment (Fig. 1.6).

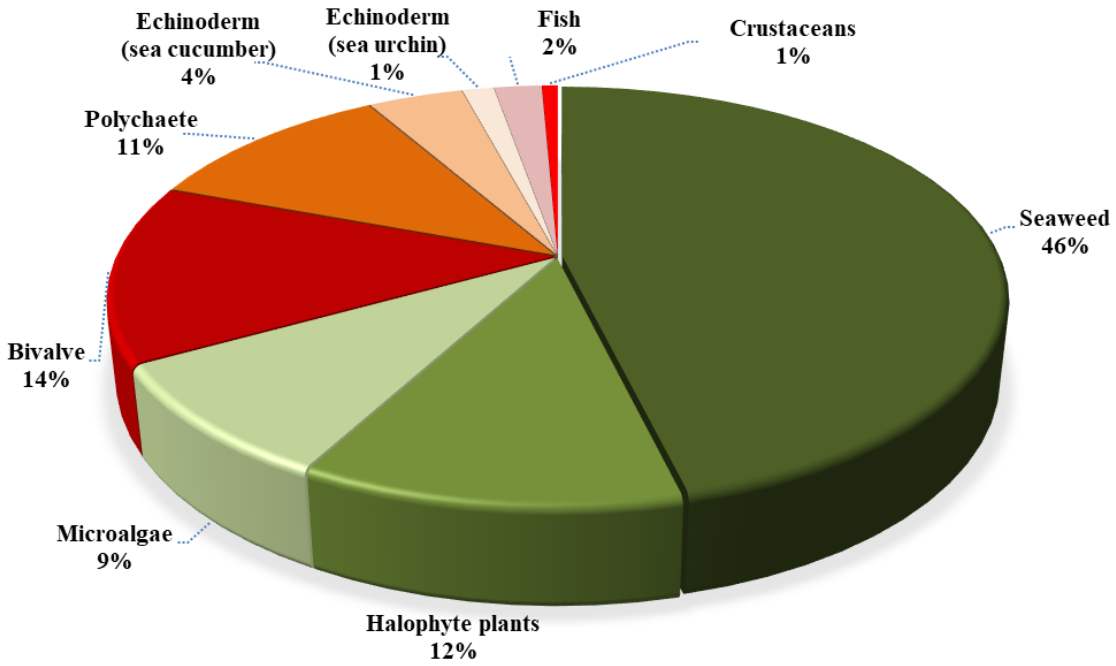


Figure 1.5. Percentage of each of the trophic groups included in lab, pilot or commercial scale marine land-based IMTA designs

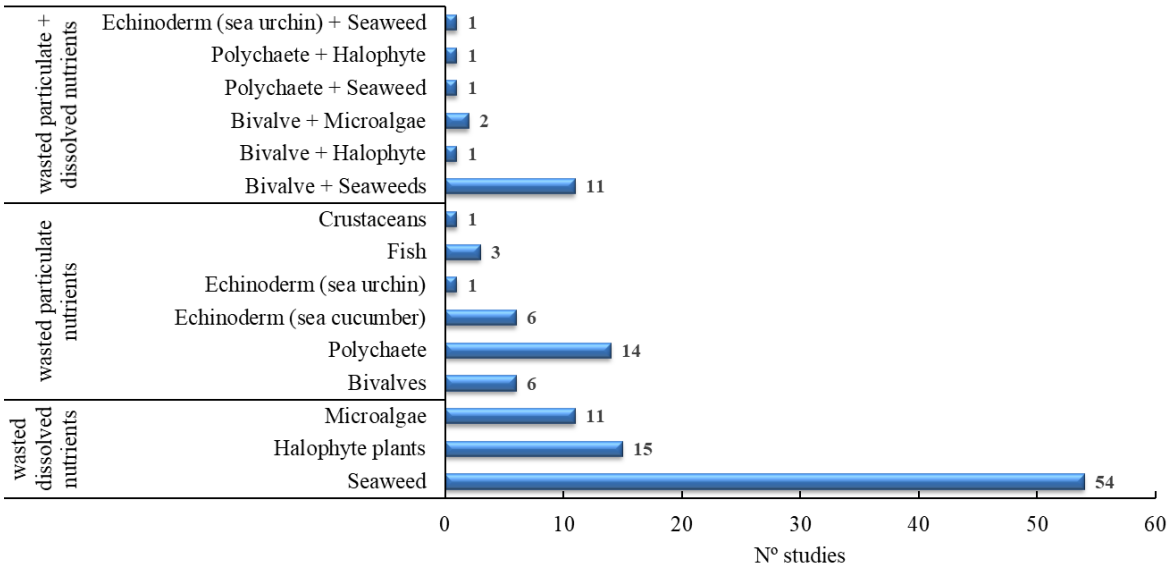


Figure 1.6. Studies performed under lab, pilot or commercial scale marine land-based IMTA designs for each trophic group considered.

The development of production models for polychaetes allows to fulfil the growing demand for these marine invertebrates and contribute to somehow avoid the over-exploitation of their natural stocks and the multitude of negative environmental impacts associated with their capture from the wild[35-39].

The polychaete *Hediste diversicolor* O.F. Müller, 1776 (Fig. 1.7), popularly known as ragworm, is one of the most well-represented marine worms in IMTA designs (included in approx. 40% of all designs including polychaetes – Table 1.2). This polychaete exhibits a wide distribution along the shallow marine and brackish waters of European and North American estuaries, being an infaunal species that produces a three-dimensional burrow network in sandy mud bottoms[40]. This species is classified as presenting free movement via its burrow system and as a biodiffusor in sediment reworking, thus featuring an important role in bioturbation (i.e., the biogenic transport of sediment particles and pore water which destroys sediment stratigraphy[41]) and bioirrigation (i.e., the ventilation of burrows and diffusion of oxidized solutes by infauna[41,42]). This biogenic modification of sediments through particle reworking and burrow ventilation, is a key mediating process of many important geochemical processes in marine ecosystems[43]. This polychaete species is omnivorous, being classified as an active predator[44]. However, it also exhibits a deposit-feeder behaviour that allows it to mainly consume organic matter present in the unconsolidated substrate[45,46]. The two main feeding strategies it displays are crawling on the sediment surface prospecting for food, catching it with its jaws and ingesting it immediately, as well as capturing food with mucous secretions that are deposited at the entrance of its burrow[45]. Bacteriolytic activity in their digestive tract demonstrates that this species is a significant bacteriovore as well[47]. Juveniles can accumulate plant detritus in their burrow where constant irrigation holds aerobic conditions that favour the decay process of plant debris by stimulating bacterial growth[48]. Ragworms can also be facultative filter-feeders, which meet metabolic requirements on a pure diet of phytoplankton, much like a typical obligate filter-feeder species[49,50]. Its life cycle is characterized by females brooding their embryos in the maternal burrow, where a short pelagic larval life takes place[45]. Its environmental engineering behaviour and biomass rich in EFA makes them an appealing extractive species for IMTA designs.

Figure 1.7. Image of the polychaete *Hediste diversicolor* used in the studies performed within this thesis. **Photo credits Daniel Jerónimo**

Table 1.2. List of polychaete species considered in lab, pilot or commercial scale integrated multi-trophic aquaculture (IMTA) designs.

Polychaetes	Tested IMTA design
Abarenicola pusilla [51,52]	*Polychaete assisted sand filters (PASFs)*
Branchiomma luctuosum [53]	
Capitella sp.[54]	
Hediste diversicolor[8,55-61]	
Alitta virens[62]	
Ophryotrocha craigsmithi[54]	
Perinereis aibuhitensis[63]	
Perinereis helleri[64]	
Perinereis nuntia[64]	
Perinereis vallata [65]	
Sabella spallanzanii [53,66,67]	

Alitta virens cited as *Nereis virens; Perinereis vallata* cited as *Perinereis nuntia vallata*

Polychaete assisted sand filters (PASFs), the most common reference of polychaete filters, allow to combine the high retention efficiency of POM promoted by the substrate with the subsequent incorporation of available nutrients into valuable worm biomass. Bioturbation and bioirrigation promoted by polychaetes are essential to maintain these filters

operational and ensure the maintenance of water percolation through the substrate housing polychaetes burrows. The treatment of aquaculture effluents at flow rates of approximately 4300 L m^{-2} d^{-1} (continuous flow) has been reported for PASFs stocked with *H. diversicolor*, with retentions above 70% of inflowing POM being achieved[56]. *Hediste diversicolor* has adapted to IMTA conditions by exhibiting excellent growth and productivity performances when fed only on unused nutrients derived from aquaculture facilities (i.e., ingesting faeces, uneaten aquafeeds and bacterial biofilms). Specific growth rates between 2 and 6% have been previously reported[55]. In works performed during longer periods, it was possible to record the occurrence of reproduction, with the biomass being harvested corresponding to a newly generated population of polychaetes with final densities being approximately 18-times higher than that of initially stocked specimens[56]. So far there are no studies that relate the reproductive success of *H. diversicolor* with the nutrient load present in the aquaculture effluents filtered by PASFs, neither as been documented the efficiency of integrating these filters in aquaculture systems more exposed to natural conditions, such as semi-intensive aquaculture performed in earthen ponds.

Polychaete assisted sand filters can integrate IMTA designs, which may also include subsequent biofilters stocked with extractive species from different trophic levels (e.g., seaweeds, microalgae, halophyte plants), thus contributing to a complete IMTA design that allow to simultaneously recover particulate and dissolved nutrients from aquaculture effluents. Production systems with these features, were rarely assessed to date, although some examples can be referenced, such as the combined use of PASFs and halophyte plants in aquaponics (*H. diversicolor* and *Halimione portulacoides*, respectively)[56], as well as the combined culture of polychaetes and seaweed or microalgae (*H. diversicolor*, *Solieria chordalis* or *Nannochloropsis* sp., respectively)[55,68]. In these studies, extractive species from different trophic groups were cultured in separate tanks, thus contributing to the increment of the operational area required to implement such IMTA designs. The operational footprint is often identified as one of the greatest limitations of IMTA[33], being therefore essential to evaluate if and how can production per unit area optimized. For example, one may study the efficiency of culturing in the same tank species from different trophic levels with a complementary bioremediation action. Biofilters with vegetable crops (e.g., microalgae, seaweed, and halophyte plants) will benefit if they receive the effluent water previously filtered by PASFs, since most POM that can be harmful to these crops has already been

removed. Besides this advantage, the bioturbation and bio-irrigation activity promoted by polychaetes present in sand filters can enhance the mineralization of POM, thus contributing to increment the concentration of dissolved forms in the effluent waters of PASFs (as already reported for dissolved inorganic phosphorus)[56].

The polychaete *H. diversicolor* is one of the most valued polychaete species used as bait for sports fishing[36,37,39,69]. The potential market value to produce this marine worm in PASFs under IMTA conditions (final productivities: 7000 ind. m^{-2} – 2300 g fresh weight biomass) was evaluated in approximately 90 € m^{-2} (if sold as live bait)[56]. When cultured under IMTA conditions it has the ability to recover incorporate valuable nutrients, such as unused *n*-3 HUFA (e.g., EPA and DHA)[8,58,61,70,71]. This fact is of great relevance if we consider the great demand for biomass rich in lipids and fatty acids (namely *n*-3 HUFA) for both human and animal nutrition[8]. From the studies performed to date which characterized the fatty acid profile of *H. diversicolor*, a large variability in the total pool of FA has been reported, with values ranging between 50 and 280 µg mg^{-1} DW for specimens tested with commercial aquafeeds[58,59,71-73] or between 24 and 110 µg mg^{-1} DW for specimens tested with aquaculture effluents[8,58,59,61,70,72]. Also, the proportion of *n*-3 and *n*-6 FA reported to date for this species is highly variable, with the above-mentioned works reporting values ranging between 5 to 33% for *n*-3 FA and 9 to 27% to *n*-6 FA. This variability is a consequence of several factors, such as the duration of experimental trials, the maturation stage of polychaetes at the beginning and during experiments, the composition of the supplied diet and the abiotic conditions experienced during culture (e.g., temperature, salinity, photoperiod…). Due to these reasons, there is still some uncertainty on how the FA biosynthesis pathways take place on this species. It is under this scenario that it is essential to develop further studies which aim to clarify the biochemical valorisation of *H. diversicolor* when cultured under an IMTA framework.

This polychaete species has already been shown to perform *de novo* biosynthesis of some EFA from acetyl coenzyme A, by using several FA desaturase and enlogase enzymes; as such, it is common to detect higher concentrations of PUFA and HUFA in ragworms biomass than on their diet[61,71,74]. It is because of presenting a biomass rich in EFA[8,57-59,61], as well as containing a considerable proportion of proteins and lipids (49-60% and 11-22% dry weight biomass, respectively[57,59]), that *H. diversicolor* is one of the most promising raw materials to integrate new premium aquafeed formulations (e.g., finishing and breeding diets). The

production of pathogens free and DHA-rich polychaetes biomass to be sold frozen (or dehydrated) and included in aquafeeds formulations must therefore be a top priority. These organisms are already known to play a central dietary role on the nutrition and production of some fish and crustacean species (e.g., soles, shrimps and crabs), being often used to trigger gonad maturation and spawning[75-80]. Also, their biomass contains essential ingredients that can act as important supplements for aquafeeds formulation, such as amino acids and other odorants that elicit a feeding response for several fish species (e.g., Senegalense sole)[58,81].

In this thesis, the efficiency of the polychaete species *Arenicola marina* Linnaeus, 1758 (Fig. 1.8), commonly known as lugworm, was also tested in PASFs. This polychaete exhibits a wide distribution in north-western European coasts, from the British Isles to the Iberian Peninsula, with its southern limit of distribution being close to 40ºN[82]. It is found from middle to lower shores and reaches high abundances in sheltered estuarine sediments where it lives in U or J-shaped burrow (0.2-0.4 m deep)[83]. In the wild, these polychaetes can reach densities between 100-150 ind. m^{-2} and tolerate salinities from 12-35[84].

Figure 1.8. Image of the polychaete *Arenicola marina* used in integrated multi-trophic aquaculture (IMTA) studies performed within this thesis. **Photo credits Daniel Jerónimo**

Adults of *A. marina* can reach between 120 to 200 mm in length, with lugworms being considered a premium bait for sea anglers[83,84]. *Arenicola marina* exhibits a completely distinct life cycle from the one described above for *H. diversicolor*, since it can reproduce several times throughout its life cycle (iteroparous species), it attains sexual maturity at 2-3

years of age, has separated sexes and displays external fertilization, with different populations releasing eggs and sperm in a synchronized period of 2 weeks that commonly runs from October to November[83,85]. Also, the bioturbation strategy differs completely to the one described for *H. diversicolor*, given that *A. marina* ingests sediment/substrate with the subsequent absorption of debris and microorganisms present in them, and after absorbing all organic content it releases its characteristics worm cast[84]. The bioturbation promoted by this species, along with a growing interest in the biotechnological use of its biomass (e.g., production of extracellular hemoglobin [HBL Hb] as a promising substitute for human blood[86] and use in solutions for organ preservation[87]) makes this polychaete species a promising candidate for IMTA. The integration of both the above-mentioned polychaete species aimed to evaluate the performance of PASFs (i.e., bioremediation and biomass generation) stocked with two species that display contrasting life cycles, distinct bioturbation strategies and biochemical profiles.

In this thesis the description of the FA profiles of *Diopatra neapolitana*, *Sabella* cf. *pavonina* and *Terebella lapidaria* was also performed for the first-time, with the larvae of these three polychaete species having naturally colonized PASFs and adapted well to IMTA culture conditions.

Regarding halophyte plants, these are naturally evolved salt-resistant plants adapted to grow in saline environments, and, in some cases, require an exposure to salinity to survive[88-91]. The development of production models for these plants came up as an answer to various interconnected needs, such as: 1) having to provide food to a growing undernourished population[5,92]; 2) the decrease in freshwater resources and increase salinized soils in many parts of the world[93-96]; 3) the valorisation of unused resources by conventional crop production[90,96-99]. According to Koyro *et al.* (2011)[100], population growth, water shortage and land degradation in arid and semi-arid regions are interlinked and jointly cause problems of poverty, social insecurity, and environmental refugee situations. Currently 70% of freshwater worldwide is consumed for irrigation, with this consumption having increased by more than 600% over the last century[100]. Nowadays, this resource is scarce in developing countries, where about 40% of the world´s population live and is currently expected that 50% of mankind may experience a scarcity of freshwater by the year 2025[100]. Of the above-mentioned facts emerged an important question that needs to be answered urgently, "How to satisfy the basic food needs of a growing world population?". It is in this scenario that the

production of valuable vegetable crops, such as halophyte plants, can play an important role, namely by targeting less explored resources (e.g., nutrient-rich water derived from aquaculture). The effluents derived from marine or brackish water aquaculture production contain valuable nutrients and halophyte plants can present a key role as extractive species of inorganic dissolved nutrients (e.g., nitrogen and phosphorous) when cultured under IMTA conditions. The almost infinite availability of saline water highlights the importance of this plants as a renewable bioresource, particularly because they do not compete with glycophytic food crops[97]. Halophytes have gained their space as new vegetable products with several applications (e.g., food, fodder, nutraceuticals, pharmaceuticals, biodiesel)[101,102]. These salt tolerant plants have also been tested under IMTA conditions, which used substrate, such as constructed wetlands and drainage lysimeters, as efficient and cost-effective systems for the purification of aquaculture effluents[103-105]. Usually, these systems are tested as an end-of-pipe unit that allow to remove with great efficiency particulate and dissolved nutrients present in effluent waters of aquaculture facilities [97,105-107]. Non-substrate dependent designs have also been tested, such as aeroponics with roots absorbing nutrients provided by aerossol, deep water culture techniques (DWC) on which roots are completely submerged in a nutrient-rich medium, or cultivated under a nutrient film technique (NFT) on which only a thin water sheet passes through the roots and supplies nutrients to the plants being farmed [89]. These two last-mentioned culture methods may be more suitable for filtering RAS processed water, with their integration into these systems offering the advantage of culturing crops in a medium with a constant supply of nutrients and stable environmental conditions. Non-substrate dependent designs allow to achieve: 1) production in areas where soil is unavailable or unsuitable; 2) a reduction of intensive labour which is inherent to traditional crop methods; 3) the conservation of water and nutrients (mainly in closed systems such as RAS); 4) an easier eradication of plant diseases (mainly in closed systems such as RAS); and 5) the possibility of operating at maximum yields[89,108].

More than 50% of all IMTA studies performed with halophyte plants included *Salicornia* s.l. (Table 1.4), with these plants being widely distributed in boreal, temperate and subtropical regions of the northern hemisphere and in South Africa[109,110]. These halophytes produce succulent shoots, which are highly appreciated in gourmet cuisine due to their salty taste, being consumed as uncooked vegetables or as pickles, and also being a source of oilseed[90,111-113]. The species tested during this work, *Salicornia ramosissima* J. Woods, 1851

(Fig. 1.9), is seen as a candidate with great potential for the development of production models based on exploitation of saline resources (e.g., use of brackish water and salinized soils). Their nutritional profile reveals high protein content (5.20 g/100 g DW), *n*-3 PUFA (mainly α-linolenic and linoleic acid) and the presence of multiple important minerals (such as sodium, potassium, calcium, magnesium, iron and manganese)[112]. *Salicornia* spp. also exhibit a significant antioxidant and anti-inflammatory potential due to their total phenolics content[102,112,114,115]. In addition, seeds of *Salicornia* spp. contain considerable levels of oil and protein (e.g., *S. bigelovii* seeds present 26-33% oil and 31% protein[116]). Oil yielding crop plants are very important for economic growth of the agriculture sector, with many of the FA identified in plant seeds being highly demanded for several industrial sectors (e.g., plastics, textile, pharmaceuticals, cosmetics)[117,118].

Figure 1.9. Image of halophyte plant *Salicornia ramosissima* used in integrated multi-trophic aquaculture (IMTA) study performed within this thesis. **Photo credits Daniel Jerónimo**

Table 1.3. Relevant examples of halophyte plants considered in lab, pilot or commercial scale integrated multi-trophic aquaculture (IMTA) designs.

Halophyte plants	IMTA design
Atriplex barclayana[103]	Drainage lysimeters
Batis maritima[119,120]	Deep water culture
Bolboschoenus maritimus[121]	Drainage lysimeters
Crithmum maritimum[122]	Deep water culture
Halimione portulacoides[56]	Deep water culture
Plantago coronopus[123]	Deep water culture
Phragmites australis[121]	Drainage lysimeters
Salicornia bigelovii[103,124]	Constructed wetlands; Drainage lysimeters
Salicornia europaea[89,105,125-127]	Aeroponics; Constructed wetland; Deep water culture
Salicornia persica[104]	Constructed wetlands
Salicornia procumbens[123]	Deep water culture
Salicornia virginica[128]	Drainage lysimeters
Sarcocornia ambigua[129,130]	Nutrient film technique
Schoenoplectus tabernaemontani[121]	Drainage lysimeters
Sesuvium portulacastrum[119,120]	Deep water culture
Suaeda esteroa[103,131]	Drainage lysimeters
Triglochin palustris[121]	Drainage lysimeters
Tripolium pannonicum[121,123]	Deep water culture; Drainage lysimeters
Typha angustifolia[121]	Drainage lysimeters

Schoenoplectus tabernaemontani cited as *Scirpus tabernaemontani; Salicornia procumbens* cited as *Salicornia dolichostachya; Tripolium pannonicum* cited as *Aster tripolium.*

To date few studies have evaluated the growth and bioremediation performances of *Salicornia* s.l. using the deep-water culture technique, also termed as raft or float systems (e.g., Waller *et al.*, 2015[123] and Chen *et al.* 2017[127]). Also, to date, have halophyte plants rarely been integrated in IMTA designs which include species belonging to other trophic groups. This approach was followed by Marques *et al.* (2017)[56] which integrated in the same IMTA design PASFs (stocked with *H. diversicolor*) and halophytes cultured in aquaponics (*Halimione portulacoides*). Also, Chen *et al.* (2017)[127] integrated in the same IMTA design a biofilter with bivalves (*Scaphara subcrenata*) and halophytes floating rafts (stocked with *Salicornia europaea*). In both studies the extractive species of both trophic levels played a key role in the simultaneous recovery of particulate and dissolved nutrients that remained unused in aquaculture effluents. As already referred, the study of IMTA designs that integrate species from different trophic levels, such as polychaetes and halophyte plants,

should be prioritised, with emphasis on the optimization of the operational area required to successfully employ these IMTA designs.

Aim and outline of the thesis

To successfully achieve the objectives of the present thesis, the work plan was divided into 5 complementary tasks, which correspond to Chapters 2 to 6.

Chapter 2 aimed to evaluate the performance of PASFs stocked with the ragworm *H. diversicolor* in different locations of an open marine land-based IMTA facility. These locations were selected to ensure that PASFs were supplied with effluent water with contrasting loads of nutrients, in order to better understand how these would limit or improve the successful production of ragworms. To achieve this goal, a first set of PASFs was installed to filter the raw fish farm effluent originating from earthen ponds stocked with gilthead seabream (*Sparus aurata*); the second set of PASFs filtered the same effluent but after it being screened by a drum filter (45-µm mesh size) and finally the third set of PASFs filtered the same effluent screened by the drum filter and subsequently by a macroalgae biofilter (stocked with sea lettuce, *Ulva rigida*).

Chapter 3 aimed to evaluate the potential valorisation of several polychaete species produced through IMTA conditions. These polychaete species were cultured in PASFs which filtered an organic rich effluent from earthen ponds used for semi-intensive finfish grow-out (*Sparus aurata*). The polychaete biomass here considered for biochemical analysis was the resulted from the study described in chapter 2 of this thesis. The fatty acid profile of IMTA cultured *H. diversicolor* were also compared with the one exhibited by wild conspecifics to evaluate any potential shift promoted by the culture conditions being tested. The FA profiles of *H. diversicolor* stocked in PASFs was also compared with that of the most representative polychaete species whose planktonic larvae successfully settled on the sand beds, namely *Diopatra neapolitana*, *Sabella* cf. *pavonina* and *Terebella lapidaria*, three polychaete species whose FA profile was described for the first-time in the present work. Finally, the FA profiles of cultured polychaete species were compared to that of the formulated aquafeed provided to the finfish farmed in the earthen ponds, in order to

determine which one of them displayed the FA profile that most closely resembled that of the aquafeed.

Chapter 4 aimed to shed some light over the evolution of the FA profile of *H. diversicolor* when fed with a commercial aquafeed (with a well-known FA composition) during 10, 20 and 40 days and under different combinations of water temperature (20 and 25 ºC) and salinity (15, 20 and 25). These are optimal abiotic conditions for the culture of ragworms, which are also within the range of temperature and salinity commonly employed by warm-temperate aquaculture systems operating with brackish water. A comparison between cultured and wild polychaetes over time was also performed to confirm that the evolution of the FA profile of cultured polychaetes being fed on aquafeeds was not influenced by natural cycles. The feeding and growth performances of *H. diversicolor* cultured under the different combinations of water temperature and salinity were evaluated over time as well.

Chapter 5 aimed to evaluate the bioremediation performance and biomass production of the combined culture of polychaetes (*Arenicola marina* or *Hediste diversicolor*) with halophytes (*Salicornia ramosissima*) using the effluent water from a RAS facility performing zootechnical trials on shrimp (*Litopenaeus vannamei*) and operating using pre-treated saline groundwater (*ca.* 20 g L^{-1} of salt). These different IMTA designs were tested using different operational footprints by culturing polychaetes and halophytes in the same tank *vs.* polychaetes and halophytes cultured in two separated tanks (with 0.3 and 0.6 m^2 of operational footprint, respectively).

Chapter 6 aimed to evaluate the growth and bioremediation performances, along with the elemental composition of the halophyte *Salicornia ramosissima* when cultured under different brackish water salinities within the species tolerance range (15, 20 and 25) and under different concentrations of iron (Fe) which mimicked a deficiency scenario promoted by aquaculture treatments (e.g., ozonation and chemical oxidation), a scenario with iron concentrations equal to natural brackish water, and a scenario in which this element was enriched (5 - 10, 10 - 30 and 250 - 500 µg Fe^{2+} L^{-1}, respectively). To shed light over these issues two independent experiments were performed simultaneously under controlled conditions: a salinity and an iron experiment.

Lastly, in **Chapter 7,** a summary, integration, and overall conclusions of results from previous chapters is provided, along with future guidelines and suggestions for further research.

1.1.5. Chapter 1 - References

1. FAO. The state of world fisheries and aquaculture: Sustainability in action. ROME (2020).
2. FAO, FAO Online Query Panels (2020). (https://www.fao.org/fishery/statistics/global-aquaculture-production/query/en)
3. FAO 1988. Definition of aquaculture. Seventh Session of the IPFC Working Party of Expects on Aquaculture, IPFC/WPA/WPZ. p. 1–3. RAPA/FAO, Bangkok
4. FAO. The State of World. Fisheries and Aquaculture 2014. Livre (2016). doi:92-5-105177-1.
5. United Nations. The future we want - Outcome document of the United Nations Conference on Sustainable Development. 41 (2012) doi:10.1126/science.202.4366.409.
6. FAO, IFAD, UNICEF, WFP, W. The state of food security and nutrition in the World. IEEE Journal of Selected Topics in Applied Earth Observations and Remote Sensing (2020).
7. Tocher, D. R. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture* **449**, 94–107 (2015).
8. Bischoff, A. A., Fink, P. & Waller, U. The fatty acid composition of *Nereis diversicolor* cultured in an integrated recirculated system: Possible implications for aquaculture. *Aquaculture* **296**, 271–276 (2009).
9. Sargent, J. R., Tocher, D. R. & Bell, J. G. 4 - The Lipids. in Fish Nutrition (Third Edition) (eds. Halver, J. E. & Hardy, R. W.) 181–257 (Academic Press, 2003).
10. Tocher, D. R. Metabolism and functions of lipids and fatty acids in teleost fish. *Rev. Fish. Sci.* **11**, 107–184 (2003).
11. Gebauer, S., Psota, T., Harris, W. & Kris-Etherton, P. *n*-3 fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. *Am J Clin Nutr.* **83**, 1526S–1535S (2006).
12. Aranceta, J. & Pérez-Rodrigo, C. Recommended dietary reference intakes, nutritional goals and dietary guidelines for fat and fatty acids: A systematic review. *Br. J. Nutr.* **107**, S8–S22 (2012).
13. EFSA. Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). *EFSA J.* **10**, 1–48 (2012).
14. Tacon, A. G. J. Global Trends in Aquaculture and Compound Aquafeed Production. *World Aquac.* 33–46 (2018).
15. Cashion, T., Le Manach, F., Zeller, D. & Pauly, D. Most fish destined for fishmeal production are food-grade fish. *Fish Fish.* **18**, 837–844 (2017).
16. Tacon, A. G. J. & Metian, M. Feed matters: Satisfying the feed demand of aquaculture. *Rev. Fish. Sci. Aquac.* **23**, 1–10 (2015).
17. Tacon, A. G. J. & Metian, M. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture* **285**, 146–

158 (2008).

18. Tacon, A. “Feeding tomorrow’s fish: Keys for sustainability”.Proceedings of the workshop of the CIHEAM Network on Technology of Aquaculture in the Mediterranean (TECAM)(1996)

19. Pateiro, M. *et al.* Nutritional Profiling and the Value of Processing By-Products from Gilthead Sea Bream (*Sparus aurata*). *Mar. Drugs* **18**, 1–18 (2020).

20. EUMOFA. Fishmeal and fish oil - Production and trade flows in the EU. 1–31 (2021).

21. https://www.iffo.com/feeding-growing-population; 05/08/2021, 6 PM

22. Ytrestøyl, T., Aas, T. S. & Åsgård, T. Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture* **448**, 365–374 (2015).

23. Stevens, J. R., Newton, R. W., Tlusty, M. & Little, D. C. The rise of aquaculture by-products: Increasing food production, value, and sustainability through strategic utilisation. *Mar. Policy* **90**, 115–124 (2018).

24. Lane, A., Hough, C. & Bostock, J. The Long-Term Economic and Ecologic Impact of Larger Sustainable Aquaculture. http://www.ncbi.nlm.nih.gov/pubmed/15003161 (2014)

25. EUMOFA. Blue bioeconomy: Situation report and perspectives. 1–152 (2018).

26. Turchini, G. M., Torstensen, B. E. & Ng, W. K. Fish oil replacement in finfish nutrition. *Rev. Aquac.* **1**, 10–57 (2009).

27. Wan, A. H. L., Davies, S. J., Soler-Vila, A., Fitzgerald, R. & Johnson, M. P. Macroalgae as a sustainable aquafeed ingredient. *Rev. Aquac.* **11**, 458–492 (2019).

28. Oliva-Teles, A., Enes, P. & Peres, H. Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish. in *Feed and Feeding Practices in Aquaculture* (2015). doi:10.1016/b978-0-08-100506-4.00008-8.

29. Barrington, K., Chopin, T. & Robinson, S. Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. Integr. Maric. - A Glob. Rev. - *FAO Fish. Aquac. Tech. Pap.* **529** 7–46 (2009).

30. Chopin, T. *et al.* Multitrophic Integration for Sustainable Marine Aquaculture. *Encycl. Ecol.* **3**, 2463–2475 (2008).

31. Troell, M. *et al.* Ecological engineering in aquaculture - Potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. *Aquaculture* **297**, 1–9 (2009).

32. OECD & Chopin, T. Integrated Multi-Trophic Aquaculture. in Advancing the Aquaculture Agenda: Workshop Proceedings 195–217 (OECD publishing, 2010).

33. Hughes, A. D. & Black, K. D. Going beyond the search for solutions: understanding trade-offs in European integrated multi-trophic aquaculture development. *Aquac. Environ. Interact.* **8**, 191–199 (2016).

34. Watson, G. J., Murray, J. M., Schaefer, M. & Bonner, A. Bait worms: a valuable and important fishery with implications for fisheries and conservation management. *Fish Fish.* **18**, 374–388 (2016).

35. Olive, P. J. W. Polychaete aquaculture and polychaete science: A mutual synergism. *Hydrobiologia* **402**, 175–183 (1999).

36. Sá, E. *et al.* Trade of live bait in Portugal and risks of introduction of non-indigenous species associated to importation. *Ocean Coast. Manag.* **146**, 121–128 (2017).

37. Fidalgo E. Costa, P. *et al.* The market features of imported non-indigenous polychaetes in Portugal and consequent ecological concerns. *Sci. Mar.* **70**, 287–292 (2006).

38. Xenarios, S., Queiroga, H., Lillebø, A. I. & Aleixo, A. Introducing a regulatory policy framework of bait fishing in European coastal lagoons: The case of ria de aveiro in Portugal. *Fishes* **3**, 1–19 (2018).

39. Pombo, A. *et al.* Insight into aquaculture's potential of marine annelid worms and ecological concerns: A Review. *Rev. Aquac.* 1–15 (2018) doi:10.1111/raq.12307.

40. Dashtgard, S. E. & Gingras, M. K. Marine Invertebrate Neoichnology. *Dev. Sedimentol.* **64**, 273–295 (2012).

41. Shull, D. H. Bioturbation. *in* Encyclopedia of Ocean Sciences (ed. Steele, J. H. B. T.) 395–400 (Academic Press, 2009).

42. Quintana, C. O. *et al.* Carbon mineralization pathways and bioturbation in coastal Brazilian sediments. *Sci. Rep.* **5**, 1–13 (2015).

43. Queirós, A. M. *et al.* A bioturbation classification of European marine infaunal invertebrates. *Ecol. Evol.* **3**, 3958–3985 (2013).

44. Fauchald, K. & Jumars, P. A. The diet of worms: a study of polychaete feeding guilds. *Oceanogr. Mar. Biol. Annu. Rev.* **17**, 193–284 (1979).

45. Scaps, P. A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O. F. Müller) (Annelida: Polychaeta). *Hydrobiologia* **470**, 203–218 (2002).

46. Reise, K. Spatial configurations generated by motile benthic polychaetes. *Helgoländer Wiss. Meeresunters.* **32**, 55–72 (1979).

47. Lucas, F. & Bertru, G. Bacteriolysis in the gut of *Nereis diversicolor* (O.F. Muller) and effect of the diet. *J. Exp. Mar. Bio. Ecol.* **215**, 235–245 (1997).

48. Olivier, M., Desrosiers, G., Caron, A., Retière, C. & Caillou, A. Réponses comportementales des polychètes *Nereis diversicolor* (O.F. Müller) et *Nereis virens* (Sars) aux stimuli d'ordre alimentaire: utilisation de la matière organique particulaire (algues et halophytes). *Can. J. Zool.* **73**, 2307–2317 (1995).

49. Riisgård, H. U. Filter-feeding in the polychaete *Nereis diversicolor*: a review. *Netherlands J. Aquat. Ecol.* **28**, 453–458 (1994).

50. Vedel, A., Andersen, B. B. & Riisgard, H. U. Field investigations of pumping activity off the facultatively filter-feeding polychaete *Nereis diversicolor* using an improved infrared phototransducer system. *Mar. Ecol. Prog. Ser.* **103**, 91–102 (1994).

51. Gómez, S., Hurtado, C. F. & Orellana, J. Bioremediation of organic sludge from a marine recirculating aquaculture system using the polychaete *Abarenicola pusilla* (Quatrefages,

1866). *Aquaculture* **507**, 377–384 (2019).

52. Gómez, S., Hurtado, C. F., Orellana, J., Valenzuela-Olea, G. & Turner, A. Abarenicola pusilla (Quatrefages, 1866): A novel species for fish waste bioremediation from marine recirculating aquaculture systems. *Aquac. Res.* 1–5 (2017) doi:10.1111/are.13562.

53. Cavallo, D., Pusceddu, A., Danovaro, R. & Giangrande, A. Particulate organic matter uptake rates of two benthic filter-feeders (*Sabella spallanzanii* and *Branchiomma luctuosum*) candidates for the clarification of aquaculture wastewaters. *Mar. Pollut. Bull.* **54**, 602–625 (2007).

54. Nederlof, M. *et al.* Application of polychaetes in (de)coupled integrated aquaculture: production of a high-quality marine resource. *Aquac. Environ. Interact.* **11**, 221–237 (2019).

55. Bischoff, A. Solid waste reduction of closed recirculated aquaculture systems by secondary culture of detritivorous organisms. (Christian-Albrechts-Universität Zu Kiel, 2007).

56. Marques, B., Calado, R. & Lillebø, A. I. New species for the biomitigation of a super-intensive marine fish farm effluent: Combined use of polychaete-assisted sand filters and halophyte aquaponics. 600, 1922–1928 (2017).

57. Yousefi-Garakouei, M., Kamali, A. & Soltani, M. Effects of rearing density on growth, fatty acid profile and bioremediation ability of polychaete *Nereis diversicolor* in an integrated aquaculture system with rainbow trout (*Oncorhynchus mykiss*). *Aquac. Res.* **50**, 725–735 (2019).

58. Wang, H. *et al.* Growth and nutritional composition of the polychaete *Hediste diversicolor* (OF Müller, 1776) cultivated on waste from land-based salmon smolt aquaculture. *Aquaculture* **502**, 232–241 (2019).

59. Pajand, Z. O., Soltani, M., Bahmani, M. & Kamali, A. The role of polychaete *Nereis diversicolor* in bioremediation of wastewater and its growth performance and fatty acid composition in an integrated culture system with *Huso huso* (Linnaeus, 1758). *Aquac. Res.* **48**, 5271–5279 (2017).

60. García-Alonso, J., Hardege, J. D. & Müller, C. T. Influence of food regimes and seasonality on fatty acid composition in the ragworm. *Aquat. Biol.* **4**, 7–13 (2008).

61. Marques, B. *et al.* Adding value to ragworms (*Hediste diversicolor*) through the bioremediation of a super-intensive marine fish farm. *Aquac. Environ. Interact.* **10**, 79–88 (2018).

62. Brown, N., Eddy, S. & Plaud, S. Utilization of waste from a marine recirculating fish culture system as a feed source for the polychaete worm, *Nereis virens*. *Aquaculture* 322–323, 177–183 (2011).

63. Yang, D., Cao, C., Wang, G., Zhou, Y. & Xiu, Z. The Growth Study of *Perinereis aibuhitensis* in Airlift Recirculating Aquaculture System. *Open Biotechnol. J.* **9**, 143–149 (2015).

64. Palmer, P. J. Polychaete-assisted sand filters. *Aquaculture* **306**, 369–377 (2010).

65. Honda, H. & Kikuchi, K. Nitrogen budget of polychaete *Perinereis nuntia vallata* fed on the feces of Japanese flounder. *Fish. Sci.* **68**, 1304–1308 (2002).

66. Stabili, L., Schirosi, R., Licciano, M., Mola, E. & Giangrande, A. Bioremediation of bacteria in aquaculture waste using the polychaete *Sabella spallanzanii*. *N. Biotechnol.* **27**, 774–781 (2010).

67. Giangrande, A. *et al.* Utilization of the filter feeder polychaete *Sabella spallanzanii* Gmelin (Sabellidae) as bioremediator in aquaculture. *Aquac. Int.* **13**, 129–136 (2005).

68. Kube, N. The integration of microalgae photobioreactors in a recirculation system for low water discharge mariculture. 189 (2006).

69. Carvalho, A. N., Vaz, A. S. L., Sérgio, T. I. B. & Santos, P. J. T. Sustainability of bait fishing harvesting in estuarine ecosystems – Case study in the Local Natural Reserve of Douro Estuary, Portugal. *J. Integr. Coast. Zo. Manag.* **13**, 157–168 (2013).

70. Yousefi-Garakouei, M., Kamali, A. & Soltani, M. Effects of rearing density on growth, fatty acid profile and bioremediation ability of polychaete *Nereis diversicolor* in an integrated aquaculture system with rainbow trout (*Oncorhynchus mykiss*). *Aquac. Res.* **50**, 725–735 (2019).

71. Santos, A. *et al.* Effect of three diets on the growth and fatty acid profile of the common ragworm *Hediste diversicolor* (O.F. Müller, 1776). *Aquaculture* **465**, 37–42 (2016).

72. García-Alonso, J., Müller, C. T. & Hardege, J. D. Influence of food regimes and seasonality on fatty acid composition in the ragworm. *Aquat. Biol.* **4**, 7–13 (2008).

73. Fidalgo e Costa, P., Narciso, L. & Cancela da Fonseca, L. Growth, survival and fatty acid profile of *Nereis diversicolor* (O. F. Muller, 1776) fed on six different diets. *Bull. Mar. Sci.* **67**, 337–343 (2000).

74. Vásquez, V., Krieg, M., Lockhead, D. & Goodman, M. B. Phospholipids that contain polyunsaturated fatty acids enhance neuronal cell mechanics and touch sensation. *Cell Rep.* **6**, 70–80 (2014).

75. Dinis, M. T. Aspects of the potential of *Solea senegalensis* Kaup for aquaculture: larval rearing and weaning to an artificial diet. *Aquac. Res.* **23**, 515–520 (1992).

76. Dinis, M. T., Ribeiro, L., Soares, F. & Sarasquete, C. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture* **176**, 27–38 (1999).

77. Luis, O. J. & Ponte, A. C. Control of Reproduction of the Shrimp *Penaeus kerathurus* held in captivity. *J. World Aquac. Soc.* **24**, 31–39 (1993).

78. Lytle, J. S., Lytle, T. F. & Ogle, J. T. Polyunsaturated fatty acid profiles as a comparative tool in assessing maturation diets of *Penaeus vannamei*. *Aquaculture* **89**, 287–299 (1990).

79. Meunpol, O., Meejing, P. & Piyatiratitivorakul, S. Maturation diet based on fatty acid content for male *Penaeus monodon* (Fabricius) broodstock. *Aquac. Res.* **36**, 1216–1225 (2005).

80. Luis, O. J. & Passos, A. M. Seasonal changes in lipid content and composition of the polychaete *Nereis* (*Hediste*) *diversicolor*. *Comp. Biochem. Physiol.* **111B**, 579–586 (1995).

81. Velez, Z., Hubbard, P. C., Hardege, J. D., Barata, E. N. & Canário, A. V. M. The contribution of amino acids to the odour of a prey species in the Senegalese sole (*Solea senegalensis*). *Aquaculture* **265**, 336–342 (2007).

82. Pires, A. *et al.* Expansion of lugworms towards southern European habitats and their identification using combined ecological, morphological and genetic approaches. *Mar. Ecol. Prog. Ser.* **533**, 177–190 (2015).

83. Howie, D. I. D. The reproductive biology of the lugworm, *Arenicola marina* L. *in* Polychaete Reproduction (eds. Fischer, A. & Pfannenstiel, H. D.) 247–263 (G. Fischer, 1984).

84. Fish, J. & Fish, S. Annelida. in A student's guide to the seashore 141–189 (Cambridge University Press, 2011). Fish J.D., Fish S. (1989) Annelida. *In*: A Student's Guide to the Seashore. Springer, Dordrecht.

85. Alvarez-Claudio, C. & Anadón, N. Reproductive biology of the polychaete Arenicola marina (L.) in the Villaviciosa estuary (North of Spain). *Bull. la Soc. Zool. Fr.* **124**, 227–237 (1999).

86. Rousselot, M. *et al. Arenicola marina* extracellular hemoglobin: A new promising blood substitute. *Biotechnol. J.* **1**, 333–345 (2006).

87. Thuillier, R. *et al.* Supplementation with a new therapeutic oxygen carrier reduces chronic fibrosis and organ dysfunction in kidney static preservation. *Am. J. Transplant.* **11**, 1845–1860 (2011).

88. Singh, D., Buhmann, A. K., Flowers, T. J., Seal, C. E. & Papenbrock, J. Salicornia as a crop plant in temperate regions: Selection of genetically characterized ecotypes and optimization of their cultivation conditions. *AoB Plants* **6**, (2014).

89. Gunning, D., Maguire, J. & Burnell, G. The Development of Sustainable Saltwater-Based Food Production Systems: A Review of Established and Novel Concepts. *Water* **8**, 598 (2016).

90. Panta, S. *et al.* Halophyte agriculture: Success stories. *Environ. Exp. Bot.* **107**, 71–83 (2014).

91. Flowers, T. J. & Colmer, T. D. Plant salt tolerance: adaptations in halophytes. *Ann. Bot.* **115**, 327–331 (2015).

92. Flett, I. How aquaponics can improve aquaculture and help feed a hungry world. (2017).

93. Turcios, A. E. & Papenbrock, J. Sustainable treatment of aquaculture effluents-What can we learn from the past for the future? *Sustain.* **6**, 836–856 (2014).

94. Shahid, M., Jaradat, A. A. & Rao, N. K. Use of Marginal Water for *Salicornia bigelovii* Torr. Planting in the United Arab Emirates. (2013).

95. Gilliham, M. Salinity tolerance of crops - what is the cost? Tansley insight (2015)

96. Scagel, C. F., Lee, J. & Mitchell, J. N. Salinity from NaCl changes the nutrient and polyphenolic composition of basil leaves. *Ind. Crops Prod.* **127**, 119–128 (2019).

97. Ventura, Y., Eshel, A., Pasternak, D. & Sagi, M. The development of halophyte-based agriculture: Past and present. *Ann. Bot.* **115**, 529–540 (2015).

98. Buhmann, A. & Papenbrock, J. An economic point of view of secondary compounds in halophytes. *Funct. Plant Biol.* **40**, 952–967 (2013).

99. Ventura, Y. & Sagi, M. Halophyte crop cultivation: The case for salicornia and sarcocornia. *Environ. Exp. Bot.* **92**, 144–153 (2013).

100. Koyro, H. W., Khan, M. A. & Lieth, H. Halophytic crops: A resource for the future to reduce the water crisis? *Emirates J. Food Agric.* **23**, 1–16 (2011).

101. Ksouri, R. *et al.* Medicinal halophytes: potent source of health promoting biomolecules with medical, nutraceutical and food applications. *Crit. Rev. Biotechnol.* **32**, 1–38 (2011).

102. Lopes, A. *et al.* Natural products from extreme marine environments: Searching for potential industrial uses within extremophile plants. *Ind. Crops Prod.* **94**, 299–307 (2016).

103. Brown, J. J., Glenn, E. P., Fitzsimmons, K. M. & Smith, S. E. Halophytes for the treatment of saline aquaculture effluent. *Aquaculture* **175**, 255–268 (1999).

104. Shpigel, M. *et al.* Constructed wetland with Salicornia as a biofilter for mariculture effluents. *Aquaculture* **412–413**, 52–63 (2013).

105. Webb, J. M. *et al.* Halophyte filter beds for treatment of saline wastewater from aquaculture. *Water Res.* **46**, 5102–5114 (2012).

106. Lin, Y. F. *et al.* Performance of a constructed wetland treating intensive shrimp aquaculture wastewater under high hydraulic loading rate. *Environ. Pollut.* **134**, 411–421 (2005).

107. Lin, Y.-F., Jing, S.-R. & Lee, D.-Y. The potential use of constructed wetlands in a recirculating aquaculture system for shrimp culture. *Environ. Pollut.* **123**, 107–113 (2003).

108. Jones, J. B. Hydroponics: A Practical Guide for the Soilless Grower. (CRC Press, 2016).

109. Davy, A. J., Bishop, G. F. & Costa, C. S. B. Salicornia L. (*Salicornia pusilla* J. Woods, *S. ramosissima* J. Woods, *S. europaea* L., *S. obscura* P.W. Ball & Tutin, *S. nitens* P.W. Ball & Tutin, *S. fragilis* P.W. Ball & Tutin and *S. dolichostachya* Moss). *J. Ecol.* **89**, 681–707 (2001).

110. Muscolo, A., Panuccio, M. R. & Piernik, A. Ecology, Distribution and Ecophysiology of Salicornia europaea L. In Sabkha Ecosystems (ed. Khan M.A., Böer B., Öztürk M., Al Abdessalaam T.Z., Clüsener-Godt M., G. B.); Volume IV: Cash Crop Halophyte and Biodiversity Conservation: 233–240 (Springer, 2014).

111. Ventura, Y. *et al.* Effect of seawater concentration on the productivity and nutritional value of annual Salicornia and perennial Sarcocornia halophytes as leafy vegetable crops. *Sci. Hortic.* 189–196 (2011).

112. Barreira, L. *et al.* Halophytes: Gourmet food with nutritional health benefits? *J. Food Compos. Anal.* **59**, 35–42 (2017).

113. Feng, L., Ji, B. & Su, B. Economic value and exploiting approaches of sea asparagus, a seawater-irrigated vegetable. *Agric. Sci.* **04**, 40–44 (2013).

114. Rodrigues, M. J. *et al.* Unravelling the antioxidant potential and the phenolic composition of different anatomical organs of the marine halophyte *Limonium algarvense*. *Ind. Crops Prod.* **77**, 315–322 (2015).

115. Rodrigues, M. J. *et al.* Growth performance, in vitro antioxidant properties and chemical composition of the halophyte *Limonium algarvense* Erben are strongly influenced by the

irrigation salinity. *Ind. Crops Prod.* **143**, 111930 (2020).

116. Glenn, E. P., Coates, W. E., Riley, J. J., Kuehl, R. O. & Swingle, R. S. *Salicornia bigelovii* Torr.: a seawater-irrigated forage for goats. *Anim. Feed Sci. Technol.* **40**, 21–30 (1992).

117. Eganathan, P., Subramanian, H. M., Latha, R. & Rao, C. S. Oil analysis in seeds of *Salicornia brachiata*. *Ind. Crops Prod.* **23**, 177–179 (2006).

118. Hosamani, K. M. & Sattigeri, R. M. Industrial utilization of *Rivea ornata* seed oil: A moderate source of vernolic acid. *Ind. Crops Prod.* **12**, 93–96 (2000).

119. Boxman, S. E., Nystrom, M., Ergas, S. J., Main, K. L. & Trotz, M. A. Evaluation of water treatment capacity, nutrient cycling, and biomass production in a marine aquaponic system. *Ecol. Eng.* **120**, 299–310 (2018).

120. Boxman, S. E. *et al.* Effect of support medium, hydraulic loading rate and plant density on water quality and growth of halophytes in marine aquaponic systems. *Aquac. Res.* 1–15 (2016)

121. Hegedűs, R. *et al.* Potential role of halophytic macrophytes in saline effluent treatment. *Int. J. Biol. Biomol. Agric. Food Biotechnol. Eng.* **4**, 273–277 (2010).

122. Vlahos, N. *et al.* An experimental brackish aquaponic system using juvenile gilthead sea bream (*Sparus aurata*) and rock samphire (*Crithmum maritimum*). *Sustain.* **11**, (2019).

123. Waller, U. *et al.* Integrated multi-trophic aquaculture in a zero-exchange recirculation aquaculture system for marine fish and hydroponic halophyte production. *Aquacult Int* **23**, 1473–1489 (2015).

124. Ma, X. *et al.* Characterization of Microbial Communities in Pilot-Scale Constructed Wetlands with Salicornia for Treatment of Marine Aquaculture Effluents. *Hindawi archaea*, 1–12 (2018).

125. Gunning, D. Cultivating Salicornia europaea (Marsh Samphire). (University College Cork, Ireland, 2016).

126. Webb, J. M. *et al.* The effect of halophyte planting density on the efficiency of constructed wetlands for the treatment of wastewater from marine aquaculture. *Ecol. Eng.* **61**, 145–153 (2013).

127. Chen, X. *et al.* A novel combined recirculating treatment system for intensive marine aquaculture. *Aquac. Res.* **48**, 5062–5071 (2017).

128. Watanabe, W. O. & Farnell, R. D. Experimental evaluation of the halophyte, *Salicornia virginica*, for biomitigation of dissolved nutrients in effluent from a recirculating aquaculture system for marine finfish. *J. World Aquac. Soc.* (2017).

129. Poli, M. A. *et al.* Integrated multitrophic aquaculture applied to shrimp rearing in a biofloc system. *Aquaculture* **511**, 1–6 (2019).

130. Pinheiro, I. *et al.* Production of the halophyte *Sarcocornia ambigua* and Pacific white shrimp in an aquaponic system with biofloc technology. *Ecol. Eng.* **100**, 261–267 (2017).

131. Brown, J. J. & Glenn, E. P. Reuse of highly saline aquaculture effluent to irrigate a potential forage halophyte, *Suaeda esteroa*. *Aquac. Eng.* **20**, 91–111 (1999).

1.1.6. Chapter 1 - Supporting Information

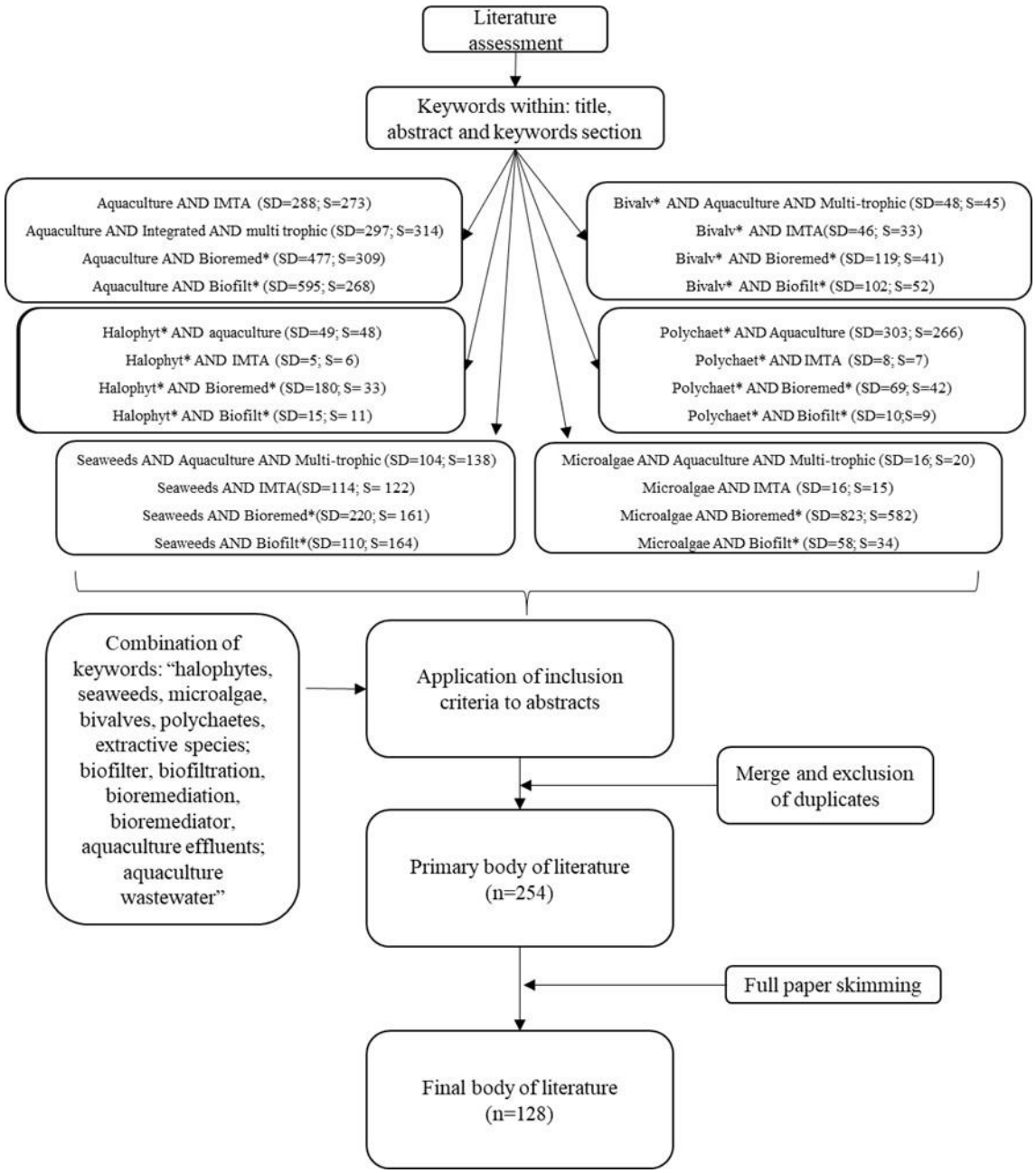


Figure S1.1. Process employed for the selection of relevant literature. Review performed between October-December 2019.

Table S.1.1. Criteria defined for the selection of relevant literature.

Criteria	Description
A	IMTA works that included marine or brackish water species.
B	IMTA works developed in land-based commercial units that included in their configuration the main species that produced waste (e.g., fish or shrimp) or that used wasted nutrients collected in other commercial/experimental production units.
C	IMTA works developed in experimental systems on a pilot or laboratory scale that included in its configuration the main species that produces waste (e.g., fish or shrimp) or that used wasted nutrients collected in other commercial / experimental fish production units.
D	Works developed in an off-shore environment were excluded
E	Studies whose scope was to test bacterial biofilters were excluded

Table S.1.2. List of works that included seaweeds in integrated multi-trophic aquaculture (IMTA) designs.

Species	Year	Reference	Country
Ulva rigida; Gracilaria conferta; Hypnea musciformis	2019	Ashkenazi, D. Y. A novel two-stage seaweed integrated multi-trophic aquaculture. *Rev. Aquac.* **11**, 246–262 (2019).	Israel
Caulerpa lentillifera	2019	Bambaranda, B. V. A. S. M., Tsusaka, T. W., Chirapart, A., Salin, K. R. & Sasaki, N. Capacity of *Caulerpa lentillifera* in the Removal of Fish Culture Effluent in a Recirculating Aquaculture System. *Processes* **7**, 440 (2019).	Thailand
Gracilaria birdiae	2019	Brito, L. O. *et al.* Bioremediation of shrimp biofloc wastewater using clam, seaweed and fish. *Chem. Ecol.* **34**, 901–913 (2018).	Brazil
Ulva-Periphyton	2019	Guttman, L. *et al.* An integrated Ulva-periphyton biofilter for mariculture effluents: Multiple nitrogen removal kinetics. *Algal Res.* **42**, 101586 (2019).	Israel
Gracilaria verrucosa	2019	Ihsan, Y. N., Subiyanto, Pribadi, T. D. K. & Schulz, C. Nitrogen assimilation potential of seaweed (*Gracilaria verrucosa*) in polyculture with pacific white shrimp (*Penaeus vannamei*). *AACL Bioflux* **12**, 51–62 (2019).	Indonesia
Ulva ohnoi	2019	Oca, J., Cremades, J., Jiménez, P., Pintado, J. & Masaló, I. Culture of the seaweed *Ulva ohnoi* integrated in a *Solea senegalensis* recirculating system: influence of light and biomass stocking density on macroalgae productivity. *J. Appl. Phycol.* **31**, 2461–2467 (2019).	Spain
Gracilaria tenuistipitata	2019	Sarkar, S., Rekha, P. N. & Balasubramanian, C. P. Bioremediation Potential of the Brackishwater Macroalga *Gracilaria tenuistipitata* (Rhodophyta) co-cultured with Pacific White Shrimp *Penaeus vannamei* (Boone). *J. Coast. Res.* **86**, 248–254 (2019).	India
Ulva lactuta	2018	Elizondo-González, R., Quiroz-Guzmán, E., Escobedo-Fregoso, C., Magallón-Servín, P. & Peña-Rodríguez, A. Use of seaweed *Ulva lactuca* for water bioremediation and as feed additive for white shrimp *Litopenaeus vannamei*. *PeerJ* **6**, e4459 (2018).	México
Saccharina latíssima	2016	Azevedo, I. C., Marinho, G. S., Silva, D. M. & Sousa-Pinto, I. Pilot scale land-based cultivation of *Saccharina latissima* Linnaeus at southern European climate conditions: Growth and nutrient uptake at high temperatures. *Aquaculture* **459**, 166–172 (2016).	Portugal
Gracilaria birdiae; Gracilaria domingensis	2016	Brito, L. O., Chagas, A. M., Pereira, E. & Borda, R. Water quality, Vibrio density and growth of Pacific white shrimp *Litopenaeus vannamei* (Boone) in an integrated biofloc system with red seaweed *Gracilaria birdiae* (Greville). *Aquac. Res.* **47**, 940–950 (2016).	Brazil
Ulva lactuta	2015	Al-Hafedh, Y. S., Alam, A. & Buschmann, A. H. Bioremediation potential, growth and biomass yield of the green seaweed, *Ulva lactuca* in an integrated marine aquaculture system at the Red Sea coast of Saudi Arabia at different stocking densities and effluent flow rates. *Rev. Aquac.* **7**, 161–171 (2015).	Saudi Arabia
Mastocarpus stellatus	2015	Azevedo, G. *et al.* Impact of cultivation of *Mastocarpus stellatus* in IMTA on the seaweeds chemistry and hybrid carrageenan properties. *Carbohydr. Polym.* **116**, 140–148 (2015).	Portugal
Codium tomentosum	2015	da Costa, E. *et al.* Decoding bioactive polar lipid profile of the macroalgae *Codium tomentosum* from a sustainable IMTA system using a lipidomic approach. *Algal Res.* **12**, 388–397 (2015)	Portugal
Mastocarpus stellatus	2015	Domingues, B., Abreu, M. H. & Sousa-Pinto, I. On the bioremediation efficiency of *Mastocarpus stellatus* (Stackhouse) Guiry, in an integrated multi-trophic aquaculture system. *J. Appl. Phycol.* **27**, 1289–1295 (2015).	Portugal

Continued…

Species	Year	Reference	Country
Gracilaria tikvahiae	2015	Samocha, T. M., Fricker, J., Ali, A. M., Shpigel, M. & Neori, A. Growth and nutrient uptake of the macroalga *Gracilaria tikvahiae* cultured with the shrimp *Litopenaeus vannamei* in an Integrated Multi-Trophic Aquaculture (IMTA) system. *Aquaculture* **446**, 263–271 (2015).	USA
Ulva rigida; Enteromorpha clathrata	2014	Aníbal, J. *et al.* Macroalgae mitigation potential for fish aquaculture effluents: an approach coupling nitrogen uptake and metabolic pathways using *Ulva rigida* and *Enteromorpha clathrata*. *Environ. Sci. Pollut. Res.* **21**, 13324–13334 (2014).	Portugal
Ulva lactuta	2014	Ben-Ari, T. *et al.* Management of *Ulva lactuca* as a biofilter of mariculture effluents in IMTA system. *Aquaculture* **434**, 493–498 (2014).	Israel
Gracilariopsis bailiniae	2014	Carton-Kawagoshi, R. J. *et al.* Low water exchange *Gracilariopsis bailiniae* Zhang & B.M. Xia culture in intensive milkfish culture effluents: Effects of seaweed density on seaweed production and effluent treatment. *Aquac. Int.* **22**, 573–584 (2014).	Phillipines
Palmaria palmata	2014	Corey, P., Kim, J. K., Duston, J. & Garbary, D. J. Growth and nutrient uptake by *Palmaria palmata* integrated with Atlantic halibut in a land-based aquaculture system. *Algae* **29**, 35–45 (2014).	Nova Scotia
Gracilariopsis longíssima	2014	He, Q., Zhang, J., Chai, Z., Wen, S. & He, P. *Gracilariopsis longissima* as biofilter for an Integrated Multi-Trophic aquaculture (IMTA) system with *Sciaenops ocellatus* : Bioremediation efficiency and production in a recirculating system. *Indian J. Geo-Marine Sci.* **43**, 528–537 (2014).	China
Ulva lactuta; Gracilaria edulis	2014	Lavania-Baloo, Azman, S., Mohd Said, M. I., Ahmad, F. & Mohamad, M. Biofiltration potential of macroalgae for ammonium removal in outdoor tank shrimp wastewater recirculation system. *Biomass and Bioenergy* **66**, 103–109 (2014).	Malaysia
Gracilaria chilensis; Ulva lactuta	2014	Macchiavello, J. & Bulboa, C. Nutrient uptake efficiency of *Gracilaria chilensis* and *Ulva lactuca* in an IMTA system with the red abalone *Haliotis rufescens*. *Lat. Am. J. Aquat. Res* **42**, 523–533 (2014).	Chile
Ulva reticulata	2014	Rabiei, R. *et al.* Bioremediation efficiency and biochemical composition of *Ulva reticulata* Forsskål (Chlorophyta) cultivated in shrimp (*Penaeus monodon*) hatchery effluent. *Iran. J. Fish. Sci.* **13**, 621–639 (2014).	Malaysia
Hydropuntia córnea	2014	Robledo, D., Navarro-Angulo, L., Lozano, D. V & Freile-Pelegrín, Y. Nutrient removal efficiency of *Hydropuntia cornea* in an integrated closed recirculation system with pink shrimp *Farfantepenaeus brasiliensis*. *Aquac. Res.* **45**, 1648–1658 (2014).	México
Palmaria palmata; Chondrus crispus	2013	Kim, J. K., Duston, J., Corey, P. & Garbary, D. J. Marine finfish effluent bioremediation: Effects of stocking density and temperature on nitrogen removal capacity of *Chondrus crispus* and *Palmaria palmata* (Rhodophyta). *Aquaculture* **414**–**415**, 210–216 (2013).	Nova Scotia
Ulva lactuta; Gracilaria arcauta	2012	Al-Hafedh, Y. S., Alam, A., Buschmann, A. H. & Fitzsimmons, K. M. Experiments on an integrated aquaculture system (seaweeds and marine fish) on the Red Sea coast of Saudi Arabia: Efficiency comparison of two local seaweed species for nutrient biofiltration and production. *Rev. Aquac.* **4**, 21–31 (2012).	Saudi Arabia
Gracilaria vermiculophylla	2011	Abreu, M. H., Pereira, R., Yarish, C., Buschmann, A. H. & Sousa-Pinto, I. IMTA with *Gracilaria vermiculophylla*: Productivity and nutrient removal performance of the seaweed in a land-based pilot scale system. *Aquaculture* **312**, 77–87 (2011).	Portugal
Gracilaria salicornia; Caulerpa letillifera	2011	Chaitanawisuti, N., Santhaweesuk, W. & Kritsanapuntu, S. Performance of the seaweeds *Gracilaria salicornia* and *Caulerpa lentillifera* as biofilters in a hatchery scale recirculating aquaculture system for juvenile spotted babylons (*Babylonia areolata*). *Aquac. Int.* **19**, 1139–1150 (2011).	Thailand

Continued…

Species	Year	Reference	Country
Undaria pinnatifida; Gracilaria vermiculophylla	2011	Skriptsova, A. V. & Miroshnikova, N. V. Laboratory experiment to determine the potential of two macroalgae from the Russian Far-East as biofilters for integrated multi-trophic aquaculture (IMTA). *Bioresour. Technol.* **102**, 3149–3154 (2011).	Russia
Ulva lactuta; Undaria pinnatifida	2010	Cahill, P. L., Hurd, C. L. & Lokman, M. Keeping the water clean - Seaweed biofiltration outperforms traditional bacterial biofilms in recirculating aquaculture. *Aquaculture* **306**, 153–159 (2010).	New Zealand
Asparagopsis armata; Ulva rigida	2010	Mata, L., Schuenhoff, A. & Santos, R. A direct comparison of the performance of the seaweed biofilters, *Asparagopsis armata* and *Ulva rigida*. *J. Appl. Phycol.* **22**, 639–644 (2010).	Portugal
Ulva lactuta	2010	Msuya, F. E. & Neori, A. The performance of spray-irrigated *ulva lactuca* (Ulvophyceae, chlorophyta) as a crop and as a biofilter of fishpond effluents. *J. Phycol.* **46**, 813–817 (2010).	Israel
Ulva clathrata	2009	Copertino, M. D. S., Tormena, T. & Seeliger, U. Biofiltering efficiency, uptake and assimilation rates of *Ulva clathrata* (Roth) J. Agardh (Clorophyceae) cultivated in shrimp aquaculture waste water. *J. Appl. Phycol.* **21**, 31–45 (2009).	Brazil
Gracilaria caudata	2009	Marinho-Soriano, E., Panucci, R. A., Carneiro, M. A. A. & Pereira, D. C. Evaluation of *Gracilaria caudata* J. Agardh for bioremediation of nutrients from shrimp farming wastewater. *Bioresour. Technol.* **100**, 6192–6198 (2009).	Brazil
Gracilaria birdiae	2009	Marinho-Soriano, E., Nunes, S. O., Carneiro, M. A. A. & Pereira, D. C. Nutrients' removal from aquaculture wastewater using the macroalgae *Gracilaria birdiae*. *Biomass and Bioenergy* **33**, 327–331 (2009).	Brazil
Porphyra yezoensis	2009	Kang, Y. H. *et al.* Physiological responses of *Porphyra yezoensis* Ueda (Bangiales, Rhodophyta) exposed to high ammonium effluent in a seweed-based integrated aquaculture system. *J. Fish. Sci. Technol.* **12**, 70–77 (2009	Korea
Kappaphycus alvarezii	2008	Hayashi, L. *et al.* Nutrients removed by *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae) in integrated cultivation with fishes in recirculating water. *Aquaculture* **277**, 185–191 (2008).	Brazil
Ulva pertusa	2007	Wang, H., Liu, C. F., Qin, C. X., Cao, S. Q. & Ding, J. Using a macroalgae *Ulva pertusa* biofilter in a recirculating system for production of juvenile sea cucumber *Apostichopus japonicus*. *Aquac. Eng.* **36**, 217–224 (2007).	China
Kappaphycus alvarezii; Kappaphycus sp.; K. striatum	2007	Rodrigueza, M. R. C. & Montaño, M. N. E. Bioremediation potential of three carrageenophytes cultivated in tanks with seawater from fish farms. *J. Appl. Phycol.* **19**, 755–762 (2007).	Phillipines
Gracilariopsis longíssima	2006	Hernández, I. *et al.* Studies on the biofiltration capacity of *Gracilariopsis longissima*: From microscale to macroscale. *Aquaculture* **252**, 43–53 (2006).	Spain
Chondrus crispus; Gracilaria bursa pastoris; Palmaria palmata	2006	Matos, J., Costa, S., Rodrigues, A., Pereira, R. & Sousa Pinto, I. Experimental integrated aquaculture of fish and red seaweeds in Northern Portugal. *Aquaculture* **252**, 31–42 (2006).	Portugal
Ulva reticulata	2006	Msuya, F. E., Kyewalyanga, M. S. & Salum, D. The performance of the seaweed *Ulva reticulata* as a biofilter in a low-tech, low-cost, gravity generated water flow regime in Zanzibar, Tanzania. *Aquaculture* **254**, 284–292 (2006).	Tanzania
Gracilaria lemaneiformis	2006	Zhou, Y. *et al.* Bioremediation potential of the macroalga *Gracilaria lemaneiformis* (Rhodophyta) integrated into fed fish culture in coastal waters of north China. *Aquaculture* **252**, 264–276 (2006).	China

Continued…

Species	Year	Reference	Country
Ulva rotundata; Gracilariopsis longíssima	2005	Hernández, I., Fernández-Engo, M. A., Pérez-Lloréns, J. L. & Vergara, J. J. Integrated outdoor culture of two estuarine macroalgae as biofilters for dissolved nutrients from *Sparus aurata* waste waters. *J. Appl. Phycol.* **17**, 557–567 (2005).	Spain
Ulva lactuta	2003	Neori, A. *et al.* A novel three-stage seaweed (*Ulva lactuca*) biofilter design for integrated mariculture. *J. Appl. Phycol.* **15**, 543–553 (2003).	Israel
Ulva lactuta	2003	Schuenhoff, A. *et al.* A semi-recirculating, integrated system for the culture of fish and seaweed. *Aquaculture* **221**, 167–181 (2003).	Israel
Ulva rotundata, Ulva intestinalis (=*Enteromorpha intestinalis*)*; Gracilaria gracilis*	2002	Hernandez, I., Martinez-Aragon, J. F., Tovar, A., Terez-Llorens, J. L. & Vergara, J. J. Biofiltering efficiency in removal of dissolved nutrients by the three species of estuarine macroalgae with sea bass (*Dicentrarchus labrax*) wate waters 1. ammonium. *Jounal Appl. Phycol.* **14**, 375–384 (2002). Martinez-Aragon, J. F., Hernandez, I., Tovar, A., Terez-Llorens, J. L. & Vergara, J. J. Biofiltering efficiency in removal of dissolved nutrients by the three species of estuarine macroalgae with sea bass (*Dicentrarchus labrax*) wate waters 2. Ammonium. *Jounal Appl. Phycol.* **14**, 375–384 (2002).	Spain
Ulva lactuta; Gracilaria conferta	2000	Neori, A., Shpigel, M. & Ben-Ezra, D. A sustainable integrated system for culture of fish, seaweed and abalone. *Aquaculture* **186**, 279–291 (2000).	Israel
Gracilaria chilensis; Ulva lactuta	1996	Buschmann, A. H., Troell, M., Kautsky, N. & Kautsky, L. Integrated tank cultivation of salmonids and *Gracilaria chilensis* (Gracilariales, Rhodophyta). *Hydrobiologia* **326**–**327**, 75–82 (1996).	Chile
Ulva rígida	1996	Río, M. J., Ramazanov, Z. & García-Reina, G. *Ulva rigida* (Ulvales, Chlorophyta) tank culture as biofilters for dissolved inorganic nitrogen from fishpond effluents. *Hydrobiologia* **326**–**327**, 61–66 (1996).	Spain
Ulva lactuta	1995	Krom, M. D., Ellner, S., van Rijn, J. & Neori, A. Nitrogen and phosphorus cycling and transformations in a prototype non-polluting' integrated mariculture system, Eilat, Israel. *Mar. Ecol. Prog. Ser.* **118**, 25–36 (1995).	Israel
Ulva lactuta	1991	Cohen, I. & Neori, A. *Ulva lactuca* Biofilters for Marine Fishpond Effluents I. Ammonia Uptake Kinetics and Nitrogen Content. *Bot. Mar.* **34**, 475–482 (1991). Neori, A., Cohen, I. & Gordin, H. *Ulva lactuca* Biofilters for Marine Fishpond Effluents II. Growth Rate, Yield and C:N Ratio. *Bot. Mar.* **34**, 483–490 (1991).	Israel

Table S1.3. List of works that included halophyte plants in integrated multi-trophic aquaculture (IMTA) designs.

Species	Year	Reference	Country
Sarcocornia ambígua	2019	Poli, M. A. *et al.* Integrated multitrophic aquaculture applied to shrimp rearing in a biofloc system. *Aquaculture* **511**, 1–6 (2019).	Brazil
Crithmum maritimum	2019	Vlahos, N. *et al.* An experimental brackish aquaponic system using juvenile gilthead sea bream (*Sparus aurata*) and rock samphire (*Crithmum maritimum*). *Sustain.* **11**, (2019).	Greece
Sesuvium portulacastrum; Batis marítima	2018	Boxman, S. E., Nystrom, M., Ergas, S. J., Main, K. L. & Trotz, M. A. Evaluation of water treatment capacity, nutrient cycling, and biomass production in a marine aquaponic system. *Ecol. Eng.* **120**, 299–310 (2018).	USA
Salicornia bigelovii	2018	Ma, X. *et al.* Characterization of Microbial Communities in Pilot-Scale Constructed Wetlands with Salicornia for Treatment of Marine Aquaculture Effluents. *Hindawi archaea* **2018**, 1–12 (2018).	China
Sarcocornia ambígua	2017	Pinheiro, I. *et al.* Production of the halophyte *Sarcocornia ambigua* and Pacific white shrimp in an aquaponic system with biofloc technology. *Ecol. Eng.* **100**, 261–267 (2017).	Brazil
Salicornia virginica	2017	Watanabe, W. O. & Farnell, R. D. Experimental Evaluation of the Halophyte, *Salicornia virginica*, for Biomitigation of Dissolved Nutrients in Effluent from a Recirculating Aquaculture System for Marine Finfish. *J. World Aquac. Soc.* (2017).	USA
Sesuvium portulacastrum; Batis marítima	2016	Boxman, S. E. *et al.* Effect of support medium, hydraulic loading rate and plant density on water quality and growth of halophytes in marine aquaponic systems. *Aquac. Res.* 1–15 (2016).	USA
Salicornia europaea	2016	Gunning, D., Maguire, J. & Burnell, G. The Development of Sustainable Saltwater-Based Food Production Systems: A Review of Established and Novel Concepts. *Water* **8**, 598 (2016).	Ireland
Salicornia dolichostachya; Tripolium pannonicum; Plantago coronopus	2015	Waller, U. *et al.* Integrated multi-trophic aquaculture in a zero-exchange recirculation aquaculture system for marine fish and hydroponic halophyte production. *Aquacult Int* **23**, 1473–1489 (2015).	Germany
Salicornia pérsica	2013	Shpigel, M. *et al.* Constructed wetland with Salicornia as a biofilter for mariculture effluents. *Aquaculture* **412**–**413**, 52–63 (2013).	Israel
Salicornia europaea	2013	Webb, J. M. *et al.* The effect of halophyte planting density on the efficiency of constructed wetlands for the treatment of wastewater from marine aquaculture. *Ecol. Eng.* **61**, 145–153 (2013).	UK
Salicornia europeaea	2012	Webb, J. M. *et al.* Halophyte filter beds for treatment of saline wastewater from aquaculture. *Water Res.* **46**, 5102–5114 (2012).	UK
Phragmites australis; Typha angustifolia; Glyceria maxima; Scirpus tabernaemontani; Aster tripolium; Bolbo schoenus maritimus; Triglochin palustris; Carex vulpine	2010	Hegedűs, R. *et al.* Potential Role of Halophytic Macrophytes in Saline Effluent Treatment. *Int. J. Biol. Biomol. Agric. Food Biotechnol. Eng.* **4**, 273–277 (2010).	Hungary
Salicornia bigelovii; Suaeda esteroa; Atriplex barclayana	1999	Brown, J. J., Glenn, E. P., Fitzsimmons, K. M. Smith, S. E. Halophytes for the treatment of saline aquaculture effluent. *Aquaculture* **175**, 255–268 (1999).	USA
Suaeda esteroa	1999	Brown, J. J. & Glenn, E. P. Reuse of highly saline aquaculture effluent to irrigate a potential forage halophyte, *Suaeda esteroa*. *Aquac. Eng.* **20**, 91–111 (1999).	USA

Table S1.4. List of works that included microalgae in integrated multi-trophic aquaculture (IMTA) designs

Species	Year	Reference	Country
Tetraselmis suecica; Dunaliella tertiolecta	2019	Andreotti, V., Solimeno, A., Chindris, A., Marazzi, F. & García, J. Growth of Tetraselmis suecica and Dunaliella tertiolecta in Aquaculture Wastewater: Numerical Simulation with the BIO_ALGAE Model. *Water. Air. Soil Pollut.* **230**, (2019).	Italy
Tetraselmis sp. and others	2019	Li, M. *et al.* Bioremediation of fishpond effluent and production of microalgae for an oyster farm in an innovative recirculating integrated multi-trophic aquaculture system. *Aquaculture* **504**, 314–325 (2019).	France
Spirulina platensis	2018	Nogueira, S. M. S., Junior, J. S., Maia, H. D., Saboya, J. P. S. & Farias, W. R. L. Use of *Spirulina platensis* in treatment of fish farming wastewater. *Rev. Ciência Agronômica* **49**, 599–606 (2018).	Brazil
Tetraselmis suecica; Isochrysis galbana; Dunaliella tertiolecta	2017	Andreotti, V. *et al.* Bioremediation of aquaculture wastewater from *Mugil cephalus* (Linnaeus, 1758) with different microalgae species. *Chem. Ecol.* **33**, 750–761 (2017).	Italy
Platymonas helgolandica; Chlorella vulgaris; Chaetoceros mulleri	2016	Ge, H. *et al.* Effect of microalgae with semicontinuous harvesting on water quality and zootechnical performance of white shrimp reared in the zero water exchange system. *Aquac. Eng.* **72**–**73**, 70–76 (2016).	China
Picochlorum maculatum	2016	Kumar, S. D., Santhanam, P., Park, M. S. & Kim, M. K. Development and application of a novel immobilized marine microalgae biofilter system for the treatment of shrimp culture effluent. *J. Water Process Eng.* **13**, 137–142 (2016)	India
Chlorella sp.	2014	Kumar, S. D., Santhanam, P., Lewis-Oscar, F. & Thajuddin, N. A Dual Role of Marine microalga *Chlorella* sp. (PSDK01) in aquaculture effluent with emphasis on initial population density. *Arab. J. Sci. Eng.* **40**, 29–35 (2014).	India
Chlorella sp.	2014	Lananan, F. *et al.* Symbiotic bioremediation of aquaculture wastewater in reducing ammonia and phosphorus utilizing Effective Microorganism (EM-1) and microalgae (*Chlorella* sp.). *Int. Biodeterior. Biodegradation* **95**, 127–134 (2014).	Malaysia
Tetraselmis suecica	2014	Michels, M. H. A., Vaskoska, M., Vermuë, M. H. & Wijffels, R. H. Growth of *Tetraselmis suecica* in a tubular photobioreactor on wastewater from a fish farm. *Water Res.* **65**, 290–296 (2014)	Netherlands
Nannochloropsis oculata; Tetraselmis chuii	2014	Sirakov, I. N. & Velichkova, K. N. Bioremediation of wastewater originate from aquaculture and biomass production from microalgae species - *Nannochloropsis oculata* and *tetraselmis chuii*. *Bulg. J. Agric. Sci.* **20**, 66–72 (2014).	Bulgaria
Isochrysis galbana; Phaedactilum tricornutum; tetraselmis suecica	2005	Borges, M. T., Silva, P., Moreira, L. & Soares, R. Integration of consumer-targeted microalgal production with marine fish effluent biofiltration - A strategy for mariculture sustainability. *J. Appl. Phycol.* **17**, 187–197 (2005).	Portugal

Table S1.5. List of works that included polychaetes in integrated multi-trophic aquaculture (IMTA) designs.

Species	Year	Reference	Country
Abarenicola pusilla	2019	Gómez, S., Hurtado, C. F. & Orellana, J. Bioremediation of organic sludge from a marine recirculating aquaculture system using the polychaete *Abarenicola pusilla* (Quatrefages, 1866). *Aquaculture* **507**, 377–384 (2019).	Chile
Hediste diversicolor	2019	Yousefi-Garakouei, M., Kamali, A. & Soltani, M. Effects of rearing density on growth, fatty acid profile and bioremediation ability of polychaete *Nereis diversicolor* in an integrated aquaculture system with rainbow trout (*Oncorhynchus mykiss*). *Aquac. Res.* **50**, 725–735 (2019).	Iran
Capitella sp.; Ophryotrocha craigsmithi	2019	Nederlof, M. *et al.* Application of polychaetes in (de)coupled integrated aquaculture: production of a high-quality marine resource. *Aquac. Environ. Interact.* **11**, 221–237 (2019).	Norway
Hediste diversicolor	2019	Wang, H. *et al.* Growth and nutritional composition of the polychaete *Hediste diversicolor* (OF Müller, 1776) cultivated on waste from land-based salmon smolt aquaculture. *Aquaculture* **502**, 232–241 (2019).	Norway
Abarenicola pusilla	2017	Gómez, S., Hurtado, C. F., Orellana, J., Valenzuela-Olea, G. & Turner, A. *Abarenicola pusilla* (Quatrefages, 1866): A novel species for fish waste bioremediation from marine recirculating aquaculture systems. *Aquac. Res.* 1–5 (2017).	Chile
Hediste diversicolor	2017	Pajand, Z. O., Soltani, M., Bahmani, M. & Kamali, A. The role of polychaete *Nereis diversicolor* in bioremediation of wastewater and its growth performance and fatty acid composition in an integrated culture system with *Huso huso* (Linnaeus , 1758). *Aquac. Res.* **48**, 5271–5279 (2017).	Iran
Perinereis aibuhitensis	2014	Yang, D., Cao, C., Wang, G., Zhou, Y. & Xiu, Z. The Growth Study of *Perinereis aibuhitensis* in Airlift Recirculating Aquaculture System. *Open Biotechnol. J.* **9**, 143–149 (2015).	China
Nereis virens	2011	Brown, N., Eddy, S. & Plaud, S. Utilization of waste from a marine recirculating fish culture system as a feed source for the polychaete worm, *Nereis virens*. *Aquaculture* **322**–**323**, 177–183 (2011).	USA
Perinereis nuntia; P. helleri	2010	Palmer, P. J. Polychaete-assisted sand filters. *Aquaculture* **306**, 369–377 (2010).	Australia
Sabella spallanzanii	2010	Stabili, L., Schirosi, R., Licciano, M., Mola, E. & Giangrande, A. Bioremediation of bacteria in aquaculture waste using the polychaete *Sabella spallanzanii*. *N. Biotechnol.* **27**, 774–781 (2010).	Italia
Nereis diversicolor	2008	García-Alonso, J., Hardege, J. D. & Müller, C. T. Influence of food regimes and seasonality on fatty acid composition in the ragworm. *Aquat. Biol.* **4**, 7–13 (2008).	UK
Sabella spallanzanii; Branchiomma luctuosum	2007	Cavallo, D., Pusceddu, A., Danovaro, R. & Giangrande, A. Particulate organic matter uptake rates of two benthic filter-feeders (*Sabella spallanzani*i and *Branchiomma luctuosum*) candidates for the clarification of aquaculture wastewaters. *Mar. Pollut. Bull.* **54**, 602–625 (2007).	Italy
Sabella spallanzanii	2005	Giangrande, a. *et al.* Utilization of the filter feeder polychaete *Sabella spallanzanii* Gmelin (Sabellidae) as bioremediator in aquaculture. *Aquac. Int.* **13**, 129–136 (2005).	Italy
Perinereis nuntia vallata	2002	Honda, H. & Kikuchi, K. Nitrogen budget of polychaete *Perinereis nuntia vallata* fed on the feces of Japanese flounder. *Fish. Sci.* **68**, 1304–1308 (2002).	Japan

Table S1.6. List of works that included other particulate organic matter (POM) extractive species integrated in multi-trophic aquaculture (IMTA) designs.

Species	Year	Reference	Country
Bivalves			
Pecten maximus	2019	Bergvik, M. *et al.* Incorporation of feed and fecal waste from salmon aquaculture in great scallops (*Pecten maximus*) co-fed by different algal concentrations. *Front. Mar. Sci.* **5**, 1–14 (2019).	Norway
Anomalocardia brasiliana	2019	Brito, L. O. *et al.* Bioremediation of shrimp biofloc wastewater using clam, seaweed and fish. *Chem. Ecol.* **34**, 901–913 (2018).	Brazil
Crassostrea rhizophorae	2014	Oliveira, K. F. De & Azevedo, R. V. De. Use of the oyster *Crassostrea rhizophorae* as a biological filter for effluent treatment to shrimp farm. *Ciências Agrárias* **35**, 2789–2798 (2014).	Brazil
Crassostrea gigas;Mytilus galloprovincialis	2014	Zhou, Y., Zhang, S., Liu, Y. & Yang, H. Biologically induced deposition of fine suspended particles by filter-feeding bivalves in land-based industrial marine aquaculture wastewater. *PLoS One* **9**, 1–6 (2014).	China
Mytilus edulis and Mytilus trossulus	2010	Reid, G. K. *et al.* Absorption efficiency of blue mussels (*Mytilus edulis* and *M . trossulus*) feeding on Atlantic salmon (*Salmo salar*) feed and fecal particulates: Implications for integrated multi-trophic aquaculture. *Aquaculture* **299**, 165–169 (2010).	Canada
Crassostrea corteziensis; Anadara tuberculosa	2009	Peña-Messina, E., Martínez-Cordova, L. R., Bückle-Ramírez, L. F., Segovia-Quintero, M. A. & Zertuche-González, J. A. A preliminary evaluation of physiological filtration variables for *Crassostrea corteziensis* (Hertlein , 1951) and *Anadara tuberculosa* (Sowerby , 1833) in shrimp aquaculture effluents. *Aquac. Res.* **40**, 1750–1758 (2009).	México
Sea cucumbers			
Holothura arguinensis	2019	Domínguez-Godino, J. A. & González-Wangüemert, M. Assessment of *Holothuria arguinensis* feeding rate, growth and absorption efficiency under aquaculture conditions. *New Zeal. J. Mar. Freshw. Res.* **53**, 60–76 (2019).	Portugal
Apostichopus japonicas	2019	Gao, X. *et al.* N and P budgets of *Haliotis discus hanai*, *Apostichopus japonicas*, and *Sebastes schlegeli* in a polyculture system. *Aquac. Res.* **50**, 2398–2409 (2019).	China
Actinopyga bannwarthi	2019	Israel, D., Lupatsch, I. & Angel, D. L. Testing the digestibility of seabream wastes in three candidates for integrated multi-trophic aquaculture: Grey mullet, sea urchin and sea cucumber. *Aquaculture* **510**, 364–370 (2019).	Israel
Holothuria scabra	2019	Robinson, G., Caldwell, G. S., Jones, C. L. W. & Stead, S. M. The effect of resource quality on the growth of *Holothuria scabra* during aquaculture waste bioremediation. *Aquaculture* **499**, 101–108 (2019).	South Africa
Holothuria forskali	2013	MacDonald, C., Stead, S. & Slater, M. Consumption and remediation of European Seabass (*Dicentrarchus labrax*) waste by the sea cucumber *Holothuria forskali*. *Aquac. Int.* **21**, 1279–1290 (2013).	UK
Cucumaria frondosa	2012	Nelson, E. J., Macdonald, B. A. & Robinson, S. M. C. The absorption ef fi ciency of the suspension-feeding sea cucumber , Cucumaria frondosa , and its potential as an extractive integrated multi-trophic aquaculture (IMTA) species. *Aquaculture* **370**–**371**, 19–25 (2012).	USA
Sea urchins			
Paracentrotus lividus	2019	Israel, D., Lupatsch, I. & Angel, D. L. Testing the digestibility of seabream wastes in three candidates for integrated multi-trophic aquaculture: Grey mullet, sea urchin and sea cucumber. *Aquaculture* **510**, 364–370 (2019).	Israel
Arthropodes			
Caprella equilibra; Caprella scaura	2016	Guerra-García, J. M. *et al.* Towards Integrated Multi-Trophic Aquaculture: Lessons from Caprellids (Crustacea: Amphipoda). *PLoS One* **11**, 1–26 (2016).	Spain
Fish			
Mugil cephalus	2019	Israel, D., Lupatsch, I. & Angel, D. L. Testing the digestibility of seabream wastes in three candidates for integrated multi-trophic aquaculture: Grey mullet, sea urchin and sea cucumber. *Aquaculture* **510**, 364–370 (2019).	Israel
Mugil cephalus	2016	Shpigel, M., Ari, T. Ben, Shauli, L., Odintsov, V. & Ben-Ezra, D. Nutrient recovery and sludge management in seabream and grey mullet co-culture in Integrated Multi-Trophic Aquaculture (IMTA). *Aquaculture* **464**, 316–322 (2016).	Israel
Sebastes schlegeli	2019	Gao, X. *et al.* N and P budgets of *Haliotis discus hanai*, *Apostichopus japonicas*, and *Sebastes schlegeli* in a polyculture system. *Aquac. Res.* **50**, 2398–2409 (2019).	China

Table S1.7. List of works that combined extractive species from different trophic levels integrated in multi-trophic aquaculture (IMTA) design.

Species	Year	Reference	Country
Bivalve + Seaweeds			
Crassostrea cuttackensis **+** *Ulva* spp.*****	2019	Biswas, G., Kumar, P., Kailasam, M., Ghoshal, T. K. & Bera, A. Application of Integrated Multi Trophic Aquaculture (IMTA) concept in brackishwater ecosystem: The first exploratory trial in the Sundarban, India. *J. Coast. Res.* **86**, 49–55 (2019).	India
Meretrix lusoria + *Gracilaria sp.*	2019	Chang, B. V. *et al.* Investigation of a farm-scale multitrophic recirculating aquaculture system with the addition of *Rhodovulum sulfidophilum* for milkfish (*Chanos chanos*) coastal aquaculture. *Sustain.* **11**, 1–15 (2019).	Taiwan
Perna perna **+** *Ulva lactuta*	2019	Nardelli, A. E., Chiozzini, V. G., Braga, E. S. & Chow, F. Integrated multi-trophic farming system between the green seaweed *Ulva lactuca*, mussel, and fish: a production and bioremediation solution. *J. Appl. Phycol.* **31**, 847–856 (2019).	Brazil
Crassostrea gigas **+** *Gracilaria lemaneiformis*	2019	Song, X., Pang, S., Guo, P. & Sun, Y. Evaluation of carrying capacity for shrimp pond culture with integrated bioremediation techniques. *Aquac. Res.* **00**, 1–9 (2019).	China
Crassostrea angulata **+** *Gracilaria verrucosa*	2017	Yeh, S., Dahms, H., Chiu, Y., Chang, S. & Wang, Y. Increased production and water remediation by land-based farm-scale sequentially integrated multi-trophic aquaculture systems — An example from Southern Taiwan. *Sustainability* **9**, 2–13 (2017).	Taiwan
Argopecten irradians **+** *Gracilaria lemaneiformis*	2016	Xianli, S. *et al.* Integrated bioremediation techniques in a shrimp farming environment under controlled conditions. *Acta Ocean. Sin.* **35**, 88–94 (2016).	China
*Crassostrea iredale*i + *Gracilaria* sp.	2006	Shimoda, T., Suryati, E. & Ahmad, T. Evaluation in a shrimp aquaculture system using mangroves, oysters, and seaweed as biofilters based on the concentrations of nutrients and Chlorophyll *a*. *Japan Agric. Res. Q.* **40**, 189–193 (2006).	Indonesia
Saccostrea commercialis **+** *Gracilaria edulis*	2002	Jones, A. B., Preston, N. P. & Dennison, W. C. The efficiency and condition of oysters and macroalgae used as biological filters of shrimp pond effluent. *Aquac. Res.* **33**, 1–19 (2002).	Australia
Saccostrea commercialis + *Gracilaria edulis*	2001	Jones, A. B., Dennison, W. C. & Preston, N. P. Integrated treatment of shrimp effluent by sedimentation, oyster filtration and macroalgal absorption: A laboratory scale study. *Aquaculture* **193**, 155–178 (2001).	Australia
Tapes philippinarum + *Ulva lactuta and Gracilaria sp.*	1996	Shpigel, M. & Neori, A. The integrated culture of seaweed, abalone, fish and clams in modular intensive land-based systems: I . Proportions of size and projected revenues. *Aquac. Eng.* **15**, 313–326 (1996).	Israel
Crassostrea gigas and/or Tapes semidecussatus + *Ulva lactuta*	1993	Shpigel, M., Neori, A., Popper, D. M. & Gordin, H. A proposed model for 'environmentally clean' land-based culture of fish, bivalves and seaweeds. *Aquaculture* **117**, 115–128 (1993).	Israel
Bivalve + Microalgae			
Chiome fluctifraga **+** *Navicula sp.*	2011	Martínez-Córdova, L. R., López-Elías, J. A., Martínez-Porchas, M., Bernal-Jaspeado, T. & Miranda-Baeza, A. Studies on the bioremediation capacity of the adult black clam, *Chione fluctifraga*, of shrimp culture effluents. *Rev. Biol. Mar. Oceanogr.* **46**, 105–113 (2011). Martínez-Córdova, L. R., López-Elías, J. A., Leyva-Miranda, G., Armenta-Ayón, L. & Martínez-Porchas, M. Bioremediation and reuse of shrimp aquaculture effluents to farm whiteleg shrimp, *Litopenaeus vannamei*: A first approach. *Aquac. Res.* **42**, 1415–1423 (2011).	México
Bivalves + Halophytes			
Scaphara subcrenata **+** *Salicornia europea*	2017	Chen, X. *et al.* A novel combined recirculating treatment system for intensive marine aquaculture. *Aquac. Res.* **48**, 5062–5071 (2017).	China
Polychaetes + Seaweeds			
Hediste diversicolor **+** *Ulva lactuca or Solieria chordalis*	2009	Bischoff, A. A., Fink, P. & Waller, U. The fatty acid composition of *Nereis diversicolor* cultured in an integrated recirculated system: Possible implications for aquaculture. *Aquaculture* **296**, 271–276 (2009).	Germany
Polychaetes + Halophytes			
Hediste diversicolor **+** *Halimione portulacoides*	2017	Marques, B., Calado, R. & Lillebø, A. I. New species for the biomitigation of a super-intensive marine fish farm effluent: Combined use of polychaete-assisted sand filters and halophyte aquaponics. *Sci. Total Environ.* **600**, 1922–1928 (2017).	Portugal
Echinoderms + Seaweeds			
Paracentrotus lividus + *Ulva lactuta*	2018	Shpigel, M. *et al.* The sea urchin, *Paracentrotus lividus*, in an integrated multi-trophic aquaculture (IMTA) system with fish (*Sparus aurata*) and seaweed (*Ulva lactuca*): Nitrogen partitioning and proportional configurations. *Aquaculture* **490**, 260–269 (2018).	Israel

Chapter 2

2.1. Performance of polychaete assisted sand filters under contrasting nutrient loads in an integrated multi-trophic aquaculture (IMTA) system

Jerónimo, D., Lillebø, A.I., Santos, A., Cremades, J., Calado, R. Performance of polychaete assisted sand filters under contrasting nutrient loads in an integrated multi-trophic aquaculture (IMTA) system.
Scientific Reports **10,** 20871 (2020). https://doi.org/10.1038/s41598-020-77764-x

2.1. Performance of polychaete assisted sand filters under contrasting nutrient loads in an integrated multi-trophic aquaculture (IMTA) system

Abstract

Polychaete assisted sand filters (PASFs) allow to combine a highly efficient retention of particulate organic matter (POM) present in aquaculture effluent water and turn otherwise wasted nutrients into valuable worm biomass, following an integrated multi-trophic aquaculture (IMTA) approach. This study evaluated the bioremediation and biomass production performances of three sets of PASFs stocked with ragworms (*Hediste diversicolor*) placed in three different locations of an open marine land-based IMTA system. The higher organic matter (OM) recorded in the substrate of the systems which received higher POM content (Raw and Df PASFs – filtered raw and screened by drum filter effluent, respectively) likely prompted a superior reproductive success of stocked polychaetes (final densities 2-7 times higher than initial stock; ≈1000-3000 ind. m^{-2}). Bioremediation efficiencies of ≈70% of supplied POM (≈1.5-1.8 mg L^{-1}) were reported in these systems. The PASFs with lower content of OM in the substrate (Df+Alg PASFs – filtered effluent previously screened by drum filter and macroalgae biofilter) differed significantly from the other two, with stocked polychaetes displaying a poorer reproductive success. The PASFs were naturally colonized with marine invertebrates, with the polychaetes *Diopatra neapolitana*, *Terebella lapidaria* and *Sabella* cf. *pavonina* being some of the species identified with potential for IMTA.

2.1.1. Introduction

Marine and brackish water aquaculture production contribute significantly for the world food security and in 2018 represented approximately 56% and 45% of the volume and value generated by this sector (values above 111 million tonnes and USD 250 billions)[1]. The production of fish contributed greatly for these values being reported productions of ≈12% and 31% of the volume and value of saltwater production in 2018 (diadromous species included)[1]. The intensive production of the majority of these organisms require well nutritionally balanced formulated feeds. The total use of aquafeeds estimated for 2016 alone was ≈49.6 million tonnes, being expected to rise to 76.2 million tonnes by 2025[2]. Not all these feeds are fully converted into biomass of cultured species and a non-negligible portion of these nutrients are often wasted in the form of uneaten feed, or due to the inability of farmed species to fully assimilate ingested nutrients (being commonly excreted through faeces)[3-6]. Some studies pointed that only 25 - 40% of the whole nitrogen and phosphorus (N and P, respectively) available in aquafeeds is truly assimilated in the form of biomass by fed species[6,7]. Carnivorous finfish excrete between 50 to 80% of feed N and 35 to 85% of feed P[8]. These nutrients are present in the water in the form of particulate organic matter (POM), dissolved organic matter (DOM) (include dissolved organic nitrogen [DON] and phosphorus [DOP]) and dissolved inorganic nutrients (include dissolved inorganic nitrogen [DIN] = NO_x-N + NH_4-N and dissolved inorganic phosphorus [DIP] = PO_4-P)[9,10]. In this way, the investment made by producers in aquafeeds is not fully recovered in the form of biomass by the target species being farmed, and wasted nutrients commonly need to be eliminated from the productive process (including effluent water) by more or less complex processes that ultimately represent another financial burden. The recovery of these nutrients into valuable biomass and the consequent reduction of capital loss from uneaten aquafeeds (> 50% of operating costs[11]) are goals that can be achieved by adopting an integrated multi-trophic aquaculture (IMTA) approach. This concept considers the integrated production of commercially valuable species that rank in different levels of the trophic chain, in order to maximize the recovery of nutrients initially supplied through aquafeeds to the production system, but are not fully used by the target species being fed. This concept, and their main potentialities and limitations has been the topic of numerous reviews in recent years[12-19]. In marine land-based production, the recovery of valuable nutrients present in effluent waters can be pursued through the integration of extractive species capable of recovering available POM. Marine polychaetes have been often pinpointed as holding great potential to recover

wasted nutrients from POM with species such as *Hediste diversicolor*[9,20-25], *Perinereis aibuhitensis*[26], *Alitta virens*[26,27], *Perinereis nuntia*[28,29], *Perinereis helleri*[29,30], *Arenicola marina*[26], *Abarenicola pusilla*[31,32], *Capitella* sp. and *Ophryotrocha craigsmithi*[33] already being tested under IMTA designs. These marine worms have already been successfully combined with organisms that may retain dissolved inorganic nutrients efficiently, such as macro and microalgae[22,23], as well as halophytes[9].

The ragworm, *Hediste diversicolor* (O.F. Müller, 1776), has been a key species to include in IMTA models, as it allows the recovery of otherwise wasted nutrients in the form of a biomass rich in highly unsaturated fatty acids (HUFA), namely eicosapentaenoic (EPA, 20:5 *n* - 3), docosahexaenoic (DHA, 22:6 *n* - 3) and arachidonic (ARA, 20:4 *n* - 6)[20-25]. The ability of this species to synthesise *de novo* polyunsaturated fatty acids (PUFA) and HUFA[34] is an important feature when selecting the different trophic compartments that will be included in an IMTA design. The recovery of nutrients from aquafeeds into ragworms biomass is of great relevance if one considers the high demand for lipids and FA (namely *n*-3 HUFA) for both human and animal nutrition[22]. Additionally, this species is also commonly used in marine finfish and crustaceans maturation diets[22,34] and is one of the most prized baits for sports fishing[35]. The development of production models for these organisms allows to suppress their growing demand and avoid over-exploitation of natural stocks[36-38]. By reworking the substrate, ragworms can be termed as biodiffusors with important ecosystem engineering functions[39,40]. These organisms build extensive burrows and promote bioturbation (i.e., biogenic transport of sediment particles and pore water which destroys sediment stratigraphy[41]) and bioirrigation (i.e., ventilation of burrows and diffusion of oxidized solutes by infauna[41,42]). Microenvironments with steep gradients between reduced and oxidized compounds are created in polychaetes burrows, which act as transition zones that support enhanced microbial activities and are favour reoxidation processes[39,42,43]. Biogeochemical processes, such as carbon oxidation reactions (e.g., denitrification), manganese, iron and sulphate reduction are highly dependent on reoxidation and transport processes associated to bioturbation[39,42,44].

Polychaete assisted sand filters (PASFs), are sand column filters stocked with marine worms that are highly efficient in the recovery of POM in the form of these species biomass[9,20,21,29,30]. By fostering the retention of POM and contributing to its mineralization, thus enhancing the availability of dissolved inorganic nutrients, PASFs can play a key role in IMTA designs including macro/micro algae and/or halophytes, if integrated as the first extractive unit[9,23]. In order to fine tune the use of PASFs in IMTA, the present work tested

the performance of PASFs stocked with the ragworm *H. diversicolor* in different locations of an open marine land-based IMTA facility. These locations were selected to ensure that PASFs were supplied with effluent water with contrasting loads of nutrients, in order to better understand how these would limit or improve the successful production of ragworms. To achieve this goal a first set of PASFs was installed to filter the raw fish farm effluent originating from earthen ponds stocked with gilthead seabream (*Sparus aurata*); the second set to filter the same effluent but screened by a drum filter (45-µm mesh size) and finally the third set to filter the same effluent screened by a drum filter and subsequently by a macroalgae biofilter (stocked with sea lettuce, *Ulva rigida*).

2.1.2. Material and methods

2.1.2.1. Selected extractive species

The polychaete *Hediste diversicolor*, popularly known as ragworm, was selected for the present study 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 produce three-dimensional burrow network in sandy-mud bottoms[49]. This species is classified as presenting free movement via the burrow system and as a biodiffusor in the sediment reworking, thus presenting an important action in bioturbation and bioirrigation - the biogenic modification of sediments through particle reworking and burrow ventilation, a key mediating process of many important geochemical processes in marine systems[40]. This polychaete species is omnivorous, being classified as an active predator[50]. However, it also exhibits a deposit-feeding behaviour that allows it to mainly consume organic matter present in the substrate[47,51]. The two main feeding strategies it displays are crawling on the sediment surface prospecting for food, catching it with its jaws and ingesting it immediately, as well as capturing food with mucous secretions that are deposited at the entrance of its burrow[47]. Bacteriolytic activity in their digestive tract demonstrates that this species is a significant bacteriovore as well[52]. Juveniles can accumulate plant detritus in their burrow where constant irrigation holds aerobic conditions that favour the decay process of plant debris by stimulating bacterial growth[53]. Ragworms can also be facultative filter-feeders, which meet metabolic requirements on a pure diet of phytoplankton, much like a typical obligate filter-feeder species[54,55]. Its life cycle is characterized by females

brooding their embryos in the maternal burrow, where its short pelagic larval life takes place[47]. The environmental engineering behaviour, along with the fact of exhibiting a biomass rich in essential fatty acids (EFA) makes them an appealing extractive species for IMTA systems.

2.1.2.2. IMTA experimental design

The organic rich effluent used in the present study resulted from the semi-intensive production of gilthead seabream (*Sparus aurata*) (≈ 12.000 specimens with average weight ≈ 400 g) stocked in an earthen pond and fed twice a day (SFR ≈ 1.5% day^{-1}) with a commercial diet that present 43% of crude protein, 17% of crude fat and 10% of crude fiber (Standard Orange 4; AQUASOJA). The effluent water used was collected at the end of this earthen pond. The first set of PASFs was supplied with the raw effluent water without any type of filtration (Raw PASFs), while the second set received the raw effluent but mechanically filtered by a drum filter (45µm mesh size) (Df PASFs). The third set received the raw effluent filtered by the drum filter and after passing through a macroalgae biofilter stocked with *Ulva rigida* (Df+Alg PASFs). The algae biofilter presented a volume of 36 m^3 (surface area of 24 m^2), with a flow rate varying between 50 to 100 L h^{-1} (3.3%-7% renewal day^{-1}) and *U. rigida* being cultured at a density between 2.5 – 5 Kg FW m^{-2}. Each of the above-mentioned sets of PASFs consisted of 5 tanks each arranged in a parallel set-up. Each replicate tank from each of the three sets of PASFs presented a volume of 0.1 m^3 and a surface area of 0.3 m^2 and featured a 200 mm bottom sand bed (0.7 - 1 mm grain size) and a superficial 100 mm water column. To allow a complete percolation of the effluent water being supplied through the sand bed, each tank was equipped with a bottom draining pipe bellow the sand bed. Each tank received an effluent flow of 25 L h^{-1} (0.5 renewal each hour) and the treated water was not re-used, thus being the system employed an open-IMTA. The schematic representation of the experimental set-up adopted is presented in Fig.2.1. The present study was run for a total of 15 weeks (from July 2017 to November 2017), during which no additional feed was provided to any of the three sets of PASFs.

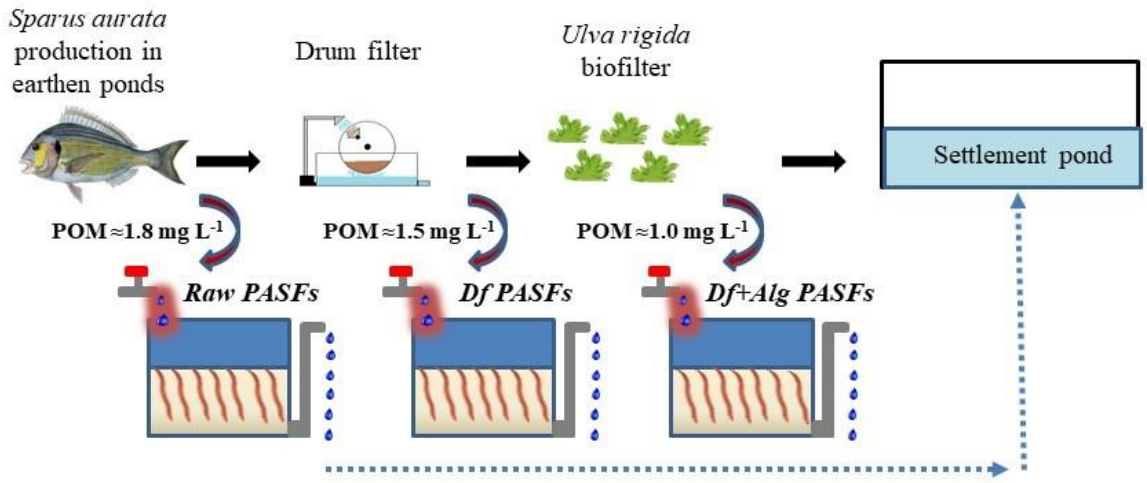


Figure 2.1. Schematic representation of the experimental set-up adopted with polychaete assisted sand filters (PASFs) placed in different locations of an open marine land-based IMTA facility: Raw PASFs – received the raw effluent from the earthen pond stocked with Sparus aurata; Df PASFs - received the raw effluent after being screened by a drum filter; and Df+Alg PASFs – received effluent after being screened by a drum filter and a macroalgae biofilter stocked with Ulva rigida.

2.1.2.3. IMTA extractive species cultivation

Wild specimens of *H. diversicolor* were collected in Aveiro coastal lagoon by local fisherman and each sand bed was inoculated with an initial density of 440 ind. m^{-2} (167 g m^{-2}) of polychaetes with a length superior to 40 mm. Fifteen weeks post-stocking, the polychaetes biomass was evaluated by performing five hand core samples (Ø 75 mm, 150 mm depth) from each replicate tank on each of the three sets of PASFs, with their content being preserved in buffered 4% formaldehyde for latter analysis. In the laboratory, specimens of *H. diversicolor* were sorted in two distinct groups (new generation biomass, displaying a length < 5 mm and original stocking biomass, with a length > 40 mm). Other polychaete species that naturally colonised the PASFs were also sorted and identified to species level according to Fauvel (1923)[56,57]. Ash free dry weight (AFDW) of *H. diversicolor* and other polychaete species that naturally colonized the PASFs were determined by loss of ignition method (LOI%; 5 h combustion at 450 ºC of samples previously dried at 90 ºC, until constant weight was recorded). Sediment samples from each replicate tank of each of the three sets of PASFs were collected in triplicate at the beginning and at the end of experiment to determine organic matter (OM) content. This content was determined by the difference between dry weight and ash free dry weight, using the LOI% determination described above.

2.1.2.4. IMTA monitoring

Temperature, pH, dissolved oxygen (DO) and salinity determined in the inflowing water of each of the three sets of PASFs was monitored weekly. Due to differences promoted by seaweed biofilter during daytime, each parameter was monitored at three distinct periods: 10 AM, 2 PM and 6 PM. The monitoring was performed using a multi-parameter probe Lovibond SensoDirect 150. Samples from the inflowing water of each of the five tanks of the three sets of PASFs, as well as the outflowing water of each PASFs tank after having percolated through the sand bed, were collected every week, in order to determine suspended particulate matter (SPM), particulate organic matter (POM), total nitrogen (TN), total phosphorus (TP), dissolved inorganic nitrogen (DIN = NOx-N + NH_4-N) and dissolved inorganic phosphorus (DIP = PO_4-P). Water samples were transported to the laboratory under dark and refrigerated conditions and immediately filtered (Whatman GF/C, Ø47mm dehydrated (105 °C) and pre-weighed filters) and subsequently frozen (−20 °C) until further analysis. Filters containing SPM were processed following the EPA method 160.2 (USEPA) and POM was determined by loss of ignition method (LOI%; 5 h combustion at 450 ºC of samples previously dried at 90 ºC, until constant weight was recorded), resulting from the difference between dry weight and ash free dry weight (AFDW). Water samples were analysed using an automated continuous flow analyser (Skalar San^{++}) to determine the content of TN, TP, NH_4-N and PO_4-P. The oxidized forms of NOx-N were determined using a flow injection system (FIAstar 5000 Analyser). The analytical quality control was ensured by using calibration curves that result from running standard solutions at the beginning and in parallel with blanks and samples. All analyses were performed according to the protocols made available to each parameter by the equipment´s manufacturer.

2.1.2.5. Statistical analysis

Data retrieved over 15 consecutive weeks on the environmental parameters (Temp., oxygen, pH, salinity) of the inflowing water supplying each of the three sets of PASFs being compared (Raw, Df and Df+Alg PASFs) were used to prepare three independent resemblance matrixes. A first resemblance matrix was prepared for environmental parameters being monitored in triplicate at 10 AM (n=3), a second one for parameters monitored at 2 PM (n=3) and a third matrix for parameters monitored at 6 PM (n=3). The

rationale for assembling these three independent resemblance matrixes was the shifts known to occur a priori on the environmental parameters of the inflowing water caused by time of day. Each of these three resemblance matrixes was compared separately using permutational multivariate analysis of variance (PERMANOVA), with PASFs being used on each of them as a fixed predictive factor (with three levels: Raw, Df and Df+Alg). A fourth resemblance matrix was also prepared using data retrieved over 15 consecutive weeks on the following parameters of the inflowing water being supplied to each of the three sets of PASFs: SPM, POM, TN, DIN, TP and DIP. Samples were always collected in triplicate (n=3) at 10 AM. This resemblance matrix was also compared using PERMANOVA, with PASFs also being used as a fixed predictive factor (with three levels: Raw, Df and Df+Alg). All resemblance matrixes were prepared using Euclidean distances of data previously normalized. Whenever significant differences (p<0.05) were detected, these were further examined using a posteriori pair-wise comparison. Similarity percentage (SIMPER) analysis (cut-off 90%) were also performed to evaluate the percentage that each environmental parameter (Temp., oxygen, pH, salinity) or water composition parameters (SPM, POM, TN, DIN, TP and DIP) contributed to the dissimilarity recorded between PASFs. PERMANOVA and SIMPER analysis were performed using PRIMER v6 with the PERMANOVA+ add-on (PRIMER-E, UK), according to the procedures described by Anderson, Gorley & Clarke (2006)[58].

To compare POM retention efficiency in each of PASFs tested, the level of POM present in the outflowing water of each of the five tanks (n=5) from the three sets of PASFs being compared (Raw, Df and Df+Alg PASFs) were determined over 15 consecutive weeks at 10 AM. The existence of significant differences was tested using the non-parametric test of Kruskal-Wallis (p<0.05) with PASFs being used as fixed predictive factor (with three levels). The organic matter recorded in the sand beds of each of the five tanks (n=5) from the three sets of PASFs being compared at the end of experiment, as well as differences in the final abundance (ind. m^{-2}) of *H. diversicolor*, were compared between each pair of PASFs using the non-parametric test of Kruskal-Wallis (p<0.05). Data were previously checked for normality (Anderson-Darling test) and homogeneity of variances (Bartlett´s and Levene´s tests). These statistical analyses were performed using Minitab 18 Statistical Software (State College, PA).

A cluster analysis of the ratio between initial stocking and abundance of newly generated *H. diversicolor* recorded in each tank was also performed using PRIMER v6 with the PERMANOVA+ add-on (PRIMER-E, UK).

The statistical results of the tests mentioned above are summarized in supplementary Tables S2.1-S2.6.

2.1.3. Results

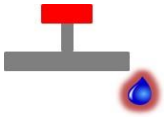
2.1.3.1. Characterization of inflowing water and POM bioremediation promoted by polychaete assisted sand filters (PASFs)

Table 2.1 summarizes the average values (± SD) of pH, concentration of dissolved oxygen (DO), temperature and salinity monitored in the inflowing water of each PASFs over 15 weeks at 10 AM, 2 PM and 6 PM (weekly characterization – Supplementary Fig. S2.1 – S2.4). The PERMANOVA analysis revealed significant differences in the environmental conditions of inflowing water supplying each set of PASFs at 10 AM (p = 0.001), 2 PM (p = 0.001) and 6 PM (p = 0.001) (Supplementary Table S2.1). The SIMPER analysis (cut-off level 90%) revealed the existence of variable dissimilarities between, Raw – Df (4.2 - 7.9%), Raw – Df+Alg (10.2 - 12.8%) and Df – Df+Alg (6.3 - 7.3%), respectively. The following parameters that contributed the most for the dissimilarities recorded are summarized in Supplementary Table S2.2. The lowest average value of pH and oxygen were measured in Raw, while the lowest variance of both parameters between periods was recorded in Df system. The DO measured at 2 PM in Df+Alg PASFs was approximately twice that recorded at Raw PASFs.

Figure 2.2 summarizes the average values (± SD) of suspended particulate matter and particulate organic matter (SPM and POM, respectively), total and dissolved inorganic nitrogen (TN and DIN, respectively) and total and dissolved inorganic phosphorus (TP and DIP, respectively) in inflowing and outflowing water of each PASFs.

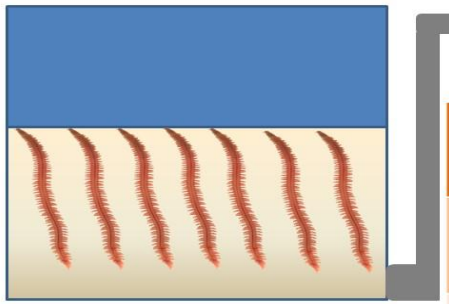
Table 2.1. Average values (±SD) of pH, dissolved oxygen (DO), temperature and salinity measured weekly (n=5) in the inflowing water of each set of polychaete assisted sand filters (PASFs) at 10 AM, 2 PM and 6 PM. Raw PASFs – received the raw effluent from the earthen pond stocked with *Sparus aurata*; Df PASFs - received the raw effluent after being screened by a drum filter; and Df+Alg PASFs – received effluent after being screened by a drum filter and a macroalgae biofilter stocked with *Ulva rigida*.

	10 AM				**2 PM**			**6 PM**		
PASFs	pH	O_2 (mg L^{-1})	Temp. (°C)	Salinity	pH	O_2 (mg L^{-1})	Temp. (°C)	pH	O_2 (mg L^{-1})	Temp. (°C)
Raw	7.66 ± 0.14	5.52 ± 0.94	19.28 ± 2.29	40.08 ± 1.01	7.64 ± 0.12	5.98 ± 0.66	20.96 ± 2.28	7.73 ± 0.17	6.47 ± 1.26	20.49 ± 4.00
Df	7.78 ± 0.15	7.95 ± 0.25	19.44 ± 2.28	40.07 ± 1.01	7.81 ± 0.16	8.01 ± 0.42	20.81 ± 2.44	7.83 ± 0.19	7.94 ± 0.77	20.31 ± 3.99
Df+Alg	8.20 ± 0.24	8.40 ± 0.80	18.87 ± 2.29	40.04 ± 1.11	8.61 ± 0.19	10.11 ± 0.94	21.27 ± 2.41	8.81 ± 0.14	8.68 ± 0.83	20.76 ± 2.97



Inflowing water

PASFs	Particulate matter	Nitrogen	Phosphorus
Raw	SPM: 12.96 ± 9.84 POM: 1.82 ± 1.01	TN: 0.95 ± 0.21 DIN: 0.59 ± 0.13	TP: 0.13 ± 0.04 DIP: 0.11 ± 0.03
Df	SPM: 9.75 ± 6.45 POM: 1.54 ± 0.73	TN: 0.78 ± 0.24 DIN: 0.51 ± 0.17	TP: 0.11 ± 0.03 DIP: 0.10 ± 0.03
Df+Alg	SPM: 4.12 ± 1.71 POM: 1.00 ± 0.44	TN: 0.32 ± 0.09 DIN: 0.09 ± 0.04	TP: 0.07 ± 0.02 DIP: 0.05 ± 0.02



Outflowing water

PASFs	Particulate matter	Nitrogen	Phosphorus
Raw	SPM: 1.38 ± 0.78 **(-89%)** POM: 0.51 ± 0.34 **(-72%)**	TN: 0.83 ± 0.14 **(-13%)** DIN : 0.66 ± 0.14 **(+12%)**	TP: 0.14 ± 0.02 **(+8%)** DIP: 0.13 ± 0.02 **(+18%)**
Df	SPM: 1.68 ± 1.04 **(-83%)** POM: 0.48 ± 0.29 **(-69%)**	TN: 0.78 ± 0.23 **(0%)** DIN : 0.62 ± 0.17 **(+22%)**	TP: 0.12 ± 0.03 **(+9%)** DIP: 0.11 ± 0.02 **(+10%)**
Df+Alg	SPM: 1.73 ± 1.19 **(-58%)** POM: 0.60 ± 0.42 **(-40%)**	TN: 0.33 ± 0.09 **(+3%)** DIN: 0.16 ± 0.05 **(+78%)**	TP: 0.07 ± 0.02 **(0%)** DIP: 0.06 ± 0.02 **(+20%)**

Figure 2.2. Average values (±SD) of suspended particulate matter (SPM), particulate organic matter (POM), total nitrogen (TN), dissolved inorganic nitrogen (DIN), total phosphorus (TP) and dissolved inorganic phosphorus (DIP) of the values determined over 15 consecutive weeks (n=15) in each of the three sets of polychaete assisted sand filters (PASFs): Raw PASFs – received the raw effluent from the earthen pond stocked with *Sparus aurata;* Df PASFs - received the raw effluent after being screened by a drum filter; and Df+Alg PASFs – received effluent after being screened by a drum filter and a macroalgae biofilter stocked with *Ulva rigida*.

No significant differences were recorded in the composition of inflowing water supplying Raw and Df PASFs (PERMANOVA: $p = 0.240$) (Supplementary Table S2.3). SIMPER analysis (cut-off 90%) revealed dissimilarities of 8.35% (Supplementary Table S2.4). The composition of inflowing water supplying Df+Alg PASFs differed significantly from Raw PASFs (PERMANOVA: $p = 0.001$) and Df PASFs (PERMANOVA: $p = 0.001$). SIMPER analysis (cut-off 90%) revealed dissimilarities of 21% and 14.5%, respectively.

Raw, Df and Df+Alg PASFs promoted the retention of approximately 72%, 69% and 40% of supplied POM. These values represent removal efficiencies of 4.4, 3.5 and 1.3 mg L^{-1} m^{-2} (for Raw, Df and Df+Alg PASFs and refer to weekly characterizations - Supplementary Fig. S2.5). No significant differences were found for POM content present in the outflowing water of all PASFs (Kruskal-Wallis: $p = 0.335$) (Supplementary Table S2.5). During the study the concentration of OM (%LOI) at the sand bed of the PASFs was maintained between 0.42 - 0.69%, 0.63 - 0.75% and 0.36 - 0.58% (for Raw, Df and Df+Alg PASFs, respectively) and at the end of experiment no significant differences were recorded between the OM content of Raw and Df PASFs (Kruskal-Wallis: $p = 0.917$) (0.69 ± 0.03% and 0.75 ± 0.16%, respectively) (Supplementary Table S2.5). However, the content reported for Df+Alg (0.58 ± 0.05%) was significantly lower to that recorded in the other PASFs tested (Kruskal-Wallis: $p = 0.009$ and $p = 0.028$, respectively).

The outflowing water of each PASFs displayed higher levels of DIN than inflowing water (+12%, +22% and +78% for Raw, Df and Df+Alg PASFs, respectively). The predominant form of DIN in inflowing water was NH_4-N, while in the outflowing water of PASFs the most abundant form was NO_x-N (sum of NO_2-N and NO_3-N) (Supplementary Fig. S2.6 and S2.7, respectively). Concerning phosphorus, the outflowing water presented higher concentrations of TP (+8%, +9%, +0%, for Raw, Df and Df+Alg PASFs, respectively) and DIP (+18%, +10% and +20%, for Raw, Df and Df+Alg PASFs, respectively) compared to the concentrations in the inflowing water.

2.1.3.2. Biomass generation

Table 2.2 summarizes the average density (± SD) of *H. diversicolor* determined in each PASFs at the end of experimental period (15 weeks). No significant differences were found between final densities of Raw and Df PASFs (Kruskal-Wallis: $p = 0.117$) (Supplementary Table S2.6). These PASFs presented average values of 996 ± 627 and 3015 ± 2485 ind. m^{-}

^{2} (respectively), which corresponded to ≈ 2 - 7-fold increases of the initial stocking density. The final density reported for Df+Alg PASFs was significantly lower (79 ± 64 ind. m^{-2}) from that recorded for the other two sets of PASFs (Kruskal-Wallis: p = 0.009 and p = 0.009, respectively), with ≈ 6-fold decrease of initial stocking density. In respect to the proportion between initially stocked polychaetes and newly generated ones, 90% and 100% of the specimens identified in the Raw and Df PASFs (respectively) were classed as newly generated biomass (<5 mm). Most specimens identified in Df+Alg PASFs corresponded to adult polychaetes (≈ 86%) belonging to the initial stock. Figure 2.3 displays the cluster analysis of *H. diversicolor* group composition (initially stocked and newly generated specimens) which allow to verify that all the replicates of Raw and Df PASFs were represented in separate and well-defined groups, with a similarity between them higher than 88%. In Df+Alg PASFs, three of the replicates do not present any signs of reproduction (Supplementary Table S2.7) and therefore exhibited less than 30% similarity with other PASFs. The biomass of *H. diversicolor* recorded at the end of the experiment was 1.2 ± 0.8, 0.3 ± 0.2 and 0.6 ± 0.8 g Ash-Free Dry Weight (AFDW) m^{-2} for Raw, Df and Df+Alg PASFs, respectively.

Table 2.2. Average values (±SD) of density (ind. m^{-2}) and biomass (g. AFDW m^{-2}) of *H. diversicolor* determined at each polychaete assisted sand filters (PASFs) at the end of experimental period. Raw PASFs – received the raw effluent from the earthen pond stocked with *Sparus aurata*; Df PASFs - received the raw effluent after being screened by a drum filter; and Df+Alg PASFs – received effluent after being screened by a drum filter and a macroalgae biofilter stocked with *Ulva rigida*.

PASFs	**Original stock**	**New generation**	**Total**	
	Density (ind. m^{-2})	Density (ind. m^{-2})	Density (ind. m^{-2})	Biomass (g. AFDW m^{-2})
Raw	100 ± 59	896 ± 642	996 ± 626 [a]	1.16 ± 0.80
Df	ND	3015 ± 2485	3015 ± 2485 [a]	0.28 ± 0.22
Df+Algae	73 ± 51	18 ± 25	91 ± 55 [b]	0.63 ± 0.82

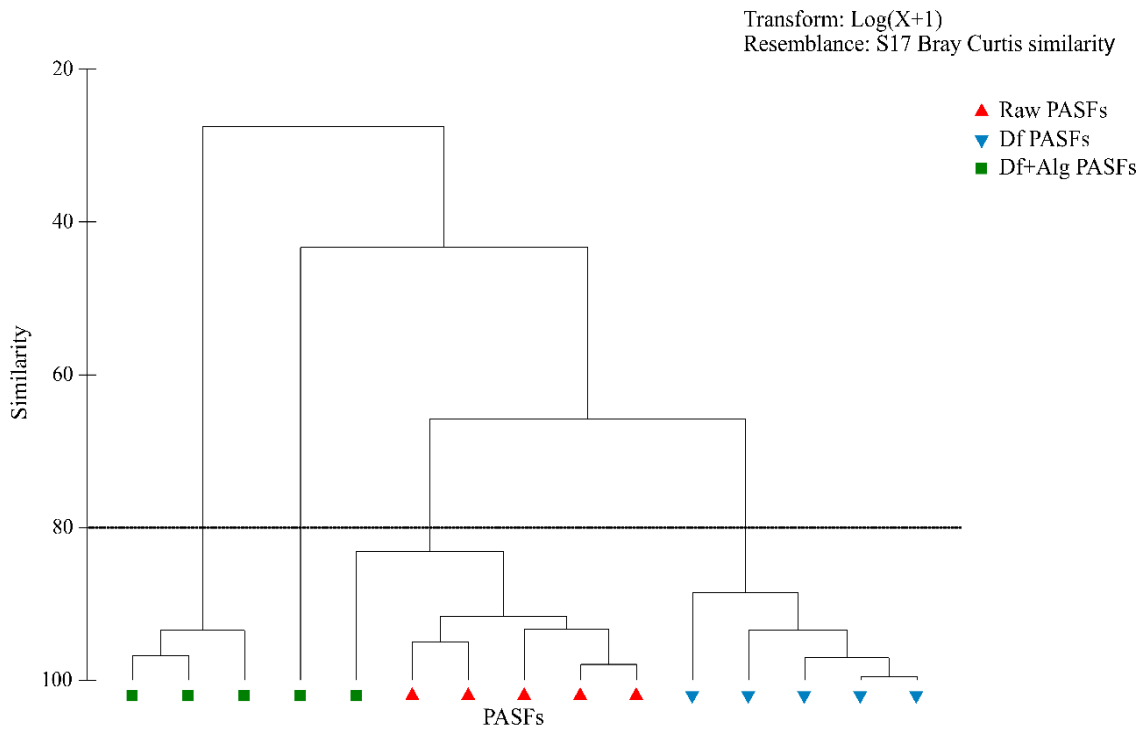


Figure 2.3. CLUSTER analysis of *H. diversicolor* groups composition (initially stocked and new generation specimens) recorded in each polychaete assisted sand filter (PASFs). Raw PASFs – received the raw effluent from the earthen pond stocked with *Sparus aurata*; Df PASFs - received the raw effluent after being screened by a drum filter; and Df+Alg PASFs – received effluent after being screened by a drum filter and a macroalgae biofilter stocked with *Ulva rigida.*

The three sets of PASFs were exposed to a potential colonization of other species occurring in the coastal lagoon supplying the earthen pond stocked with seabream. Table 2.3 summarizes the densities and biomass of the most well-represented species in each PASFs, with emphasis to polychaetes *Diopatra neapolitana* (Onuphidae), *Terebella lapidaria* (Terebellidae) and *Sabella* cf. *pavonina* (Sabellidae) (represented in Fig. 2.4). The total biomass (all species included) accounted for approximately 8.4, 6.1 and 5.2 g AFDW m^{-2} at the Raw, Df and Df+Alg, respectively.

Table 2.3. Biomass (g. AFDW m^{-2}) and density (ind. m^{-2}) of the most represented species (excluding *Hediste diversicolor*) present in different polychaete assisted sand filters (PASFs). Raw PASFs – received the raw effluent from the earthen pond stocked with *Sparus aurata*; Df PASFs - received the raw effluent after being screened by a drum filter; and Df+Alg PASFs – received effluent after being screened by a drum filter and a macroalgae biofilter stocked with *Ulva rigida*.

PASFs	Density (ind. m^{-2})	Biomass (g AFDW m^{-2})
Raw		
Capitella capitata	7415 ± 2017	1.63 ± 0.73
Terebella lapidaria	4237 ± 941	2.80 ± 0.28
Chorophium sp.	2906 ± 1513	0.16 ± 0.11
Melita palmata	1476 ± 861	0.48 ± 0.30
Hydrobia acuta	1177 ± 326	0.42 ± 0.12
Sabella cf. *pavonina*	444 ± 117	1.20 ± 0.52
Phallusia mammillata	308 ± 137	0.56 ± 0.30
Actiniaria sp.	163 ± 109	0.40 ± 0.41
Diopatra neapolitana	82 ± 74	0.73 ± 0.97
Df		
Capitella capitata	3468 ± 1083	0.61 ± 0.27
Terebella lapidaria	2327 ± 556	2.85 ± 0.98
Hydrobia acuta	1286 ± 640	0.95 ± 0.97
Actiniaria sp.	353 ± 152	0.17 ± 0.06
Phallusia mammillata	199 ± 76	1.16 ± 0.91
Sabella cf. *pavonina*	81 ± 74	0.36 ± 0.41
Df+Alg		
Hydrobia acuta	5586 ± 2056	1.63 ± 0.40
Boccardia polybranchia	2363 ± 796	0.25 ± 0.12
Haminoea sp.	1087 ± 1245	1.06 ± 0.67
Malacoceros sp.	1014 ± 840	0.16 ± 0.12
Terebella lapidaria	290 ± 214	1.95 ± 1.19
Phallusia mammillata	45 ± 45	0.18 ± 0.24

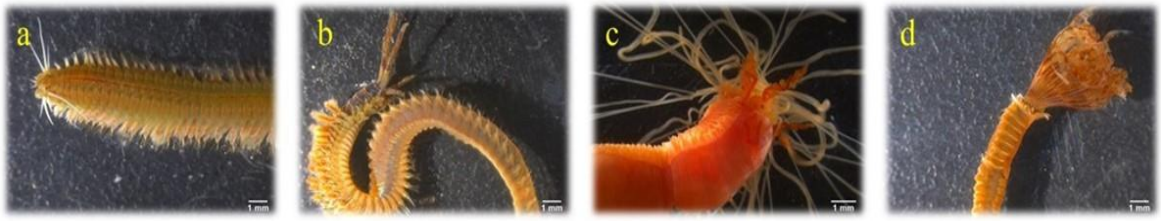


Figure 2.4. Polychaete species presented in polychaete assisted sand filters (PASFs): A) *Hediste diversicolor*; B) *Diopatra neapolitana*; C) *Terebella lapidaria* and D) *Sabella* cf. *pavonina*.

2.1.4. Discussion and conclusions

In the present work, the three sets of PASFs successfully recovered POM present in effluent waters in the form of valuable worm biomass. The Raw and Df PASFs, which filtered the raw effluent water from the production ponds of gilthead seabream and the same effluent but previously screened by a drum filter, respectively, retained approximately 70% of POM (1.8 and 1.5 mg L^{-1}, respectively). The lowest efficiency in POM retention (≈ 40% of 1.0 mg L^{-1}) was displayed by Df+Alg PASFs, most likely due to these tanks receiving smaller-sized particulate matter. This prevalence of smaller-sized particles resulted from the joint action of mechanical filtration (which fragmented larger-sized particles) and the deposition of larger particles in the macroalgae biofilter. It is also important to highlight that the nature of POM present in Df+Alg PASFs was certainly different from that in other PASFs, mostly resulting from the macroalgae biofilter (essentially macroalgae biomass) instead of fish feed/faeces. The use of the different systems tested will filter approximately 2000 L m^{-2} day^{-1}. Based on filtering efficiencies recorded, POM retention will vary between 2.1 - 2.6 g m^{-2} day^{-1} using Raw and Df PASFs, as long as the composition of the inflowing water is maintained throughout the day. A lower efficiency is expected to occur for Df+Alg PASFs (0.8 g m^{-2} day^{-1}).

To prevent the build-up of OM, and consequently preserve the filter function of sand bed, it is paramount that polychaetes successfully secure bioturbation and bioirrigation processes[9,45]. In a previous study also employing *H. diversicolor* in sand beds to filter the effluent derived from super-intensive production of Senegalese sole (*Solea senegalensis*), up to 70% of OM was removed after a 24-weeks trial[9]. In this previous study, PASFs secured a higher filtering rate (4320 L m^{-2} day^{-1}) than that reported in the present work (2000 L m^{-2} day^{-1}). PASFs stocked with polychaetes *Perinereis nuntia* and *P. helleri* to filter a shrimp farm effluent (culturing *Penaeus monodon*) were able to reduce SPM by 50% at a flow rate similar to that used in the present study[29].

Regarding the effect of PASFs in the dynamics of dissolved inorganic nutrients, it was recorded that these promoted the mineralization of OM, thus increasing the concentrations of DIN and DIP, a process that had already been reported in previous studies[9,29]. By employing these sand filters stocked with polychaetes the same level of nitrogenous and organic compounds degradation can be obtained as when employing other filtration systems more commonly used in aquaculture (e.g., plastic biological ball filters)[46]. The high efficiency in POM retention and the contribution to enhance the bioavailability of dissolved

inorganic nutrients (DIN and DIP) makes PASFs an appealing option for IMTA designs. Indeed, this approach allows to consider the integration of a second extractive unit receiving the outflowing water and targeting the uptake of dissolved inorganic nutrients (e.g., by using marine macro/micro algae or halophytes). A complete design integrating PASFs (*H. diversicolor*) and halophytes in aquaponics (*Halimione portulacoides*) was already successfully tested[9], with the second extractive unit being able to recover 67% of the DIN present in the outflowing water of PASFs. The polychaete *H. diversicolor* had already been tested to filter the water of a RAS under a complete IMTA design that also included macroalgae biofilters (*Ulva lactuta* or *Solieria chordalis*)[23]. The results obtained in the present study reinforce the biomitigation potential of *H. diversicolor* when aiming to impair the loss of nutrients available in the effluents of fish farms. Indeed, at the end of the experimental period (15 weeks) only the Raw and Df PASFs that presented the highest OM content in their sand beds also displayed a high reproductive success of stocked polychaetes (90% to 100% of specimens with a size < 5 mm). The lower reproductive success recorded for polychaetes stocked in Df+Alg PASFs may partly be explained by the higher fluctuations in the pH and oxygen recorded in inflowing water, as well as a much lower input of POM. The final densities of polychaetes recorded for Raw and Df PASFs were lower than the ones reported in previous studies (e.g., 7000 ind. m^{-2} using and initial stocking density of ≈ 400 ind. m^{-2} after 24 weeks)[9]. This difference may be explained by the shorter duration of the present study, as well as by the lower nutrient loads present in the effluent water being supplied to the PASFs. The full harvesting of the fish being farmed, and the consequent loss of IMTA conditions, made it impossible to extend the study period beyond 15 weeks. As such the evaluation of generated biomass was mostly performed based in small sized polychaetes larvae (< 5 mm), as the reproductive behaviour of *H. diversicolor* results in the death of mature worms (monotelic species)[47]. It is therefore recommended that the evaluation of growth and biomass generation of this polychaete should be performed over a longer period after the initial stocking with adult biomass (> 5 months). This procedure will guarantee a correct stabulation, reproduction and growth of new cohorts of polychaetes. Another approach is to use nectochaetes to initially stock PASFs, as this would likely allow a faster evaluation of growth performances and biomass generation. This strategy has already been successfully used in a previous study, where PASFs were stocked with juvenile forms of *Perinereis helleri* and *P. nuntia* and productivities of approximately 300 - 400 g FW m^{-2} were reported after ≈ 5 months[29].

Other polychaete species naturally colonizing the PASFs, such as *Diopatra neapolitana*, *Terebella lapidaria*, and *Sabella* cf. *pavonina*, adapted very well to the culture conditions. These polychaetes may eventually be tested in future trials featuring them as extractive species in IMTA. While *D. neapolitana* already presents a well-defined market potential, as it is commonly collected to be used as bait in sports fishing[48], the potential use of *Sabella* cf. *pavonina* and *T. lapidaria* is yet to be evaluated. It is worth highlighting that *T. lapidaria*, was able to successfully adapt to each of the three PASFs tested, being the average dry weight (AFDW) recorded for this species 10 times higher in the Df+Alg PASFs. This result was likely due to the lower specific richness recorded and subsequent lower trophic competition. Overall, the present findings allow us to conclude that the best locations to position PASFs stocked with *H. diversicolor* were Raw and Df PASFs positions, systems which showed the best efficiency in retaining POM into valuable polychaete biomass. The PASFs also favoured biogeochemical processes to increase the concentration of DIN and DIP thus revealing a potential to enhance the growth of micro/macroalgae and halophyte plants positioned in subsequent extractive units.

2.1.5. Chapter 2 - References

1. FAO. The State of World Fisheries and Aquaculture Meeting the Sustainable Development Goals. *Food and Agriculture Organization of the United Nations* (2018). doi:10.1364/OE.17.003331
2. Tacon, A. G. J. Global Trends in Aquaculture and Compound Aquafeed Production. *World Aquac. June*, **33–46** (2018).
3. Piedecausa, M. A., Aguado-Giménez, F., Cerezo-Valverde, J., Hernández-Llorente, M. D. & García-García, B. Simulating the temporal pattern of waste production in farmed gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*) and Atlantic bluefin tuna (*Thunnus thynnus*). *Ecol. Modell.* **221**, 634–640 (2010).
4. Mallekh, R., Boujard, T. & Lagardere, J. P. Evaluation of retention and environmental discharge of nitrogen and phosphorus by farmed turbot (*Scophthalmus maximus*). *N. Am. J. Aquac.* **61**, 141–145 (1999).
5. Ballestrazzi, R., Lanari, D. & D'Agaro, E. Performance, nutrient retention efficiency, total ammonia and reactive phosphorus excretion of growing European sea-bass (*Dicentrarchus labrax*, L.) as affected by diet processing and feeding level. *Aquaculture* **161**, 55–65 (1998).
6. Wang, X., Olsen, L. M., Reitan, K. I. & Olsen, Y. Discharge of nutrient wastes from salmon farms : environmental effects , and potential for integrated multi-trophic aquaculture. *Aquac. Environ. Interact.* **2**, 267–283 (2012).
7. Endut, A., Jusoh, A. & Ali, N. Nitrogen budget and effluent nitrogen components in aquaponics recirculation system. *Desalin. Water Treat.* **52**, 744–752 (2014).
8. Schneider, O., Sereti, V., Eding, E. H. & Verreth, J. A. J. Analysis of nutrient flows in integrated intensive aquaculture systems. *Aquac. Eng.* **32**, 379–401 (2005).
9. Marques, B., Calado, R. & Lillebø, A. I. New species for the biomitigation of a super-intensive marine fish farm effluent: Combined use of polychaete-assisted sand filters and halophyte aquaponics. *Sci. Total Environ.* **599–600**, 1922–1928 (2017).
10. Worsfold, P. J. *et al.* Characterisation and quantification of organic phosphorus and organic nitrogen components in aquatic systems: A Review. *Anal. Chim. Acta* **624**, 37–58 (2008).
11. Huntington, T.C. and Hasan, M. R. Fish as feed inputs for aquaculture: Practices, sustainability and implications: A global synthesis. *In* M.R. Hasan and M. Halwart (eds). Fish as feed inputs for aquaculture: practices, sustainability and implications. *FAO Fish. Aquac. Tech. Pap.* **518**, 1–61 (2009).
12. Gunning, D., Maguire, J. & Burnell, G. The Development of Sustainable Saltwater-Based Food Production Systems: A Review of Established and Novel Concepts. *Water* **8**, 598 (2016).
13. Granada, L., Sousa, N., Lemos, M. F. L. & Lopes, S. Is integrated multitrophic aquaculture the solution to the sectors' major challenges? – a review. *Rev. Aquac.* **8**, 283–300 (2016).

14. Chopin, T. Integrated Multi-Trophic Aquaculture. in Advancing the Aquaculture Agenda (OECD workshop) 195–217 (2010). doi:DOI: 10.1787/9789264088726-15-en

15. Chopin, T. *et al.* Multitrophic Integration for Sustainable Marine Aquaculture. *Encycl. Ecol.* **3**, 2463–2475 (2008).

16. Troell, M. *et al*. Ecological engineering in aquaculture — Potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. *Aquaculture* **297**, 1–9 (2009).

17. Custódio, M., Villasante, S., Cremades, J., Calado, R. & Lillebø, A. I. Unravelling the potential of halophytes for marine integrated multi-trophic aquaculture (IMTA)— a perspective on performance, opportunities and challenges. *Aquac. Environ. Interact.* **9**, 445–460 (2017).

18. Hughes, A. D. & Black, K. D. Going beyond the search for solutions: understanding trade-offs in European integrated multi-trophic aquaculture development. *Aquac. Environ. Interact.* **8**, 191–199 (2016).

19. Sanz-Lazaro, C. & Sanchez-Jerez, P. Regional Integrated Multi-Trophic Aquaculture (RIMTA): Spatially separated, ecologically linked. *J. Environ. Manage.* **271**, 110921 (2020).

20. Yousefi-Garakouei, M., Kamali, A. & Soltani, M. Effects of rearing density on growth, fatty acid profile and bioremediation ability of polychaete *Nereis diversicolor* in an integrated aquaculture system with rainbow trout (*Oncorhynchus mykiss*). *Aquac. Res.* **50**, 725–735 (2019).

21. Wang, H. *et al.* Growth and nutritional composition of the polychaete *Hediste diversicolor* (OF Müller, 1776) cultivated on waste from land-based salmon smolt aquaculture. *Aquaculture* **502**, 232–241 (2019).

22. Bischoff, A. A., Fink, P. & Waller, U. The fatty acid composition of *Nereis diversicolor* cultured in an integrated recirculated system: Possible implications for aquaculture. *Aquaculture* **296**, 271–276 (2009).

23. Bischoff, A. Solid waste reduction of closed recirculated aquaculture systems by secondary culture of detritivorous organisms. (Christian-Albrechts-Universität Zu Kiel, 2007).

24. Marques, B. *et al.* Adding value to ragworms (*Hediste diversicolor*) through the bioremediation of a super-intensive marine fish farm. *Aquac. Environ. Interact.* **10**, 79–88 (2018).

25. Pajand, Z. O., Soltani, M., Bahmani, M. & Kamali, A. The role of polychaete *Nereis diversicolor* in bioremediation of wastewater and its growth performance and fatty acid composition in an integrated culture system with *Huso huso* (Linnaeus , 1758). *Aquac. Res.* **48**, 5271–5279 (2017).

26. Guerrero, S. & J. Cremades (eds.) (2012). Integrated Multi-trophic Aquaculture (IMTA): A sustainable, pioneering alternative for marine cultures in Galicia. XUNTA DE GALICIA,111pp (2012).

27. Brown, N., Eddy, S. & Plaud, S. Utilization of waste from a marine recirculating fish culture system as a feed source for the polychaete worm, *Nereis virens*. *Aquaculture* **322–323**, 177–183 (2011).

28. Honda, H. & Kikuchi, K. Nitrogen budget of polychaete *Perinereis nuntia vallata* fed on the feces of Japanese flounder. *Fish. Sci.* **68**, 1304–1308 (2002).

29. Palmer, P. J. Polychaete-assisted sand filters. *Aquaculture* **306**, 369–377 (2010).

30. Palmer, P. J., Wang, S., Houlihan, A. & Brock, I. Nutritional status of a nereidid polychaete cultured in sand filters of mariculture wastewater. *Aquac. Nutr.* **20**, 675–691 (2014).

31. Gómez, S., Hurtado, C. F. & Orellana, J. Bioremediation of organic sludge from a marine recirculating aquaculture system using the polychaete *Abarenicola pusilla* (Quatrefages, 1866). *Aquaculture* **507**, 377–384 (2019).

32. Gómez, S., Hurtado, C. F., Orellana, J., Valenzuela-Olea, G. & Turner, A. *Abarenicola pusilla* (Quatrefages, 1866): A novel species for fish waste bioremediation from marine recirculating aquaculture systems. *Aquac. Res.* 1–5 (2017). doi:10.1111/are.13562

33. Nederlof, M. *et al.* Application of polychaetes in (de)coupled integrated aquaculture: production of a high-quality marine resource. *Aquac. Environ. Interact.* **11**, 221–237 (2019).

34. Santos, A. *et al.* Effect of three diets on the growth and fatty acid profile of the common ragworm *Hediste diversicolor* (O.F. Müller, 1776). *Aquaculture* **465**, 37–42 (2016).

35. Watson, G. J., Murray, J. M., Schaefer, M. & Bonner, A. Bait worms: a valuable and important fishery with implications for fisheries and conservation management. *Fish Fish.* **18**, 374–388 (2016).

36. Olive, P. J. W. Polychaete aquaculture and polychaete science: A mutual synergism. *Hydrobiologia* **402**, 175–183 (1999).

37. Sá, E. *et al.* Trade of live bait in Portugal and risks of introduction of non-indigenous species associated to importation. *Ocean Coast. Manag.* **146**, 121–128 (2017)

38. Fidalgo e Costa, P. *et al.* The market features of imported non-indigenous polychaetes in Portugal and consequent ecological concerns. *Sci. Mar.* **70**, 287–292 (2006).

39. Mermillod-Blondin, F. The functional significance of bioturbation and biodeposition on biogeochemical processes at the water-sediment interface in freshwater and marine ecosystems. *J. North Am. Benthol. Soc.* **30**, 770–778 (2011).

40. Queirós, A. M. *et al.* A bioturbation classification of European marine infaunal invertebrates. *Ecol. Evol.* **3**, 3958–3985 (2013).

41. Shull, D. H. Bioturbation. in *Encyclopedia of Ocean Sciences* (ed. Steele, J. H. B. T.) 395–400 (Academic Press, 2008).

42. Quintana, C. O. *et al.* Carbon mineralization pathways and bioturbation in coastal Brazilian sediments. *Sci. Rep.* **5**, 1–13 (2015).

43. Bertics, V. J. & Ziebis, W. Biodiversity of benthic microbial communities in bioturbated coastal sediments is controlled by geochemical microniches. *ISME J.* **3**, 1269–1285 (2009).

44. Nielsen, O. I., Kristensen, E. & Holmer, M. Impact of *Arenicola marina* (Polychaeta) on sediment sulfur dynamics. *Aquat. Microb. Ecol.* **33**, 95–105 (2003).

45. Bergström, P. *et al.* Testing the potential for improving quality of sediments impacted by mussel farms using bioturbating polychaete worms. *Aquac. Res.* **48**, 161–176 (2017).

46. Foysal, M. J., Fotedar, R., Gupta, S. K. & Chaklader, M. R. Biological ball filters regulate bacterial communities in marron (*Cherax cainii*) culture system. *Lett. Appl. Microbiol.* **68**, 455–463 (2019).

47. Scaps, P. A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O. F. Müller) (Annelida: Polychaeta). *Hydrobiologia* **470**, 203–218 (2002).

48. Cunha, T., Hall, A. & Queiroga, H. Estimation of the *Diopatra neapolitana* annual harvest resulting from digging activity in Canal de Mira, Ria de Aveiro. *Fish. Res.* **76**, 56–66 (2005).

49. Dashtgard, S. E. & Gingras, M. K. Marine Invertebrate Neoichnology. *Dev. Sedimentol.* **64**, 273–295 (2012).

50. Fauchald, K. & Jumars, P. a. The diet of worms: a study of polychaete feeding guilds. Oceanogr. *Mar. Biol. An Annu. Rev.* **17**, 193–284 (1979).

51. Reise, K. Spatial configurations generated by motile benthic polychaetes. *Helgoländer Wiss. Meeresunters.* **32**, 55–72 (1979).

52. Lucas, F. & Bertru, G. Bacteriolysis in the gut of *Nereis diversicolor* (O.F. Muller) and effect of the diet. *J. Exp. Mar. Bio. Ecol.* **215**, 235–245 (1997).

53. Olivier, M. *et al.* Réponses comportementales des polychètes *Nereis diversicolor* (O.F. Müller) et *Nereis virens* (Sars) aux stimuli d'ordre alimentaire : utilisation de la matière organique particulaire (algues et halophytes). *Canadian J. of Zoology*, **73**, 2307-2317 (1995).

54. Riisgård, H. U. Filter-feeding in the polychaete *Nereis diversicolor*: a review. *Netherlands J. Aquat. Ecol.* **28**, 453–458 (1994).

55. Vedel, A., Andersen, B. B. & Riisgard, H. U. Field investigations of pumping activity off the facultatively filter-feeding polychaete *Nereis diversicolor* using an improved infrared phototransducer system. *Mar. Ecol. Prog. Ser.* **103**, 91–102 (1994).

56. Fauvel, P. Faune de France - Polychaetes sedentaires. *Fédération Française des Sociétés de Sciences Naturelles*, **16**, 494pp (1927).

57. Fauvel, P. Faune de France - Polychètes Errantes. *Fédération Française des Sociétés de Sciences Naturelles*, **5**, 491pp (1923).

58. Anderson, M. J., Gorley, R. N. & Clarke, K. R. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. *Plymouth UK*, 1–214 (PRIMER.E Ltd, 2008).

2.1.6. Chapter 2 - Supporting Information

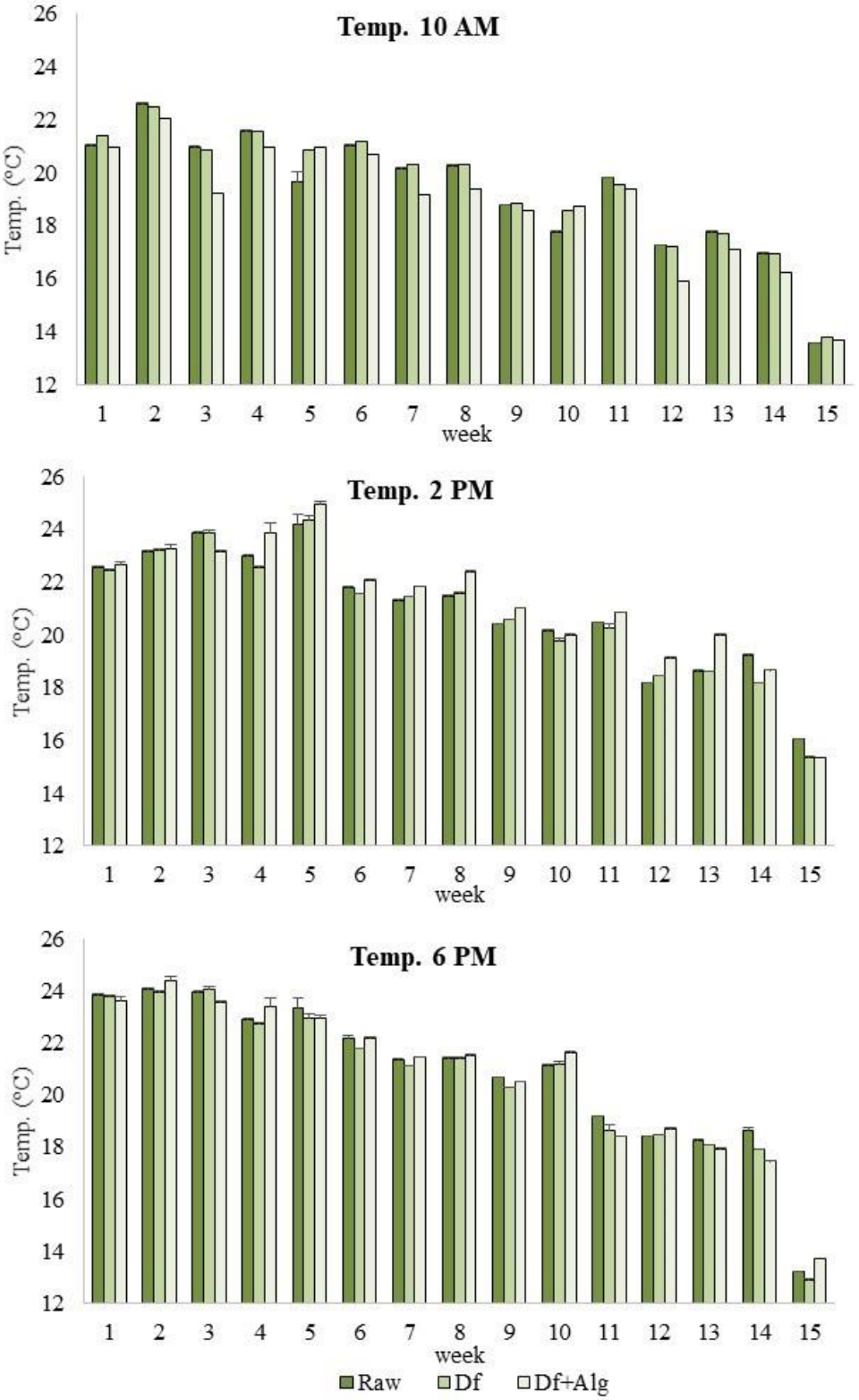


Figure S2.1. Weekly characterisation of temperature monitored in the inflowing water of each polychaete assisted sand filter (PASFs) at 10 AM, 2 PM and 6 PM. Average values (±SD) (n=3).

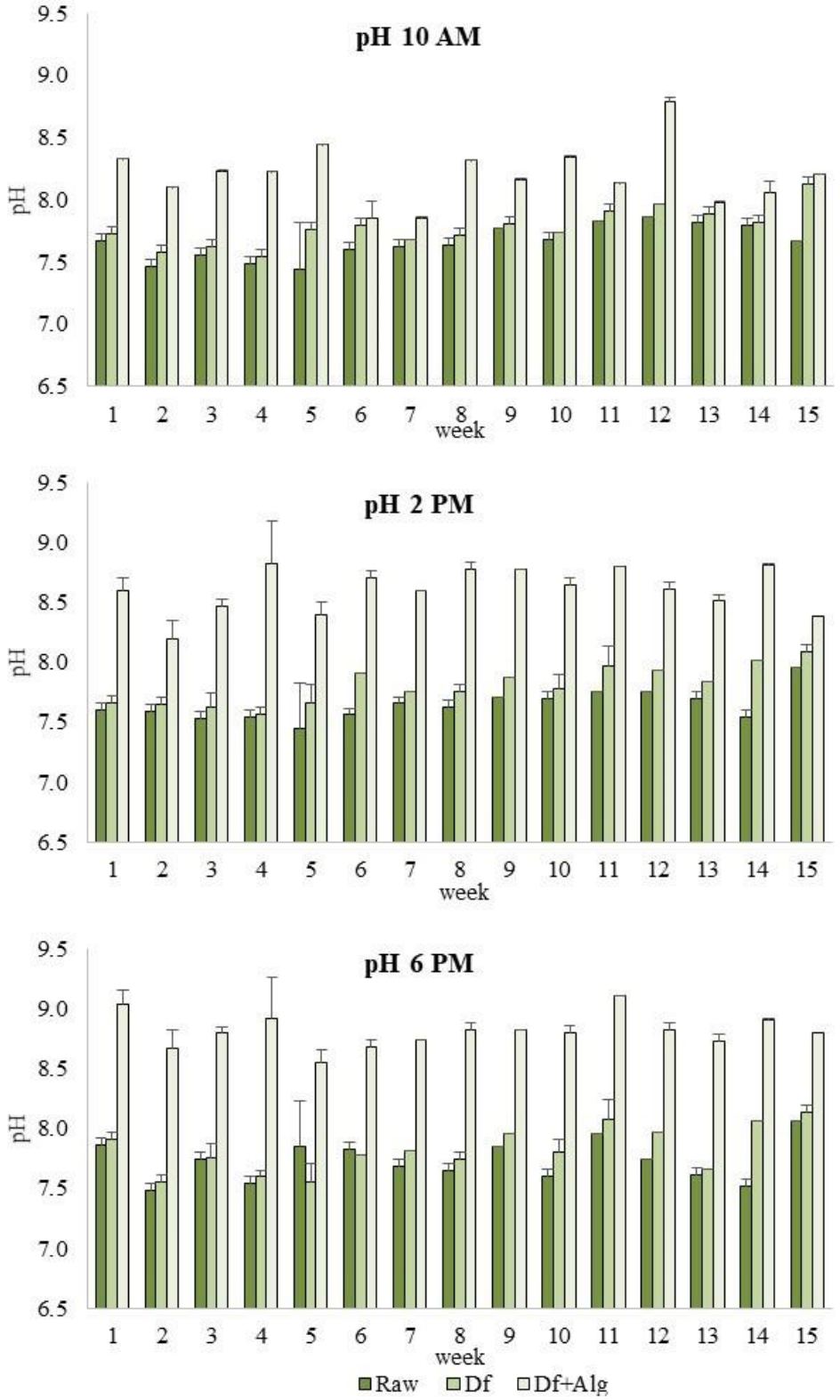


Figure S2.2. Weekly characterisation of pH monitored in the inflowing water of each polychaete assisted sand filter (PASFs) at 10 AM, 2 PM and 6 PM. Average values (±SD) (n=3).

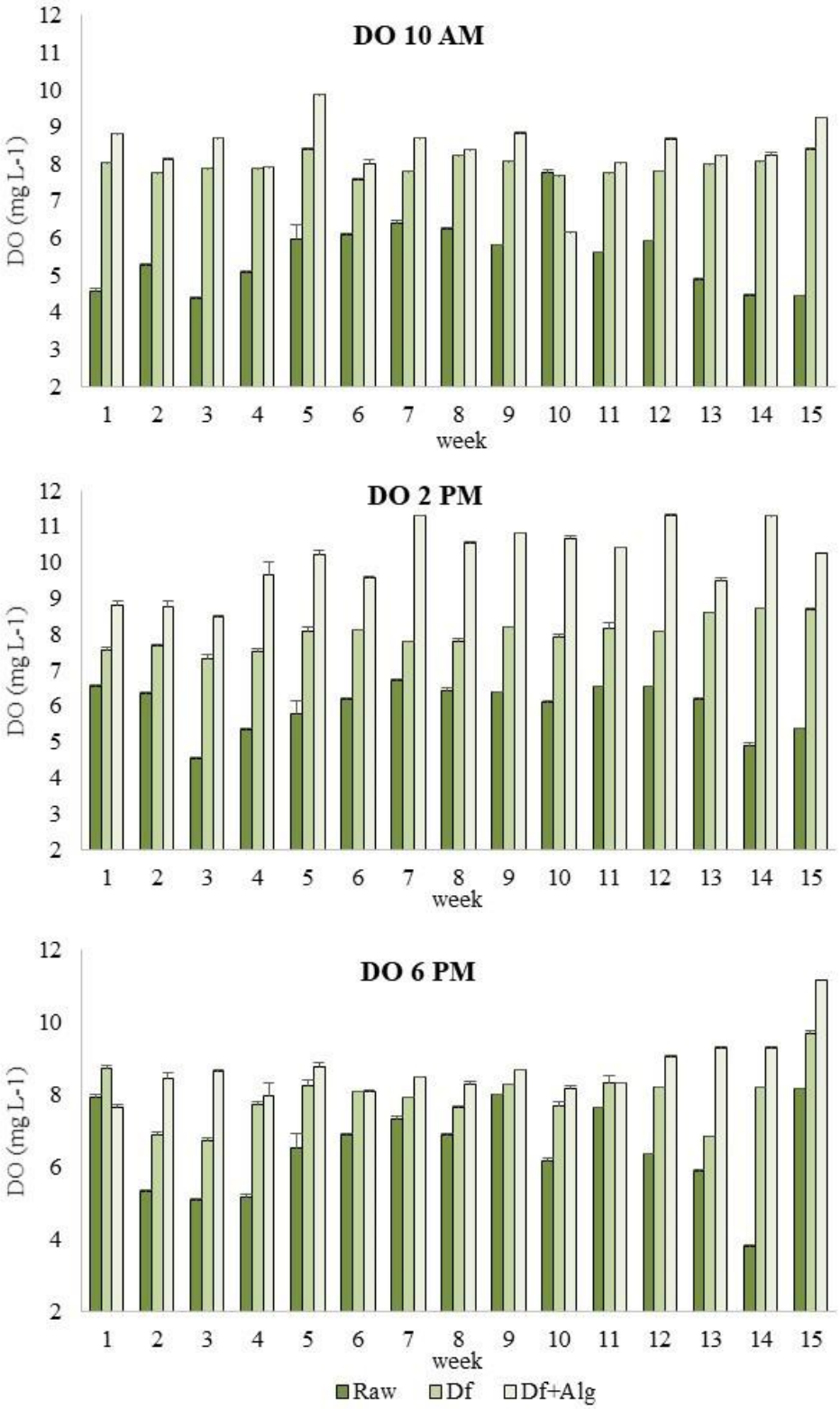


Figure S2.3. Weekly characterisation of dissolved oxygen (DO) concentration monitored in the inflowing water of each polychaete assisted sand filter (PASFs) at 10 AM, 2 PM and 6 PM. Average values (±SD) (n=3).

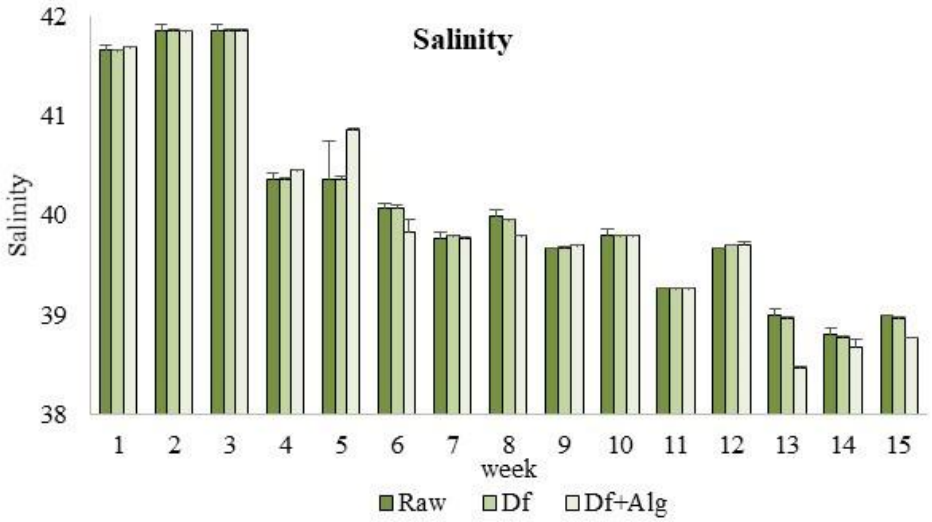


Figure S2.4. Weekly characterisation of salinity measured in the inflowing water of each polychaete assisted sand filter (PASFs). Average values (±SD) (n=3).

Table S2.1. Permutational multivariate analysis of variance (PERMANOVA) to evaluate variations in the environmental parameters (Temp., oxygen, pH, salinity) monitored in inflowing water between different polychaetes assisted sand filters (PASFs) in each of the monitored periods: 10 AM, 2 PM and 6 PM. Significant differences when $p<0.05$

	*Pseudo-F and t-value**	*P(perm)*
10AM		
Global Test		
PASFs	35.670	0.001
Pairwise Tests		
Raw - Df	5.240*	0.001
Raw - Df+Alg	7.632*	0.001
Df - Df+Alg	4.365*	0.001
2PM		
Global Test		
PASFs	90.291	0.001
Pairwise Tests		
Raw - Df	5.028*	0.001
Raw - Df+Alg	12.844*	0.001
Df - Df+Alg	8.677*	0.001
6PM		
Global Test		
PASFs	56.330	0.001
Pairwise Tests		
Raw - Df	3.837*	0.001
Raw - Df+Alg	9.892*	0.001
Df - Df+Alg	8.044*	0.001

Table S2.2. Similarity percentage analysis (SIMPER) (cut-off 90%) to evaluate contributions of each parameter to dissimilarities between polychaetes assisted sand filters (PASFs) in each of the monitored periods: 10 AM, 2 PM and 6 PM.

10 AM			**2 PM**			**6 PM**		
Raw & Df			Raw & Df			Raw & Df		
Avg. Dissimilarity = 7.9			*Avg. Dissimilarity = 4.2*			*Avg. Dissimilarity = 5.5*		
Parameter	Contrib. (%)	Cum. (%)	Parameter	Contrib. (%)	Cum. (%)	Parameter	Contrib. (%)	Cum. (%)
O_2	43.4	43.4	*T*	47.0	47.0	O_2	48.8	48.8
T	24.7	68.1	O_2	44.8	91.8	*T*	46.0	94.8
Sal	23.4	91.5						
Raw & Df+Alg			Raw & Df+Alg			Raw & Df+Alg		
Avg. Dissimilarity = 12.8			*Avg. Dissimilarity = 12.1*			*Avg. Dissimilarity = 10.2*		
Parameter	Contrib. (%)	Cum. (%)	Parameter	Contrib. (%)	Cum. (%)	Parameter	Contrib. (%)	Cum. (%)
O_2	34.8	34.8	O_2	44.3	44.3	*pH*	44.4	44.4
pH	33.3	68.2	*pH*	39.8	84.2	O_2	38.7	83.1
Sal	16.0	84.2	*T*	15.8	100	*T*	16.9	100
T	15.8	100						
Df & Df+Alg			Df & Df+Alg			Df & Df+Alg		
Avg. Dissimilarity = 7.3			*Avg. Dissimilarity = 6.7*			*Avg. Dissimilarity = 6.3*		
Parameter	Contrib. (%)	Cum. (%)	Parameter	Contrib. (%)	Cum. (%)	Parameter	Contrib. (%)	Cum. (%)
pH	40.0	40.0	*pH*	50.8	50.8	*pH*	60.9	60.9
Sal	28.3	68.3	*T*	31.4	82.1	*T*	27.5	88.4
T	27.7	96.0	O_2	17.9	100	O_2	11.6	100

Table S2.3. Permutational multivariate analysis of variance (PERMANOVA) to evaluate variations in the inflowing water composition (SPM, POM, TN, DIN, TP and DIP) between different polychaetes assisted sand filters (PASFs). Significant differences when $p<0.05$

	*Pseudo-F and t-value**	*P(perm)*
Global Test		
PASFs	17.984	0.001
Pairwise Tests		
Raw - Df	1.201*	0.240
Raw - Df+Alg	5.817*	0.001
Df - Df+Alg	5.363*	0.001

Table S2.4. Similarity percentage analysis (SIMPER) (cut-off 90%) to evaluate contributions of each parameter to dissimilarities verified in composition of water supplied to different polychaetes assisted sand filters (PASFs).

Raw & Df			Raw & Df+Alg			Df & Df+Alg		
Avg. Dissimilarity = 8.4			*Avg. Dissimilarity = 21*			*Avg. Dissimilarity = 14.5*		
Parameter	Contrib. (%)	Cum. (%)	Parameter	Contrib. (%)	Cum. (%)	Parameter	Contrib. (%)	Cum. (%)
POM	24.6	24.6	*DIN*	20.7	20.7	*DIN*	23.2	23.2
SPM	23.4	47.9	*TN*	20.4	41.1	*TN*	19.2	42.4
TP	17.2	65.2	*TP*	16.9	58.0	*DIP*	16.6	58.9
DIP	16.9	82.1	*DIP*	16.3	74.3	*TP*	15.5	74.5
TN	11.2	93.2	*SPM*	13.5	87.7	*SPM*	13.3	87.8
			POM	12.3	100	*POM*	12.2	100

Table S2.5. Kruskal-Wallis test to evaluate variations in particulate organic matter (POM) monitored in outflowing water and to evaluate variations in organic matter (OM) content monitored in sand beds between different polychaetes assisted sand filters (PASFs). Significant differences when $p<0.05$.

	p-value
POM monitored in outflowing water between PASFs	
Global Test	
PASFs	0.335
OM recorded in sand bed between PASFs	
Global Test	
PASFs	0.018
Pairwise Tests	
Raw - Df	0.917
Raw - Df+Alg	0.009
Df - Df+Alg	0.028

Table S2.6. Kruskal-Wallis test to evaluate variations in density (ind. m-2) of *Hediste diversicolor* determined in each polychaete assisted sand filters (PASFs) at the end of experimental period (15 weeks). Significant differences when $p<0.05$

	p-value
Density of *H. diversicolor*	
Global Test	
PASFs	0.005
Pairwise Tests	
Raw - Df	0.117
Raw - Df+Alg	0.009
Df - Df+Alg	0.009

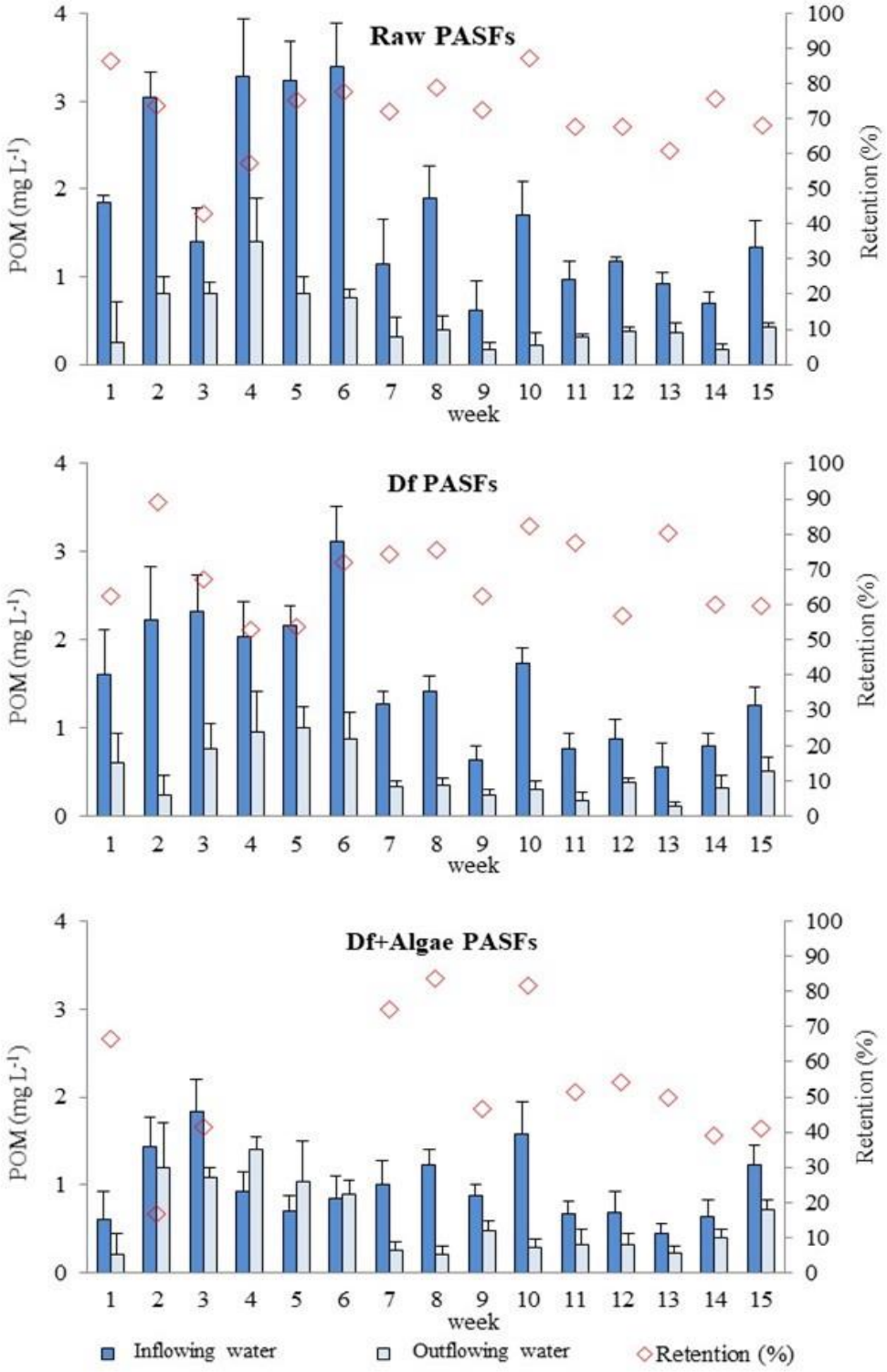


Figure S2.5. Weekly characterisation of particulate organic matter (POM) measured in the inflowing and outflowing water of Raw, Df and Df+Alg polychaete assisted sand filters (PASFs). The red diamond represent the percentage of retention (left axis; difference between inflowing and outflowing) in PASFs. Average values (±SD) (n=5).

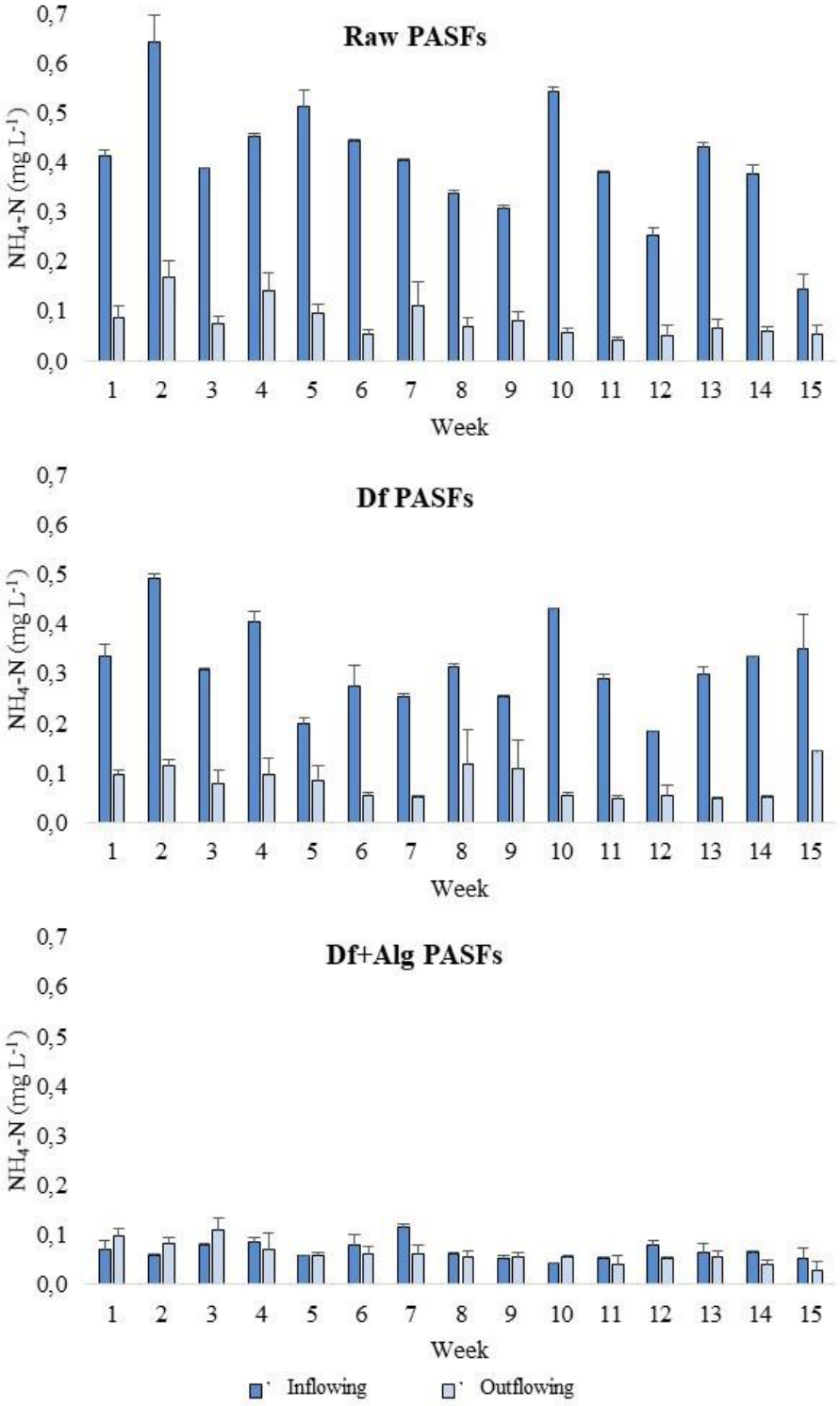


Figure S2.6. Weekly characterisation of ammonium-nitrogen (NH4-N) monitored in the inflowing and outflowing water of Raw, Df and Df+Alg polychaete assisted sand filters (PASFs). Average values (±SD) (n=5).

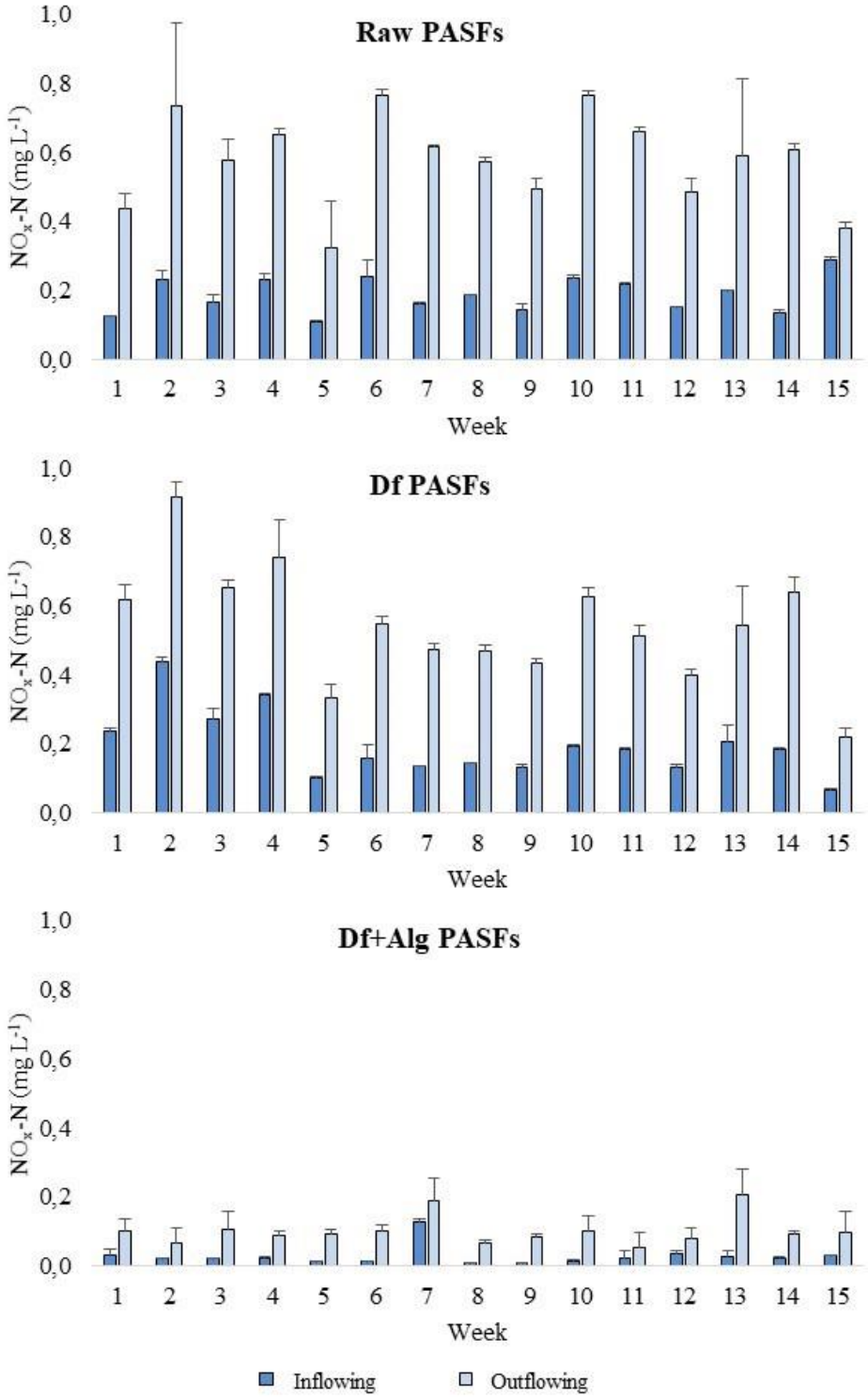


Figure S2.7. Weekly characterisation of oxidized forms of dissolved inorganic nitrogen (NOx-N) monitored in the inflowing and outflowing water of Raw, Df and Df+Alg polychaete assisted sand filters (PASFs). Average values (±SD) (n=5).

Table S2.7. Density of *H. diversicolor* (ind. m^{-2}) determined at each replicate (Tk) of polychaete assisted sand filters (PASFs) at the end of experimental period.

	Raw PASFs					Df PASFs					Df+Alg PASFs				
Group	Tk 1	Tk 2	Tk 3	Tk 4	Tk 5	Tk 1	Tk 2	Tk 3	Tk4	Tk 5	Tk 1	Tk 2	Tk 3	Tk 4	Tk 5
Initial stock (ind. m^{-2})	181	45	91	45	136	ND	ND	ND	ND	ND	91	ND	91	45	136
New generation (ind. m^{-2})	452	407	1901	1177	543	1765	2897	2671	589	7153	ND	45	ND	ND	45
Total (ind. m^{-2})	633	453	1992	1222	679	1765	2897	2671	589	7153	91	45	91	45	181

Chapter 3

3.1. Unravelling the fatty acid profiles of different polychaete species cultured under integrated multi-trophic aquaculture (IMTA)

Jerónimo, D., Lillebø, A.I., Maciel, E., Domingues, M.R.M., Cremades, J., Calado, R. Unravelling the fatty acid profiles of different polychaete species cultured under integrated multi-trophic aquaculture (IMTA).
Scientific Reports **11,** 10812 (2021). https://doi.org/10.1038/s41598-021-90185-8

3.1. Unravelling the fatty acid profiles of different polychaete species cultured under integrated multi-trophic aquaculture (IMTA)

Abstract

Polychaetes can be successfully employed to recover otherwise wasted nutrients present in particulate organic matter (POM) of aquaculture effluents. The present study describes the fatty acid (FA) profile of four different polychaete species cultured in sand filters supplied with effluent water from a marine fish farm. The FA profile of cultured and wild *Hediste diversicolor* was compared and revealed a ≈24.2% dissimilarity, with cultured biomass displaying a higher content in two essential *n*-3 highly unsaturated FA (HUFA) (EPA [20:5 *n*-3] and DHA [22:6 *n*-3] – eicosapentaenoic and docosahexaenoic acid, respectively). The comparison of the FA profile of cultured *H. diversicolor* with that of other polychaete species whose larvae successfully settled on the sand filters (*Diopatra neapolitana*, *Sabella* cf. *pavonina* and *Terebella lapidaria*) revealed that their FA profile, which is here described for the first time, displayed high levels of EPA and DHA (≈ 1.5 – 4.8 and 1.0 – 1.1 µg mg^{-1} DW, respectively). The highest concentration of total FA per biomass of polychaete was recorded in *H. diversicolor* and *T. lapidaria*, with both species being the ones whose FA profiles revealed a lowest level of dissimilarity and more closely resembled that of the aquafeed used in the fish farm. In the present work it was demonstrated that it is possible to produce polychaetes biomass with high nutritional value through an eco-design concept such as integrated multi-trophic aquaculture (IMTA). Indeed, this framework promotes a cleaner production and, in this specific case, allowed to recover essential fatty acids that are commonly wasted in aquaculture effluents.

3.1.1. Introduction

Aquaculture has grown globally 5.8% per year during the period 2001-2016 and continues to grow faster than any other food production sector[1]. In 2016, this industry produced 110.1 million tonnes of food fish and aquatic plants with an estimated value of USD 243.3 billion[1]. It is through the growth and development of this industry that can be possible to supplement human needs in *n*-3 highly unsaturated fatty acids (HUFA). A dose of 500 mg/day of eicosapentaenoic (20:5 *n*-3 [EPA]) and docosahexaenoic (22:6 *n*-3 [DHA]), *n*-3 HUFA, is recommended to reduce the risk of cardiovascular disease[2-5]. Based on this recommended dose to maintain a good cardiac wellness, there is a global requirement of approximately 0.4 million metric tonnes of *n*-3 HUFA per year[5]. Our needs in these essential fatty acids (EFA) are due to limitations that vertebrate species exhibit in the de novo synthesis of these molecules due to the lack of desaturases (Δ12 and Δ15) responsible to produce polyunsaturated fatty acids (PUFA) from oleic acid (18:1 *n*−9), thereby making their inclusion in the aquafeeds essential[5-7]. Marine fish for example incorporate in their tissues with little or no modification the fatty acids (FA) from lower trophic levels and, as such, some species may present well-defined FA signatures depending on their diet[6]. These EFA are included in formulated aquafeeds to satisfy the needs of cultured species, but especially so that these at the end of a productive cycle exhibit an optimal profile for human nutrition[5]. Presently, balanced aquafeeds are formulated using fish meal and fish oil (mainly for marine finfish and shrimp), two increasingly scarcer and costly marine based resources[1]. Their inclusion has been optimized over time and today´s formulas contain less than 10% and 20% of their protein and oil-based composition derived from these sources, respectively[8,9]. Nonetheless, to sustain the expected global growth of aquaculture the search for new sources of EFA is of utmost importance.

Polychaete species can play a key-role on this quest for new sources of valuable EFA. These species can uptake nutrients present in aquaculture effluents in the form of particulate organic matter (POM) and, therefore, their culture under integrated multi-trophic aquaculture (IMTA) conditions has gained a growing attention. In marine IMTA systems, extractive organisms act at different trophic levels targeting the recovery of particulate organic matter (POM deposit feeders such as detritivores fish or invertebrates), dissolved organic matter (DOM filter feeders such as invertebrates) and dissolved inorganic nutrients (primary producers such as micro or macroalgae and halophytes)[10-17]. This concept enables POM-extractive organisms to incorporate otherwise wasted *n*-3 HUFA contained in the

uneaten fraction of aquafeeds supplied to farmed species[18-20]. Indeed, as POM deposit feeders, polychaetes can play an important role in the recovery of these EFA (e.g., EPA [20:5 *n*-3] and DHA [22:6 *n*-3]). Species such as *Hediste diversicolor*[6,15,21-28], *Perinereis nuntia* and *P. helleri*[29], *Nereis virens*[30], *Abarenicolla pusilla*[31], *Sabella spallanzanii*[32] and *Arenicola marina*[27] have already been tested as IMTA extractive organisms. The ragworm *H. diversicolor* in particular revealed a significantly ability to retain valuable HUFA (such as EPA [20:5 *n*-3] and DHA [22:6 *n*-3]) from uneaten fish feeds that would otherwise be lost to the environment and negatively impact adjacent aquatic ecosystems[6,23]. Some polychaete species evidenced *de novo* EFA biosynthesize, while their fat content also reflected the fat content of the diet[33]. These organisms are already known to play a central dietary role on the nutrition and production of some fish and crustacean species (e.g., soles, shrimps and crabs), being often used to trigger gonad maturation and spawning[17,34,35]. The development of production models that include polychaete species appears as an opportunity to meet the growing demand for these worm's biomass. The potential market to produce for example *H. diversicolor* in polychaete assisted sand filters (PASFs) under IMTA conditions (final productivities: 7000 ind. m^{-2} – 2300 g fresh weight biomass) was evaluated in approximately 90 € m^{-2} (if sold as bait)[15]. Unfortunately, there is no reference value available that may allow us to estimate what would be the tentative price of this DHA-rich polychaetes biomass if it was to be sold frozen (or dehydrated) and free of pathogens for premium aquafeeds formulation (e.g., finishing and breeding diets). The values of global harvest of polychaetes in 2016 (approx. 121,000 tonnes) are comparable to many of the world´s most important fisheries[36]. It has also already been acknowledged that their collection from the wild is likely insufficient to satisfy the global market demands (either as bait for sports fishing or as feed for aquaculture) and that this practice drives a multitude of negative environmental impacts[37]. Multiple objectives were target with the development of polychaete production models, such as the reduction of indiscriminate harvesting, reduction of imports of non-native species, development of new aquaculture products and the unravelling of new market and products[17,38,39].

The present study evaluated the valorisation potential of several polychaete species produced through IMTA, a concept which promotes a cleaner production, as otherwise wasted nutrients can be converted into valuable polychaete biomass. This eco-design concept maximizes and diversifies production and increases efficiency in the use of nutrients, water and energy. Therefore, the first objective of the present study was to identify the FA profile of *H. diversicolor* stocked in tanks with a sand bed being supplied

with an organic rich effluent from earthen ponds used for semi-intensive finfish grow-out and compare it with the FA profile of wild conspecifics. The FA profiles of *H. diversicolor* stocked in the tanks was also compared with that of the most representative polychaete species whose planktonic larvae successfully settled on the sand beds, namely *Diopatra neapolitana*, *Sabella* cf. *pavonina* and *Terebella lapidaria.* Finally, the FA profiles of cultured polychaetes were compared to that of the formulated aquafeed provided to the finfish in earthen ponds, in order to identify which species mimicked more closely the FA profile of the aquafeed, hence holding a greater potential to be more readily incorporated in its formulation.

3.1.2. Material and methods

3.1.2.1. Experimental setup

The biomass of polychaetes whose FA profiles were evaluated in present work resulted from an IMTA study performed at AlgaPlus (40° 36′ 43″ N, 8° 40′ 43″ W), an aquaculture company operating in Ria de Aveiro coastal Lagoon watershed area (western Atlantic coast of Portugal)[27]. The present study used the POM fraction of the effluent water from a semi-intensive production pond stocked with gilthead seabream (*Sparus aurata*). Approximately ≈12.000 fish with an average weight of 400 g were stocked, being fed twice a day (specific feeding rate ≈ 1.2% day^{-1}) on a commercial diet with 43% crude protein, 17% crude fat and 10% crude fibre (Standard orange 4; Sorgal). The effluent water was pumped to 5 tanks arranged in a parallel set-up. Each tank had a volume of 0.1 m^3, a surface area of 0.3 m^2 and its bottom was covered by a 200-mm tall sand bed (0.7 - 1 mm grain size). A 100 mm water column was used on each tank by placing an external standpipe regulating the water level. The standpipe was also connected to a bottom draining pipe that allowed full water percolation through the sand bed. Each tank received a water flow of 25 L h^{-1} (0.5 tank volume renewal h^{-1}). An image of the experimental set-up is presented in Figure 3.1. The experimental trial was run for 15 weeks, from (July 2017 to November 2017) and no additional feed was supplied to the tanks with the sand bed besides the fish farm effluent. The characterisation of the environmental (Temp., oxygen, pH, salinity) and water composition (SPM, POM, TN, DIN, TP and DIP) conditions of effluent filtered by PASFs,

as well as the efficiency of bioremediation and productivity achieved in these systems are described in detail in Jerónimo *et al.* (2020)[27].

Figure 3.1. a) Polychaete assisted sand filters (PASFs) used in the present study; b) *Hediste diversicolor* in sand bed; c) Schematic representation of PASFs

3.1.2.2. Polychaetes stocking and sampling

Wild specimens of *H. diversicolor* were collected at Ria de Aveiro (40° 47′ 23″ N, 8° 40′ 23″ W) by local fisherman and each of the 5 tanks with a sand bed was inoculated with 440 ind. m^{-2} (≈167 g FW m^{-2}). As the effluent originated from earthen ponds supplied by the coastal lagoon (Ria de Aveiro) was not pre-filtered, the presence of other polychaete species, namely in their larv planktonic phase was expected to co-occur in the experimental units. At the end of the experimental period, polychaetes were collected with hand core samples (Ø 75 mm, 150 mm depth; N=5). Specimens were sorted in situ and transported to the laboratory for taxonomic identification while alive and further processing. All specimens were depurated overnight in aerated containers holding pre-combusted sterilized sand and artificial seawater to safeguard empty guts and no potential bias of FA analysis. Following depuration, all polychaetes were freeze-dried and stored at -80 ºC before further analysis.

The FA profiles of *H. diversicolor* stocked in the tanks was also compared with that of other polychaete species whose planktonic larvae successfully settled on the sand beds, namely *Diopatra neapolitana* (Onuphidae), *Sabella* cf. *pavonina* (Sabellidae) and *Terebella lapidaria* (Terebellidae). For each species, a composite sample per tank was used for FA analysis. The same procedure was applied to generate 5 composite samples of wild specimens of *H. diversicolor* from the collection site at Ria de Aveiro. For the species *H. diversicolor*, *D. neapolitana* and *S.* cf. *pavonina* 5 polychaetes were considered for each

composite sample, while for *T. lapidaria* 20 polychaetes were considered due to the lower size of their specimens. In addition, 5 samples of fish feed were freeze dried and stored at -80 ºC before FA analysis.

3.1.2.3. Fatty acid extraction and analysis

The FA content was quantified by screening the fatty acid methyl esters (FAME) obtained through gas chromatography-mass spectrometry (GC-MS) following a well-established method currently on use in our laboratory[59-61]. To prepare the FAME all freeze-dried samples were powdered and homogeneized. Then, 1 mL of *n*-hexane containing 10 µg mL^{-1} of the internal standard C19:0 was added to 10 mg of biomass. Then, 200 µl of methalonic (MeOH) KOH solution (2M) was added, and the tube was sealed and mixed vigorously in a vortex shaker for 2 min. Following this procedure, 2 ml of saturated NaCl solution (aqueous solution of 1 g NaCl in 100 mL Milli-Q water) was added to the tube, and the mixture was centrifuged for 5 min at 2000 rpm. Following centrifugation, 20 µL of the organic phase was transferred into another tube, was dried under a stream of nitrogen gas and store at -20 ºC until FAME analysis. Immediately before analysis, the FAME were dissolved in 100 µL of hexane and 2 µL of this solution was analysed by gas chromatography-mass spectrometry system (GC-MS) (Agilent Technologies, USA) connected to an Agilent 5973 Network Mass Selective Detector (70 eVand and m/z 50–550 in a 1 s cycle), and equipped with a DB-FFAP column (30 m long, 0.32 mm internal diameter, and 0.25 µm film thickness) (J & W Scientific, Folsom, CA).The oven temperature programmed were as follows: 1) initial temperature of 80 °C for 3 min; 2) linear increase to 160ºC (25 °C min^{-1}); 3) linear increase to 210ºC (2ºC min^{-1}); 4) linear increase to 250ºC (30 °C min^{-1}); 5) standing at 250 °C for 10 min. The temperatures of injector and detector were 220 and 280 °C, respectively. Helium was used as the carrier gas (1.7 mL min^{-1}). The FA content of the fish feed was determined, using 1 mL of *n*-hexane containing 0.75 µg mL^{-1} of internal standard (C19:0) added to 15 µg of the lipid extract. All remaining procedures were identical as described above. The FA identification was performed by matching with a previously injected standards mixture (Supelco37 Component FAME Mix, Sigma-Aldrich), as well as by comparing each MS spectrum with a database (AOCS lipid library). The FA content (µg mg^{-1} dry weight, DW) in the samples analysed was calculated based on an external calibration curve using a certified standard mixture (Supelco37 Component FAME Mix, Sigma-Aldrich) and C19:0 as internal

standard. The FA 18:4 *n*-3, $^{\Delta 7,13,16}$22:3, 22:4 *n*-6, 22:5 *n*-3, 22:5 *n*-6, 16:3 *n*-3, 24:2, 13-methyl-14:0 iso, 14-methyl-15:0 iso &13-methyl-15:0 anteiso, 14-methyl-16:0 anteiso, 10-methyl-16:0, 7-methyl-hexadec-6-enoate and 16-methyl-17:0 iso were determined based on the reference values of the FA 18:3 *n*-3, 22:2, 23:0, 22:6 *n*-3, 22:6 *n*-3, 16:0, 24:1 *n*-9, 15:0, 16:0, 17:0, 17:0, 17:0 and 18:0, respectively. In the present study, PUFA were defined as all FA with two or more double bounds, while HUFA refers to all FA with four or more double bonds.

3.1.2.4. Statistical analysis

Statistical analysis was performed using PRIMER v6 with the PERMANOVA+ add-on. In order to ascertain differences in the FA content (µg mg^{-1} DW) of wild and cultured *H. diversicolor*, a 1-way analysis of similarities (ANOSIM) was performed on a resemblance matrix produced using Bray Curtis similarity coefficient of data previously transformed using the formula log (x+1). A SIMPER analysis was also performed to evaluate which FA contributed the most to the dissimilarities recorded between samples mentioned above until a total of 50% cumulative dissimilarity was achieved. A 1-way ANOSIM and SIMPER analysis using the same criteria was used to highlight the differences in FA profiles between stocked *H. diversicolor* and other polychaete species whose planktonic larvae successfully settled in the experimental units (*D. neapolitana*, *S.* cf. *pavonina* and *T. lapidaria*). To determine which species displayed the FA profile that most closely resembled the FA source (aquafeed provided to fish), resemblance matrixes of the 16 most common FA between feed and polychaetes (common with at least one species) were prepared using Bray Curtis similarity coefficient of the data previously log (x+1) transformed and then a principal coordinates analysis (PCO) plot was performed.

Those FA known to be related to the microbiome (15:0, 17:0, 17:1 *n*-8 and 17:1 *n*-9) and others (13-methyl-14:0, 14-methyl-15:0 + 13-methyl-15:0, 10-methyl-16:0, 7-methyl-hexadec-6-enoate, 16-methyl-17:0) were not included in the above-mentioned analysis. For a detailed description of all the statistical methods referred employed above please see Anderson, Gorley & Clarke (2008)[62].

3.1.3. Results

3.1.3.1. Comparison of fatty acid profiles of wild and IMTA-cultured HEDISTE DIVERSICOLOR.

The FA profiles of wild and IMTA-cultured *H. diversicolor* are detailed in Table 3.1 (FA from microbiome and iso and anteiso are presented in Supplementary Table S3.1). Significant differences were found between the FA profiles (ANOSIM test; R=1; p=0.008), with the SIMPER analysis 50% cut-off (Table 3.2) revealing an average dissimilarity of 24.2%. The higher content of alpha-linolenic (18:3 *n*-3 [ALA]), arachidonic (20:4 *n*-6 [ARA]) and adrenic (22:4 *n*-6 [AdA]) acids recorded in wild polychaetes biomass contributed greatly for these differences, as well as the higher content of linoleic acid (18:2 *n*-6 [LA]) and DHA (22:6 *n*-3) recorded in IMTA-cultured specimens. The 7,10,13-hexadecatrienoic acid (16:3 *n*-3) and gamma-linolenic acid (18:3 *n*-6) were identified only in wild polychaetes biomass, while DHA (22:6 *n*-3) was only identified in cultured polychaetes biomass. In general, IMTA-cultured polychaetes exhibited a FA profile with a higher unsaturated/saturated FA (UFA/SFA) ratio (Fig. 3.2a). By analysing the HUFA profile, it was also possible to verify that IMTA-cultured polychaetes exhibited a higher *n*-3/*n*-6 HUFA ratio, featuring an increment of *n*-3 HUFA (including EPA and DHA) and a reduction of *n*-6 HUFA (Fig. 3.2b and 3.2c, respectively).

Table 3.1. Fatty acid composition (µg mg^{-1} DW) of wild and IMTA-cultured polychaete species and aquafeed added to fish. Average values ± (SD).

Fatty acid	*Hediste diversicolor* (Wild)	*Hediste diversicolor*	*Diopatra neapolitana*	*Sabella* cf. *pavonina*	*Terebella lapidaria*	Aquafeed
14:0	0.39 ± 0.20	0.85 ± 0.15	0.27 ± 0.08	1.19 ± 0.71	0.46 ± 0.08	1.30 ± 0.31
16:0	8.64 ± 0.71	6.70 ± 1.49	1.09 ± 0.22	4.31 ± 1.58	5.69 ± 0.48	16.78 ± 2.58
18:0	2.27 ± 0.17	1.86 ± 0.43	1.22 ± 0.17	2.55 ± 0.66	1.47 ± 0.15	6.47 ± 1.83
20:0	ND	ND	ND	ND	ND	0.28 ± 0.03
22:0	ND	ND	ND	ND	ND	0.14 ± 0.01
∑SFA	**11.30 ± 1.07**	**9.40 ± 2.04**	**2.58 ± 0.44**	**8.05 ± 2.93**	**7.62 ± 0.69**	**24.97 ± 4.25**
16:1 *n*-9	ND	ND	ND	ND	0.14 ± 0.03	3.50 ± 0.38
16:1 *n*-7	ND	ND	ND	ND	ND	0.24 ± 0.02
16:1 *n*-5	0.67 ± 0.11	1.70 ± 0.41	0.26 ± 0.04	1.18 ± 0.51	1.44 ± 0.25	ND
18:1 *n*-14	1.73 ± 0.09	1.33 ± 0.25	ND	ND	0.59 ± 0.03	ND
18:1 *n*-9 & n-7	3.76 ± 0.34	8.02 ± 2.43	0.67 ± 0.11	4.79 ± 0.83	7.22 ± 1.64	36.14 ± 3.66
20:1 *n*-13 & n-11	1.90 ± 0.07	2.29 ± 0.56	0.53 ± 0.09	0.21 ± 0.02	1.96 ± 0.28	ND
20:1 *n*-9	0.20 ± 0.11	0.05 ± 0.04	ND	0.61 ± 0.16	0.01 ± 0.01	2.33 ± 0.25
20:1 *n*-7	ND	ND	ND	ND	ND	0.18 ± 0.04
22:1	0.04 ± 0.01	0.24 ± 0.13	0.05 ± 0.02	0.04 ± 0.00	0.09 ± 0.03	2.25 ± 0.14
∑MUFA	**8.30 ± 0.48**	**13.63 ± 3.36**	**1.50 ± 0.20**	**6.82 ± 1.48**	**11.44 ± 2.11**	**43.86 ± 4.31**
16:3 *n*-3	0.07 ± 0.02	ND	ND	ND	ND	ND
18:2 *n*-6 (LA)	1.19 ± 0.10	3.78 ± 1.21	0.22 ± 0.04	0.71 ± 0.03	2.29 ± 0.61	16.72 ± 1.85
18:3 *n*-6	0.05 ± 0.01	ND	ND	0.08 ± 0.03	ND	0.10 ± 0.03
18:3 *n*-3 (ALA)	3.55 ± 0.27	0.63 ± 0.25	0.04 ± 0.01	0.11 ± 0.04	0.34 ± 0.08	2.80 ± 0.32
$^{\Delta 5,11}$ 20:2	0.25 ± 0.03	0.53 ± 0.10	ND	ND	0.15 ± 0.03	ND
$^{\Delta 5,13}$20:2	0.07 ± 0.01	0.07 ± 0.03	ND	ND	0.24 ± 0.02	ND
20:2 *n*-6	ND	ND	ND	ND	ND	0.49 ± 0.06
$^{\Delta 8,11}$ 20:2	1.27 ± 0.06	1.11 ± 0.36	0.37 ± 0.06	0.23 ± 0.02	1.23 ± 0.26	ND
20:3 *n*-6	1.23 ± 0.61	0.58 ± 0.19	0.54 ± 0.28	0.78 ± 0.52	0.59 ± 0.18	ND
20:3 *n*-3	0.32 ± 0.02	0.10 ± 0.03	0.03 ± 0.01	0.05 ± 0.03	0.03 ± 0.01	0.15 ± 0.01
$^{\Delta 7,13}$ 22:2 *n*-9	0.34 ± 0.06	0.72 ± 0.11	0.99 ± 0.22	ND	0.34 ± 0.05	ND
$^{\Delta 5,13}$ 22:2 *n*-9	ND	ND	ND	1.50 ± 0.22	ND	ND
$^{\Delta 7,13,16}$ 22:3	0.22 ± 0.08	0.22 ± 0.06	0.10 ± 0.03	ND	0.30 ± 0.03	ND
24:2	ND	ND	ND	0.40 ± 0.07	ND	ND
∑PUFA	**8.58 ± 0.96**	**7.75 ± 1.85**	**2.30 ± 0.39**	**3.86 ± 0.79**	**5.52 ± 1.11**	**20.27 ± 2.24**
18:4 *n*-3	ND	ND	ND	ND	ND	0.58 ± 0.07
20:4 *n*-6 (ARA)	3.45 ± 0.07	0.65 ± 0.22	0.45 ± 0.07	0.71 ± 0.06	1.12 ± 0.11	0.39 ± 0.04
20:4 *n*-3	0.40 ± 0.05	0.09 ± 0.04	ND	0.03 ± 0.01	0.13 ± 0.03	0.29 ± 0.03
20:5 *n*-3 (EPA)	3.68 ± 0.13	4.83 ± 0.99	3.06 ± 0.36	1.46 ± 0.47	3.00 ± 0.32	2.11 ± 0.18
22:4 *n*-6 (AdA)	2.86 ± 0.25	0.55 ± 0.25	0.21 ± 0.04	0.04 ± 0.02	1.58 ± 0.08	ND
22:5 *n*-6	ND	ND	0.03 ± 0.02	0.02 ± 0.01	0.23 ± 0.03	ND
22:5 *n*-3 (DPA)	0.85 ± 0.06	0.64 ± 0.19	0.47 ± 0.11	0.08 ± 0.02	0.89 ± 0.14	0.55 ± 0.03
22:6 *n*-3 (DHA)	ND	0.99 ± 0.30	1.10 ± 0.22	1.00 ± 0.17	1.10 ± 0.21	4.42 ± 0.26
∑HUFA	**11.24 ± 0.45**	**7.76 ± 1.76**	**5.32 ± 0.77**	**3.33 ± 0.68**	**8.05 ± 0.65**	**8.34 ± 0.52**
∑Others	**2.16 ± 0.46**	**1.23 ± 0.20**	**0.81 ± 0.21**	**3.15 ± 1.27**	**3.56 ± 0.24**	**0.04 ± 0.02**
∑ Total FA	**41.58 ± 2.74**	**39.78 ± 8.48**	**12.51 ± 1.62**	**25.22 ± 7.04**	**36.19 ± 4.43**	**97.44 ± 11.23**

Abbreviations: SFA - saturated FA; MUFA - mono-unsaturated FA; PUFA - polyunsaturated FA; HUFA - highly unsaturated FA; AdA – adrenic acid; ALA -alpha-linolenic acid; ARA – arachidonic acid; DHA – docosahexaenoic acid; DPA – docosapentaenoic acid; EPA – eicosapentaenoic acid; LA – linoleic acid. Others – sum of FA identified from microbiome and iso and anteiso (Supplementary Table S1); ND - FA not detected. PUFA defined as all FA with ≥ 2 double bonds and HUFA all FA with ≥ 4 double bonds (not considered within ∑PUFA).

Table 3.2. SIMPER overall average dissimilarities (%) between fatty acids (FA) profile of wild and cultured polychaete *Hediste diversicolor*.

H. diversicolor		
Wild & IMTA-cultured		
FA	Contrib.%	Cum.%
18:3 *n*-3 (ALA)	11.90	11.90
20:4 *n*-6 (ARA)	11.48	23.38
22:4 *n*-6 (AdA)	10.61	33.99
18:2 *n*-6 (LA)	8.56	42.55
22:6 *n*-3 (DHA)	7.69	50.24

Abbreviations: AdA – adrenic acid; ALA -alpha-linolenic acid; ARA – arachidonic acid; DHA – docosahexaenoic acid; LA – linoleic acid.

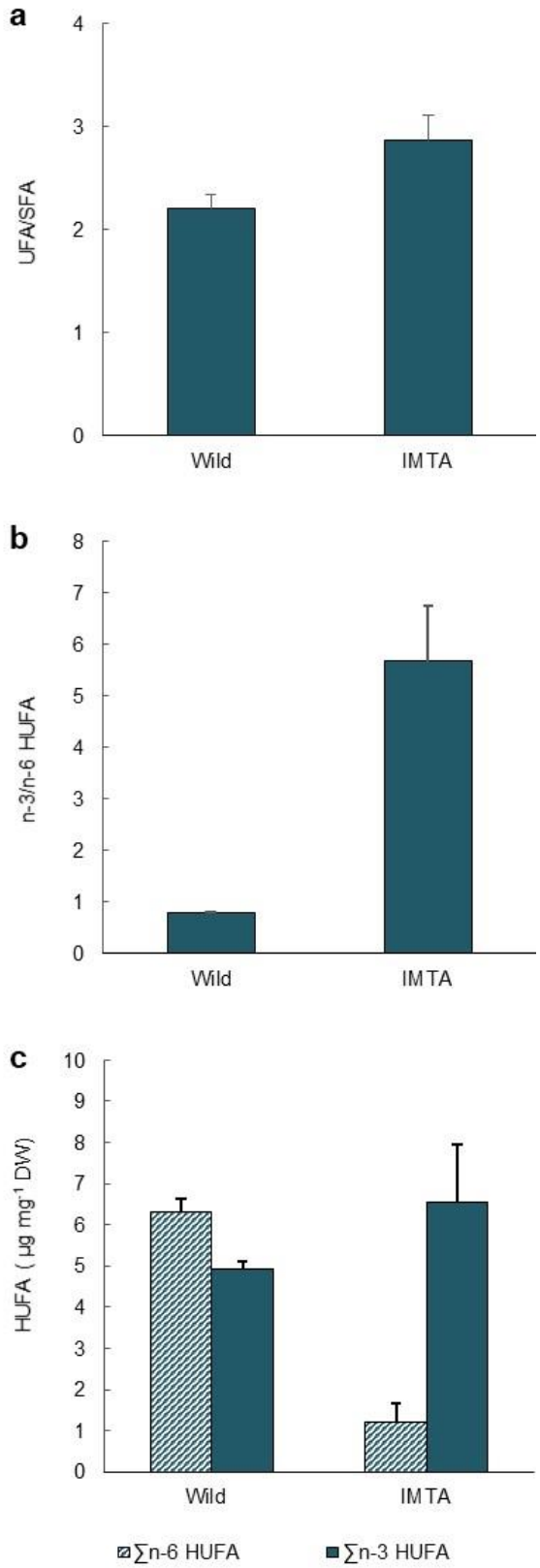


Figure 3.2. Fatty acid profile of wild and IMTA-cultured *Hediste diversicolor*: a) unsaturated and saturated fatty acids ratio (UFA/SFA); b) *n*-3/*n*-6 highly unsaturated fatty acids ratio (*n*-3/*n*-6 HUFA); c) sum of *n*-3 and *n*-6 highly unsaturated fatty acids content (∑*n*-3 and *n*-6 HUFA; values in µg mg^{-1} DW). Average values ± SD (n=5)

3.1.3.3. Comparison of fatty acid profiles of different IMTA-cultured polychaete species

The FA profile of IMTA-cultured polychaetes *H. diversicolor*, *D. neapolitana*, *S.* cf. *pavonina* and *T. lapidaria* (Fig. 3.3) are summarized in Table 3.1.

Figure 3.3. Polychaete species surveyed during the present study: a) *Hediste diversicolor*; b) *Diopatra neapolitana*; c) *Sabella* cf. *pavonina* and d) *Terebella lapidaria*.

Apart from *H. diversicolor*, the FA profile of all other polychaete species is here described for the first time. A total of 22, 25 and 28 FA (excluding FA from microbiome and iso and anteiso – Supplementary Table S3.1) were identified for *D. neapolitana*, *S.* cf. *pavonina* and *T. lapidaria*, respectively. Significant differences were found between the FA profiles of the four IMTA-cultured polychaete species (ANOSIM test; R=0.968; p=0.001), with SIMPER analysis at a cut-off of 50% revealing the FA that most contributed to dissimilarities between them (Table 3.3). *Terebella lapidaria* exhibited the FA profile with the lowest dissimilarity for *H. diversicolor*, followed by *S.* cf. *pavonina* and *D. neapolitana* (Table 3.3). The polychaetes *H. diversicolor* and *T. lapidaria* exhibited the highest concentration of total FA per mg DW biomass. Palmitic (16:0), sum of oleic and

vaccenic (18:1 *n*-9 & *n*-7), LA (18:2 *n*-6) and EPA (20:5 *n*-3) were the SFA, MUFA, PUFA and HUFA (respectively) that revealed the highest content for both polychaete species. The majority of these FA were also the ones most abundant for the other two polychaete species, except stearic (18:0) and 7,13-docosadienoate (22:2 *n*-9) which were the most abundant SFA and PUFA in the FA profile of *D. neapolitana*, and 5,13-docosadienoate (22:2 *n*-9) which was the most abundant PUFA in the FA profile of *S.* cf. *pavonina*. The concentration of DHA (22:6 *n*-3) was similar between the four IMTA-cultured polychaete species (0.99 – 1.10 µg mg^{-1} DW). *Hediste diversicolor*, *D. neapolitana* and *T. lapidaria* exhibited similar and higher UFA/SFA ratios (Fig. 3.4a). When analysing the HUFA profile, it was possible to verify that *H. diversicolor* and *D. neapolitana* exhibited the highest *n*-3/*n*-6 HUFA ratio (Fig. 3.4b), while the highest *n*-3 and *n*-6 HUFA contents were determined in *H. diversicolor* and *T. lapidaria* biomass (Fig. 3.4c).

Table 3.3. SIMPER overall average dissimilarities (%) between fatty acid (FA) profile of different polychaete species cultured in sand beds using an open integrated multi-trophic aquaculture (IMTA) approach. The FA identified with bold superscript: Hd, Dn, Tl and Sp were only identified in the species *Hediste diversicolor*, *Diopatra neapolitana*, *Terebella lapidaria* and *Sabella* cf. *pavonina.* respectively.

H. Diversicolor* & *D. neapolitana			***H. Diversicolor* & *S.* cf. *pavonina***			***H. diversicolor* & *T. lapidaria***		
Avg. Dissimilarity: 40.8%			Avg. Dissimilarity: 36.5%			Avg. Dissimilarity: 15.3%		
FA	Contrib. %	Cum. %	FA	Contrib. %	Cum. %	FA	Contrib. %	Cum. %
18:1 *n*-9 & n-7	16.01	16.01	18:2 *n*-6 (LA)	9.28	9.28	22:4 *n*-6 (AdA)	10.14	10.14
18:2 *n*-6 (LA)	12.90	28.92	20:1 *n*-13 & *n*-11	9.18	18.47	18:1 *n*-14	7.30	17.44
16:0	12.44	41.36	$^{\Delta 5,13}$ 22:2 n-9 **Sp**	8.51	26.97	18:2 *n*-6 (LA)	7.10	24.55
18:1 *n*-14 **Hd**	8.12	49.48	20:5 *n*-3 (EPA)	8.09	35.07	20:5 *n*-3 (EPA)	7.00	31.54
20:1 *n*-13 & *n*-11	7.26	56.74	18:1 *n*-14 **Hd**	7.84	42.91	$^{\Delta 5,11}$20:2	5.57	37.12
			$^{\Delta 7,13}$ 22:2 *n*-9 **Hd**	5.03	47.94	20:4 *n*-6 (ARA)	5.07	42.19
			$^{\Delta 8,11}$20:2	4.89	52.84	18:1 *n*-9 & n-7	4.96	47.15
						$^{\Delta 7,13}$ 22:2 *n*-9	4.81	51.95
D. neapolitana* & *S.* cf. *pavonina			***D. neapolitana* & *T. lapidaria***			***T. lapidaria* & *S.* cf. *pavonina***		
Avg. Dissimilarity: 43.2%			Avg. Dissimilarity: 39.7%			Avg. Dissimilarity: 35.8%		
FA	Contrib. %	Cum. %	FA	Contrib. %	Cum. %	FA	Contrib. %	Cum. %
18:1 *n*-9 & *n*-7	14.57	14.57	18:1 *n*-9 & *n*-7	16.39	16.39	22:4 *n*-6 (AdA)	9.06	9.06
$^{\Delta 5,13}$ 22:2 *n*-9 **Sp**	10.72	25.29	16:0	12.14	28.53	$^{\Delta 5,13}$ 22:2 *n*-9 **Sp**	9.01	18.08
16:0	10.55	35.83	18:2 *n*-6 (LA)	10.10	38.63	20:1 *n*-13 + *n*-11	8.83	26.90
$^{\Delta 7,13}$ 22:2 *n*-9 **Dn**	8.06	43.90	22:4 *n*-6 (AdA)	7.92	46.54	18:2 *n*-6 (LA)	6.25	33.15
20:5 *n*-3 (EPA)	6.16	50.05	20:1 *n*-13 & *n*-11	6.86	53.40	$^{\Delta 8,11}$20:2	5.84	38.99
						22:5 *n*-3 (DPA)	5.52	44.52
						20:5 *n*-3 (EPA)	5.00	49.52
						18:1 *n*-14	4.57	54.09

Abbreviations: AdA – adrenic acid; ARA – arachidonic acid; DPA – docosapentaenoic acid; EPA – eicosapentaenoic acid; LA – linoleic acid.

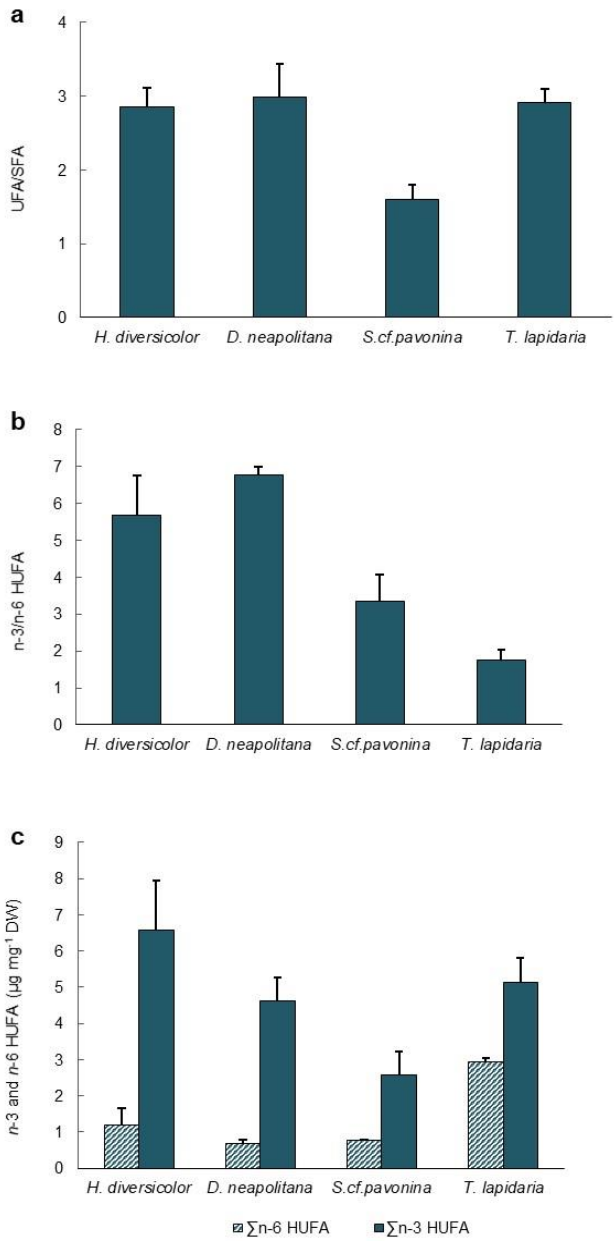


Figure 3.4. Fatty acid profile of different IMTA-cultured polychaetes (*Hediste diversicolor*, *Diopatra neapolitana*, *Sabella* cf. *pavonina* and *Terebella lapidaria*): a) unsaturated and saturated fatty acids ratio (UFA/SFA); b) *n*-3/*n*-6 highly unsaturated fatty acids ratio (*n*-3/*n*-6 HUFA); c) sum of *n*-3 and *n*-6 highly unsaturated fatty acids content (∑*n*-3 and *n*-6 HUFA; values in µg mg^{-1} DW). Average values ± SD (n=5)

3.1.3.4. Comparison of fatty acid profiles of IMTA-cultured polychaete species and the aquafeed provided to fish in earthen ponds

The FA content of the aquafeed provided to the fish is detailed in Table 3.1. Palmitic acid (16:0), along with the sum of oleic and vaccenic acid (18:1 *n*-9 & *n*-7), LA (18:2 *n*-6) and DHA (22:6 *n*-3) were the SFA, MUFA, PUFA and HUFA (respectively) that exhibited the highest levels in the aquafeed.

The most represented UFA class in the aquafeed was MUFA (≈ 60.5% of all UFA), while in polychaetes the sum of PUFA and HUFA accounted for most UFA (53.2% for *H. diversicolor*, 83.5% for *D. neapolitana*, 51.4% for *S.* cf. *pavonina* and 54.3% for *T. lapidaria*). The FA profile of the aquafeed exhibited a content of *n*-3 HUFA (7.94 ± 0.49 µg mg^{-1} DW) similar to the one reported for *H. diversicolor* and, to a lesser extent, to the one reported for *T. lapidaria*. The levels of DHA (22:6 *n*-3) in the aquafeed per DW biomass was approximately 4-times higher than that recorded in all IMTA-cultured polychaete species. The EPA (20:5 *n*-3) content of all polychaete species, except *S.* cf. *pavonina*, was higher than the one present in the aquafeed. The principal coordinates analysis (PCO) revealed that the FA profiles that more closely resembled that of the aquafeed supplied to the fish being farm in earthen ponds were those of *H. diversicolor* and *T. lapidaria* (Fig. 3.5). The FA profile of *D. neapolitana* was the less similar to the aquafeed. The two PCO axis explained more than 87% of the variation recorded between samples from different groups.

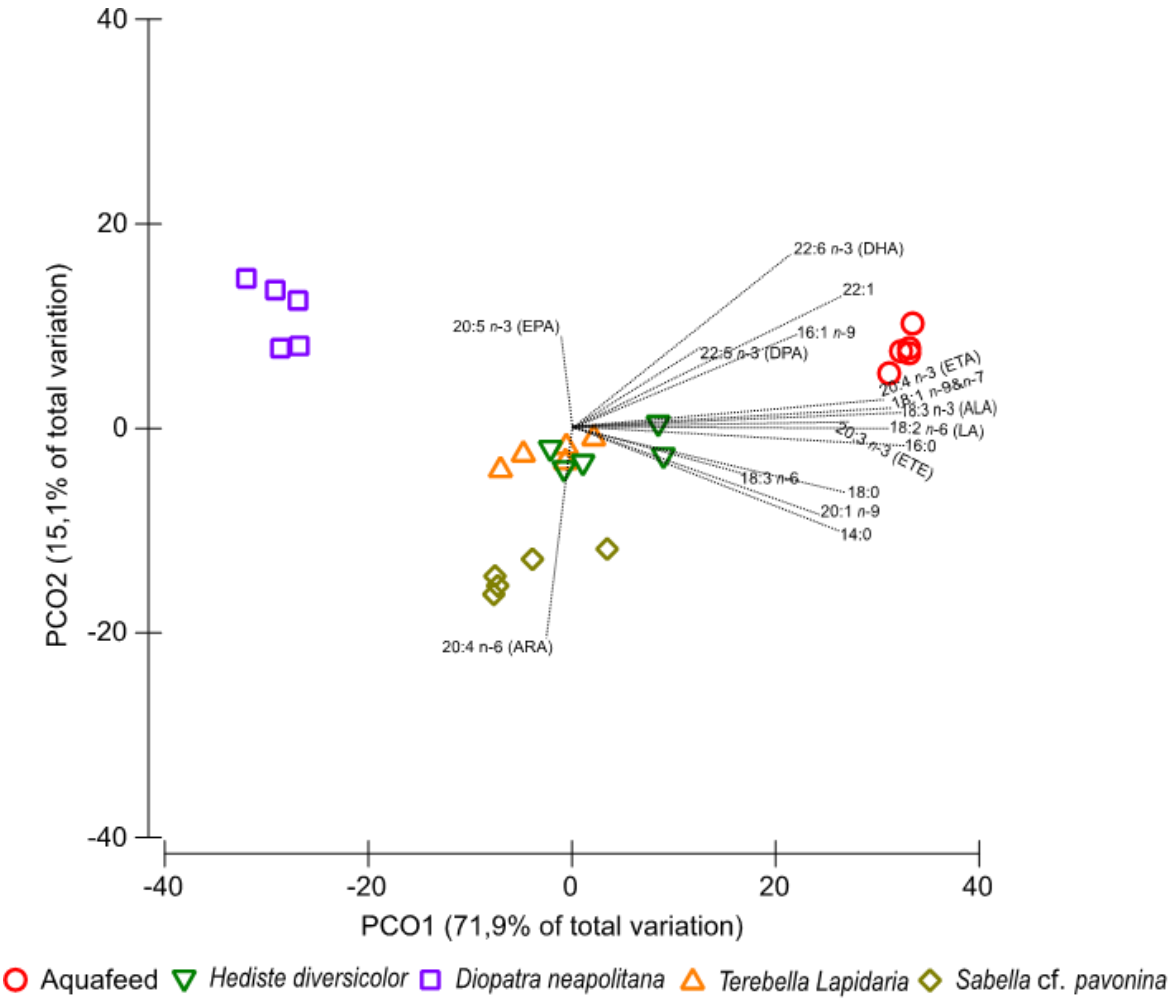


Figure 3.5. Principal coordinates analysis (PCO) of common fatty acids present in the aquafeed supplied to fish being farmed and the four IMTA-cultured polychaetes (*Hediste diversicolor*, *Diopatra neapolitana*, *Sabella* cf. *pavonina* and *Terebella lapidaria*) (common with at least one of the species). Average values (± SD) (n = 5). Abbreviations: ALA -alpha-linolenic acid; ARA – arachidonic acid; DHA – docosahexaenoic acid; DPA – docosapentaenoic acid; EPA – eicosapentaenoic acid; ETA – eicosatetraenoic acid; ETE – eicosatrienoic acid; LA – linoleic acid

3.1.4. Discussion and conclusions

The current scarcity of new sources of *n*-3 HUFA (mainly EPA and DHA) makes paramount the search for new ingredients from where these EFA can be derived[5]. Polychaetes are likely in the frontline of alternative sources of EFA that can be explored, namely through their integration in IMTA systems as extractive species to recover these valuable nutrients[6,23,25,26,40].

Hediste diversicolor is well represented in multiple IMTA designs that have already featured its potential to recover nutrients from organic rich effluents[6,15,23-28]. The biomass of *H. diversicolor*, whose FA profile was evaluated in present work, was cultured in PASFs installed to filter the effluent water of earthen ponds stocked with gilthead seabream (*S.*

aurata) during 15 weeks[27]. From an initial inoculum of approximately 400 ind. m^{-2} a density of approximately 1000 ind. m^{-2} (2.5-fold increase) was achieved, with PASFs contributing to retain with high efficiency the POM present in the aquaculture effluent (approx. 1.8±1 mg L^{-1})[27]. The significant differences recorded between the FA profile of IMTA-cultured and wild *H. diversicolor* (with an overall dissimilarity of 24.2%) were mainly due to shifts in the concentration of common FA (e.g., ALA [18:3 *n*-3], ARA [20:4 *n*-6], AdA [22:4 *n*-6] and LA [18:2 *n*-6]). This dissimilarity was also due to the presence of less common FA, such as 7,10,13-hexadecatrienoic (16:3 *n*-3) and gamma-linolenic (18:3 *n*-6) which were only identified in wild polychaetes, and DHA (22:6 *n*-3) which was only identified in cultured polychaetes. In general, a total of 35 and 34 FA were identified in wild and IMTA-cultured *H. diversicolor* (respectively) (27 and 26 if FA from microbiome, Iso and anteiso are excluded). In the present study, it was not possible to conclude that the culture conditions benefit the enrichment of FA profile if evaluated only in terms of total FA content, as the values recorded for wild and IMTA-cultured polychaetes was very similar (≈41.6 and 39.8 µg mg^{-1} DW, respectively). Comparing the results recorded in the present study with previous ones which have characterised the FA profile of IMTA-cultured and wild *H. diversicolor* (Table 3.4), it is possible to verify that total FA content was slightly higher to that displayed by polychaetes supplied with the effluent water of a super-intensive farm of *S. senegalensis* (≈37.6 µg mg^{-1} DW)[23], as well as that recorded for conspecifics supplied with processed water from a *S. aurata* RAS (27.1 µg mg^{-1} DW)[6]. On the other side, Wang *et al.* (2019)[25] reported a slightly higher FA content (56.9 µg mg^{-1} DW) in polychaetes filtering the effluent water from a salmon smolt facility. Conversely to our results, these studies reported increases between 30-50% in total FA content of cultured organisms in respect to wild conspecifics (24.4, 17.8 and 41.6 µg mg^{-1} DW, respectively). Pajand *et al.* (2017)[24] also reported a higher FA content (109.9 mg g^{-1} DW) for *H. diversicolor* that filtered the effluent water of beluga sturgeon (*Huso huso*), although no comparison was performed with the FA profile of wild conspecifics. Different size classes of *H. diversicolor* can present different FA profiles (<30 mm: ≈25.4, 30–50 mm: 27.3 and >50mm: ≈37.6 µg mg^{-1} DW)[15]. In the present study the FA characterisation was performed in specimens with a size >40 mm and differences recorded with the above-mentioned studies could also be due to different maturation stages and not solely a consequence of contrasting culture conditions (environmental and effluent water nutrient load). The higher concentration of MUFA detected in IMTA-cultured biomass, may be likely a consequence of the FA profile exhibited by the main source of nutrients present in effluent water (the

aquafeed provided to *S. aurata*). Pajand *et al.* (2017)[24] obtained a similar result with MUFA and HUFA being the most and least represented FA classes, respectively, in *H. diversicolor* (≈39.4% and 4.6% of total FA, respectively) reflecting the formulation of the aquafeed supplied to *H. huso* (≈40.5% and 0.6% of total FA, respectively) (Table 4). Bischoff *et al.* (2009)[6] and Marques *et al.* (2018)[23] verified that HUFA was the major FA class in IMTA cultured polychaetes (≈ 34% and 37.8% of total FA, respectively) when aquafeeds being supplied to fish displayed a higher proportion of HUFA (24% and 20 - 28% of total FA, respectively) (Table 4). From *H. diversicolor* production under the culture conditions tested in the present work, it can be predicted a generation of approximately 39.8 g of total FA per Kg DW biomass, of which ≈ 6.6 g corresponded to *n*-3 HUFA (≈ 4.8 g EPA and 1.0 g DHA). The levels of EPA and DHA measured in IMTA-cultured specimens in the present study differed from the values reported by Marques *et al.* (2018)[23], as well as those reported by Pajand *et al.* (2017)[24] (Table 4). These differences likely reflect different culture conditions, mainly the intensification of fish culture conditions and different aquafeeds formulation. In the present work, IMTA-cultured polychaetes displayed EPA (20:5 *n*-3), DHA (22:6 *n*-3), ALA (18:3 *n*-3) and ARA (20:4 *n*-6), with only DHA not being detected in wild conspecifics. Marques *et al.* (2018)[23] did not detect ALA in IMTA-cultured specimens, nor DHA in wild *H. diversicolor*. Bischoff *et al.* (2009)[6] reported that wild specimens did not exhibit any detectable levels of DHA, ALA and ARA. These finds are likely explained by the seasonal shifts in the lipid content and FA profile that *H. diversicolor* is known to display, with maximum level of lipid content being detected in the winter (19.3% DW) and the lowest during the summer (6.6% DW)[34].

Table 3.4. Summary of the results of FA characterisation obtained in studies where the species *H. diversicolor* where included in IMTA designs. Table summarizes the FA characterisations of wild and IMTA-cultured *Hediste diversicolor* (Hd) depending on the origin of wasted nutrients: SsW - *Solea senegalensis* waste; Hh W - *Huso huso* waste; Om W - *Onchorhynchus mykiss* waste; Ssm W - salmon smolt waste; Sa W - *Sparus aurata* waste. Other FA characterisations corresponded to fish W (waste - faeces and uneaten feed) and fish feed. PUFA defined as all FA with ≥ 2 double bonds and HUFA all FA with ≥ 4 double bonds (not considered within ∑PUFA). The values between brackets were estimated based on the FA profile reported in each work.

	Absolute values (µg mg^{-1} DW biomass)							**Relative values (% FAMEs)**							
	Marques *et al.* 2018					**Pajand *et al.* 2016**		**Garakouei *et al.* 2019**		***Wang et al.* 2019**			**Bischoff *et al.* 2009**		
FA Class	*Hd (wild)*	*Hd (SsW)*	*Fish W*	*Fish Feed A*	*Fish Feed B*	*Hd (Hh W)*	*Fish feed*	*Hd (Om W)*	*Fish feed*	*Hd (wild)*	*Hd (Ssm W)*	*Fish W*	*Hd (wild)*	*Hd (Sa W)*	*Fish Feed*
Nº FA identified	19	19	18	17	17	19	20	16	17	19	20	18	10	14	11
SFA	6.5	9.00	13.2	18.8	36.5	(26.9)	(57.4)	34.0	22.9	29.4	29.5	40.9	(36.0)	(34.0)	(36.0)
MUFA	6.7	10.1	14.2	37.5	28.8	(43.29	(62.5)	24.8	31.5	24.4	25.4	36.9	(24.0)	(23.0)	(26.0)
PUFA	2.3	4.0	5.3	14.5	10.8	(34.7)	(34.46)	(33.6)	*(38.8)*	(14.0)	(13.9)	(10.1)	(1.0)	(9.0)	(14.0)
HUFA	8.8	14.2	2.5	17.5	29.5	(5.1)	(0.97)	(7.1)	(6.8)	(32.2)	(31.2)	(12.3)	(39.0)	(34.0)	(24.0)
20:5 n-3 (EPA)	5.5	8.3	0.1	7.1	16.2	3.4	0.3	5.6	2.8	22.8	19.1	0.6	(39.0)	(24.0)	(11.4)
22:6 n-3 (DHA)	ND	0.8	1.7	8.3	10.6	1.6	0.6	2.1	4.0	1.4	5.4	6.2	-	(4.0)	(13.0)
n-3 HUFA	(7.5)	(10.9)	(2.1	(16.6)	(28.1)	(5.1)	(1.0)	(7.7)	(6.8)	(28.2)	(27.9)	(11.8)	(40)	(32)	(25)
n-6 HUFA	(1.2)	(3.4)	(0.5)	(0.9)	(1.4)	ND	ND	ND	ND	4.1	3.3	0.5	ND	(6)	(ND)
n-3/n-6 HUFA	(6.3)	(3.2)	(4.2)	(18.4)	(20.0)	-	-	-	-	(6.9)	(8.5)	(23.6)	-	(5)	-
Total FA (µg mg^{-1} DW)	(24.4)	(37.6)	(35.3)	(88.3)	(105.6)	(109.9)	(155.3)	-	-	41.6	56.9	47.2	17.8	27.1	24.5
Total lipid (mg g^{-1} DW)	-	-	-	-	-	-	-	-	-	125.5	123.6	-	-	-	-
Total lipid (% DW)	-	-	-	-	-	11.6	20.4	22.2	15.6	-	-	-	-	-	-
Total Protein (%DW)	-	-	-	-	-	49.3	41.8	59.7	41.5	-	-	-	-	-	-

In this study it was also possible to compare the FA profile of *H. diversicolor* with that of other polychaete species (*D. neapolitana*, *S.* cf. *pavonina* and *T. lapidaria*) which adapted to the conditions in PASFs and were identified as potential candidates to integrate IMTA designs as extractive species[27]. The planktonic larvae of the three polychaete species mentioned above successfully colonized the sand beds of PASFs, most likely because the substrate of these filters provided the specific cues required for their larvae to settle and metamorphose (e.g., free FA have been suggested to favour the settlement of some species [43]). To date, the performance of *D. neapolitana* had never been tested under an IMTA framework. The adults of this species can present sizes ranging between 150 and 500 mm in length, being one of the species most intensively harvested in the coastal lagoon where the present study was performed[44]. This polychaete reveals an iteroparous reproduction behaviour with a discontinuous reproductive season (spawning: March - July; resting: August - September)[45]. From the four polychaete species whose FA profiles were evaluated in the present work, it was *D. neapolitana* that exhibited the lowest content of total FA with the *n*-3/*n*-6 HUFA ratio being similar to that of *H. diversicolor*. Despite this similarity, in overall, *D. neapolitana* was the species whose FA profile showed a greater dissimilarity to that of *H. diversicolor*. An analysis of *D. neapolitana* productivity in terms of FA profile allowed us to estimate the generation of approximately 12.5 g of total FA per Kg DW biomass produced, of which approximately 40% corresponded to *n*-3 HUFA (including EPA and DHA). As the FA profile of wild specimens of *D. neapolitana* has never been determined, it is impossible to verify if IMTA conditions enhance their value in EFA. Previous studies showed that this species reveals a lower capacity to grow in highly organic enriched areas[46], a fact that may constraint its use in more intensive IMTA systems. The development of sustainable production models for *D. neapolitana* is paramount[45], as the level of exploitation (eventually even overexploitation) and inherent digging activity may result in an enhanced biodiversity loss in the benthos[44]. In terms of bioremediation, it must be highlighted that these organisms are ecosystem engineers that stabilise the sediment with the tubes they secrete and thus increase the structural complexity and biodiversity of their habitat[45,47,48], a feature that may contribute for a less pronounced bioturbation. For this reason, this species is likely less suitable to promote safeguard the functionality of PASFs tested in present work, as these required complete percolation of water through the substrate[27].

The species *S.* cf. *pavonina* inhabits the tubes that the worm secretes, and it feeds by using crowns of ciliated filaments on their heads[49]. This polychaete can achieve a size of

270 mm, with an additional 45 mm of its branchial crown[50,51]. It displays a filter feeding behaviour and is a gonochoristic broadcaster that displays an annual reproductive cycle (spawning period in May/June)[51]. There is no evidence of this species having ever been included in IMTA designs as an extractive species. The total FA content detected in this polychaete species was lower than that of *H. diversicolor*, being also the species which exhibited the lowest *n*-3/*n*-6 HUFA ratio. *Sabella* cf. *pavonina* exhibited a FA profile slightly more similar to that of *H. diversicolor* than the one observed for *D. neapolitana*. An analysis of *S.* cf. *pavonina* productivity in terms of FA profile allowed us to estimate the generation of approximately 25.2 g of total FA per Kg DW biomass produced, of which approximately 10% corresponded to *n*-3 HUFA (including EPA and DHA). It is known that this species can filter more than 70 L of seawater per hour[52]. However, no major enhancement of bioturbation in PASFs could be perceived for this tubiculous polychaete, which, like *D. neapolitana* and for the same reasons, does not appear to be a species indicated to promote the functionality of PASFs.

Concerning the polychaete *T. lapidaria*, this species can achieve a size of 100 mm[53] and is characterized by the presence of a feed collecting apparatus formed by numerous tentacles that secrete mucus to trap different feed items[54]. Until the present study, it has never been considered for culture or tested using IMTA conditions. The high culture density recorded in the present study at the end of experimental period (>4000 ind. m^{-2}) revealed the great potential that this worm presents to adapt to these systems[27]. The results here reported are the first FA characterization for *T. lapidaria*. This polychaete species exhibited the most similar FA profile to *H. diversicolor*, concerning total FA content and FA composition. Despite having a concentration of *n*-3 HUFA similar to *H. diversicolor* and *D. neapolitana*, this species exhibited the lowest *n*-3/*n*-6 HUFA ratio, due to the fact that it has a concentration of *n*-6 HUFA higher than all other polychaete species tested in the present work. An analysis of *T. lapidaria* productivity in terms of FA profile allowed us to estimate the generation of approximately 36.2 g of total FA per Kg DW biomass produced, of which approximately 15% correspond to *n*-3 HUFA (including EPA and DHA).

In the evaluation of which of the four IMTA-cultured polychaete species exhibited the FA profile that most resemble that of aquafeed formula (diet supplied to *S. aurata* produced in earthen ponds) it was concluded that *H. diversicolor* and *T. lapidaria* were the species whose FA profile displayed the highest similarity. These polychaetes revealed the higher contents of *n*-3 and *n*-6 HUFA in their composition. This allowed us to assume that both

species featured an EFA profile more suitable to be integrated in premium aquafeeds formulation. Here it is important to bear in mind that the differences in *n*-3 and *n*-6 FA profile exhibited by the different polychaete species may likely be explained by its contrasting abilities to produce FA *de novo*.

The four polychaete species display different feeding habits and explore different trophic niches. *Hediste diversicolor* is considered a discrete motile polychaete, classified as an active predator[55]. This omnivorous species may exhibit a deposit-feeding behaviour and mainly consumes organic matter from substrate[55,56]. *Diopatra neapolitana* is considered a discrete motile polychaete, omnivorous, a scavenger and detritus feeder[39,55,57]. *Terebella lapidaria* is sessile or a discretely motile polychaete and a surface deposit feeder[55] that traps detritus, including unicellular algae (e.g., diatoms), and various small invertebrates (including larvae) with the mucus secreted by its tentacles, which transfers feed to its mouth[54,55]. This species also benefits from sediment enrichment in POM derived from fish production[58]. The polychaete *S.* cf. *pavonina* is a sessile species that display filter feeding behaviour and can feed both on phytoplankton (e.g., pelagic diatoms, dinoflagellates, other unicellular algae), small invertebrates (including larvae) and POM dissolved in water column, thus contributing to making the water clearer[32,50,55]. Due to these different feeding habits, the nutrition of the four polychaete species surveyed may be more or less on dependent on the POM fraction derived from uneaten fish feed.

The present work demonstrated that it is possible to produce polychaetes biomass with high nutritional value through an eco-design concept such as IMTA, a framework that promotes a cleaner production and, in this case, allowed to recover EFA commonly wasted in aquaculture effluents. The potential of using *H. diversicolor* to recover nutrients, namely EFA, present in the effluent water of earthen ponds used for finfish aquaculture was confirmed. It was also shown that it is feasible to co-culture several other polychaete species in deep sand beds stocked with *H. diversicolor* through the natural settling of planktonic larvae (e.g., *D. neapolitana*, *S.* cf. *pavonina* and *T. lapidaria*). All species displayed different FA profiles, but all hold the potential to recover available nutrients in the effluent water and give origin to value-added biomass, rich in EFA (namely *n*-3 HUFA, such as EPA and DHA). The FA profile of *D. neapolitana*, *S.* cf. *pavonina* and *T. lapidaria* was described here for the first time demonstrating that it is feasible to diversify the polychaete species to be included in PASFs. As polychaetes with planktonic larvae will likely always appear in IMTA designs similar to the ones described in the present work,

further studies are necessary to maximize the polyculture potential of marine polychaetes using PASFs.

3.1.5. Chapter 3 - References

1. FAO. The State of World Fisheries and Aquaculture - Meeting the Sustainable Development Goals. *Food and Agriculture Organization of the United Nations* (2018).
2. Gebauer, S., Psota, T., Harris, W. & Kris-Etherton, P. n-3 fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. *Am. J. Clin. Nutr.* **83**, 1526S–1535S (2006).
3. Aranceta, J. & Pérez-Rodrigo, C. Recommended dietary reference intakes, nutritional goals and dietary guidelines for fat and fatty acids: A systematic review. *Br. J. Nutr.* **107**, S8-S22 (2012).
4. EFSA. Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA*). EFSA J.* **10(7)**, 1–48 (2012).
5. Tocher, D. R. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture* **449**, 94–107 (2015).
6. Bischoff, A. A., Fink, P. & Waller, U. The fatty acid composition of Nereis diversicolor cultured in an integrated recirculated system: Possible implications for aquaculture. *Aquaculture* **296**, 271–276 (2009).
7. Sargent, J. R., Tocher, D. R. & Bell, J. G. The Lipids. In Fish Nutrition 3rd Edition (eds. Halver, J. E. & Hardy, R. W.).181–257 (Academic Press, 2003).
8. Ytrestøyl, T., Aas, T. S. & Åsgård, T. Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. *Aquaculture* **448**, 365–374 (2015).
9. Stevens, J. R., Newton, R. W., Tlusty, M. & Little, D. C. The rise of aquaculture by-products: Increasing food production, value, and sustainability through strategic utilisation. *Mar. Policy* **90**, 115–124 (2018).
10. Barrington, K., Chopin, T. & Robinson, S. Integrated multi-trophic aquaculture (IMTA) in marine temperate waters In Integrated Mariculture: A Global Review (ed. D. Soto). FAO Fish. Aquac. Tech. Pap. **529**, 7–46 (2009).
11. Chopin, T. *et al.* Multitrophic Integration for Sustainable Marine Aquaculture. *Encycl. Ecol.* **1-5**, 2463–2475 (2008).
12. Chopin, T. *et al.* Integrating seaweeds into marine aquaculture systems: a key towards sustainability. *J. Appl. Phycol.* **37**, 975–986 (2001).
13. Custódio, M., Villasante, S., Cremades, J., Calado, R. & Lillebø, A. I. Unravelling the potential of halophytes for marine integrated multi-trophic aquaculture (IMTA)— a perspective on performance, opportunities and challenges. *Aquac. Environ. Interact.* **9**, 445–460 (2017).
14. Gunning, D., Maguire, J. & Burnell, G. The Development of Sustainable Saltwater-Based Food Production Systems: A Review of Established and Novel Concepts. *Water* **8**, 598 (2016).
15. Marques, B., Calado, R. & Lillebø, A. I. New species for the biomitigation of a super-intensive marine fish farm effluent: Combined use of polychaete-assisted sand filters and halophyte aquaponics. *Sci. Total Environ.* **599–600**, 1922–1928 (2017).

16. Nardelli, A. E., Chiozzini, V. G., Braga, E. S. & Chow, F. Integrated multi-trophic farming system between the green seaweed Ulva lactuca, mussel, and fish: a production and bioremediation solution. *J. Appl. Phycol.* **31**, 847–856 (2019).

17. Pombo, A. *et al.* Insight into aquaculture's potential of marine annelid worms and ecological concerns: a review. *Rev. Aquac.*, 1–15 (2018).

18. Schneider, O., Sereti, V., Eding, E. H. & Verreth, J. A. J. Analysis of nutrient flows in integrated intensive aquaculture systems. *Aquac. Eng.* **32**, 379–401 (2005).

19. Wang, X., Olsen, L. M., Reitan, K. I. & Olsen, Y. Discharge of nutrient wastes from salmon farms: environmental effects, and potential for integrated multi-trophic aquaculture. *Aquac. Environ. Interact.* 2, 267–283 (2012).

20. Endut, A., Jusoh, A. & Ali, N. Nitrogen budget and effluent nitrogen components in aquaponics recirculation system. *Desalin. Water Treat.* **52**, 744–752 (2014).

21. Bergström, P., Hällmark, N., Larsson, K. J. & Lindegarth, M. Biodeposits from Mytilus edulis: a potentially high-quality food source for the polychaete, Hediste diversicolor. *Aquac. Int.* **27**, 89–104 (2019).

22. Bergström, P. *et al.* Testing the potential for improving quality of sediments impacted by mussel farms using bioturbating polychaete worms. *Aquac. Res.* **48**, 161–176 (2017).

23. Marques, B. *et al.* Adding value to ragworms (Hediste diversicolor) through the bioremediation of a super-intensive marine fish farm. *Aquac. Environ. Interact.* **10**, 79–88 (2018).

24. Pajand, Z. O., Soltani, M., Bahmani, M. & Kamali, A. The role of polychaete Nereis diversicolor in bioremediation of wastewater and its growth performance and fatty acid composition in an integrated culture system with Huso huso (Linnaeus, 1758). *Aquac. Res.* **48**, 5271–5279 (2017).

25. Wang, H. *et al.* Growth and nutritional composition of the polychaete Hediste diversicolor (OF Müller, 1776) cultivated on waste from land-based salmon smolt aquaculture. *Aquaculture* **502**, 232–241 (2019).

26. Yousefi-Garakouei, M., Kamali, A. & Soltani, M. Effects of rearing density on growth, fatty acid profile and bioremediation ability of polychaete Nereis diversicolor in an integrated aquaculture system with rainbow trout (Oncorhynchus mykiss). *Aquac. Res.* **50**, 725–735 (2019).

27. Jerónimo, D., Lillebø, A. I., Santos, A., Cremades, J. & Calado, R. Performance of polychaete assisted sand filters under contrasting nutrient loads in an integrated multi-trophic aquaculture (IMTA) system. *Sci. Rep.* **10**, 1–10 (2020).

28. Jerónimo, D., Lillebø, A. I., Cremades, J., Cartaxana, P. & Calado, R. Recovering wasted nutrients from shrimp farming through the combined culture of polychaetes and halophytes. *Sci. Rep.* 1–16 (2021)

29. Palmer, P. J. Polychaete-assisted sand filters. Aquaculture **306**, 369–377 (2010).

30. Brown, N., Eddy, S. & Plaud, S. Utilization of waste from a marine recirculating fish culture system as a feed source for the polychaete worm, Nereis virens. *Aquaculture* **322–323**, 177–183 (2011).

31. Gómez, S., Hurtado, C. F., Orellana, J., Valenzuela-Olea, G. & Turner, A. Abarenicola pusilla (Quatrefages, 1866): A novel species for fish waste bioremediation from marine recirculating aquaculture systems. *Aquac. Res.*, 1–5 (2017).

32. Giangrande, A. *et al.* Utilization of the filter feeder polychaete Sabella spallanzanii Gmelin (Sabellidae) as bioremediator in aquaculture. *Aquac. Int.* **13**, 129–136 (2005).

33. Santos, A. *et al.* Effect of three diets on the growth and fatty acid profile of the common ragworm Hediste diversicolor (O.F. Müller, 1776). *Aquaculture* **465**, 37–42 (2016).

34. Luis, O. J. & Passos, A. M. Seasonal changes in lipid content and composition of the polychaete Nereis (Hediste) diversicolor. *Comp. Biochem. Physiol.* **111B**, 579–586 (1995).

35. Luis, O. J. & Ponte, A. C. Control of reproduction of the shrimp Penaeus kerathurus held in captivity. *J. World Aquac. Soc.* **24**, 31–39 (1993).

36. Watson, G. J., Murray, J. M., Schaefer, M. & Bonner, A. Bait worms: a valuable and important fishery with implications for fisheries and conservation management. *Fish Fish.* **18**, 374–388 (2016).

37. Fidalgo e Costa, P. *et al.* The market features of imported non-indigenous polychaetes in Portugal and consequent ecological concerns. *Sci. Mar.* **70**, 287–292 (2006).

38. Olive, P. J. W. Polychaete aquaculture and polychaete science: A mutual synergism. *Hydrobiologia* **402**, 175–183 (1999).

39. Xenarios, S., Queiroga, H., Lillebø, A. & Aleixo, A. Introducing a Regulatory Policy Framework of Bait Fishing in European Coastal Lagoons: The Case of Ria de Aveiro in Portugal. *Fishes*, 1-19 (2018).

40. Palmer, P. J., Wang, S., Houlihan, A. & Brock, I. Nutritional status of a nereidid polychaete cultured in sand filters of mariculture wastewater. *Aquac. Nutr.* **20**, 675–691 (2014).

41. Fidalgo e Costa, P., Sarda, R. & Cancela da Fonseca, L. Life cycle, growth and production of the polychaete *Nereis diversicolor* O. F. Müller in three lagoonal estuarine systems of the southwestern Portuguese coast (Odeceixe, Aljezur and Carrapateira). *Ecologie* **29**, 523–533 (1998).

42. Scaps, P. A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O. F. Müller) (Annelida: Polychaeta). *Hydrobiologia* **470**, 203–218 (2002).

43. Qian, P. Reproductive Strategies and Developmental Patterns in Annelids. *Hydrobiologia* **402**, 239-253 (1999).

44. Cunha, T., Hall, A. & Queiroga, H. Estimation of the *Diopatra neapolitana* annual harvest resulting from digging activity in Canal de Mira, Ria de Aveiro. *Fish. Res.* **76**, 56–66 (2005).

45. Arias, A., Paxton, H. & Budaeva, N. Redescription and biology of *Diopatra neapolitana* (Annelida: Onuphidae), a protandric hermaphrodite with external spermaducal papillae. *Estuar. Coast. Shelf Sci.* **174**, 1–17 (2016).

46. Carregosa, V. *et al.* Physiological and biochemical responses of the Polychaete *Diopatra neapolitana* to organic matter enrichment. *Aquat. Toxicol.* **155**, 32–42 (2014).

47. Bailey-Brock, J. H. Ecology of the tube-building polychaete *Diopatra leuckarti* Kinberg, 1865 (Onuphidae) in Hawaii: community structure, and sediment stabilizing properties. *Zool. J. Linn. Soc.* **1865**, 191–199 (1984).

48. Thomsen, M. S., Muth, M. F. & McGlathery, K. J. Tube-forming polychaetes enhance invertebrate diversity and abundance in sandy sediments of Mozambique, Africa. *African J. Mar. Sci.* **33**, 327–332 (2011).

49. Wells, G. P. On the behaviour of sabella. R. Soc. **138**, 278–299 (1951).

50. Knight-jones, P. & Perkins, T. H. A revision of Sabella, Bispira and Stylomma (Polychaeta: Sabellidae). *Zool. J. Linn. Soc.* **123**, 385–467 (1998).

51. Murray, J. M., Watson, G. J., Giangrande, A., Bentley, M. G. & Farrell, P. Reproductive biology and population ecology of the marine fan worm *Sabella pavonina* (savigny) (polychaeta: Sabellidae). *Invertebr. Reprod. Dev.* **55**, 183–196 (2011).

52. Dales, R. P. Some Quantitative Aspects of Feeding in Sabellid and Serpulid fan worms. *J. mar. biol. Ass. U.K.* **36**, 309–316 (1956).

53. Rouse, G. W. & Pleijel, F. Polychaetes (ed. Westheide, W.) 345pp (Oxford University Press, 2001).

54. Sutton, M. F. The feeding mechanism, functional morphology and histology of the alimentary canal of *Terebella lapidaria* L. (Polychaeta). *Proc. Zool. Soc. London* **129**, 487–523 (1957).

55. Fauchald, K. & Jumars, P. A. The diet of worms: a study of polychaete feeding guilds. *Oceanogr. Mar. Biol.* **17**, 193–284 (1979).

56. Reise, K. Spatial configurations generated by motile benthic polychaetes. *Helgoländer Wiss. Meeresunters.* **32**, 55–72 (1979).

57. Dağli, E., Ergen, Z. & Çinar, M. E. One-year observation on the population structure of *Diopatra neapolitana* Delle Chiaje (Polychaeta: Onuphidae) in Izmir Bay (Aegean Sea, eastern Mediterranean). *Mar. Ecol.* **26**, 265–272 (2005).

58. Carvalho, S., Barata, M., Gaspar, M. B., Pousão-Ferreira, P. & Cancela da Fonseca, L. Enrichment of aquaculture earthen ponds with *Hediste diversicolor*: Consequences for benthic dynamics and natural productivity. *Aquaculture* **262**, 227–236 (2007).

59. Aued-Pimentel, S., Lago, J. H. G., Chaves, M. H. & Kumagai, E. E. Evaluation of a methylation procedure to determine cyclopropenoids fatty acids from *Sterculia striata* St. Hil. Et Nauds seed oil. *J. Chromatogr. **A*** **1054**, 235–239 (2004).

60. Maciel, E. *et al.* Polar lipidome profiling of *Salicornia ramosissima* and *Halimione portulacoides* and the relevance of lipidomics for the valorization of halophytes. *Phytochemistry* **153**, 94–101 (2018).

61. Melo, T. *et al.* Lipidomics as a new approach for the bioprospecting of marine macroalgae - unraveling the polar lipid and fatty acid composition of *Chondrus crispus*. *Algal Res.* **8**, 181–191 (2015).

62. Anderson, M. J., Gorley, R. N. & Clarke, K. R. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. Plymouth UK, 1–214

3.1.6. Chapter 3 - Supporting Information

Table S3.1. Average values (±SD) (n=5) of fatty acid composition of total lipids (µg mg^{-1} DW) identified as others (microbiome and iso and anteiso FA) in wild and IMTA-cultured polychaete species (*Hediste diversicolor*, *Diopatra neapolitana*, *Sabella* cf. *pavonina* and *Terebella lapidaria*,) and aquafeed supplied to farmed fish.

Fatty acid	*H. diversicolor* (Wild)	*H. diversicolor*	*D. neapolitana*	*S.* cf. *pavonina*	*T. lapidaria*	Fish feed
15:0	0.59 ± 0.06	0.25 ± 0.05	0.08 ± 0.02	0.35 ± 0.14	0.29 ± 0.02	ND
17:0	1.01 ± 0.30	0.55 ± 0.08	0.43 ± 0.12	0.70 ± 0.35	0.75 ± 0.10	ND
21:0	ND	ND	ND	ND	ND	0.04 ± 0.02
17:1 n-8 $^{\Delta 9}$	0.08 ± 0.00	0.01 ± 0.01	ND	ND	0.16 ± 0.01	ND
17:1 n-9 $^{\Delta 8}$	0.02 ± 0.02	0.07 ± 0.02	ND	ND	0.08 ± 0.02	ND
13-methyl-C14:0 (iso)	0.18 ± 0.10	0.14 ± 0.05	0.14 ± 0.05	0.43 ± 0.18	0.49 ± 0.03	ND
14-methyl-C15:0 (iso)/ 13-methyl-C15:0 (anteiso)	0.04 ± 0.03	0.04 ± 0.02	ND	0.27 ± 0.12	0.21 ± 0.02	ND
14-methyl-C16:0 (anteiso)	0.12 ± 0.01	0.16 ± 0.04	0.10 ± 0.02	1.07 ± 0.39	0.92 ± 0.11	ND
10-methyl-C16:0	0.11 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.25 ± 0.12	0.21 ± 0.05	ND
7-methyl-hexadec-6-enoate	ND	ND	0.04 ± 0.02	ND	0.42 ± 0.04	ND
16-methyl-C17:0 (iso)	ND	ND	ND	0.08 ± 0.02	0.03 ± 0.01	ND
∑Others	**2.16 ± 0.46**	**1.23 ± 0.20**	**0.81 ± 0.21**	**3.15 ± 1.27**	**3.56 ± 0.24**	**0.04 ± 0.02**

Chapter 4

4.1. Optimizing the timeframe to produce polychaetes (*Hediste diversicolor*) enriched with essential fatty acids under different combinations of temperature and salinity

Jerónimo D, Lillebø AI, Rey F, Ii HK, Domingues MRM and Calado R (2021) Optimizing the Timeframe to Produce Polychaetes (*Hediste diversicolor*) Enriched With Essential Fatty Acids Under Different Combinations of Temperature and Salinity.
Frontiers in Marine Science. 8:671545. doi: 10.3389/fmars.2021.671545

4.1. Optimizing the timeframe to produce polychaetes (*Hediste diversicolor*) enriched with essential fatty acids under different combinations of temperature and salinity

Abstract

Polychaetes can be successfully employed to recover essential fatty acids (EFA) from wasted uneaten aquafeeds present in aquaculture effluents. The optimization of the timeframe required to produce premium ragworms (*Hediste diversicolor*) biomass rich in EFA is paramount to make available to the aquafeeds industry another alternative ingredient to fish meal and fish oil. The present study aimed to evaluate the potential enrichment of ragworms fatty acid (FA) profile when fed a commercial aquafeed during 10, 20 and 40 days (D10, D20 and D40) under different combinations of water temperature (20 and 25ºC) and salinity (15, 20 and 25). Total FA incremented progressively overtime, with D40 polychaetes exhibiting average values ranging between 70 and 90 µg mg^{-1} DW. The average values of *n*-6 FA ranged between 13 and 17 µg mg^{-1} DW, while that of *n*-3 FA varied between 17 and 19 µg mg^{-1} DW at D40. No significant differences were found in the FA profile of *H. diversicolor* cultured under different combinations of temperature and salinity. The FA profile of cultured polychaetes exhibited between 28 - 31% dissimilarity from that of wild conspecifics and displayed a higher content of two essential *n*-3 FA: eicosapentaenoic (20:5 *n*-3, EPA) and docosahexaenoic acids (22:6 *n*-3, DHA) (values ranging between 9.6 - 11.2% and 4.3 -5.0% of total FA, respectively). A higher similarity in FA profile was recorded between D40 polychaetes and aquafeed than with initially stocked or wild specimens. Palmitic (16:0), oleic (18:1 *n*-9), linoleic (18:2 *n*-6), eicosadienoic (20:2 *n*-6), EPA (20:5 *n*-3) and DHA (22:6 *n*-3) were the FA whose concentration exhibited the highest increment. Evidence of *de novo* FA biosynthesis was observed through the formation of some FA that were neither present in the initially stocked biomass, nor in the aquafeed supplied, such as 5,11-eicosadienoate ($^{\Delta 5,11}$20:2), 7,13,16-docosatrienoate ($^{\Delta 7,13,16}$22:3), dihomo-gamma-linolenic (20:3 *n*-6), eicosatrienoic (20:3 *n*-3) and eicosatetraenoic (20:4 *n*-3) acids. A plateau of total FA, *n*-6, and *n*-3 FA was not reached over the study period. Overall, the present study highlights the potential of *H. diversicolor* as an extractive species for integrated multi-trophic aquaculture (IMTA) applications.

4.1.1. Introduction

World aquaculture production reached an all-time record of 114.5 million tonnes in live weight in 2018 (USD 263.6 billion)[1]. This figure includes the production of 54.2 million tonnes of fish (USD 139.7 billion) and 9.4 million tonnes of crustaceans (USD 69.3 billion), with 13% and 61% of these productions resulting from saltwater aquaculture (which includes marine and brackish water species), respectively[1]. The majority of farmed species of both groups are produced using commercial aquafeeds (~ 57 million tonnes in 2018)[1]. Most marine teleosts (and other vertebrate species including humans) display a limited ability to perform de novo synthesis of *n*-3 highly unsaturated fatty acids (HUFA) due to the lack of desaturases Δ12 and Δ15, the enzymes responsible to produce polyunsaturated fatty acids (PUFA) from oleic acid (18:1 *n*−9)[2,3]. As such, it is paramount to include in aquafeeds a balanced profile of HUFA and not their FA precursors[4–6]. Aquaculture of marine fishes and crustaceans thus depends on a rich supply of *n*-3 HUFA, including eicosapentaenoic (20:5 *n*-3, EPA) and docosahexaenoic (22:6 *n*-3, DHA) acids, through the formulation and supply of well-balanced aquafeeds commonly employing fishmeal and fish oil, two increasingly scarcer and costly resources for aquafeeds[1,7–9]. The progressive reduction in the supply of these marine origin raw materials has been coupled with a surging demand driven by a fast-growing aquaculture industry (farmed aquatic animals grew on average 5.3% per year between 2001 and 2018)[1]. There is a global requirement of approximately 0.4 million metric tonnes of *n*-3 HUFA per year[5]. Formulated aquafeeds have to both satisfy the nutritional needs of cultured species and safeguard that at the end of a productive cycle farmed species display an optimal biochemical profile for human nutrition.

Polychaetes display a significant commercial value for the culture of vertebrates and invertebrates[10,11]. According to reported values, approximately 121.000 tonnes of polychaetes (with an estimated value of USD 8.39 billion) were harvested globally in 2015, with these figures being comparable to some of the most important world fisheries[12]. It has already been acknowledged that the collection of polychaetes from the wild is likely insufficient to satisfy global market demands and that this practice drives a multitude of negative environmental impacts[13–15]. The polychaete *Hediste diversicolor* O.F. Müller, 1776, popularly known as ragworm, is a highly valued bait species for sports fishing[13,14,16,17]. It also plays a key dietary role on the nutrition and production of some fish and crustacean species (e.g., soles, shrimps and crabs), being often used to trigger gonad maturation and

spawning[18-22]. This omnivorous species is an active predator[23] that is also able to perform deposit-feeding[24,25] and filter-feeding[26,27]. Their deposit-feeding behaviour allows them to consume considerable amounts of organic matter present in the substrate where it burrows[24,25]. The biomass of ragworms contains essential ingredients that can act as important supplements for aquafeeds formulation, such as amino acids and other odorants that elicit a feeding response for several fish species (e.g., Senegalense sole)[28,29]. Ragworms also display high proteic and lipidic contents (54 - 60% and 11 - 22% of DW biomass, respectively)[29,30]. This species has already been shown to perform *de novo* biosynthesis of some essential fatty acids (EFA) from acetyl coenzyme A, by using several fatty acids (FA) desaturase and enlogase enzymes; as such, it is usual to detect higher concentrations of PUFA and HUFA in ragworms biomass than on their diet[31,32]. The abundance of EFA and ragworms ability to recycle *n*-3 HUFA (such as EPA and DHA) from uneaten aquafeeds and sludge from aquaculture effluents, that would otherwise be lost to the environment, make *H. diversicolor* a key extractive species for integrated multi-trophic aquaculture (IMTA) applications[29–31,33,34]. A large variability in the total pool of FA determined for *H. diversicolor* biomass has been reported to date, with values ranging between 50 and 280 µg mg^{-1} DW for specimens fed commercial aquafeeds[10,29,31,35,36] or between 24 and 110 µg mg^{-1} DW when cultured using aquaculture effluents[10,29,30,33,34,36]. Also, the proportion of *n*-3 and *n*-6 FA reported to date for this species is also highly variable, with the above-mentioned works reporting values ranging between 5 to 33% for *n*-3 FA and 9 to 27% to *n*-6 FA. This variability is a consequence of several factors, such as the duration of experimental trials, the maturation stage of polychaetes at the beginning and during experiments, the composition of the supplied diet and the abiotic conditions experienced during culture (e.g., temperature, salinity, photoperiod). The main source of EFA in IMTA designs will always be the aquafeed supplied to upstream species. However, the majority of studies performed to date including polychaetes as extractive species under IMTA frameworks fails to characterize the proportion of faeces and uneaten aquafeed present in the particulate fraction of nutrients being supplied. Additionally, only a few studies have evaluated the initial FA profile of polychaetes in order to assess how these biomolecules evolve when ragworms are supplied a certain pool of nutrients[29,33,34]. It also remains to be clarified how the enrichment in EFA occurs in ragworms biomass over time under different combinations of water temperature and salinity, and whether it is possible to reach a plateau for enrichment in total

FA, *n*-6 and *n*-3 FA. All this information is paramount to optimize the timeframe required to produce a premium polychaete biomass rich in EFA.

In order to shed some light over the above-mentioned questions, the present study aimed to evaluate the evolution of the FA profile of *H. diversicolor* fed a commercial aquafeed (with a well-known FA composition) during 10, 20 and 40 days (D10, D20 and D40) under different combinations of water temperature (20 and 25 ºC) and salinity (15, 20 and 25). These are optimal conditions for the culture of ragworms, which are within the range of temperature and salinity commonly employed by warm-temperate aquaculture systems operating with brackish water. A comparison between cultured and wild polychaetes over time was also performed to confirm that the evolution of FA profile of polychaetes fed on aquafeeds was not influenced by natural cycles. The feeding and growth performances of *H. diversicolor* cultured under the different combinations of water temperature and salinity were evaluated over time as well.

4.1.2. Material and methods

4.1.2.1. Experimental set-up

To evaluate the evolution of FA profile of *H. diversicolor*, five independent replicates were considered for each of the six-temperature x salinity combinations, at each of the three-time sampling points considered (D10, D20, D40). Each replicate consisted of ten polychaetes stocked on a glass flask (100 mm x 100 mm x 180 mm, ~1-L volume) filled with 80 mm of sand (0.5 – 0.7 mm grain size) (approximately 1000 ind. m^{-2}) and aerated artificial sea water (see below for details). Five water baths were used for each of the temperatures being tested, with 100-W Eheim® thermocontrol 3612 aquarium heaters being used to control this parameter. Replicates of the different salinities were randomly distributed over these water baths. The different salinities were previously prepared using artificial sea water (prepared by mixing Red Sea® salt with tap water purified by a reverse osmosis unit), with the whole water volume of each glass flask being changed every 2 days. Constant water aeration was secured by air stones connected to an aeration pump (Hailea® vortex blower) and a 16:8 h light:dark photoperiod was employed using fluorescent white

tubes connected to automatic timers. The schematic representation of the experimental set-up is represented in Figure 4.1.

Polychaetes were fed, once a day, a commercial grow-out diet for flatfish that present 62% of crude protein, 18% of crude fat and 0.3% of crude fiber (WINFlat - SPAROS). Aquafeed was supplied *ad libitum* until polychaetes lost interest in the pellets and these remained untouched at the top of the sand in the glass flasks. Uneaten feed was never removed from the glass flasks, with its presence or absence being used to adjust the feed being supplied the next day.

Water parameters, namely dissolved oxygen (DO), pH, temperature and salinity were monitored daily using a manual probe (pH/Cond 3320, WTW, Weilheim, Germany), with ammonia and nitrites being determined once a week using colorimetric tests (Salifert Profi Test).

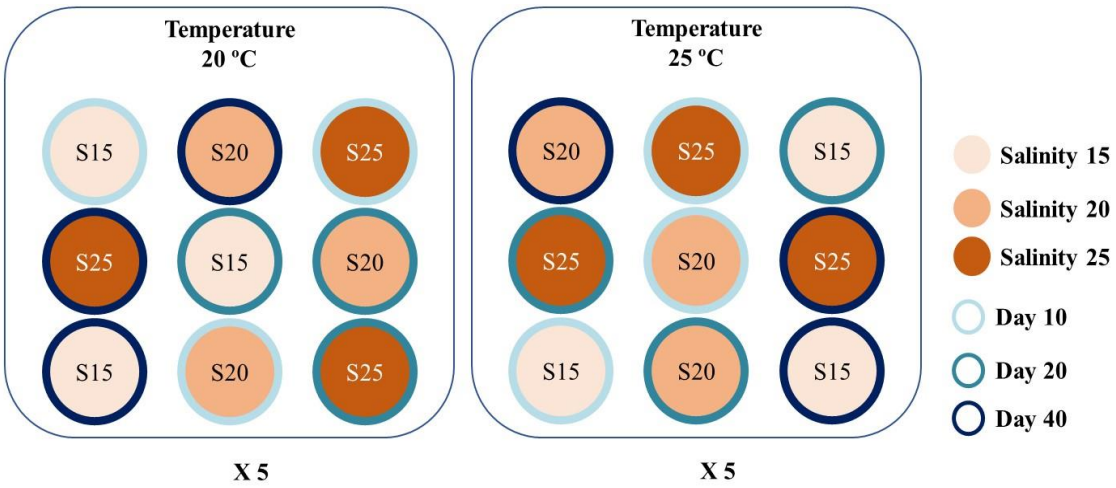


Figure 4.1. Schematic representation of one of the five replicate water baths used to control temperatures (20 and 25 ºC) housing randomly distributed glass flasks stocked with 10 *Hediste diversicolor* at different salinities (S – 15, 20 and 25) to evaluate the evolution of their fatty acid profiles after being fed a commercial aquafeed during 10, 20 and 40 days (D10, D20 and D40).

4.1.2.2. Polychaetes stocking and sampling

Wild specimens of *H. diversicolor* were collected from Ria de Aveiro coastal lagoon (Portugal) at Espinheiro Channel (40° 38′ 8.4″ N, 8° 39′ 41.8″ W) at the end of February 2019 and transported to the laboratory. An acclimation period of 24 h to experimental conditions was carried out and no feed was supplied during this period, so as not to modify the FA profile of wild polychaetes. The polychaetes were randomly selected and weighed individually and distributed per each of the five replicates of the different experimental conditions being tested. At the end of each sampling point (D10, D20, D40), polychaetes were depurated for 24 h in aerated glass flaks holding pre-combusted sterilized sand and artificial seawater (prepared as detailed above) to safeguard empty guts and avoid potential bias when performing FA analysis. The same temperature x salinity combination was maintained for each condition during the depuration process. Following depuration, all samples were freeze-dried and stored at -80 ºC until further analysis.

Five composite samples of polychaetes (10 specimens per composite sample) were collected from the wild at the beginning of the experiment (February 2019) to characterize the FA profile of the initially stocked biomass (Initial). To clarify if the evolution of the FA profile of polychaetes being supplied with aquafeeds in the laboratory was not influenced by natural cycles, another five composite samples of wild specimens (10 specimens per composite sample) were collected in the same site at each sampling time point of the experiment (Wild10, Wild20 and Wild40) (February-March 2019). Depuration and storage processes for these samples were the same as detailed above.

In Fig. 4.2 is displayed the flowchart of *H. diversicolor* samples collection for fatty acid analysis. Five composite samples of the aquafeed being used were also freeze dried and stored at -80 ºC for FA analysis.

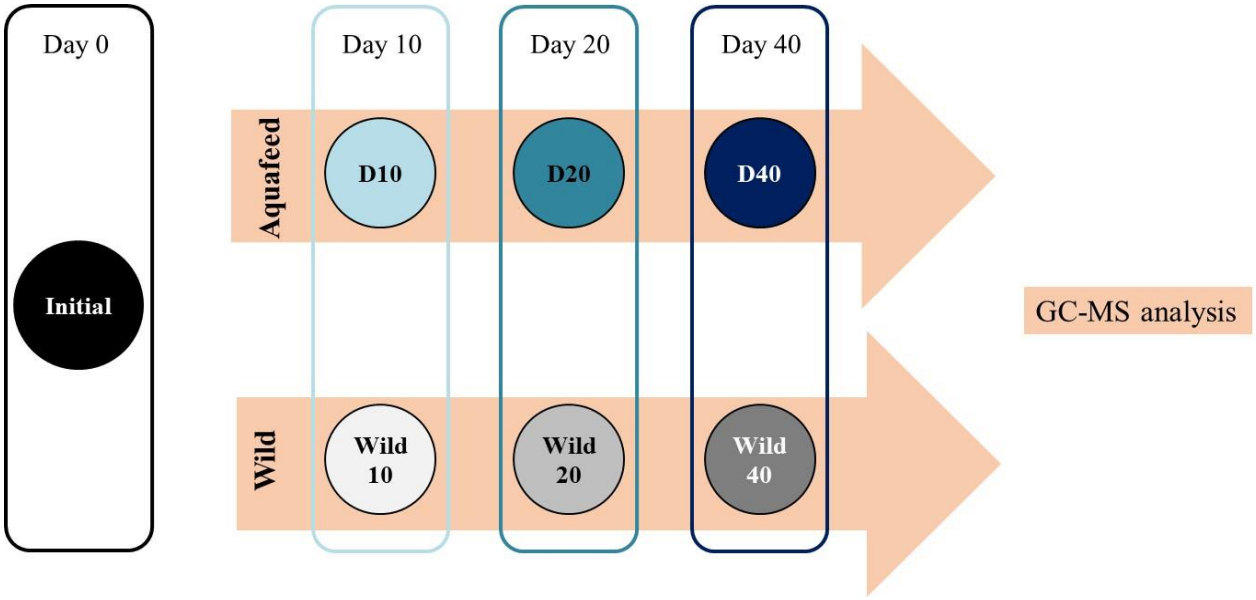


Figure 4.2. Flowchart of *Hediste diversicolor* samples collection for fatty acid analysis by gas chromatography-mass spectrometry (GC-MS). Initially stocked polychaetes (Initial); polychaetes fed with commercial aquafeed for 10, 20 and 40 days (D10, D20 and D40); polychaetes collected on the wild on the same dates of laboratorial trials (Wild10, Wild20 and Wild40).

4.1.2.3. Lipid extraction and fatty acid analysis

The FA content of both polychaetes and aquafeeds was determined by screening FA methyl esters (FAME) analysed by gas chromatography-mass spectrometry (GC-MS). Before analysis, all freeze-dried samples were powdered and homogenised. Lipid extraction was performed following the Bligh & Dyer (1959)[37] method using an initial sample biomass of 20 mg. Each sample was transferred to a glass tube, homogenized with 2500 µL methanol (MeOH) and 1250 µl dichloromethane (CH_2Cl_2), sonicated for 1 min and then incubated on ice in an orbital shaker for 30 min. Afterwards, each sample was centrifuged at 2000 rpm for 10 min and the organic phase was collected. A volume of 1250 µl CH_2Cl_2 and 1250 µL of Mili Q water was added to the total organic phase to promote phase separation, followed by centrifugation for 10 min at 2000 rpm. Organic phase aliquots of lipid extract (74 µL for polychaetes and 57 µL for aquafeeds; total lipid extract: 3000 µL) were collected for a new tube, previously washed in *n*-hexane. In order to define the final aliquot volume, a previous study was performed to calculate the total amount of lipid extract present in the different aliquots and analysing them in GC-MS. Lipid extract aliquots were dried under a nitrogen gas stream, and posteriorly used for methylation. FAME were prepared by adding 1 mL of the internal standard 19:0 FA (1.5 µg mL^{-1}) in *n*-hexane (99%) to the tube containing lipids.

Subsequently, 200 µl of methalonic (MeOH) KOH solution (2M) were added and vigorously vortexed for 2 min. Following this procedure, 2 mL of NaCl saturated solution was added to the tube, and then centrifuged for 5 min at 2000 rpm. Then 600 µL of the organic phase contained FAME were collected. To remove cholesterol from FAME, the solution was cleaned by solid-phase extraction. A column containing 0.1 g of silica was activated with 3 mL of *n*-hexane (99%) and the aliquot containing FAME (600 µL) was applied in the column. Subsequently, 3 mL of *n*-hexane 99%:diethyl ether (95:5 by volume) were eluted to recover the FAME. This fraction was dried under a nitrogen stream and stored at -20 ºC until GC-MS analysis. FAME were then dissolved in 100 µL of *n*-hexane 99% and 2 µL of this solution were used for analysis on a GC-MS system (Agilent Technologies 5977 B GC/MSD) equipped with a DB-FFAP column (123-3232, J & W Scientific, Folsom, CA, USA) with the following specifications: 30 m length, an internal diameter of 0.32 mm and a film thickness of 0.25 µm. The system employed includes a Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the mass range m/z 50–550 in a 1 s cycle in a full scan mode acquisition. The oven temperature program was as follows: 1) initial temperature of 80 °C for 2 min; 2) linear increase to 160 ºC (25 °C min^{-1}); 3) linear increase to 210º C (2 ºC min^{-1}); 4) linear increase to 225 ºC (20 °C min^{-1}); and 5) standing at 225 °C for 20 min. Helium was used as the carrier gas (1 mL min^{-1}). FA were integrated through Agilent's Masshunter Solutions Quantitative Analysis v10.0 automatic integration. Identification of FA was performed considering retention time and analysis of MS spectra in comparison with MS spectra of FA standards (Supelco 37 Component FAME Mix, ref. 47885-U, Sigma-Aldrich) and comparison with chemical databases (Wiley 275 library, AOCS lipid library, and NIST 2014 Mass spectral library). The FA content (expressed as µg of FA mg^{-1} dry weight, DW) was determined using calibration curves obtained from FAME certified standard mixture (Supelco®37 Component FAME Mix, Sigma-Aldrich) and 19:0 FA as internal standard.

In the present study, PUFA were defined as all FA with two or three double bonds, while HUFA refers to all FA with four or more double bonds.

FA increments between day 1-10, day 11-20, and day 21-40 (D1-D10, D11-D20 and D21-D40) were determined by calculating the differences in concentration between samples collected at the beginning of the experiment and D10, between samples from D10 and D20, and between samples from D20 and D40, respectively.

4.1.2.4. Feeding and growth performance of polychaetes

At each sampling point (D10, D20, D40) the whole sand of each glass flask was sieved, with polychaetes being sorted, counted and weighted individually. The specific growth rate (SGR), daily growth rate (DGR) and feeding rate (FR) were calculated as follows:

$$SGR\ (\%\ d^{-1}) = \frac{Ln\ final\ weight - Ln\ initial\ weight}{days}\ x\ 100$$

$$DGR\ (g\ d^{-1}) = \frac{Final\ wet\ weight - Initial\ wet\ weight}{days}$$

$$FR\ (\%\ ) = \frac{Total\ feed\ supplied/Initial\ biomass}{days}\ x\ 100$$

4.1.2.5. Statistical analysis

A two-way nested analysis of variance (ANOVA) was performed to determine the existence of significant differences in SGR, DGR and FR of *H. diversicolor* cultured under different combinations of water temperature and salinity. This analysis was performed for each independent group fed a commercial aquafeed during 10, 20 and 40 days (D10, D20 and D40) (n = 5). The factors being tested were as follows: "Temperature" (two fixed levels, 20 ºC and 25 ºC) and "Salinity" (three fixed levels, 15, 20 and 25) nested within temperature. Data were previously checked for normality (Anderson-Darling test) and homogeneity of variances (Bartlett´s and Levene´s tests for normal and non-normal distribution, respectively). When normality was not verified, the hypotheses were tested using non-parametric Kruskal-Wallis test. Significant differences were always considered at $p < 0.05$. These statistical analyses were performed using MINITAB 18 Software (State College, PA).

To determine the existence of significant differences in the FA profile of cultured *H. diversicolor* under the different combinations of water temperature and salinity tested in the present work, a two-way nested analysis of similarities (ANOSIM) was performed on a resemblance matrix produced using Bray Curtis similarity coefficient of data previously transformed using the formula log (x+1). This analysis was performed for each independent group at D10, D20 and D40 days (n = 5). The factors being tested were as follows:

"Temperature" (two levels, 20 ºC and 25 ºC) and "Salinity" (three levels, 15, 20 and 25) nested within temperature. The differences between FA profile of cultured polychaetes under different combinations of water temperature and salinity and the FA profile of wild conspecifics collected in the same sampling period (Wild10, Wild20 and Wild40) (n = 5) were performed using a one-way ANOSIM. When significant differences were recorded, a similarity percentages (SIMPER) analysis was performed to evaluate which FA contributed the most to the dissimilarities being recorded between samples, until a total of 50% cumulative dissimilarity was achieved. A principal coordinates analysis (PCO) plot was also used to compare the results of total FA concentration, FA classes concentration (saturated FA [SFA], monounsaturated FA [MUFA], PUFA and HUFA) and *n*-3 and *n*-6 FA concentration from D40 polychaetes cultured under different combinations of temperature and salinity, Wild40 polychaetes, initially stocked polychaetes (Initial) and the aquafeed supplied (resemblance matrix produced using Bray Curtis similarity used as input). These statistical analyses were performed using PRIMER v6 with the PERMANOVA+ add-on. For a detailed description of all the statistical methods employed please see Anderson *et al.* (2008)[38].

4.1.3. Results

4.1.3.1. Experimental conditions

The average values of DO and pH inside the glass flask varied between 6.7 – 7.9 mg L^{-1} and 8.2 – 8.4 along the duration of the experiment, respectively. The average value of ammonia (NH_4) and nitrites (NO_2) varied between 0.4 – 1.2 and 0.5 – 1.4 mg L^{-1}, respectively. Temperature and salinity were kept stable in the predefined conditions (see Supplementary Table S4.1).

4.1.3.2. Feeding and growth performance of polychaetes

The FR (%), SGR (% d^{-1}) and DGR (g d^{-1}) determined for polychaetes cultured under each combination of water temperature and salinity are summarized in Table 4.1 (complete data of biomass and aquafeed supplied summarized in Supplementary Table S4.2). No significant differences were found for FR, SGR and DGR exhibited by polychaetes at D10, D20 and D40 ($p > 0.05$ – Supplementary Table S4.3). The average values of polychaetes survival from the different treatments, D10, D20 and D40, varied between 66 – 78%, 48 – 70% and 38 – 54%, respectively (Supplementary Table S4.2).

Table 4.1. Feeding Rate (FR), Specific Growth Rate (SGR) and Daily Growth Rate (DGR) of *Hediste diversicolor* fed during 10, 20 and 40 days (D10, D20 and D40) with a commercial aquafeed under different combinations of temperature (T – 20 and 25 ºC) and salinity (S – 15, 20 and 25). Average values (± SD) (n=5).

Performance	Samples	T20S15	T20S20	T20S25	T25S15	T25S20	T25S25
FR (%)	D10	1.49 ± 0.04	1.53 ± 0.08	1.50 ± 0.06	1.48 ± 0.02	1.46 ± 0.07	1.48 ± 0.08
	D20	1.84 ± 0.08	1.87 ± 0.09	1.82 ± 0.06	1.84 ± 0.04	1.76 ± 0.08	1.79 ± 0.08
	D40	2.41 ± 0.13	2.38 ± 0.06	2.41 ± 0.09	2.33 ± 0.12	2.32 ± 0.08	2.38 ± 0.08
SGR (% d^{-1})	D10	3.47 ± 0.66	4.39 ± 1.56	3.62 ± 1.70	3.39 ± 0.67	3.34 ± 0.88	3.28 ± 1.19
	D20	4.61 ± 0.82	3.46 ± 1.73	3.54 ± 0.48	3.57 ± 0.34	2.70 ± 0.29	3.20 ± 0.95
	D40	3.14 ± 0.47	3.34 ± 0.19	2.97 ± 0.29	2.64 ± 0.61	2.44 ± 0.86	2.94 ± 0.81
DGR (g d^{-1})	D10	0.14 ± 0.01	0.16 ± 0.03	0.15 ± 0.02	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.02
	D20	0.13 ± 0.02	0.10 ± 0.04	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.00	0.10 ± 0.02
	D40	0.09 ± 0.02	0.10 ± 0.01	0.08 ± 0.01	0.07 ± 0.02	0.07 ± 0.02	0.08 ± 0.03

4.1.3.3. Fatty acid profile of the commercial aquafeed supplied to polychaetes

A total of 16 FA were identified in the aquafeed supplied to polychaetes, with palmitic (16:0), oleic (18:1 *n*-9), linoleic (18:2 *n*-6, LA), EPA (20:5 *n*-3), myristic (14:0) and DHA (22:6 *n*-3) exhibiting the highest concentration (Table 4.2).

SFA and MUFA accounted for 33.4% and 33.5% of total FA, respectively, while PUFA and HUFA accounted for 16.5% and 16.7% of total FA, respectively. DHA and EPA accounted for 6.2% and 7.6 % of total FA, respectively (accounting for 37.1% and 45.7 of total HUFA, respectively).

Table 4.2. Fatty acid (FA) profile (µg mg^{-1} DW) of the commercial aquafeed (WIN flat ® - SPAROS) supplied to *Hediste diversicolor*. Average values (± SD) (n = 5). SFA - saturated FA; MUFA - monounsaturated FA; PUFA - polyunsaturated FA; HUFA - highly unsaturated FA. PUFA are defined as all FA with 2 or 3 double bonds and HUFA as all FA with ≥ 4 double bonds.

Fatty acid	Aquafeed
14:0	7.44 ± 0.86
16:0	22.83 ± 2.03
18:0	5.17 ± 0.42
∑SFA	**35.45 ± 2.75**
16:1 *n*-7	5.86 ± 0.75
18:1 *n*-9	16.06 ± 1.68
18:1 *n*-7	5.69 ± 0.55
20:1 *n*-13	1.10 ± 0.04
20:1 *n*-11	2.73 ± 0.25
20:1 *n*-9	1.20 ± 0.03
22:1 *n*-11	2.94 ± 0.24
∑MUFA	**35.56 ± 3.46**
18:2 *n*-6 (LA)	14.32 ± 1.49
18:3 *n*-3 (ALA)	3.13 ± 0.28
∑PUFA	**17.45 ± 1.77**
20:4 *n*-6 (ARA)	1.35 ± 0.04
20:5 n-3 (EPA)	8.10 ± 0.93
22:5 *n*-3 (DPA)	1.69 ± 0.07
22:6 n-3 (DHA)	6.57 ± 0.60
∑HUFA	**17.71 ± 1.62**
Total FA	**106.17 ± 9.42**

4.1.3.4. Fatty acid dynamics of polychaetes supplied a commercial aquafeed under different combinations of temperatures and salinity

The FA profile of *H. diversicolor* from D40, along with that of initially stocked polychaetes (Initial) and conspecifics collected from the wild (Wild40) are detailed in Table 4.3 (FA profile of D10 and D20 polychaetes displayed in Supplementary Table S4.4 and S4.5, respectively). A total of 25 FA were identified in D40 polychaetes, with palmitic (16:0), EPA (20:5 *n*-3), oleic (18:1 *n*-9), LA (18:2 *n*-6), eicosadienoic (20:2 *n*-6), vaccenic (18:1 *n*-7), DHA (22:6 *n*-3) and stearic (18:0) FA displaying the highest concentrations. No significant differences were found in the FA profile of D10, D20 and D40 polychaetes ($p > 0.05$ – Supplementary Table S4.6).

A progressive increment of total FA content over time was recorded in all combinations of water temperature and salinity evaluated in the present work, with values varying from 30 µg mg^{-1} DW (Initial) to 70 - 90 µg mg^{-1} DW (D40) (Figure 4.3). The proportion of PUFA incremented approximately 10% in the whole pool of FA over the 40 days of the experimental trial, with all FA classes (SFA, MUFA, PUFA and HUFA) being present with a similar proportion at the end of the study (approx. 25% in all FA classes) (Supplementary Figure S4.1).

Table 4.3. Fatty acid profile (µg mg^{-1} DW) of *Hediste diversicolor* fed during 40 days with a commercial aquafeed under different combinations of temperature (T – 20 and 25 ºC) and salinity (S – 15, 20 and 25), along with conspecifics initially stocked (Initial) and conspecifics collected from the wild at the same time point (Wild40). Average values (± SD) (n = 5). Abbreviations: SFA - saturated FA; MUFA - monounsaturated FA; PUFA - polyunsaturated FA; HUFA - highly unsaturated FA. PUFA are defined as all FA with 2 or 3 double bonds and HUFA as all FA with ≥ 4 double bonds.

Fatty acid	Initial	T20S15	T20S20	T20S25	T25S15	T25S20	T25S25	Wild40
14:0	0.41 ± 0.01	1.66 ± 0.23	1.69 ± 0.44	1.61 ± 0.29	1.59 ± 0.37	1.39 ± 0.29	1.87 ± 0.55	0.49 ± 0.03
16:0	4.14 ± 0.31	14.08 ± 2.56	15.16 ± 2.66	13.09 ± 1.15	14.64 ± 3.44	12.28 ± 1.77	15.74 ± 3.54	7.19 ± 1.19
18:0	2.44 ± 0.09	4.37 ± 0.59	4.12 ± 0.65	4.12 ± 0.26	3.88 ± 0.77	3.66 ± 0.59	3.95 ± 0.58	2.92 ± 0.23
∑SFA	**6.99 ± 0.27**	**20.11 ± 3.32**	**20.97 ± 3.73**	**18.82 ± 1.66**	**20.11 ± 4.58**	**17.34 ± 2.49**	**21.56 ± 4.63**	**10.60 ± 1.35**
16:1 *n*-7	1.05 ± 0.18	1.73 ± 0.42	2.05 ± 0.54	1.53 ± 0.19	1.74 ± 0.45	1.26 ± 0.32	2.21 ± 0.59	1.15 ± 0.14
18:1 *n*-14	2.18 ± 0.07	2.47 ± 0.23	2.73 ± 0.18	2.74 ± 0.22	2.67 ± 0.22	2.46 ± 0.23	2.65 ± 0.12	2.32 ± 0.11
18:1 *n*-9	1.48 ± 0.06	6.28 ± 1.60	6.96 ± 2.05	6.14 ± 1.21	6.14 ± 1.80	5.22 ± 1.40	7.80 ± 2.73	1.51 ± 0.05
18:1 *n*-7	1.97 ± 0.05	3.78 ± 0.79	4.29 ± 0.75	4.03 ± 0.47	3.93 ± 0.82	3.55 ± 0.63	4.71 ± 1.07	2.32 ± 0.09
20:1 *n*-13	1.31 ± 0.11	1.52 ± 0.30	1.71 ± 0.04	1.88 ± 0.16	1.75 ± 0.13	1.76 ± 0.27	1.74 ± 0.22	1.19 ± 0.06
20:1 *n*-11	0.91 ± 0.01	2.76 ± 0.70	3.01 ± 0.66	2.80 ± 0.41	2.98 ± 0.74	2.59 ± 0.70	3.43 ± 0.79	0.90 ± 0.04
20:1 *n*-9	0.75 ± 0.02	0.83 ± 0.06	0.85 ± 0.05	0.86 ± 0.04	0.84 ± 0.04	0.81 ± 0.03	0.85 ± 0.05	0.72 ± 0.01
22:1 *n*-11	ND	1.87 ± 0.33	1.55 ± 0.25	1.63 ± 0.26	1.54 ± 0.29	1.89 ± 0.41	1.51 ± 0.39	ND
∑MUFA	**9.66 ± 0.25**	**21.24 ± 4.04**	**23.14 ± 4.26**	**21.61 ± 2.79**	**21.59 ± 4.03**	**19.54 ± 3.14**	**24.90 ± 5.76**	**10.12 ± 0.24**
18:2 *n*-6 (LA)	0.70 ± 0.03	5.96 ± 1.56	6.56 ± 2.02	5.98 ± 1.10	5.28 ± 1.55	4.41 ± 1.37	6.84 ± 2.71	0.71 ± 0.04
18:3 *n*-3 (ALA)	1.04± 0.06	1.50 ± 0.27	1.62 ± 0.28	1.62 ± 0.20	1.53 ± 0.18	1.42 ± 0.18	1.73 ± 0.35	1.06 ± 0.03
$^{\Delta 5,11}$ 20:2	ND	1.48 ± 0.13	1.71 ± 0.18	1.56 ± 0.11	1.75 ± 0.22	1.54 ± 0.21	1.88 ± 0.25	ND
20:2 *n*-6	1.04 ± 0.05	4.54 ± 1.59	5.30 ± 1.00	5.58 ± 0.62	4.94 ± 1.09	4.66 ± 1.39	6.02 ± 1.32	1.01 ± 0.07
20:3 *n*-6 (DGLA)	ND	1.46 ± 0.30	1.25 ± 0.11	1.38 ± 0.11	1.24 ± 0.11	1.36 ± 0.18	1.22 ± 0.25	ND
20:3 *n*-3 (ETE)	ND	0.91 ± 0.05	0.94 ± 0.03	0.97 ± 0.04	0.94 ± 0.03	0.93 ± 0.05	0.95 ± 0.03	ND
$^{\Delta 7,13}$ 22:2	1.12 ± 0.05	1.70 ± 0.29	1.89 ± 0.09	2.11 ± 0.08	1.97 ± 0.15	1.92 ± 0.26	2.03 ± 0.15	1.08 ± 0.03
$^{\Delta 7,13,16}$ 22:3	ND	1.27 ± 0.14	1.42 ± 0.07	1.54 ± 0.10	1.44 ± 0.15	1.41 ± 0.18	1.60 ± 0.17	ND
∑PUFA	**3.89 ± 0.09**	**18.82 ± 3.92**	**20.69 ± 3.51**	**20.74 ± 2.19**	**19.09 ± 2.91**	**17.65 ± 3.12**	**22.26 ± 4.97**	**3.86 ± 0.13**
20:4 *n*-6 (ARA)	1.77 ± 0.07	2.06 ± 0.59	2.18 ± 0.27	2.06 ± 0.20	2.18 ± 0.34	1.98 ± 0.31	2.19 ± 0.68	1.93 ± 0.26
20:4 *n*-3 (ETA)	ND	0.99 ± 0.06	1.00 ± 0.05	1.04 ± 0.07	0.97 ± 0.05	0.97 ± 0.03	1.09 ± 0.12	ND
20:5 *n*-3 (EPA)	3.31 ± 0.13	7.80 ± 2.04	8.93 ± 1.28	9.15 ± 0.89	8.17 ± 1.01	7.73 ± 1.08	8.54 ± 1.78	3.58 ± 0.35
22:4 *n*-6 (AdA)	1.39 ± 0.07	1.90 ± 0.55	2.00 ± 0.38	2.19 ± 0.29	2.23 ± 0.31	2.08 ± 0.40	2.18 ± 0.56	1.42 ± 0.16
22:5 *n*-3 (DPA)	1.34 ± 0.01	1.74 ± 0.33	1.96 ± 0.24	2.11 ± 0.14	2.01 ± 0.20	1.96 ± 0.35	2.19 ± 0.26	1.37 ± 0.11
22:6 *n*-3 (DHA)	ND	3.89 ± 0.87	4.23 ± 1.33	3.98 ± 0.64	3.40 ± 0.82	3.42 ± 0.78	4.15 ± 1.36	ND
∑HUFA	**7.81 ± 0.16**	**18.37 ± 4.34**	**20.30 ± 3.21**	**20.54 ± 1.56**	**18.96 ± 2.30**	**18.15 ± 2.42**	**20.34 ± 4.38**	**8.29 ± 0.84**
Total FA	**28.35 ± 0.50**	**78.54 ± 15.15**	**85.09 ± 14.30**	**81.71 ± 7.83**	**79.76 ± 13.56**	**72.68 ± 10.14**	**89.06 ± 19.39**	**32.87 ± 0.59**

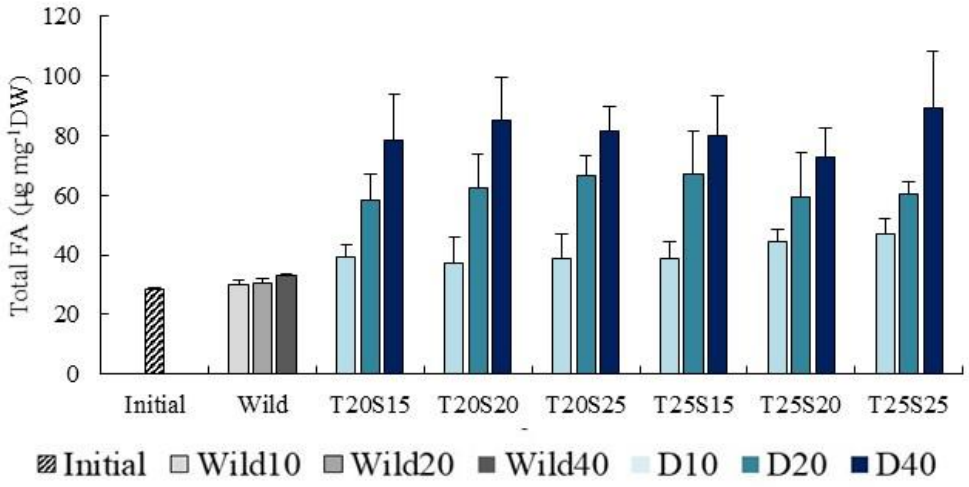


Figure 4.3. Evolution of the total amount of fatty acids (FA) of *Hediste diversicolor* fed a commercial aquafeed for 10, 20 and 40 days (D10, D20 and D40) under different combinations of water temperature (T – 20 and 25 ºC) and salinity (S –15, 20 and 25) contrasted with that of initially stocked conspecifics (Initial) and conspecifics collected from the wild at the same time points (Wild10, Wild20 and Wild40). Average values (± SD) (n = 5).

The *n*-3 FA content in D40 polychaetes was approximately 3-times higher than that of Wild40 (values ranging between 16 and 19 µg mg^{-1} DW). The highest increment in *n*-3 FA was recorded between day 20 and 40 (comparing the values of Initial, D10, D20 and D40 polychaetes) (Fig. 4.4A). The increment in *n*-6 FA content was progressive over the whole experimental period, with D40 polychaetes revealing a 2 to 3-times higher concentration than Wild40 (values between 13 - 17 µg mg^{-1} DW) (Fig. 4.4B). A lower value of *n*-3/*n*-6 ratio was reported in D20 polychaetes (0.7 - 0.9), when compared to the ones observed in other sampling periods and wild conspecifics (1.0 - 1.2) (Figure 4.4C). DHA was not detected in Initial polychaetes, while values between 3.4 - 4.2 µg mg^{-1} DW were recorded for D40 specimens (Figure 4.4D). EPA concentration in D40 polychaetes was twice the one recorded on Initial ragworms, with values ranging between 7 and 9 µg mg^{-1} DW (Fig. 4.4E). Both DHA and EPA experienced the highest increments between day 20 and 40 of the experimental trial. The DHA/EPA ratio was maintained over the sampling periods, with values averaging between 0.3 and 0.6 (Fig. 4.4F). Palmitic, oleic, linoleic, eicosadienoic, EPA and DHA were the FA which exhibited the highest increment in concentration when comparing Initial polychaetes with conspecifics from D40 (Fig. 4.5). The increment in concentration of the first three above mentioned FA (palmitic, oleic and linoleic) mainly occurred between D1 – D20 period, while the other FA (eicosadienoic, EPA and DHA) showed the highest increment between D21 – D40 period. From the above-mentioned FA, DHA was the only one that was not detected in wild polychaetes, while eicosadienoic was the only one that was not detected in the commercial aquafeed supplied. Dihomo-gamma-linolenic (20:3 *n*-6, DGLA), eicosatrienoic (20:3 *n*-3, ETE) and eicosatetraenoic (20:4 *n*-3, ETA) acids were only detected in D40 polychaetes and were not detected neither on Initial or Wild samples of polychaetes, nor in the aquafeed supplied. Significant differences were detected between the FA profile exhibited by D10, D20 and D40 polychaetes and the ones collected from the wild on the same periods ($p < 0.05$ – Supplementary Table S4.7).

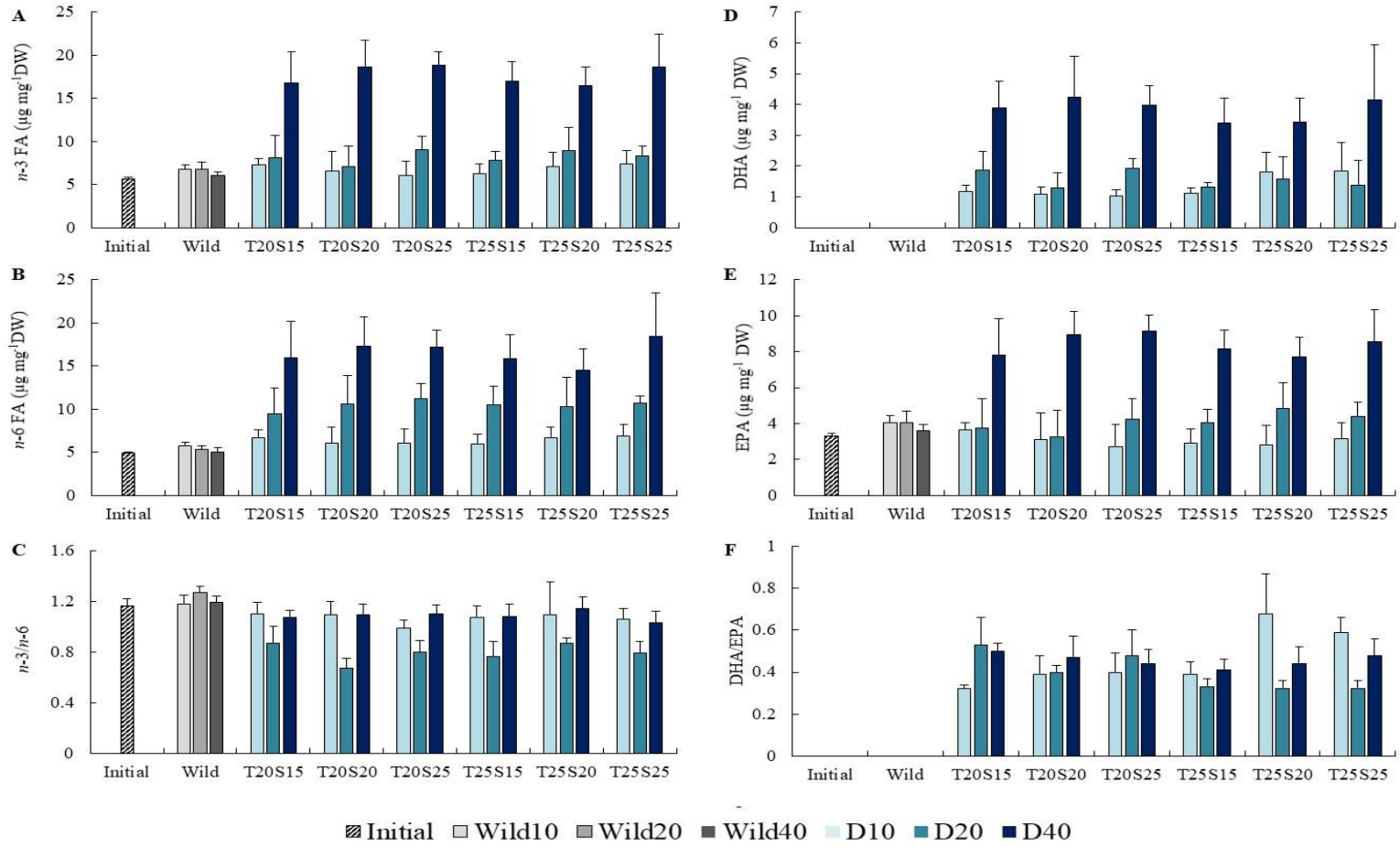


Figure 4.4. Evolution of *n*-3 fatty acids (FA) (A), *n*-6 FA (B), *n*-3/*n*-6 ratio (C), docosahexaenoic acid (DHA) (D), eicosapentaenoic acid (EPA) (E) and DHA/EPA ratio (F) of *Hediste diversicolor* fed a commercial aquafeed for 10, 20 and 40 days (D10, D20 and D40) under different combinations of water temperature (T – 20 and 25 ºC) and salinity (S –15, 20 and 25) contrasted with that of initially stocked conspecifics (Initial) and conspecifics collected from the wild at the same time points (Wild10, Wild20 and Wild40). Average values (± SD) (n = 5).

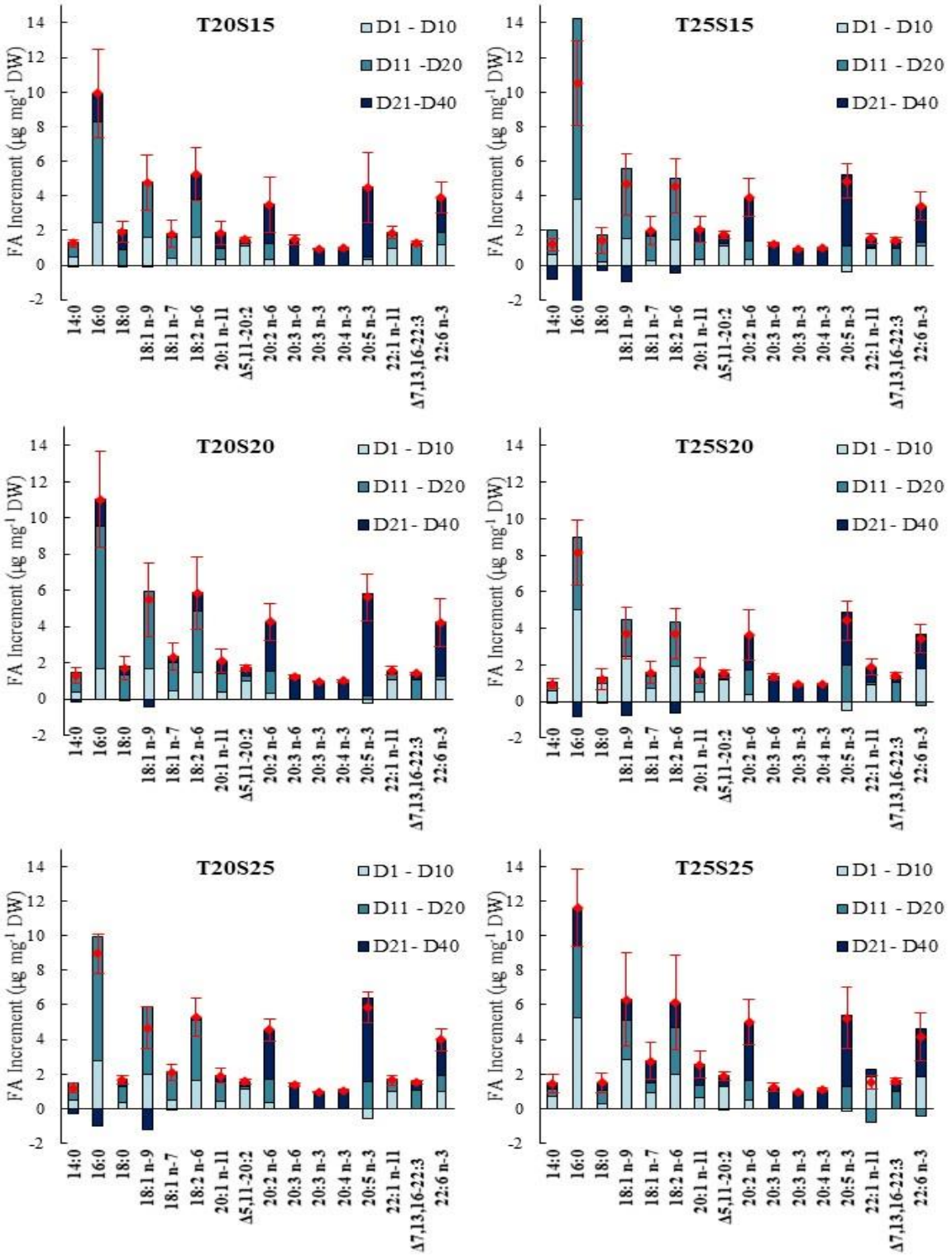


Figure 4.5. Evolution of the fatty acids (FA) in *Hediste diversicolor* fed a commercial aquafeed over time (D1 - D10, D11 - D20 and D21 - D40) under different combinations of water temperature (T – 20 and 25 ºC) and salinity (S –15, 20 and 25). Red diamond represents the final average increment recorded (± SD) (n = 5).

SIMPER analysis (cut-off 50%) revealed the existence of dissimilarities between D10 – Wild10 (15 - 20%), D20 -Wild20 (24 - 27%) and D40 -Wild40 (29 - 32%) (Supplementary Tables S4.8 – S4.10). DHA (22:6 *n*-3), LA (18:2 *n*-6), eicosadienoic (20:2 *n*-6), oleic (18:1 *n*-9), 5,11-eicosadienoate ($^{\Delta 5,11}$20:2), 11-docosenoate acid (22:1 *n*-11), DGLA (20:3 *n*-6) and 7,13,16-docosatrienoate ($^{\Delta 7,13,16}$22:3) are some of the FA that contributed the most to dissimilarities recorded between D40 and Wild40 (Supplementary Table S4.10).

PCO plots revealed two well-separated groups, one formed by D40 polychaetes and -aquafeed, and the other one formed by Initial and Wild40 polychaetes (Fig. 4.6 A-C). A similarity above 90% was recorded between D40 polychaetes and aquafeed for total FA concentration, FA classes concentration (SFA, MUFA, PUFA and HUFA) and *n*-3 and *n*-6 FA concentration. The two PCO axis explained more than 99% of the variation recorded between samples from different group.

The amount of commercial aquafeed supplied to polychaetes stocked under the different treatments performed allowed to estimate an increment of 25.0 - 37.0 µg mg^{-1} DW in the total FA present in polychaetes biomass. Concerning *n*-3 and *n*-6 FA, the increments recorded were 5.6 - 8.0 and 4.8 - 7.7 µg mg^{-1} DW in polychaetes biomass, respectively (Table 4.4). DHA and EPA increased between 2.0 - 2.6 and 2.3 - 3.2 µg mg^{-1} DW in polychaetes biomass, respectively.

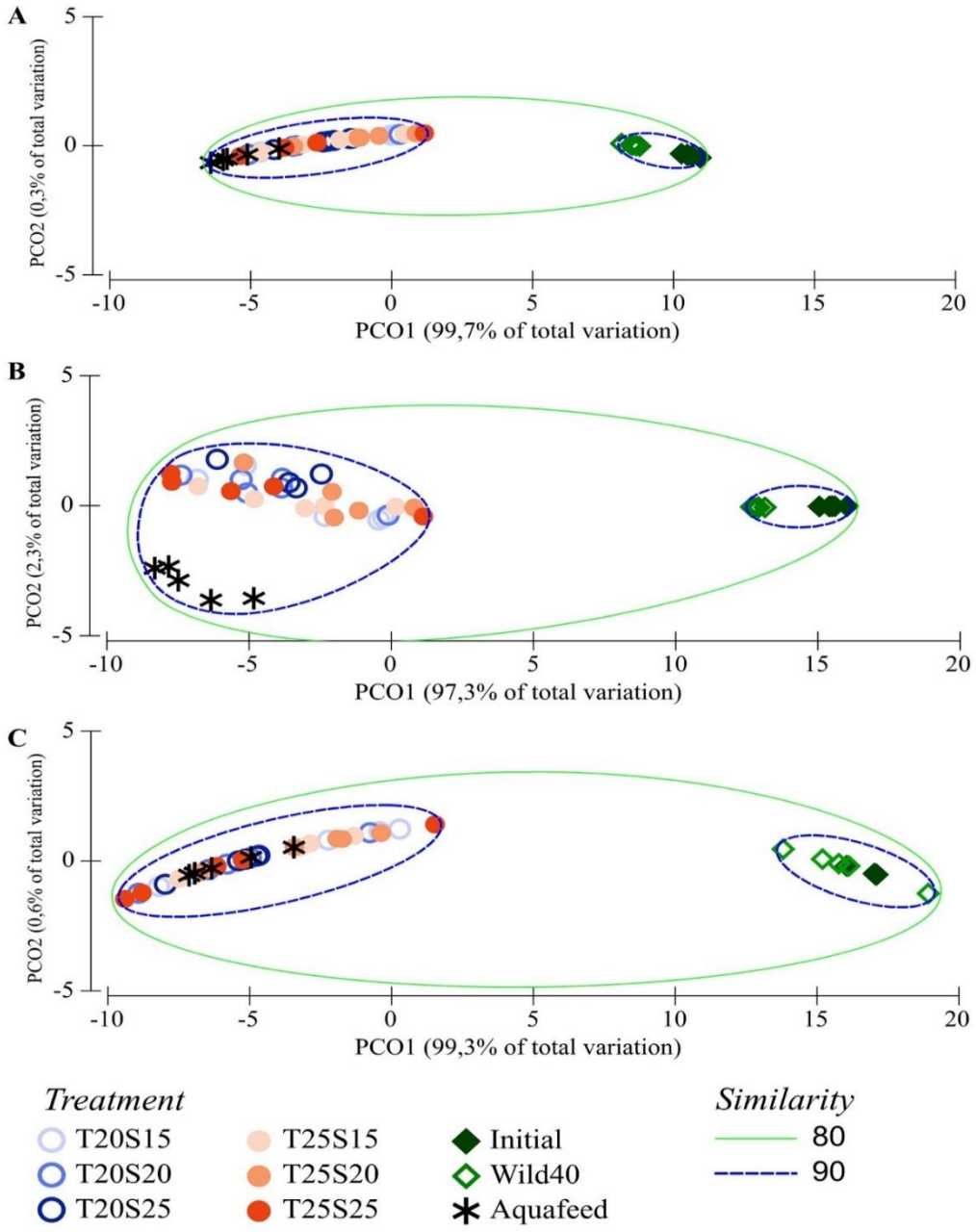


Figure 4.6. Principal coordinates analysis (PCO) of total fatty acids (FA) (A), sum of FA classes (SFA, MUFA, PUFA and HUFA) (B), sum of *n*-3 and *n*-6 FA (C) of *Hediste diversicolor* fed a commercial aquafeed for 40 days under different combinations of water temperature (T – 20 and 25 ºC) and salinity (S – 15, 20 and 25) contrasted with that wild conspecifics initially stocked (Initial) and conspecifics collected on the same date (Wild40). Average values (± SD) (n = 5). Green and blue lines define the levels of similarity between samples. PUFA are defined as all FA with 2 or 3 double bonds and HUFA as all FA with ≥ 4 double bonds.

Table 4.4. Increment in polychaetes biomass (µg mg^{-1} DW) per gram of aquafeed supplied of total fatty acid, sum of *n*-3 (Σ *n*-3) and *n*-6 fatty acids (Σ *n*-6), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Polychaetes were fed during 40 days under different combinations of temperature (T – 20 and 25 ºC) and salinity (S – 15, 20 and 25). Average values (± SD) (n = 5).

	Increment in polychaetes biomass per gram of aquafeed supplied (µg mg^{-1} DW)					
Fatty acids	T20S15	T20S20	T20S25	T25S15	T25S20	T25S25
Total	25.35 ± 8.24	27.44 ± 3.84	25.98 ± 3.98	30.23 ± 10.94	26.98 ± 7.68	37.76 ± 12.97
Σ *n*-3	5.63 ± 1.89	6.32 ± 0.80	6.42 ± 0.67	6.67 ± 2.16	6.53 ± 1.66	8.04 ± 2.40
Σ *n*-6	4.84 ± 2.12	5.36 ± 0.94	5.32 ± 0.93	5.71 ± 1.98	5.03 ± 1.77	7.68 ± 3.19
DHA	1.97 ± 0.51	2.03 ± 0.39	1.94 ± 0.29	2.01 ± 0.73	2.09 ± 0.61	2.57 ± 0.83
EPA	2.27 ± 1.05	2.75 ± 0.39	2.84 ± 0.36	2.88 ± 1.00	2.68 ± 0.74	3.24 ± 1.10

4.1.4. Discussion and conclusions

In the present study it was possible to verify that *H. diversicolor* fed a commercial aquafeed during 10, 20 and 40 days (D10, D20 and D40) under different combinations of water temperature (20 and 25ºC) and salinity (15, 20 and 25) displayed similar SGR and DGR. This finding is in line with the ecophysiological traits of this species, which is very abundant in extreme environments, such as intertidal mudflats, exposed to major shifts in water temperature and salinity[10,25]. The values of SGR reported in the present work were lower than those reported by previous studies testing commercial aquafeeds to culture this species (values ranging between 6.0 and 6.8% d^{-1})[31,35]. However, our values were higher than most of those reported when this species was fed on nutrients derived from aquaculture effluents (values ranging between 1.2 and 3.0% d^{-1})[29,36,39]. The values of DGR determined for D10 polychaetes were similar to those reported by Santos *et al.* (2016)[31] for the same species fed a commercial aquafeed for 60 days. The progressive decrease of DGR over time and the lower SGR recorded for D40 polychaetes, may be related with their sexual maturation, as detailed below. It is worth highlighting that D40 polychaetes incremented their average weight up to 4-times, confirming the potential of this species to be produced under an IMTA framework and to generate a considerable biomass in a short period of time. The progressive mortality verified in D10, D20 and D40 samples, which reached values ~50%, may have been related to the high stocking density employed at the beginning of the

experiment (1000 ind. m^{-2}). This assumption is supported by the results reported by Nesto *et al.* (2012)[40], who verified that *H. diversicolor* exhibited a higher growth and survival when cultured at a density of 300 ind. m^{-2} than at 1000 and 3000 ind. m^{-2}.

Total FA reported for D40 polychaetes in the present work are among the highest values recorded to date for *H. diversicolor* (see Table 4.5). Geographic location, abiotic conditions, diet availability and composition, and maturation state contribute to the shaping of FA profiles in this species[10,31,34,41]. Therefore, the interpretation of contrasting FA profiles reported in different studies is a challenging task. Initially stocked polychaetes, as well as wild conspecifics Wild10, Wild20 and Wild40, were collected during February-March, a transition period between winter and spring in the sampling area. Wild specimens exhibited a reddish-brown coloration (Fig. 4.7A), a characteristic feature of immature worms[25]. Two spawning periods, one in spring and the other in early autumn, were identified in wild populations of *H. diversicolor* monitored at the same sampling site of the specimens used in the present study[42]. According to Luis and Passos (1995)[41], during winter and early spring months, in the presence of a suitable diet, sexually immature worms accumulate lipids in their body. Immediately before reproduction, a bulk of FA is made available through triacylglycerol depletion, being these essential during the rapid growth of oocytes[41]. This polychaete species is therefore well-known to reflect in its body composition the FA profile of its diet[29,31,34–36]. This feature, along with maturation, are likely among the factors which most contributed for the differences in FA composition reported in previous studies testing the production of *H. diversicolor* using commercial aquafeeds or wasted nutrients derived from aquaculture effluents. In the present work it was possible to monitor the effect of diet over time (by comparing Initial, D10, D20 and D40 samples). The FA which exhibited higher increments in polychaete biomass were exactly the ones presented in higher levels in the commercial aquafeed employed. With only a few exceptions, these FA are also among the most well-represented ones reported in previous studies addressing the culture of this species[10,29–31,33–36].

Table 4.5. Summary of the fatty acid (FA) profile of *Hediste diversicolor* reported from previous studies providing this species with a commercial aquafeed or wasted nutrients derived from aquaculture effluents. Values in % of total FA methyl esters (FAME). Abbreviations: SFA - saturated FA; MUFA - monounsaturated FA; PUFA - polyunsaturated FA; HUFA - highly unsaturated FA. PUFA are defined as all FA with 2 or 3 double bonds and HUFA as all FA with ≥ 4 double bonds. NM – Value not mentioned in work.

	Commercial aquafeed						Wasted nutrients derived from aquaculture effluents (faeces and uneaten feed) (IMTA)					
	Present study (D40 samples)	Wang *et al.* 2019	Santos *et al.* 2016	Pajand *et al.* 2017	García-Alonso *et al.* 2008	Fidalgo e Costa *et al.* 2000	Yousefi-Garakouei *et al.* 2019	Wang *et al.* 2019	Marques *et al.* 2018	Pajand *et al.* 2017	Bischoff *et al.* 2009	García-Alonso *et al.* 2008
Duration (days)	40	30	60	56	50	60	60	30	150	56	500 - 600	50
Nº FA identified	24	20	17	19	28	NM	16	20	19	19	14	29
SFA (%)	23 - 26	27.8	24.2 - 28.4	23	36.2	22.4	33.9 - 34.4	29.5	22.6 - 24.2	24.4	33	34.5
MUFA (%)	27 - 28	25.3	18.9 - 19.9	38.9	28.4	38.9	24.8 - 25	25.4	26.9 - 30.3	39.3	21	26.6
PUFA (%)	22 - 24	14.2	14	35.5	18.5	10.9	33.6 - 34.8	14.0	7.0 - 10.6	31.6	11	11.9
HUFA (%)	23 - 26	32.7	26.9 - 30.3	2.5	16.9	27.8	5.8 - 7.7	31.2	38.1 - 39.9	4.6	35	27.0
n-3 (%)	21 - 24	32.9	23.4 - 26.8	5	14.9	24.8	14.3 - 15.8	30.5	27.5 - 30.8	6	32	25.1
n-6 (%)	18 - 20	14.0	8.9 - 12.1	30	15.0	12.4	25.4 - 26.3	14.6	13.6 - 15.8	26.7	14	9.2
EPA (%)	10 - 11	19.0	14.8 - 17.4	1.6	10.1	10.7	4.0 - 5.6	19.1	22.4 - 23.8	3.1	24	18.7
DHA (%)	4.3 - 5	7.8	6.7	0.8	3.0	6.7	1.7 - 2.1	5.4	1.8 - 2.5	1.5	5	4.9
Total FA ($\mu g\ mg^{-1}$ DW)	71 - 88	73.7	NM	276.3	52.1	72.7	NM	56.9	24 - 37	109.9	27.1	29.6

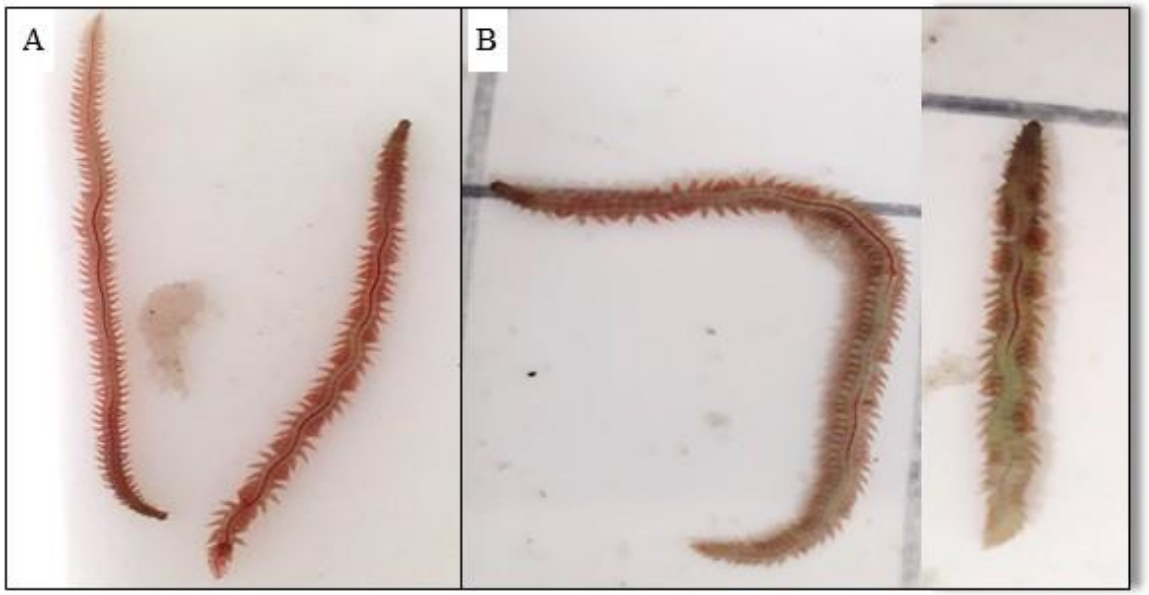


Figure 4.7. *Hediste diversicolor* at the beginning of the experiment (A) and after being fed on a commercial aquafeed for 40 days under different combinations of water temperature (20 and 25 ºC) and salinity (15, 20 and 25) (B).

An advantage of the present study in relation to previous works is that it monitored the evolution of FA profile of ragworms over time. This issue is of paramount importance if producers aim to enhance the amount of EFA of cultured ragworms by using enriched diets for a short period of time at the end of the production cycle to obtain premium polychaetes. In addition, producers may also select to enrich wild polychaetes biomass, rather than culturing these organisms over their whole life cycle. A progressive increment of total FA was recorded over time. In the first 20 days (comparing Initial, D10 and D20 polychaetes), a predominant increment of SFA and MUFA was evidenced, while in the last 20 days of the trial (comparing D20 and D40 polychaetes) an increment of PUFA and HUFA prevailed. These results may well reflect an adaptation of polychaetes to the new diet being provided and/or the experimental conditions employed, latter followed by metabolic shifts triggered by maturation. According to Nesto *et al.* (2012)[40], diets similar to the one used in the present work, with high-protein content, despite originating the best results of daily biomass production, also triggered an earlier onset of gametogenesis. Despite the increments recorded, it is worth highlighting that a plateau of total FA, *n*-6 FA and *n*-3 FA was not reached during the 40 days of the experimental trials. This finding allows us to infer that it may still be possible to further enhance *H. diversicolor* biomass in EFA if the study had been extended over a longer period. Nonetheless, one must acknowledge that when this species reaches adult size (similar to that recorded at D40 polychaetes) it starts to reproduce and die.

A comparative analysis of the FA profile of Initial, D10, D20 and D40 polychaetes also allowed to detect evidence suggesting *de novo* biosynthesis. A good example of this *de novo* biosynthesis is the 10 - 20% increment recorded in eicosadienoic (20:2 *n*-6), a FA present in Initial polychaetes but absent in the commercial aquafeed supplied. Other FA that were only detected in cultured polychaetes, being absent in initially stocked polychaetes and aquafeed, were 5,11-eicosadienoate ($^{\Delta 5,11}$20:2) (detected in D10, D20 and D40 polychaetes), 7,13,16-docosatrienoate ($^{\Delta 7,13,16}$22:3) (detected in D20 and D40 polychaetes), and DGLA (20:3 *n*-6), ETE (20:3 *n*-3) and ETA (20:4 *n*-3) (only detected in D40 polychaetes). In *de novo* FA biosynthesis, three major enzymes are involved: namely methyl-end-desaturases (ω6 - Δ12 and ω3 - Δ15, Δ17, Δ19), front-end desaturases (Δ4, Δ5, Δ6, Δ8) and elongases (Elo); their pivotal roles in PUFA and HUFA biosynthesis in aquatic invertebrates have been reviewed by Monroig and Kabeya (2018)[3] and are summarised in Figure 4.8. The detection of DGLA (20:3 *n*-6), ETE (20:3 *n*-3) and ETA (20:4 *n*-3) only in D40 polychaetes may have resulted from the activation of the respective alternative biosynthesis pathways (see Fig. 4.8). Here it is important to clarify that gamma-linolenic (18:3 *n*-6) and stearidonic acid (18:4 *n*-3) from the main biosynthesis pathway were not detected. The fact that DGLA (20:3 *n*-6), ETE (20:3 *n*-3) and ETA (20:4 *n*-3) were only detected in D40 polychaetes, along with the fact that its precursors (LA (18:2 *n*-6) and ALA (18:3 *n*-3)) were already present in the initially stocked polychaetes and in the supplied aquafeed, may result from the activation of these biosynthesis pathways due to maturation. Indeed, at the end of the experimental period some polychaetes exhibited a bright grass-green colour (see Fig. 4.7B), a common feature of mature males[25,43]. To the authors best knowledge, this is the first time that the biosynthesis pathways mentioned above are suggested to occur in this species. In recent years, several studies have highlighted *de novo* biosynthesis in annelids, and for example the action of methyl-end desaturases has been demonstrated in studies addressing *Alitta virens* (a polychaete species of family Nereididae as *H. diversicolor*), which was able to perform *de novo* biosynthesis of ARA (20:4 *n*-6) and EPA (20:5 *n*-3) when supplied with an aquafeed supplemented with 13C-labeled palmitic acid (16:0)[44]. According to Monroing and Kabeya (2018)[3], a comprehensive description of front-end-desaturases and elongase genes is still missing for annelids. However, there is enough evidence to state that these biosynthetic pathways exist in annelids, namely *Ridge piscesae* and *Protis hydrothermica*[45], *Arenicola marina*[46], *A. virens*[44] and *Perinereis aibuhitensis*[47].

The present study demonstrated how *H. diversicolor* biomass can be successfully enriched with *n*-6 and *n*-3 FA (including EPA and DHA) when provided a commercial aquafeed, even when exposed to different combinations of water temperature and salinity. This finding highlights the potential of using *H. diversicolor* as an extractive species in IMTA designs, thus allowing to recover valuable nutrients present in aquaculture effluents that would otherwise be wasted. The production of a ragworm biomass rich in EFA allows to provide the aquafeed industry with another alternative ingredient for fish meal and fish oil, at least for the formulation of premium maturation and finishing diets. The development of innovative aquaculture production models based on the integration of extractive species, such as polychaetes, to recover wasted nutrients derived from the aquaculture of fed species complies with sustainable aquaculture guidelines and is aligned with UN 2030 Sustainable Development Goals Agenda.

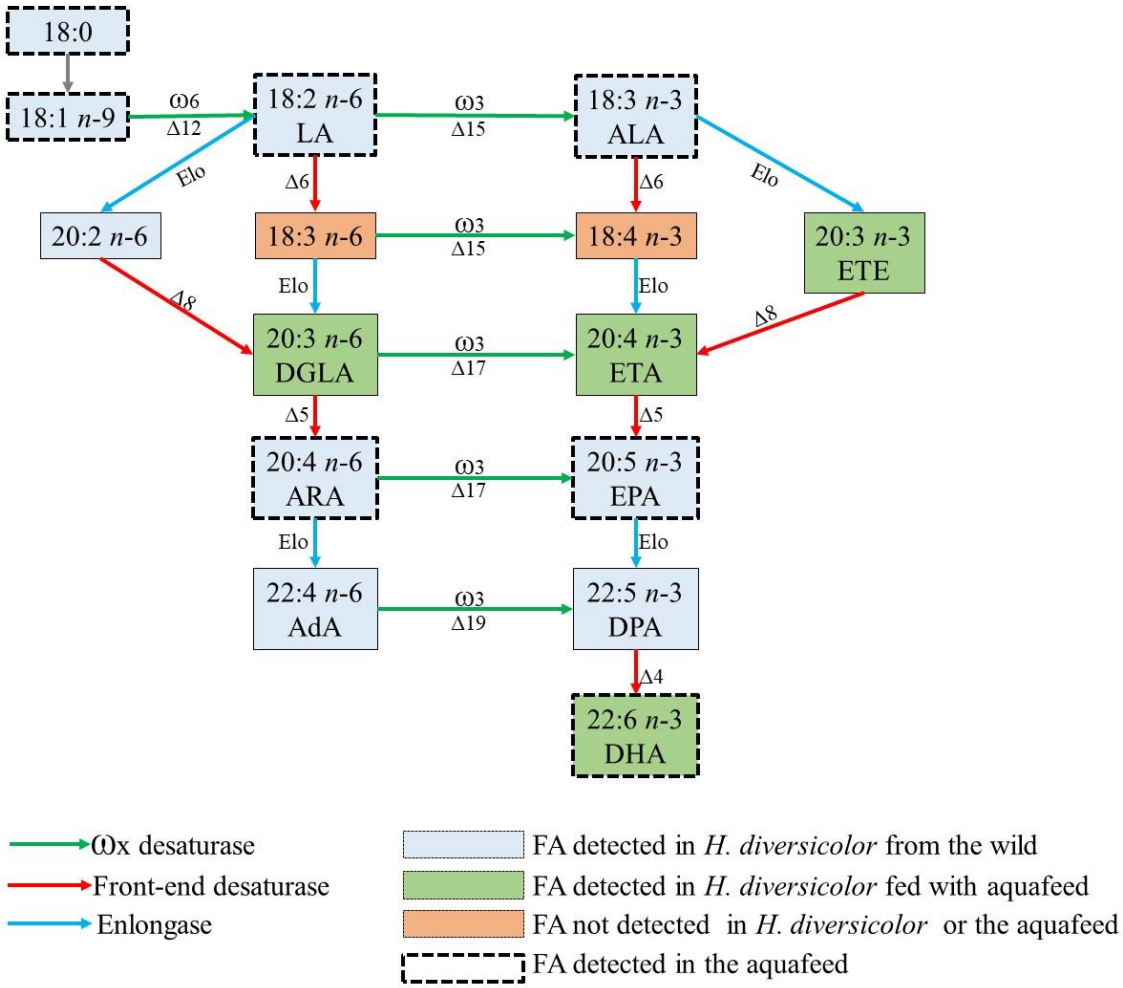


Figure 4.8. Schematic representation of putative biosynthetic pathways of some polyunsaturated fatty acids (PUFA) identified in *Hediste diversicolor* from the wild and after being fed on a commercial aquafeed for 40 days. Methyl-end desaturases (ω, green arrows); Front-end desaturases (red arrows); Elongation reactions (Elo, blue arrows). The "Δ" refers to the carbon position at which the incipient double bound (unsaturation) locates within the methyl and front ends of fatty acyl chains. AdA adrenic acid; ALA α-linolenic acid; ARA arachidonic acid; DGLA dihomo-gamma-linolenic acid; DHA docosahexaenoic acid; DPA Docosapentaenoic acid; EPA eicosapentaenoic acid; ETA eicosatetraenoic acid; ETE eicosatrienoic acid; LA linoleic acid. Scheme adapted from Monroig and Kabeya, 2018.

4.1.5. Chapter 4 - References

1. FAO. The state of world fisheries and aquaculture: Sustainability in action. ROME (2020).
2. Bell, M. V. & Tocher, D. R. Lipids in Aquatic Ecosystems. in Lipids in Aquatic Ecosystems (eds. Arts, M. T., Brett, M. T. & Kainz, M. J.) 211–237 (Springer, 2009).
3. Monroig, Ó. & Kabeya, N. Desaturases and elongases involved in polyunsaturated fatty acid biosynthesis in aquatic invertebrates: a comprehensive review. *Fish. Sci.* **84**, 911–928 (2018).
4. Sargent, J. R., Tocher, D. R. & Bell, J. G. 4 - The Lipids. in Fish Nutrition (Third Edition) (eds. Halver, J. E. & Hardy, R. W.) 181–257 (Academic Press, 2003).
5. Tocher, D. R. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture* **449**, 94–107 (2015).
6. Tocher, D. R. Metabolism and functions of lipids and fatty acids in teleost fish. *Rev. Fish. Sci.* **11**, 107–184 (2003).
7. Cashion, T., Le Manach, F., Zeller, D. & Pauly, D. Most fish destined for fishmeal production are food-grade fish. *Fish Fish.* **18**, 837–844 (2017).
8. Tacon, A. G. J. & Metian, M. Feed matters: Satisfying the feed demand of aquaculture. *Rev. Fish. Sci. Aquac.* **23**, 1–10 (2015).
9. Tacon, A. G. J. & Metian, M. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture* **285**, 146–158 (2008).
10. García-Alonso, J., Hardege, J. D. & Müller, C. T. Influence of food regimes and seasonality on fatty acid composition in the ragworm. *Aquat. Biol.* **4**, 7–13 (2008).
11. Olive, P. J. W. Polychaete aquaculture and polychaete science: A mutual synergism. *Hydrobiologia* **402**, 175–183 (1999).
12. Watson, G. J., Murray, J. M., Schaefer, M. & Bonner, A. Bait worms: a valuable and important fishery with implications for fisheries and conservation management. *Fish Fish.* **18**, 374–388 (2016).
13. Fidalgo E. Costa, P. *et al.* The market features of imported non-indigenous polychaetes in Portugal and consequent ecological concerns. *Sci. Mar.* **70**, 287–292 (2006).
14. Pombo, A. *et al.* Insight into aquaculture's potential of marine annelid worms and ecological concerns: a review. *Rev. Aquac.* 1–15 (2018).
15. Xenarios, S., Queiroga, H., Lillebø, A. I. & Aleixo, A. Introducing a regulatory policy framework of bait fishing in European coastal lagoons: The case of Ria de Aveiro in Portugal. *Fishes* **3**, 1–19 (2018).
16. Carvalho, A. N., Vaz, A. S. L., Sérgio, T. I. B. & Santos, P. J. T. Sustainability of bait fishing harvesting in estuarine ecosystems – Case study in the Local Natural Reserve of Douro Estuary, Portugal. *J. Integr. Coast. Zo. Manag.* **13**, 157–168 (2013).
17. Sá, E. *et al.* Trade of live bait in Portugal and risks of introduction of non-indigenous species associated to importation. *Ocean Coast. Manag.* **146**, 121–128 (2017).

18. Dinis, M. T. Aspects of the potential of *Solea senegalensis* Kaup for aquaculture: larval rearing and weaning to an artificial diet. *Aquac. Res.* **23**, 515–520 (1992).

19. Dinis, M. T., Ribeiro, L., Soares, F. & Sarasquete, C. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture* **176**, 27–38 (1999).

20. Luis, O. J. & Ponte, A. C. Control of Reproduction of the Shrimp *Penaeus kerathurus* Held in Captivity. *J. World Aquac. Soc.* **24**, 31–39 (1993).

21. Lytle, J. S., Lytle, T. F. & Ogle, J. T. Polyunsaturated fatty acid profiles as a comparative tool in assessing maturation diets of *Penaeus vannamei*. *Aquaculture* **89**, 287–299 (1990).

22. Meunpol, O., Meejing, P. & Piyatiratitivorakul, S. Maturation diet based on fatty acid content for male *Penaeus monodon* (Fabricius) broodstock. *Aquac. Res.* **36**, 1216–1225 (2005).

23. Fauchald, K. & Jumars, P. A. The diet of worms: a study of polychaete feeding guilds. *Oceanogr. Mar. Biol. An Annu. Rev.* **17**, 193–284 (1979).

24. Reise, K. Spatial configurations generated by motile benthic polychaetes. *Helgoländer Wiss. Meeresunters.* **32**, 55–72 (1979).

25. Scaps, P. A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O. F. Müller) (Annelida:Polychaeta). *Hydrobiologia* **470**, 203–218 (2002).

26. Riisgård, H. U. Filter-feeding in the polychaete *Nereis diversicolor*: a review. *Netherlands J. Aquat. Ecol.* **28**, 453–458 (1994).

27. Vedel, A., Andersen, B. B. & Riisgard, H. U. Field investigations of pumping activity off the facultatively filter-feeding polychaete *Nereis diversicolor* using an improved infrared phototransducer system. *Mar. Ecol. Prog. Ser.* **103**, 91–102 (1994).

28. Velez, Z., Hubbard, P. C., Hardege, J. D., Barata, E. N. & Canário, A. V. M. The contribution of amino acids to the odour of a prey species in the Senegalese sole (*Solea senegalensis*). *Aquaculture* **265**, 336–342 (2007).

29. Wang, H. *et al.* Growth and nutritional composition of the polychaete *Hediste diversicolor* (OF Müller, 1776) cultivated on waste from land-based salmon smolt aquaculture. *Aquaculture* **502**, 232–241 (2019).

30. Yousefi-Garakouei, M., Kamali, A. & Soltani, M. Effects of rearing density on growth, fatty acid profile and bioremediation ability of polychaete *Nereis diversicolor* in an integrated aquaculture system with rainbow trout (*Oncorhynchus mykiss*). *Aquac. Res.* **50**, 725–735 (2019).

31. Santos, A. *et al.* Effect of three diets on the growth and fatty acid profile of the common ragworm *Hediste diversicolor* (O.F. Müller, 1776). *Aquaculture* **465**, 37–42 (2016).

32. Vásquez, V., Krieg, M., Lockhead, D. & Goodman, M. B. Phospholipids that contain polyunsaturated fatty acids enhance neuronal cell mechanics and touch sensation. *Cell Rep.* **6**, 70–80 (2014).

33. Bischoff, A. A., Fink, P. & Waller, U. The fatty acid composition of *Nereis diversicolor* cultured in an integrated recirculated system: Possible implications for aquaculture. *Aquaculture* **296**, 271–276 (2009).

34. Marques, B. *et al.* Adding value to ragworms (*Hediste diversicolor*) through the bioremediation of a super-intensive marine fish farm. *Aquac. Environ. Interact.* **10**, 79–88 (2018).

35. Fidalgo e Costa, P., Narciso, L. & Cancela da Fonseca, L. Growth, survival and fatty acid profile of *Nereis diversicolor* (O. F. Muller, 1776) fed on six different diets. *Bull. Mar. Sci.* **67**, 337–343 (2000).

36. Pajand, Z. O., Soltani, M., Bahmani, M. & Kamali, A. The role of polychaete *Nereis diversicolor* in bioremediation of wastewater and its growth performance and fatty acid composition in an integrated culture system with *Huso huso* (Linnaeus , 1758). *Aquac. Res.* **48**, 5271–5279 (2017).

37. Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917 (1959).

38. Anderson, M. J., Gorley, R. N. & Clarke, K. R. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. Plymouth, UK (PRIMER.E_Ltd, 2008).

39. Bischoff, A. A. Solid waste reduction of closed recirculated aquaculture systems by secondary culture of detritivorous organisms. Christian-Albrechts-Universität Zu Kiel (2007).

40. Nesto, N., Simonini, R., Prevedelli, D. & Da Ros, L. Effects of diet and density on growth, survival and gametogenesis of *Hediste diversicolor* (O.F. Müller, 1776) (Nereididae, Polychaeta). *Aquaculture* **362–363**, 1–9 (2012).

41. Luis, O. J. & Passos, A. M. Seasonal changes in lipid content and composition of the polychaete *Nereis* (*Hediste*) *diversicolor*. *Comp. Biochem. Physiol.* **111B**, 579–586 (1995).

42. Abrantes, A., Pinto, F. & Moreira, M. H. Ecology of the polychaete *Nereis diversicolor* in the Canal de Mira (Ria de Aveiro, Portugal): Population dynamics, production and oogenic cycle. *Acta Oecologica* **20**, 267–283 (1999).

43. Dales, R. P. The reproduction and larval development of *Nereis diversicolor* O. F. Müller. *J. Mar. Biol. Assoc. United Kingdom* 29, 321–360 (1950).

44. Pairohakul, S. & Science, M. Evidence for Polyunsaturated Fatty Acid Biosynthesis in the Ragworm (*Nereis virens*) and the Lugworm (*Arenicola marina*). PhD Thesis. School of Marine Science and Technology - Newcastle University (2013).

45. Pond, D. W. *et al.* Origins of long-chain polyunsaturated fatty acids in the hydrothermal vent worms *Ridgea piscesae* and *Protis hydrothermica*. *Mar. Ecol. Prog. Ser.* **225**, 219–226 (2002).

46. Olive, P. J. W. *et al.* Net gain of long-chain polyunsaturated fatty acids (PUFA) in a lugworm *Arenicola marina* bioturbated mesocosm. *Mar. Ecol. Prog. Ser.* **387**, 223–239 (2009).

47. Lv, F. *et al.* The effects of five dietary lipid sources on growth, body composition and antioxidant parameters of the clamworm, *Perinereis aibuhitensis*. *Aquac. Res.* **48**, 5472–5480 (2017).

4.1.6. Chapter 4 - Supporting Information

Table S4.1. Dissolved oxygen (DO) (mg L^{-1}), pH, temperature (ºC), salinity, ammonia (NH_4) and nitrites (NO_2) monitored weekly on different treatments testing the combined effects of temperature (T – 20 and 25ºC) and salinity (S – 15, 20 and 25) in the fatty acid profile of *Hediste diversicolor* fed a commercial aquafeed during 10, 20 and 40 days (D10, D20 and D40). Average values (± SD) (n = 5). The values between brackets represented the maximum and minimum values.

Samples	Treatment	DO (mg L^{-1})	pH	Temp. (ºC)	Salinity	NH_4 (mg L^{-1})	NO_2 (mg L^{-1})
D10	T20S15	7.71 ± 0.39 (8.10 - 6.70)	8.37 ± 0.09 (8.45 - 8.08)	20.57 ± 0.76 (21.70 - 19.40)	14.87 ± 0.59 (15.60 - 13.80)	0.94 ± 0.54 (1.50 – 0.15)	0.52 ± 0.40 (1.00 – 0.10)
	T20S20	7.74 ± 0.31 (8.20 - 7.20)	8.40 ± 0.08 (8.53 - 8.26)	20.50 ± 0.83 (21.90 - 19.30)	19.92 ± 0.98 (21.00 - 18.20)	0.90 ± 0.59 (1.50 – 0.15)	0.81 ± 0.69 (2.00 – 0.10)
	T20S25	7.76 ± 0.25 (8.10 - 7.30)	8.34 ± 0.09 (8.46 - 8.12)	20.55 ± 0.75 (21.80 - 19.40)	24.89 ± 0.93 (26.00 - 23.40)	0.55 ± 0.38 (1.00 – 0.15)	1.02 ± 0.81 (2.00 – 0.10)
	T25S15	7.01 ± 0.18 (7.50 - 6.80)	8.32 ± 0.09 (8.46 - 8.16)	25.27 ± 0.78 (26.70 - 23.40)	15.59 ± 0.48 (16.40 - 14.80)	1.21 ± 0.51 (1.50 - 0.25)	0.87 ± 0.71 (2.00 – 0.10)
	T25S20	6.93 ± 0.20 (7.20 - 6.60)	8.39 ± 0.06 (8.49 - 8.31)	25.45 ± 0.82 (27.00 - 23.46)	20.55 ± 0.99 (21.60 - 18.00)	0.78 ± 0.80 (1.50 – 0.00)	0.68 ± 0.38 (1.00 – 0.10)
	T25S25	6.95 ± 0.24 (7.40 - 6.50)	8.34 ± 0.10 (8.50 - 8.12)	25.35 ± 0.78 (26.80 - 23.50)	26.01 ± 0.74 (27.20 - 24.90)	0.86 ± 0.70 (1.50 – 0.15)	0.85 ± 0.66 (2.00 – 0.10)
D20	T20S15	7.83 ± 0.29 (8.40 - 7.20)	8.36 ± 0.11 (8.59 - 8.14)	20.10 ± 0.79 (21.80 - 19.00)	15.05 ± 0.44 (15.60 - 13.60)	0.91 ± 0.56 (1.50 – 0.15)	1.21 ± 0.72 (2.00 – 0.50)
	T20S20	7.98 ± 0.30 (8.50 - 7.40)	8.39 ± 0.08 (8.52 - 8.16)	20.11 ± 0.80 (21.70 - 19.10)	19.87 ± 0.88 (21.40 - 17.60)	0.87 ± 0.52 (1.50 – 0.15)	1.23 ± 0.75 (2.00 – 0.10)
	T20S25	7.88 ± 0.28 (8.20 - 7.10)	8.40 ± 0.05 (8.50 - 8.28)	20.09 ± 0.81 (21.80 - 19.00)	24.86 ± 0.71 (25.90 - 23.40)	0.63 ± 0.54 (1.50 – 0.15)	0.81 ± 0.66 (2.00 – 0.10)
	T25S15	6.96 ± 0.28 (7.50 - 6.50)	8.28 ± 0.11 (8.46 - 7.99)	25.17 ± 0.80 (26.70 - 23.30)	15.48 ± 0.50 (16.30 - 14.10)	0.74 ± 0.65 (1.50 – 0.00)	1.05 ± 0.64 (2.00 – 0.10)
	T25S20	7.00 ± 0.31 (7.70 - 6.50)	8.27 ± 0.15 (8.49 - 7.91)	25.23 ± 0.81 (26.90 - 23.20)	20.48 ± 0.72 (21.60 - 18.80)	0.76 ± 0.67 (1.50 – 0.00)	1.13 ± 0.70 (2.00 – 0.10)
	T25S25	6.97 ± 0.33 (7.50 - 6.30)	8.28 ± 0.12 (8.46 - 8.05)	25.27 ± 0.80 (27.10 - 23.40)	25.64 ± 0.92 (27.70 - 23.90)	0.58 ± 0.53 (1.50 – 0.15)	1.09 ± 0.62 (2.00 – 0.10)
D40	T20S15	7.57 ± 0.48 (8.50 - 6.30)	8.23 ± 0.14 (8.49 - 7.95)	20.52 ± 0.91 (22.30 - 19.00)	15.16 ± 0.47 (15.60 - 13.30)	0.65 ± 0.57 (1.50 – 0.15)	1.11 ± 0.68 (2.00 – 0.25)
	T20S20	7.61 ± 0.44 (8.30 - 6.50)	8.35 ± 0.12 (8.52 - 7.97)	20.47 ± 0.92 (22.30 - 18.90)	20.19 ± 0.70 (21.20 - 18.20)	0.60 ± 0.53 (1.50 – 0.15)	1.40 ± 0.97 (2.00 – 0.10)
	T20S25	7.58 ± 0.45 (8.20 - 5.90)	8.27 ± 0.20 (8.52 - 7.73)	20.48 ± 0.89 (22.30 - 19.00)	24.90 ± 0.61 (26.20 - 23.10)	0.48 ± 0.50 (1.50 – 0.00)	1.19 ± 0.69 (2.00 – 0.25)
	T25S15	6.81 ± 0.41 (7.60 - 5.60)	8.22 ± 0.17 (8.50 - 7.81)	25.38 ± 0.70 (26.90 - 23.30)	15.65 ± 0.45 (16.60 - 14.50)	0.43 ± 0.55 (1.50 – 0.00)	0.96 ± 0.64 (2.00 – 0.10)
	T25S20	6.78 ± 0.36 (7.50 - 5.40)	8.25 ± 0.18 (8.50 - 7.71)	25.54 ± 0.71 (27.00 - 23.40)	20.43 ± 0.67 (21.60 - 18.00)	0.49 ± 0.54 (1.50 – 0.00)	1.07 ± 0.66 (2.00 – 0.00)
	T25S25	6.73 ± 0.39 (7.30 - 5.30)	8.17 ± 0.17 (8.48 - 7.79)	25.44 ± 0.71 (27.00 - 23.30)	25.58 ± 0.69 (27.00 - 24.30)	0.45 ± 0.55 (1.50 – 0.00)	1.15 ± 0.90 (2.00 – 0.25)

Table S4.2. Polychaete biomass and aquafeed supplied to different treatments testing the combined effects of temperature (T – 20 and 25ºC) and salinity (S – 15, 20 and 25) in the fatty acid profile of *Hediste diversicolor* fed a commercial aquafeed during 10, 20 and 40 days (D10, D20 and D40). Average values (± SD) (n = 5).

		Biomass Start			Biomass End			Feed supplied	Survival (%)
	Treatment	*Nº*	*Biomass (g)*	*Avg weight (g)*	*Nº*	*Biomass (g)*	*Avg weight (g)*	*Total (g)*	
D10	T20S15	10	1.86 ± 0.16	0.19 ± 0.02	7 ± 2	1.76 ± 0.56	0.26 ± 0.03	0.28 ± 0.02	66.00 ± 16.73
	T20S20	10	1.83 ± 0.22	0.18 ± 0.02	7 ± 2	1.84 ± 0.56	0.28 ± 0.02	0.28 ± 0.03	66.00 ± 21.91
	T20S25	10	1.73 ± 0.25	0.17 ± 0.03	7 ± 1	1.80 ± 0.45	0.25 ± 0.03	0.26 ± 0.04	72.00 ± 10.95
	T25S15	10	2.16 ± 0.21	0.22 ± 0.02	8 ± 1	2.36 ± 0.40	0.30 ± 0.03	0.32 ± 0.04	78.00 ± 13.04
	T25S20	10	2.14 ± 0.17	0.21 ± 0.02	7 ± 1	2.09 ± 0.31	0.30 ± 0.01	0.31 ± 0.01	70.00 ± 10.00
	T25S25	10	2.20 ± 0.24	0.22 ± 0.02	7 ± 1	2.12 ± 0.33	0.31 ± 0.04	0.32 ± 0.02	70.00 ± 12.25
D20	T20S15	10	1.76 ± 0.17	0.18 ± 0.02	6 ± 1	2.60 ± 0.27	0.45 ± 0.09	0.65 ± 0.08	60.00 ± 14.14
	T20S20	10	1.82 ± 0.19	0.18 ± 0.02	5 ± 1	1.80 ± 0.57	0.38 ± 0.11	0.68 ± 0.07	48.00 ± 8.37
	T20S25	10	1.69 ± 0.21	0.17 ± 0.02	6 ± 1	2.04 ± 0.48	0.34 ± 0.05	0.61 ± 0.08	60.00 ± 14.14
	T25S15	10	2.18 ± 0.20	0.22 ± 0.02	7 ±1	3.10 ± 0.40	0.44 ± 0.04	0.80 ± 0.08	70.00 ± 10.00
	T25S20	10	2.26 ± 0.23	0.23 ± 0.02	7 ± 1	2.64 ± 0.46	0.40 ± 0.05	0.90 ± 0.30	68.00 ± 8.37
	T25S25	10	2.06 ± 0.17	0.21 ± 0.02	7 ± 2	2.67 ± 0.32	0.40 ± 0.08	0.85 ± 0.29	70.00 ± 18.71
D40	T20S15	10	1.84 ± 0.34	0.18 ± 0.03	5 ± 1	3.21 ± 0.61	0.64 ± 0.08	1.76 ± 0.28	50.00 ± 7.07
	T20S20	10	1.76 ± 0.24	0.18 ± 0.02	5 ± 2	2.94 ± 1.04	0.67 ± 0.08	1.67 ± 0.22	46.00 ± 21.91
	T20S25	10	1.69 ± 0.26	0.17 ± 0.03	5 ± 1	2.87 ± 0.42	0.55 ± 0.08	1.63 ± 0.26	52.00 ± 4.47
	T25S15	10	2.14 ± 0.15	0.21 ± 0.02	5 ± 1	3.42 ± 1.02	0.62 ± 0.13	1.99 ± 0.15	54.00 ± 5.48
	T25S20	10	2.09 ± 0.18	0.21 ± 0.02	5 ± 1	2.86 ± 1.38	0.58 ± 0.19	2.05 ± 0.42	48.00 ± 8.37
	T25S25	10	2.17 ± 0.23	0.22 ± 0.02	4 ± 2	2.54 ± 0.91	0.71 ± 0.17	2.06 ± 0.17	38.00 ± 17.89

Table S4.3. Results of the two-way nested ANOVA or Kruskal-Wallis test to evaluate the existence of significant differences in feeding rate (FR), specific growth rate (SGR) and daily growth rate of *Hediste diversicolor* fed a commercial aquafeed during 10, 20 and 40 days (D10, D20 and D40) in different treatments of combined temperature (T – 20 and 25ºC) and salinity (S – 15, 20 and 25). S(T) - Salinity groups nested within temperature groups; The values with asterisk (*) resulted from Kruskal-Wallis test; Significant differences when $p < 0.05$.

	Test between S(T) levels	*F or H* - value*	*p – value*	*R^2 (%)*
FR	D10	0.24	0.914	10.80
	D20	0.93	0.463	21.49
	D40	0.35	0.843	13.46
SGR	D10	0.44	0.776	11.29
	D20	1.70	0.183	31.34
	D40	0.30*	0.860*	-
DGR	D10	0.51	0.730	12.80
	D20	1.69	0.186	33.13
	D40	0.85	0.510	24.44

Table S4.4. Fatty acid (FA) profile (µg mg^{-1} DW) of *Hediste diversicolor* fed a commercial aquafeed for 10 days and maintained under different combinations of water temperature (T – 20 and 25 ºC) and salinity (S – 15, 20 and 25), along with conspecifics initially stocked (Initial) and collected from the wild at the same time point (Wild10). Average values (± SD) (n = 5). SFA - saturated FA; MUFA - monounsaturated FA; PUFA - polyunsaturated FA; HUFA - highly unsaturated FA. PUFA are defined as all FA with 2 or 3 double bonds and HUFA as all FA with ≥ 4 double bonds.

FA	Initial	T20S15	T20S20	T20S25	T25S15	T25S20	T25S25	Wild10
14:0	0.41 ± 0.01	0.90 ± 0.10	0.79 ± 0.19	0.88 ± 0.27	1.02 ± 0.34	1.02 ± 0.21	1.14 ± 0.18	0.40 ± 0.02
16:0	4.14 ± 0.31	6.62 ± 1.29	5.81 ± 1.96	6.92 ± 2.39	7.97 ± 2.67	9.18 ± 1.77	9.36 ± 1.31	4.25 ± 0.34
18:0	2.44 ± 0.09	2.34 ± 0.24	2.33 ± 0.38	2.80 ± 0.31	2.60 ± 0.52	3.44 ± 1.18	2.72 ± 0.29	2.40 ± 0.16
∑SFA	**6.99 ± 0.27**	**9.87 ± 1.61**	**8.93 ± 2.52**	**10.59 ± 2.84**	**11.59 ± 3.37**	**13.63 ± 3.08**	**13.22 ± 1.72**	**7.06 ± 0.47**
16:1 *n*-7	1.05 ± 0.18	1.11 ± 0.09	1.03 ± 0.26	1.26 ± 0.42	1.07 ± 0.14	1.32 ± 0.24	1.32 ± 0.32	0.93 ± 0.14
18:1 *n*-14	2.18 ± 0.07	2.33 ± 0.15	2.20 ± 0.35	2.09 ± 0.27	1.96 ± 0.19	2.17 ± 0.15	2.19 ± 0.08	2.05 ± 0.10
18:1 *n*-9	1.48 ± 0.06	3.11 ± 0.57	3.17 ± 0.85	3.47 ± 0.77	3.04 ± 0.62	3.96 ± 0.54	4.35 ± 0.68	1.47 ± 0.02
18:1 *n*-7	1.97 ± 0.05	2.39 ± 0.23	2.44 ± 0.47	2.44 ± 0.41	2.20 ± 0.31	2.68 ± 0.25	2.88 ± 0.31	1.94 ± 0.09
20:1 *n*-13	1.31 ± 0.11	1.30 ± 0.30	1.42 ± 0.27	1.28 ± 0.12	1.29 ± 0.15	1.45 ± 0.13	1.68 ± 0.11	1.28 ± 0.09
20:1 *n*-11	0.91 ± 0.01	1.25 ± 0.17	1.30 ± 0.26	1.36 ± 0.19	1.26 ± 0.18	1.41 ± 0.11	1.57 ± 0.23	0.89 ± 0.03
20:1 *n*-9	0.75 ± 0.02	0.75 ± 0.02	0.77 ± 0.04	0.76 ± 0.02	0.74 ± 0.02	0.77 ± 0.05	0.87 ± 0.06	0.74 ± 0.02
22:1 *n*-11	ND	0.95 ± 0.11	1.03 ± 0.09	0.97 ± 0.07	0.95 ± 0.05	0.93 ± 0.07	1.86 ± 0.24	ND
∑MUFA	**9.66 ± 0.25**	**13.19 ± 1.17**	**13.35 ± 2.22**	**13.62 ± 2.14**	**12.51 ± 1.44**	**14.69 ± 0.79**	**16.73 ± 1.91**	**9.29 ± 0.45**
18:2 *n*-6 (LA)	0.70 ± 0.03	2.28 ± 0.51	2.18 ± 0.99	2.30 ± 0.86	2.16 ± 0.61	2.65 ± 0.77	2.70 ± 0.82	0.74 ± 0.03
18:3 *n*-3 (ALA)	1.04± 0.06	1.11 ± 0.06	1.11 ± 0.23	1.06 ± 0.12	1.08 ± 0.06	1.14 ± 0.11	1.10 ± 0.07	0.99 ± 0.05
$^{\Delta 5,11}$ 20:2	ND	1.10 ± 0.10	1.00 ± 0.12	1.10 ± 0.07	1.10 ± 0.05	1.20 ± 0.11	1.29 ± 0.08	ND
20:2 *n*-6	1.04 ± 0.05	1.36 ± 0.20	1.34 ± 0.27	1.38 ± 0.27	1.34 ± 0.18	1.45 ± 0.24	1.53 ± 0.27	1.04 ± 0.05
20:3 *n*-6 (DGLA)	ND	ND	ND	ND	ND	ND	ND	ND
20:3 *n*-3 (ETE)	ND	ND	ND	ND	ND	ND	ND	ND
$^{\Delta 7,13}$ 22:2	1.12 ± 0.05	1.31 ± 0.09	1.25 ± 0.15	1.25 ± 0.06	1.21 ± 0.10	1.23 ± 0.09	1.29 ± 0.08	1.23 ± 0.05
$^{\Delta 7,13,16}$ 22:3	ND	ND	ND	ND	ND	ND	ND	ND
∑PUFA	**3.89 ± 0.09**	**7.15 ± 0.87**	**6.88 ± 1.70**	**7.09 ± 1.34**	**6.89 ± 0.97**	**7.67 ± 1.01**	**7.91 ± 1.20**	**4.00 ± 0.14**
20:4 *n*-6 (ARA)	1.77 ± 0.07	1.62 ± 0.13	1.36 ± 0.34	1.31 ± 0.29	1.33 ± 0.21	1.37 ± 0.22	1.39 ± 0.19	2.09 ± 0.19
20:4 *n*-3 (ETA)	ND	ND	ND	ND	ND	ND	ND	ND
20:5 *n*-3 (EPA)	3.31 ± 0.13	3.65 ± 0.40	3.09 ± 1.48	2.73 ± 1.22	2.92 ± 0.77	3.40 ± 0.95	3.15 ± 0.92	4.06 ± 0.39
22:4 *n*-6 (AdA)	1.39 ± 0.07	1.42 ± 0.22	1.16 ± 0.27	1.13 ± 0.19	1.12 ± 0.13	1.21 ± 0.15	1.29 ± 0.14	1.85 ± 0.18
22:5 *n*-3 (DPA)	1.34 ± 0.01	1.33 ± 0.13	1.33 ± 0.27	1.22 ± 0.16	1.20 ± 0.16	1.31 ± 0.13	1.28 ± 0.16	1.69 ± 0.13
22:6 *n*-3 (DHA)	ND	1.18 ± 0.20	1.08 ± 0.24	1.02 ± 0.22	1.11 ± 0.17	1.77 ± 0.70	1.83 ± 0.48	ND
∑HUFA	**7.81 ± 0.16**	**9.20 ± 1.03**	**8.03 ± 2.58**	**7.40 ± 2.04**	**7.68 ± 1.41**	**9.05 ± 1.54**	**8.93 ± 1.84**	**9.69 ± 0.80**
Total FA	**28.35 ± 0.50**	**39.41 ± 4.03**	**37.19 ± 8.56**	**38.70 ± 8.19**	**38.67 ± 5.71**	**45.04 ± 4.70**	**46.79 ± 5.37**	**30.03 ± 1.67**

Table S4.5. Fatty acid (FA) profile (µg mg^{-1} DW) of *Hediste diversicolor* fed a commercial aquafeed for 20 days and maintained under different combinations of water temperature (T – 20 and 25 ºC) and salinity (S – 15, 20 and 25), along with conspecifics initially stocked (Initial) and collected from the wild at the same time point (Wild20). Average values (± SD) (n = 5). SFA - saturated FA; MUFA - monounsaturated FA; PUFA - polyunsaturated FA; HUFA - highly unsaturated FA. PUFA are defined as all FA with 2 or 3 double bonds and HUFA as all FA with ≥ 4 double bonds.

FA	Initial	T20S15	T20S20	T20S25	T25S15	T25S20	T25S25	Wild20
14:0	0.41 ± 0.01	1.71 ± 0.21	1.88 ± 0.34	1.88 ± 0.18	2.43 ± 1.06	1.49 ± 0.51	1.58 ± 0.14	0.45 ± 0.02
16:0	4.14 ± 0.31	12.41 ± 1.54	13.68 ± 1.89	14.08 ± 2.19	18.38 ± 5.77	13.13 ± 3.63	13.48 ± 0.83	4.98 ± 0.47
18:0	2.44 ± 0.09	3.22 ± 0.28	3.64 ± 0.51	3.68 ± 0.52	4.17 ± 0.64	3.73 ± 0.89	3.48 ± 0.23	2.11 ± 0.08
∑SFA	**6.99 ± 0.27**	**17.35 ± 1.99**	**19.20 ± 2.67**	**19.64 ± 2.88**	**24.98 ± 7.37**	**18.35 ± 4.86**	**18.54 ± 1.08**	**7.54 ± 0.56**
16:1 *n*-7	1.05 ± 0.18	1.90 ± 0.30	2.26 ± 0.51	2.20 ± 0.25	2.22 ± 0.67	1.69 ± 0.50	1.83 ± 0.18	0.88 ± 0.17
18:1 *n*-14	2.18 ± 0.07	2.38 ± 0.23	2.36 ± 0.17	2.43 ± 0.32	2.23 ± 0.35	2.14 ± 0.32	2.11 ± 0.14	2.12 ± 0.10
18:1 *n*-9	1.48 ± 0.06	6.29 ± 0.81	7.41 ± 1.65	7.34 ± 0.74	7.07 ± 1.88	5.96 ± 1.76	6.62 ± 0.83	1.50 ± 0.05
18:1 *n*-7	1.97 ± 0.05	3.54 ± 0.34	3.96 ± 0.56	4.10 ± 0.57	3.65 ± 0.79	3.36 ± 0.85	3.48 ± 0.37	2.17 ± 0.11
20:1 *n*-13	1.31 ± 0.11	1.18 ± 0.28	1.48 ± 0.13	1.66 ± 0.23	1.20 ± 0.14	1.10 ± 0.21	1.37 ± 0.22	1.28 ± 0.05
20:1 *n*-11	0.91 ± 0.01	1.89 ± 0.50	2.32 ± 0.37	2.41 ± 0.26	2.25 ± 0.55	2.21 ± 0.76	2.22 ± 0.22	0.86 ± 0.02
20:1 *n*-9	0.75 ± 0.02	0.75 ± 0.04	0.81 ± 0.02	0.89 ± 0.07	0.75 ± 0.02	0.75 ± 0.02	0.76 ± 0.04	0.76 ± 0.02
22:1 *n*-11	ND	1.74 ± 0.24	1.40 ± 0.18	1.59 ± 0.45	1.03 ± 0.08	1.02 ± 0.17	1.11 ± 0.21	ND
∑MUFA	**9.66 ± 0.25**	**19.66 ± 2.32**	**21.99 ± 3.32**	**22.63 ± 1.89**	**20.41 ± 4.31**	**18.23 ± 4.06**	**19.52 ± 1.77**	**9.57 ± 0.15**
18:2 *n*-6 (LA)	0.70 ± 0.03	4.49 ± 1.72	5.55 ± 2.12	5.84 ± 0.86	5.71 ± 1.45	5.06 ± 1.61	5.40 ± 0.36	0.72 ± 0.07
18:3 *n*-3 (ALA)	1.04± 0.06	1.27 ± 0.22	1.31 ± 0.27	1.45 ± 0.19	1.33 ± 0.14	1.32 ± 0.23	1.32 ± 0.05	1.07 ± 0.03
$^{\Delta5,11}$ 20:2	ND	1.23 ± 0.04	1.27 ± 0.11	1.28 ± 0.06	1.25 ± 0.12	1.21 ± 0.31	1.23 ± 0.09	ND
20:2 *n*-6	1.04 ± 0.05	2.29 ± 0.71	2.56 ± 0.76	2.72 ± 0.53	2.46 ± 0.64	2.73 ± 1.20	2.65 ± 0.43	1.03 ± 0.09
20:3 *n*-6 (DGLA)	ND	ND	ND	ND	ND	ND	ND	ND
20:3 *n*-3 (ETE)	ND	ND	ND	ND	ND	ND	ND	ND
$^{\Delta7,13}$ 22:2	1.12 ± 0.05	1.33 ± 0.11	1.38 ± 0.12	1.47 ± 0.15	1.18 ± 0.07	1.26 ± 0.22	1.27 ± 0.14	1.19 ± 0.05
$^{\Delta7,13,16}$ 22:3	ND	1.08 ± 0.08	1.03 ± 0.04	1.09 ± 0.06	0.95 ± 0.04	1.02 ± 0.09	1.00 ± 0.06	ND
∑PUFA	**3.89 ± 0.09**	**11.69 ± 2.75**	**13.10 ± 3.32**	**13.86 ± 1.72**	**12.87 ± 2.35**	**12.60 ± 3.58**	**12.87 ± 0.69**	**4.01 ± 0.17**
20:4 *n*-6 (ARA)	1.77 ± 0.07	1.45 ± 0.36	1.33 ± 0.34	1.44 ± 0.19	1.35 ± 0.10	1.40 ± 0.33	1.44 ± 0.21	1.96 ± 0.22
20:4 *n*-3 (ETA)	ND	ND	ND	ND	ND	ND	ND	ND
20:5 *n*-3 (EPA)	3.31 ± 0.13	3.77 ± 1.59	3.25 ± 1.47	4.26 ± 1.14	4.03 ± 0.78	4.83 ± 1.46	4.40 ± 0.80	4.03 ± 0.66
22:4 *n*-6 (AdA)	1.39 ± 0.07	1.28 ± 0.24	1.14 ± 0.26	1.25 ± 0.18	1.02 ± 0.04	1.14 ± 0.27	1.17 ± 0.16	1.62 ± 0.17
22:5 *n*-3 (DPA)	1.34 ± 0.01	1.23 ± 0.20	1.19 ± 0.17	1.34 ± 0.17	1.14 ± 0.06	1.25 ± 0.21	1.28 ± 0.14	1.67 ± 0.16
22:6 *n*-3 (DHA)	ND	1.87 ± 0.60	1.28 ± 0.50	1.94 ± 0.31	1.31 ± 0.16	1.58 ± 0.73	1.38 ± 0.19	ND
∑HUFA	**7.81 ± 0.16**	**9.59 ± 2.91**	**8.19 ± 2.70**	**10.24 ± 1.77**	**8.85 ± 1.07**	**10.21 ± 2.98**	**9.67 ± 1.35**	**9.28 ± 1.20**
Total FA	**28.35 ± 0.50**	**58.30 ± 8.58**	**62.48 ± 11.22**	**66.37 ± 7.15**	**67.11 ± 14.17**	**59.39 ± 14.89**	**60.60 ± 3.70**	**30.41 ± 1.82**

Table S4.6. Two-way nested analysis of similarities (ANOSIM) evaluating differences in the fatty acid profile of *Hediste diversicolor* fed a commercial aquafeed during 10, 20 and 40 days (D10, D20 and D40) in different treatments of combined temperature (T – 20 and 25ºC) and salinity (S – 15, 20 and 25). S(T) - Salinity groups nested within temperature groups; Significant differences when $p < 0.05$.

Samples	Tests between T and S(T) levels	*R-value*	*p-value*
D10	T	0.222	0.300
	S(T)	0.124	0.068
D20	T	0.481	0.100
	S(T)	0.101	0.089
D40	T	-0.034	0.500
	S(T)	0.051	0.241

Table S4.7. One-way analysis of similarities (ANOSIM) between the fatty acid profile of *Hediste diversicolor* fed a commercial aquafeed during 10, 20 and 40 days (D10, D20 and D40) in different treatments of combined temperature (T – 20 and 25ºC) and salinity (S – 15, 20 and 25) and wild conspecifics collected on the same date (Wild10, Wild20 and Wild40). Significant differences when $p < 0.05$.

Pairwise tests	*R*	*p*
D10 samples		
T20S15 – Wild10	1.000	0.008
T20S20 – Wild10	0.872	0.008
T20S25 – Wild10	1.000	0.008
T25S15 – Wild10	1.000	0.008
T25S20 – Wild10	1.000	0.008
T25S25 – Wild10	1.000	0.008
D20 samples		
T20S15 – Wild20	1.000	0.008
T20S20 – Wild20	1.000	0.008
T20S25 – Wild20	1.000	0.008
T25S15 – Wild20	1.000	0.008
T25S20 – Wild20	1.000	0.008
T25S25 – Wild20	1.000	0.008
D40 samples		
T20S15 – Wild40	1.000	0.008
T20S20 – Wild40	1.000	0.008
T20S25 – Wild40	1.000	0.008
T25S15 – Wild40	1.000	0.008
T25S20 – Wild40	1.000	0.008
T25S25 – Wild40	1.000	0.008

Table S4.8. Similarity percentages (SIMPER) analysis (cut-off 50%) between the fatty acid (FA) profile of *Hediste diversicolor* fed a commercial aquafeed for 10 days in different treatments of combined temperature (T – 20 and 25ºC) and salinity (S – 15, 20 and 25) and wild conspecifics collected on the same date (Wild10).

T20S15 & Wild10			T20S20 & Wild10			T20S25 & Wild10		
Avg. Dissimilarity: 14.76%			Avg. Dissimilarity: 16.12%			Avg. Dissimilarity: 16.74%		
FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%
22:6 *n*-3 (DHA)	14.14	14.14	22:6 *n*-3 (DHA)	12.34	12.34	$^{\Delta5,11}$20:2	12.08	12.08
$^{\Delta5,11}$20:2	13.51	27.65	22:1	12.08	24.42	22:6 *n*-3 (DHA)	11.32	23.39
22:1	12.17	39.82	$^{\Delta5,11}$20:2	11.78	36.20	22:1	11.09	34.48
18:2 *n*-6 (LA)	11.30	51.12	18:2 *n*-6 (LA)	9.30	45.50	18:2 *n*-6 (LA)	9.80	44.28
			$^{\Delta9}$18:1	8.44	53.94	$^{\Delta9}$18:1	9.38	53.67
T25S15 & Wild10			T25S20 & Wild10			T25S25 & Wild10		
Avg. Dissimilarity: 16.37%			Avg. Dissimilarity: 18.50%			Avg. Dissimilarity: 20.02%		
FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%
22:6 *n*-3 (DHA)	12.45	12.45	22:6 *n*-3 (DHA)	14.02	14.02	22:1	13.38	13.38
$^{\Delta5,11}$20:2	12.43	24.89	$^{\Delta5,11}$20:2	11.13	25.15	22:6 *n*-3 (DHA)	13.09	26.47
22:1	11.14	36.02	18:2 *n*-6 (LA)	10.11	35.26	$^{\Delta5,11}$20:2	10.58	37.05
18:2 *n*-6 (LA)	9.62	45.64	$^{\Delta9}$18:1	9.77	45.03	$^{\Delta9}$18:1	9.78	46.83
16:0	8.31	53.96	22:1	9.27	54.36	18:2 *n*-6 (LA)	9.32	56.15

Table S4.9. Similarity percentages (SIMPER) analysis (cut-off 50%) between the fatty acid (FA) profile of *Hediste diversicolor* fed a commercial aquafeed for 20 days in different treatments of combined temperature (T – 20 and 25ºC) and salinity (S – 15, 20 and 25), and wild conspecifics collected on the same date (Wild20).

T20S15 & Wild20			T20S20 & Wild20			T20S25 & Wild20		
Avg. Dissimilarity: 24.83%			Avg. Dissimilarity: 26.23%			Avg. Dissimilarity: 26.72%		
FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%
18:2 *n*-6 (LA)	10.57	10.57	18:2 *n*-6 (LA)	11.56	11.56	18:2 *n*-6 (LA)	11.80	11.80
$^{\Delta9}$18:1	10.25	20.82	$^{\Delta9}$18:1	10.72	22.28	$^{\Delta9}$18:1	10.30	22.10
22:6 *n*-3 (DHA)	9.96	30.78	16:0	8.03	30.30	22:6 *n*-3 (DHA)	9.23	31.32
22:1	9.70	40.47	22:1	7.85	38.16	22:1	8.11	39.43
$^{\Delta5,11}$20:2	7.76	48.23	$^{\Delta5,11}$20:2	7.38	45.53	16:0	7.87	47.31
16:0	7.74	55.97	22:6 *n*-3 (DHA)	7.22	52.75	$^{\Delta5,11}$20:2	7.10	54.41
T25S15 & Wild20			T25S20 & Wild20			T25S25 & Wild20		
Avg. Dissimilarity: 26.03%			Avg. Dissimilarity: 23.99%			Avg. Dissimilarity: 24.38%		
FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%
18:2 *n*-6 (LA)	12.13	12.13	18:2 *n*-6 (LA)	12.36	12.36	18:2 *n*-6 (LA)	12.86	12.86
$^{\Delta9}$18:1	10.37	22.50	$^{\Delta9}$18:1	9.98	22.34	$^{\Delta9}$18:1	10.83	23.69
16:0	10.22	32.72	22:6 *n*-3 (DHA)	9.16	31.50	16:0	8.66	32.35
22:6 *n*-3 (DHA)	7.59	40.31	16:0	8.34	39.84	22:6 *n*-3 (DHA)	8.44	40.79
14:0	7.36	47.68	$^{\Delta5,11}$20:2	7.84	47.68	$^{\Delta5,11}$20:2	7.83	48.61
$^{\Delta5,11}$20:2	7.32	55.00	$^{\Delta7,13,16}$22:3	7.06	54.74	22:1	7.24	55.86

Table S4.10. Similarity percentages (SIMPER) analysis (cut-off 50%) between the fatty acid (FA) profile of *Hediste diversicolor* fed a commercial aquafeed for 40 days in different treatments of combined temperature (T – 20 and 25ºC) and salinity (S – 15, 20 and 25), and wild conspecifics collected on the same date (Wild40).

T20S15 & Wild40 Avg. Dissimilarity: 30.04%			T20S20 & Wild40 Avg. Dissimilarity: 31.47%			T20S25 & Wild40 Avg. Dissimilarity: 31.34%		
FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%	FA	Contrib.%	Cum. %
22:6 *n*-3 (DHA)	10.84	10.84	22:6 *n*-3 (DHA)	10.40	10.40	22:6 *n*-3 (DHA)	10.34	10.34
18:2 *n*-6 (LA)	9.49	20.34	18:2 *n*-6 (LA)	9.28	19.68	18:2 *n*-6 (LA)	9.01	19.35
22:1	7.24	27.57	20:2 *n*-6	7.23	26.91	20:2 *n*-6	7.64	26.99
$^{\Delta 9}$18:1	7.18	34.76	$^{\Delta 9}$18:1	7.17	34.08	$^{\Delta 9}$18:1	6.68	33.67
20:2 *n*-6	6.68	41.44	$^{\Delta 5,11}$20:2	6.39	40.47	22:1	6.23	39.90
$^{\Delta 5,11}$20:2	6.27	47.79	22:1	5.97	46.44	$^{\Delta 5,11}$20:2	6.07	45.96
20:3 *n*-6	6.17	53.88	$^{\Delta 7,13,16}$22:3	5.67	52.12	$^{\Delta 7,13,16}$22:3	6.04	52.0
T25S15 & Wild40 Avg. Dissimilarity: 30.40%			**T25S20 & Wild40 Avg. Dissimilarity: 28.91%**			**T25S25 & Wild40 Avg. Dissimilarity: 32.41%**		
FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%	FA	Contrib.%	Cum.%
22:6 *n*-3 (DHA)	9.90	9.90	22:6 *n*-3 (DHA)	10.74	10.74	22:6 *n*-3 (DHA)	9.81	9.81
18:2 *n*-6 (LA)	8.57	18.47	18:2 *n*-6 (LA)	8.16	18.90	18:2 *n*-6 (LA)	8.83	18.64
20:2 *n*-6	7.20	25.68	22:1	7.68	26.58	20:2 *n*-6	7.55	26.18
$^{\Delta 9}$18:1	6.84	32.52	20:2 *n*-6	7.34	33.93	$^{\Delta 9}$18:1	7.31	33.50
$^{\Delta 5,11}$20:2	6.83	39.35	$^{\Delta 5,11}$20:2	6.78	40.70	$^{\Delta 5,11}$20:2	6.47	39.97
22:1	6.27	45.62	$^{\Delta 9}$18:1	6.43	47.14	$^{\Delta 7,13,16}$22:3	5.87	45.84
$^{\Delta 7,13,16}$22:3	6.03	51.65	$^{\Delta 7,13,16}$22:3	6.38	53.52	22:1	5.57	51.41

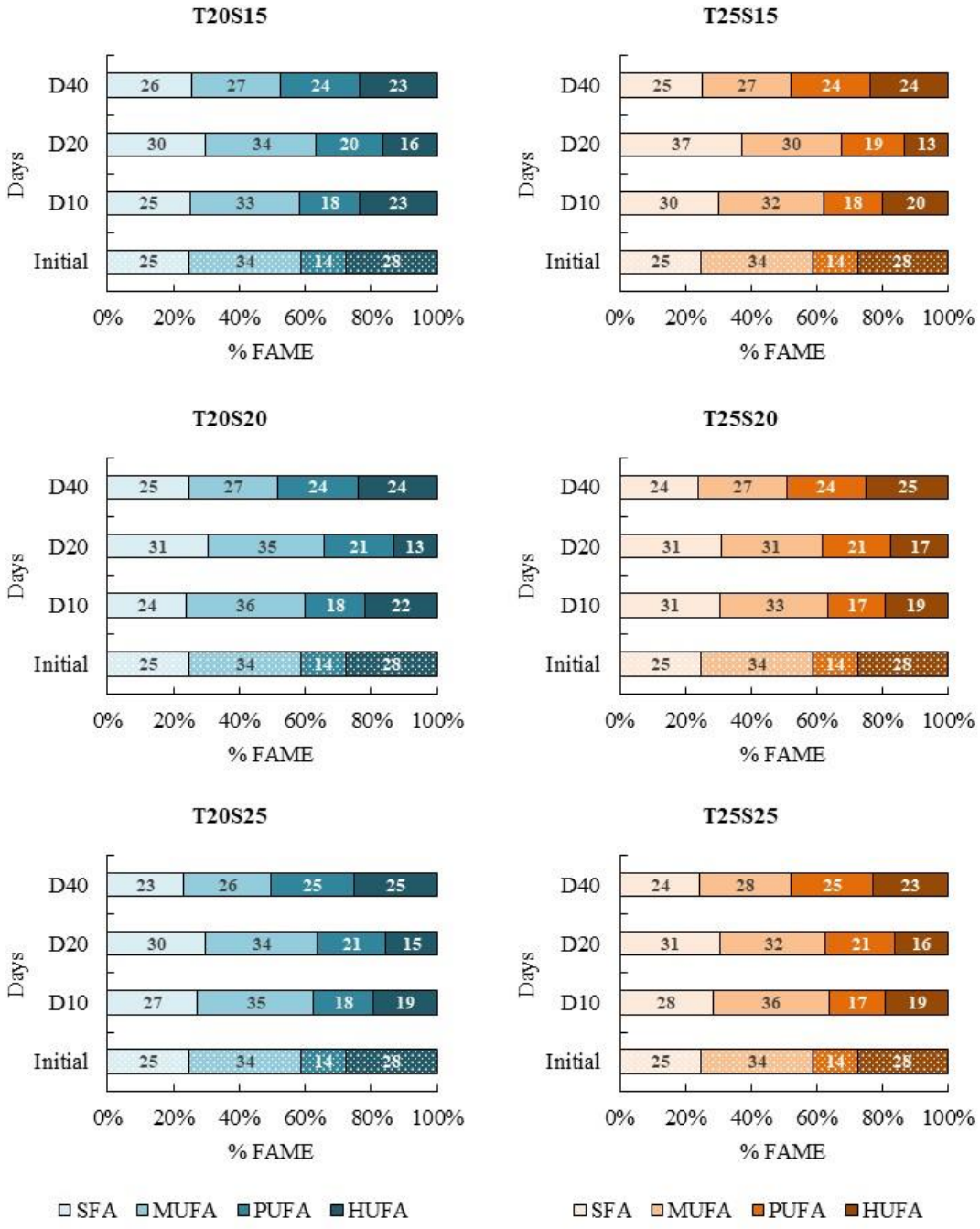
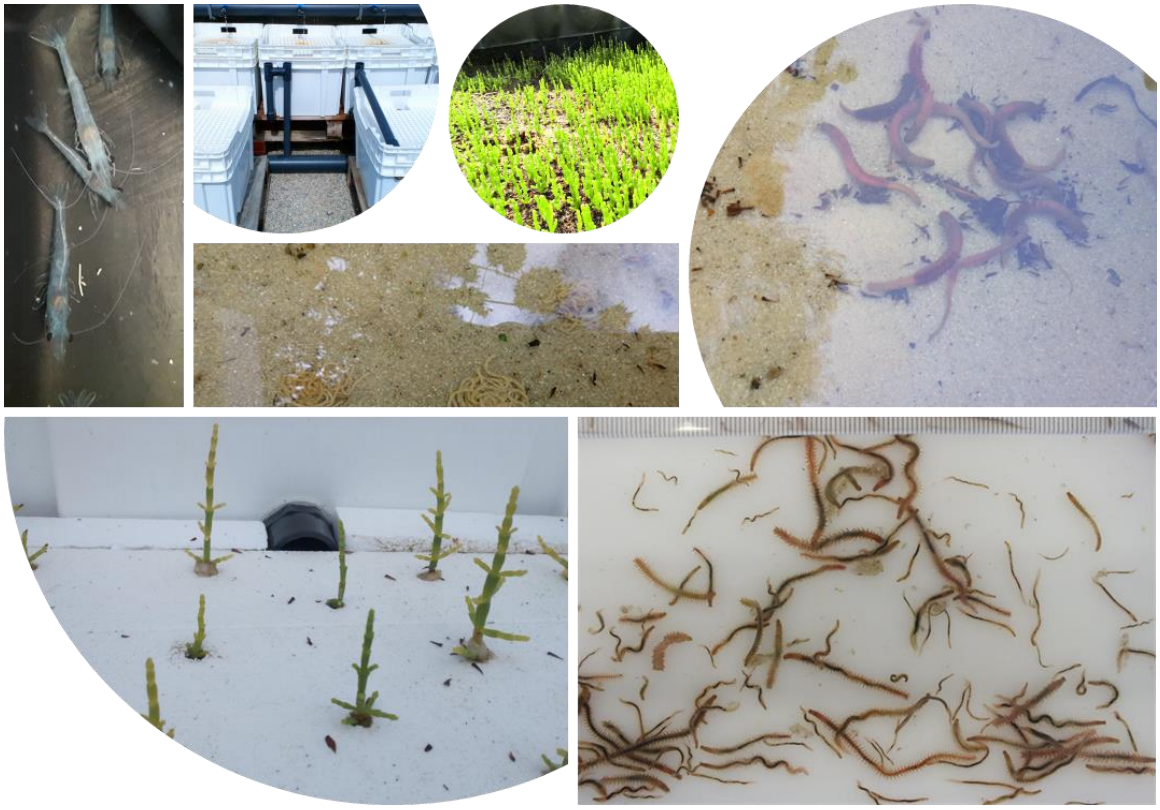


Figure S4.1. Evolution of fatty acid classes (expressed as % of total fatty acid methyl esters - FAME) of *Hediste diversicolor* fed a commercial aquafeed over time (10, 20 and 40 days) under different treatments of combined temperature (T – 20 and 25 ºC) and salinity (S – 15, 20 and 25) contrasted with that of initially stocked conspecifics (Initial). Average values (± SD) (n = 5). Abbreviations: SFA - saturated fatty acids; MUFA - monounsaturated FA; PUFA - polyunsaturated FA; HUFA - highly unsaturated FA. PUFA are defined as all FA with 2 or 3 double bonds and HUFA as all FA with ≥ 4 double bonds.

Chapter 5

5.1. Recovering wasted nutrients from shrimp farming through the combined culture of polychaetes and halophytes



Jerónimo, D., Lillebø, A.I., Cremades, Cartaxana, P., Calado, R. Recovering wasted nutrients from shrimp farming through the combined culture of polychaetes and halophytes. *Scientific Reports* **11,** 6587 (2021). https://doi.org/10.1038/s41598-021-85922-y

5.1. Recovering wasted nutrients from shrimp farming through the combined culture of polychaetes and halophytes

Abstract

The bioremediation and biomass production of organic extractive organisms (polychaetes *Arenicola marina*, *Hediste diversicolor* and halophyte *Salicornia ramosissima*) was assessed in an integrated multi-trophic aquaculture (IMTA) framework. Culture trials were performed outdoors using the nutient rich effluent from a shrimp farm employing recirculated aquaculture systems. Similar bioremediation efficiencies were obtained in cultures using a single polyculture tank (1T) or two trophic levels separated tanks (2T; ≈0.3 and 0.6 m2 operational area, respectively), with a reduction of 74-87% for particulate organic matter (POM), 56-64% for dissolved inorganic nitrogen (DIN) and 60-65% for dissolved inorganic phosphorus (DIP). *Hediste diversicolor* adapted well to culture conditions, reaching densities up to 5.000 ind. m^{-2} (≈78-98 g m^{-2}). *Arenicola marina* failed to cope with water temperature that exceeded the species thermal limits, displaying a survival <10% (20 ºC often pointed as the maximum thermal threshold for this species). Productivity of *S. ramosissima* with 1T was about twice that obtained with 2T (≈150-170 and ≈60-90 g FW m^{-2} edible aboveground biomass, respectively). The yellowish coloration of cultured plants was likely due to the chemical oxidation and rapid sand filtration pre-treatment applied to the brackish groundwater used in the aquaculture facility, that removed iron (and probably other essential elements). Overall, 1T design combining *H. diversicolor* and *S. ramosissi*ma displayed the best bioremediation performance and biomass production, while also allowing reducing in half the operational area required to implement this IMTA framework.

5.1.1. Introduction

Integrated multi-trophic aquaculture (IMTA) is an ecosystem-based approach where species of different trophic levels are integrated to maximize the recovery of nutrients introduced in the production system[1-3]. In this context, unused nutrients (uneaten feed and faeces) derived from fed species (e.g., fish and crustaceans) that are commonly wasted through farm effluents are recovered into valuable extractive species biomass, with effluent bioremediation also being achieved[1-3]. Saltwater aquaculture (marine and brackish-water) is paramount to fulfil dietary needs, contributing to food security worldwide[4]. In 2018 this sector represented approximately 56% and 46% of the volume and value generated by aquaculture in total (global values above 114 million tonnes and USD 263 billions)[5]. The vast majority of farmed fish and crustaceans (≈20% and 58% of the volume and value of saltwater aquaculture production) are carnivorous species which continue to require feeds that contain fish meal and fish oil in their composition, two increasingly scarce resources[5,6]. These raw materials, still continue to be considered the most nutritious and most digestible ingredients for major saltwater farmed species, as well as major sources of essential long-chain omega-3 fatty acids (e.g., eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA])[5]. Saltwater farmed species are known to excrete between 50 to 80% of feed nitrogen (N) and 35 to 85% of feed phosphorus (P), which represents an economic constrain, as farm effluents need to be treated to meet legal regulations to enable their release into the aquatic environment[7]. This constrain can be overcome if such excess of nutrients are extracted, e.g., through bioremediation, from saltwater aquaculture effluents.

Modern recirculating aquaculture systems (RAS) allowed culturing saltwater species everywhere, including locations away from marine water sources. However, one of the biggest concerns when operating these systems are the management and costs associated to the reduction/removal of nutrient loads present in effluent water, as saline nutrient rich effluent cannot be directly discarded/used in land to avoid salinization[8,9]. Unused nutrients that are commonly wasted in aquaculture effluents are composed by particulate organic matter (POM), dissolved organic matter (DOM) (including dissolved organic nitrogen [DON] and phosphorus [DOP]) and as dissolved inorganic nutrients (including dissolved inorganic nitrogen [DIN] = NOx-N + NH4-N and dissolved inorganic phosphorus [DIP] = PO4-P)[10,11]. The recovery of dissolved inorganic nutrients can be successfully achieved using primary producers as extractive species, such as microalgae,

seaweeds and halophytes, while the recovery of POM can be pursued using deposit-feeders and filter-feeders, like polychaetes and bivalves[3,12,13]. The integration of multiple species from different trophic levels in the same IMTA design allows to recover particulate and dissolved nutrients in the form of valuable biomass of these extra crops. This has been previously described in studies addressing the combined use of bivalves and seaweeds[14-23], bivalves and microalgae[24], bivalves and halophytes[25], echinoderms and seaweeds[26], polychaetes and seaweeds[27] and polychaetes and halophytes[11]. More than 50% of these studies were performed in the time-period between 2015-2020 evidencing that this is currently a hot-topic in aquaculture. In general, most IMTA designs addressed to date advocate the culture of different extractive species in separate tanks[11,15,17,24,25,28], following the trophic relations, which consequently require a larger operational area. This is often pointed as one of the major constrains to successfully develop an IMTA framework for new or ongoing operations. Indeed, producers need to allocate part of the area defined for the aquaculture of the target species being farmed (e.g., finfish or crustaceans) to produce the extractive species, which often have a lower commercial value[29]. As an example, one can refer that in order to recover 10% of dissolved inorganic nitrogen produced by 1,000 – 1,800 ton of salmon in 1 ha, nearly 10-23 ha of seaweed culture area are needed[29-32]. Concerning halophytes, it was estimated that approximately 10.000 m^2 of constructed wetlands planted with *Salicornia persica* would be required to recover N and total suspended solids (TSS) originated from 900 Kg of 45% crude protein fish feed during one year (11 m^2 Kg^{-1} of feed)[33]. In soilless production systems (e.g., aquaponics – using the deep-water culture technique) it was estimated that a planted area of ≈14.4 m^2 with *Salicornia dolichostachya* (1,128 plants - 78 plants m^{-2}) would be required to remove 189 g N and 29 g of P excreted by *Dicentrarchus labrax*[34]. In this way, giving the limited farming areas to culture the target fed species, it is imperative to work on the development of IMTA designs where different extractive species can be produced using innovative approaches that minimize the required operational area.

Another remaining challenge, worth highlighting, is that several modern RAS use saline groundwater which is usually supersaturated with nitrogen, argon and carbon dioxide[35]. These compounds are harmful to the main species of finfish and crustaceans being produced, and in order to increase oxygen in the water to concentrations within optimum values[36] degassing and aeration treatments must be used to pre-treat the water, safeguarding against harmful gases. Saline groundwater can also be supersaturated with iron, then treatments employing chemical oxidation are employed to promote iron oxides

to precipitate and subsequently removed using rapid sand filtration or settling basins[36]. These pre-treatments, as well as others like ozonation, needed for the production of the fed species, can remove essential micro-nutrients that might negatively affect the production of extractive primary producers like halophytes.

Polychaete worms, such as *Hediste diversicolor* O.F. Müller, 1776 and *Arenicola marina* Linnaeus, 1758, can be key extractive species to recover wasted nutrients from POM present in aquaculture effluents, while halophytes such as *Salicornia ramosissima* J. Woods, 1851 can be key extractive species to recover dissolved inorganic nutrients (mainly nitrogen and phosphorus). Therefore, the present study evaluated the bioremediation performance and biomass production of the combined culture of polychaetes and halophytes, namely, *A. marina* and *S. ramosissima* (Amar+Sram) and *H. diversicolor* and *S. ramosissima* (Hdiv+Sram) using the effluent water from a shrimp RAS operated with pre-treated saline groundwater (*ca.* 20 g L^{-1} of salt). These different IMTA designs were tested using different operational areas designated as single polyculture tank (1T) and as two trophic levels separated tanks (2T) (0.3 and 0.6 m^2 of operational area, respectively).

5.1.2. Material and methods

5.1.2.1. Selected extractive species

All extractive species tested in the present work can be easily collected and are highly abundant in the study site, Ria de Aveiro coastal lagoon (Portugal 40°44'21.1"N 8°39'40.1"W). A brief description of the main features of each extractive species is presented below:

5.1.2.1.1. Polychaete worms

The polychaete *H. diversicolor*, commonly known as ragworm, was selected to the present study 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 creates a three-dimensional burrow network in sandy-mud bottoms[37]. 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[38]. In the reproduction of this species, there is the rupture of the dissepiment in the female and by nephridies or rupture of the dissepiment in the male for gamete release; reproduction of this species is therefore followed by death of parental worms (semelparous species)[38]. The maturity takes between 1 and 2 years before spawning[38]. It is considered an active predator that displays omnivorous feeding habits, being ranked within the deposit-feeders polychaetes functional group[39]. Its bioturbation potential and high-valued biomass (rich in essential fatty acids)[39-42] makes this polychaete a well-suited species for IMTA.

The polychaete *A. marina*, commonly known as lugworm, was selected to the present study due to its wide distribution in north-western European coasts, from the British Isles to the Iberian Peninsula, with its southern limit of distribution being close to 40ºN[43]. It is found from middle to lower shores and reaches high abundances in sheltered estuarine sediments where it lives in U or J-shaped burrow (0.2-0.4 m deep)[43]. This species reproduces several times throughout its life cycle (iteroparous species) attaining sexual maturity at 2-3 years of age, having its sexes separated and displaying external fertilization, with different populations releasing eggs and sperm in a synchronized period of 2 weeks that runs from October to November[44,45]. It feeds on debris and microorganisms present in the sediment it ingests, leaving a characteristic depression on the top of the sediment (the "blow hole") and later, after absorbing all organic content, releasing a characteristic worm cast[46]. In the wild, these polychaetes can reach densities between 100-150 ind. m^{-2} and tolerate salinities from 12-35[46]. Adults can reach between 120 to 200 mm in length, with lugworms being considered a premium bait for sea anglers[45,46]. The bioturbation promoted by this species, along with a growing interest in the biotechnological use of its biomass (e.g., production of extracellular hemoglobin [HBL Hb] as a promising substitute for human blood[47] and use in solutions for organ preservation[48]) makes this polychaete species a promising candidate for IMTA.

The integration of both the above-mentioned polychaete species aimed to evaluate the performance of PASFs (i.e., bioremediation and biomass generation) stocked with two species that exhibit contrasting life cycles, distinct bioturbation strategies and biochemical profile.

5.1.2.1.2. Halophyte plants

Salicornia ramosissima is an halophyte plant popularly known as green samphire. It is widely distributed in the salt marshes of the Iberian Peninsula, western France and Serbia. It is an annual species that exhibits the best growth performances at low salinities, although it is able to tolerate high salinities[49-51]. These plants are considered a gourmet product for human consumption with their fresh branch tips being highly appreciated fresh[51-54]. Due to its saline content, this plant is also used dehydrated and grounded in preparations were it replaces traditional salt as green salt[54,55]. The nutritional profile of *S. ramosissima* is suitable for human consumption, revealing high protein content (5.20 g/100 g DW), *n*-3 and *n*-6 polyunsaturated fatty acids (mainly α-linolenic and linoleic acid)[51,54] and minerals (such as sodium, potassium, calcium, magnesium, iron and manganese)[51]. These plants also exhibit a significant antioxidant and anti-inflammatory potential due to their total phenolics content[51,56]. In addition, seeds of Salicornia spp. contain considerable levels of oil and protein (e.g., *Salicornia bigelovii* seeds present 26-33% oil and 31% protein[57]). Oil yielding crop plants are very important for economic growth of the agriculture sector, with many of the fatty acids identified in plant seeds being highly demanded for several industrial sectors (e.g., plastics, textile, pharmaceuticals, cosmetics)[58,59]. The bioremediation potential exhibited by *Salicornia* spp.[33,34,60,61] associated with the potential to produce valuable extractive biomass makes these plants key candidates for IMTA.

5.1.2.2. IMTA design

The present study was performed from June to September 2018 at RiaSearch Lda. (Portugal), a research company in the field of aquaculture nutrition operating in the coastal lagoon Ria de Aveiro (40°44'21.1"N 8°39'40.1"W). The company employs RAS to grow Pacific white shrimp (*Litopenaeus vannamei*) and operates with brackish groundwater that is pre-treated through chemical oxidation and rapid sand filtration to remove iron. Shrimp were fed twice a day with a commercial diet for flatfish that present 62% of crude protein, 18% of crude fat and 0.3% of crude fiber (WINFlat - SPAROS). Uneaten feed and faeces that were siphoned from culture tanks were collected and concentrated in a reservoir (130 L) equipped with a pump programmed to work 5 min per hour in order to promote homogenization and avoid anaerobic conditions. The water

concentrated in this reservoir was added every 3 days to an outflowing tank (0.34 m^{-3}), from which it was pumped through a plastic trickling tower installed above the inflowing tank (0.34 m^{-3}). From this inflowing tank the water was directed to the tanks where the different IMTA designs using polychaete assisted sand filters (PASFs) and halophytes in aquaponics were performed.

A schematic representation of the different IMTA designs tested in the present study is displayed in Figure 5.1a. Designs 1TAmar+Sram and 2TAmar+Sram were stocked with the polychaete *A. marina* and the halophyte *S. ramosissima*, while designs 1T Hdiv+Sram and 2T Hdiv+Sram were stocked with the polychaete *H. diversicolor* and the halophyte *S. ramosissima*. Designs with 1T cultured polychaetes and halophytes in the same tank (operational area ≈0.3 m^2), with the roots of *S. ramosissima* being maintained in the water column of PASFs. Designs with 2T cultured both extractive species in separate tanks (operational area ≈0.6 m^2) with the water passing through the sand bed then being directed to the aquaponics unit. In 1T designs, inflowing water entered PASFs tanks through a pipe whose bottom ended 0.1 m below the halophyte support plate to protect the roots from particulate organic matter. In 2T designs, this configuration was also adopted to safeguard similar conditions. Each of the four IMTA designs performed in this study was evaluated using 5 replicates, with these being distributed as represented in Figure 5.1b. Five control units with no polychaetes and no halophytes were also included in the experimental design. Each PASFs tank presented a volume of 0.05 m^3 and a surface area of 0.3 m^2 and was formed by a 150-mm sand column (0.5 – 0.7 mm grain size) in the bottom of the tank and a superficial 150-mm water column. These tanks were equipped with a bottom draining pipe to allow a complete percolation of water through the sand column. The aquaponic tank used in 2T designs presented a water volume of 0.05 m^3 and a surface area of 0.3 m^2. Each tank of the different IMTA designs tested received a continuous water flow of 25 L h^{-1} (0.5 renewal each hour), with outflowing water being redirected to the general outflowing tank and recirculated. The system assembled to perform the present study displayed a total volume of 2 m^3 and, every week, approximately 3% of this volume was added as fresh water to compensate for evaporation losses.

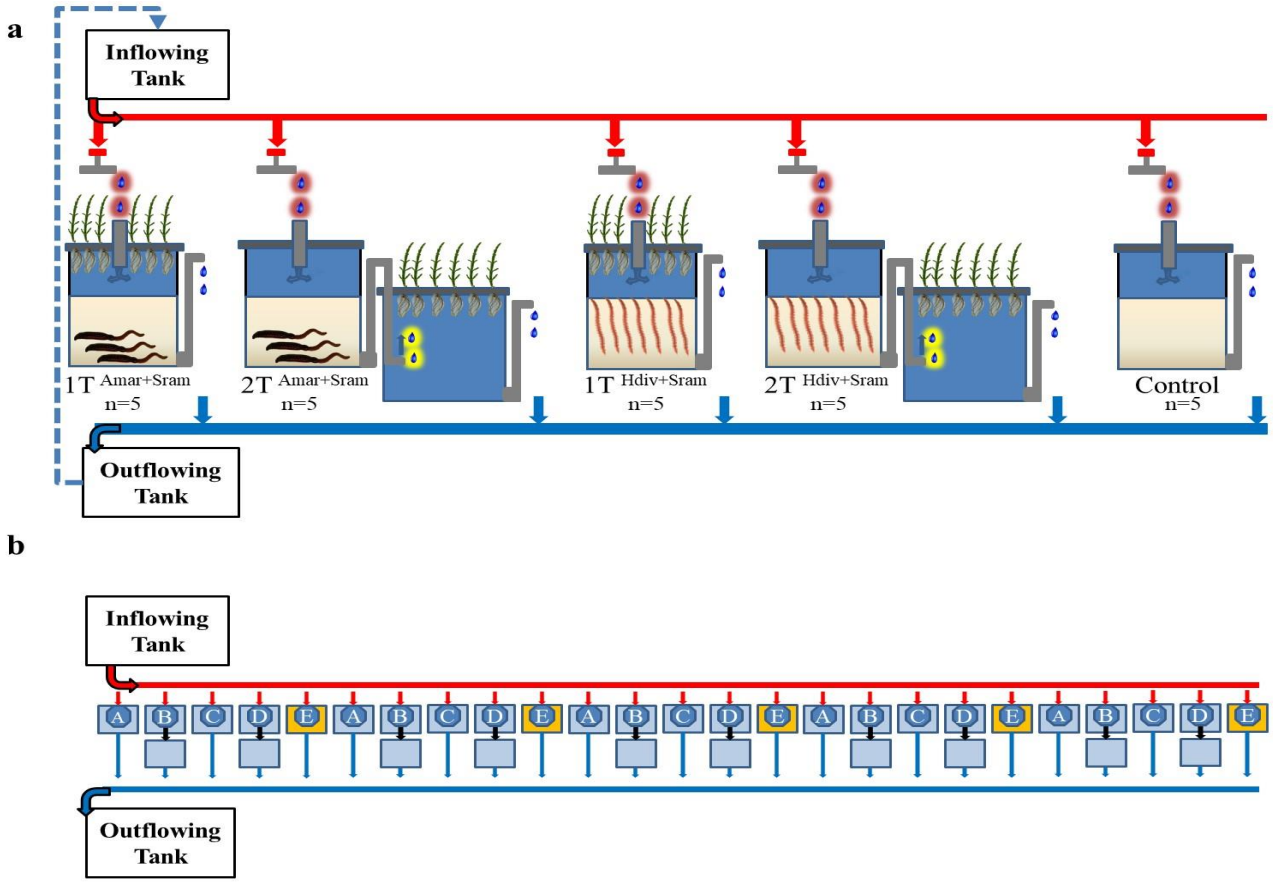


Figure 5.1. Schematic representation (1a) and distribution (1b) of different IMTA designs tested in the present study using as extractive species polychaetes (*Arenicola marina* – Amar – and *Hediste diversicolor* – Hdiv) and halophytes (*Salicornia ramosissima* – Sram) cultured in the same tank (1T – designs A and C) or in two separate tanks (2T – designs B and D): A) 1T Amar+Sram; B) 2T Amar+Sram; C) 1T Hdiv+Sram; D) 2T Hdiv+Sram; and E) control tanks with no extractive species.

5.1.2.3. IMTA extractive species culture and monitoring

Wild specimens of *A. marina* and *H. diversicolor* were collected in the coastal lagoon Ria de Aveiro by local fisherman. Culture tanks were inoculated with an initial stocking density of ≈67 ind. m^{-2} *A. marina* and 290 ind. m^{-2} of *H. diversicolor* (≈130 g FW m^{-2} for both polychaete species). Polychaetes were randomly distributed over the different IMTA designs 15 days before the beginning of the experiment for acclimation.

All plants of *S. ramosissima* used in the present work were germinated at RiaSearch Lda. Seeds were sown in trays containing a mix of coconut fibre and sand and were always kept outdoors under natural conditions of photoperiod and temperature. For 3 months, the coconut fibre was maintained wet through irrigation with ground brackish water pre-treated with chemical oxidation and rapid sand filtration seawater at a salinity 20 g L^{-1}. After this period, plants with similar weight (0.5 - 0.6 g) were randomly selected and distributed over each tank of the different IMTA designs (25 plants per tank = 83 plants m^{-2}) to start a two-week acclimation period.

Plants cultured in aquaponics were harvested 60 days after the beginning of the experiment to determine total plant biomass, as well as edible aboveground (shoots) and belowground biomass (roots). Due to the detection of *H. diversicolor* larvae in PASFs 60 days after the beginning of the experiment, the experimental period was prolonged for another 60 days (for a total of 120 days in total) but without any halophytes. During these additional 60 days, the addition of nutrient-rich water, as well as all monitoring and maintenance routines were performed exactly as during the first 60 days. At the end of experiment (120 days) the entire sand column of each PASFs was sieved and immediately transported to laboratory under refrigerated conditions where polychaetes from both species were sorted, counted and weighted.

5.1.2.4. IMTA monitoring

During the whole experimental period, pH, temperature, dissolved oxygen (DO) and salinity were monitored weekly in the inflowing water of the experimental set up using a multiparameter probe (Lovibond SensoDirect 150). Samples from inflowing and outflowing water from each replicate of the four IMTA designs being tested were collected after 15, 30, 45, 60, 90 and 120 days of the beginning of the experiment.

Samples of water entering all IMTA designs were collected after the nutrient rich water stored in the reservoir tank had been added to the outflowing tank and homogenized for at least 20 min prior being supplied to the inflowing tank (total volume of nutrient rich water = 0.68 m^3). The samples of water exiting 1T and 2T designs were collected 2 and 4 hours after the addition of nutrient-rich water, respectively, being these the times required to complete a full renewal cycle, respectively. The following parameters were determined at all sampling days: suspended particulate matter (SPM), particulate organic matter (POM), total nitrogen (TN) and phosphorus (TP) and dissolved inorganic nitrogen (DIN = NO_x-N + NH_4-N) and dissolved inorganic phosphorus (DIP = PO_4-P). After the harvesting of the whole plant biomass (at day 60), DIN and DIP were no longer determined. All water samples were transported to the laboratory under dark and refrigerated conditions and immediately filtered (Whatman GF/C, Ø 47 mm dehydrated (105 °C) and pre-weighed filters) and subsequently frozen (−20 °C) until further analysis. Water analysis were performed using an automated continuous flow analyser (Skalar San ++) to determine TN, TP, NH_4-N, NO_x-N and PO_4-P. The analytical quality control was ensured by using calibration curves that were calculated from running standard solutions at the beginning and in parallel with blanks and samples. Filters containing SPM were processed following the EPA method 160.2. Samples from control tanks were not considered, as during the study period the sand in the bottom of these tanks was clogged with particulate material and water overflowed. For this reason, no comparison of bioremediation performance was possible. Sediment samples from each sand filter were collected in triplicate at the beginning and at the end of experiment to determine the organic matter (OM) content in the sediment. This determination was performed using the loss of ignition method (LOI%; 5 h combustion at 450 ºC of substratum previously dried at 90 ºC, until a constant weight was achieved).

5.1.2.5. Determination of photosynthetic pigments of SALICORNIA RAMOSISSIMA

Samples from the edible aerial part of *S. ramosissima* (n=5) were collected from the four IMTA designs after 60 days, with samples from 1T treatments being pooled (Amar+Sram and Hdiv+Sram), as well as those from 2T treatments (Amar+Sram and Hdiv+Sram). Samples were also collected from plants used at the beginning of the experiment (n=5) and from conspecifics collected from the wild (Ria de Aveiro coastal lagoon) (n=5). The collection of halophyte plants from the wild was performed in

compliance with current Portuguese and EU guidelines, legislation and codes of good practices framing the collection of living resources from the wild. All samples were frozen in liquid nitrogen and kept at -80 ºC until freeze-dried. Samples were grounded with mortar and pestle and 7-8 mg were weighed into Eppendorf tubes. Pigments were extracted using 0.5 mL of 95% cold buffered methanol (2% ammonium acetate), followed by 45 s sonication and 20 min incubation at -20ºC in the dark. Extracts were filtered through 0.2 μm PTFE membrane filters and 50 μL were immediately injected into a HPLC system with a photodiode array detector SPD-M20A (Shimadzu, Kyoto, Japan). Chromatographic separation was carried out using a Supelcosil C18 column (25 cm length; 4.6 mm diameter; 5 μm particles; Sigma-Aldrich, St. Louis, MO, USA) following Mendes *et al.* (2007)[62]. Pigments were identified from absorbance spectra and retention times and concentrations were calculated using linear regression equations obtained from pure crystalline standards (DHI, Hørsolm, Denmark).

5.1.2.6. Statistical analysis

To evaluate the existence of significant differences in the bioremediation performance (POM, DIN and DIP concentration in outflowing water and OM present in PASFs substratum) and the production of biomass (polychaetes: final biomass; halophytes: final biomass, density, average weight, aboveground and belowground biomass) from the four different IMTA designs (n=5) two-way analysis of variance (ANOVA) were performed with polychaete species (two levels - *A. marina* and *H. diversicolor*) and operational area (1T and 2T) being used as predictive factors. To evaluate the existence of significant differences in the pigments concentration and pigments ratios exhibited between *S. ramosissima* cultured in 1T and 2T designs, in plants initially stocked in the experimental system and conspecifics collected from the wild (n=5) a one-way analysis of variance (ANOVA) was performed with biomass source (4 levels – 1T, 2T, initial and wild) being used as predictive factor. Data were previously checked for normality and homogeneity of variances through Anderson-Darling, Bartlett´s and Levene´s tests. Post-hoc Tukey's HSD tests for individual means comparison were performed whenever significance was observed. When a condition of normality was not verified, the hypotheses were tested using Johnson transformed data. Significant differences were always considered at $p < 0.05$.

All the above-mentioned statistical analysis was performed using Minitab 18 Statistical Software (State College, PA). The statistical results of the tests mentioned above are summarized in supplementary Tables S5.2-S5.7.

5.1.3. Results

5.1.3.1. Characterization of abiotic conditions and inflowing water composition

The average values of inflowing water abiotic conditions and composition monitored during the experiment are summarized in Table 5.1 (characterization over time – Supplementary Fig. S5.1a-d and S5.2a-c, respectively). Particulate organic matter (POM) and dissolved inorganic nitrogen and phosphorus (DIN and DIP) monitored in the inflowing water accounted for 75% of total suspended particulate matter (SPM), 70-75% of total nitrogen (TN) and 75-85% of total phosphorus (TP), respectively. During the polyculture trial combining polychaetes and halophytes (60 days) it was estimated that each tank filtered 490 L of effluent, which contained ≈ 5.6 g POM, 5.5 g TN (74% DIN) and 1.1 g TP (84% DIP). In the second period (60-120 days) where only polychaetes were maintained it was estimated that each tank filtered 462 L of effluent water, which contained ≈5.4 g POM, 6.9 g TN (74% DIN) and 1.4 g TP (75% DIP) (Supplementary Table S5.1).

Table 5.1. Abiotic conditions (pH, oxygen, temperature and salinity) and composition (suspended particulate matter [SPM], particulate organic matter [POM; %LOI in SPM], total nitrogen [TN], total phosphorus [TP], dissolved inorganic nitrogen [DIN] and phosphorus [DIP]) measured in inflowing water. Average values ± SD (n=5).

Parameter	**Study period**	
	1 - 60 days	**61 - 120 days**
	Polychaetes + Halophytes	*Polychaetes*
Inflowing water abiotic conditions		
pH	8.28 ± 0.07	8.25 ± 0.07
DO (mg L^{-1})	7.70 ± 0.48	8.52 ± 0.44
Temperature (ºC)	23.81 ± 1.88	19.45 ± 2.44
Salinity (ppt)	18.90 ± 0.82	17.38 ± 2.57
Inflowing water composition		
SPM (mg L^{-1})	15.14 ± 4.04	15.89 ± 0.85
POM (mg L^{-1})	11.49 ± 2.85	11.70 ± 0.42
TN (mg L^{-1})	11.13 ± 3.91	14.86 ± 0.83
DIN (mg L^{-1})	8.25 ± 3.92	10.93 ± 0.35
TP (mg L^{-1})	2.24 ± 0.86	3.09 ± 0.66
DIP (mg L^{-1})	1.89 ± 0.84	2.32 ± 0.47

5.1.3.2. Bioremediation of particulate organic matter (POM) and generation of polychaetes biomass.

The concentrations of POM quantified in the inflowing and outflowing water of 1T and 2T IMTA designs stocked with *A. marina* and *H. diversicolor* over the study period (120 days) are presented in Figure 5.2. No significant differences were found in POM concentration monitored in outflowing effluent of different IMTA designs (*Two-way*

ANOVA, $p>0.05$), with the values corresponding to retention efficiencies between 84 - 87% of inflowing POM.

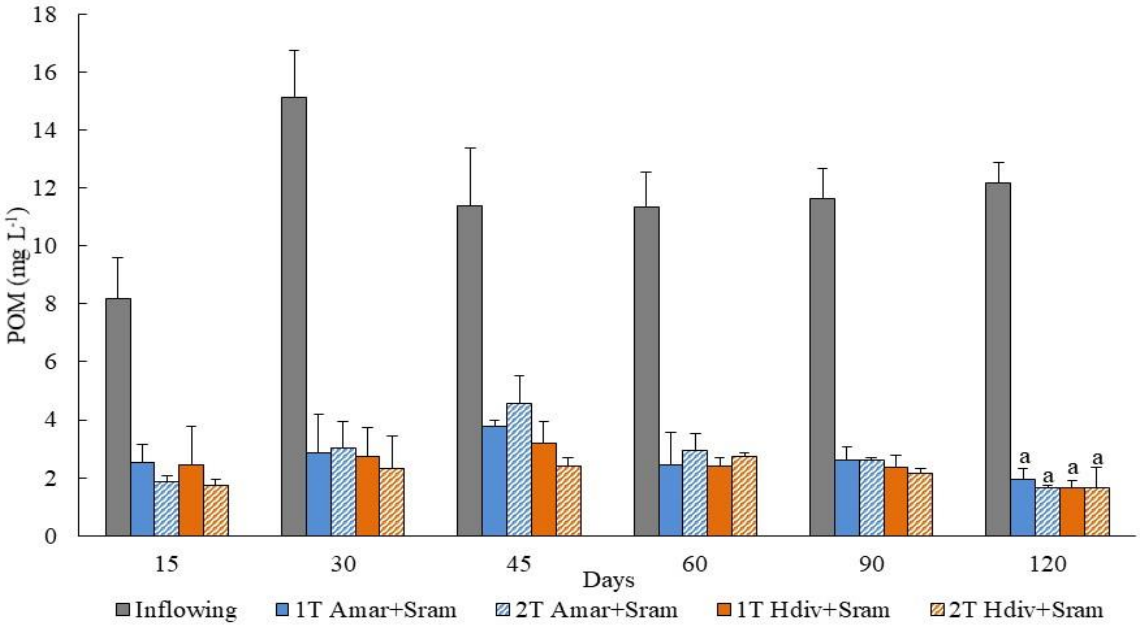


Figure 5.2. Particulate organic matter (POM) quantified in the inflowing and outflowing efluent of IMTA designs tested in the present study using as extractive species polychaetes (*Arenicola marina* – Amar – and *Hediste diversicolor* – Hdiv) and halophytes (*Salicornia ramosissima* – Sram) cultured in the same tank (1T) or in two separate tanks (2T). Average values ± SD (n=5). Statistical analysis performed only at 120 days when the biomass of extractive species was evaluated. No significant differences ($p<0.05$) between IMTA designs were observed.

The concentration of OM (determined through loss of ignition - %LOI) quantified at the end of experiment in the top 20 mm of the substratum of polychaetes assisted sand filters (PASFs) stocked with *A. marina* and *H. diversicolor* are displayed in Figure 5.3a - b. No significant differences between treatments were found in OM content monitored in the top 20 mm (*Post-hoc* Tukey HSD, $p>0.05$) and 20 – 100 mm substratum layers (*Two-way ANOVA*, $p>0.05$), with values ranging between 0.25 – 0.34 and 0.27 – 0.30% LOI, respectively.

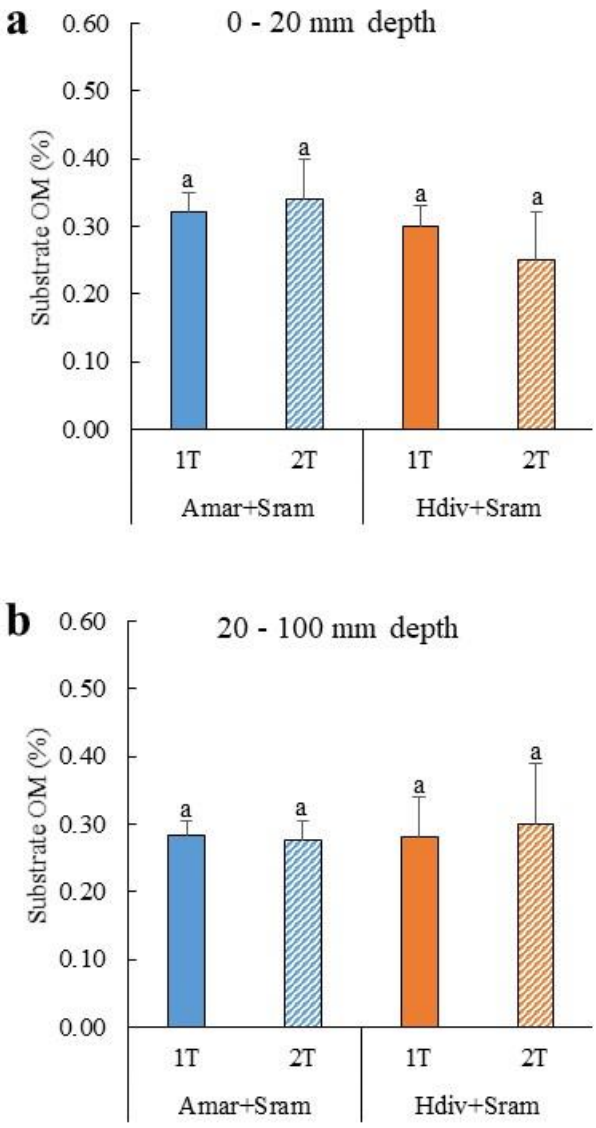


Figure 5.3. Organic matter (OM) content determined in the top 20 mm and 20–100 mm substratum layers of IMTA designs tested in the present study using as extractive species polychaetes (*Arenicola marina* – Amar – and *Hediste diversicolor* – Hdiv) and halophytes (*Salicornia ramosissima* – Sram) cultured in the same tank (1T) or in two separate tanks (2T). Average values ± SD (n=5). No significant differences (p<0.05) between IMTA designs were observed.

The average values (±SD) of biomass and density of *A. marina* and *H. diversicolor* determined at the end of the experiment are displayed in Table 5.2. For *A. marina* mortalities between 90-95% were observed revealing that the experimental conditions impaired the successful culture of this polychaete species. Regarding *H. diversicolor,* the final densities obtained for both operational IMTA designs (1T and 2T) were ≈14-15 times that of initial values. These polychaetes corresponded to newly generated biomass of different sizes, including worms with 20–30 mm length (Fig. 5.4). The biomass of *H.*

diversicolor was significantly higher to the one produced by *A. marina* independently of operational design tested (*Post-hoc* Tukey HSD, $p<0.05$). Between 1T and 2T designs stocked with the same polychaete species no significant differences were found in biomass produced (*Post-hoc* Tukey HSD, $p>0.05$).

Table 5.2. Density and total biomass of polychaetes (*Arenicola marina* – Amar – and *Hediste diversicolor* – Hdiv) cultured in the same tank with halophytes (*Salicornia ramosissima* – Sram) (1T) or in two separate tanks (2T) at day 120. Average values ± SD (n=5). Different letters indicate significant differences ($p<0.05$) between IMTA designs. The values between brackets indicate the dry weight biomass.

IMTA design	**Density** (ind. m^{-2})	**Total biomass** (g m^{-2})
1T Amar+Sram	3 ± 2	2.4 ± 2.7^{a} (0.2 ± 0.3)
2T Amar+Sram	5 ± 3	5.5 ± 3.9^{a} (0.5 ± 0.4)
1T Hdiv+Sram	3993 ± 1496	98.4 ± 25.7^{b} (13.5 ± 3.7)
2T Hdiv+Sram	4425 ± 540	77.5 ± 11.2^{b} (10.8 ± 1.5)

Figure 5.4. *Hediste diversicolor* juveniles produced after 120 days of culture.

5.1.3.3. Extraction of dissolved inorganic nitrogen and phosphorus (DIN and DIP, respectively) and generation of SALICORNIA RAMOSISSIMA *biomass.*

The concentrations of DIN and DIP monitored in the inflowing and outflowing effluent of 1T and 2T IMTA designs stocked with Amar+Sram and Hdiv+Sram at day 60 are displayed in Figure 5.5 and 5.6, respectively. The DIN concentration monitored in outflowing water of 2T Hdiv+Sram was significantly lower than the one exhibited by 1T Hdiv+Sram (*Post-hoc* Tukey HSD, $p<0.05$), while between remaining IMTA designs no significant differences were found (*Post-hoc* Tukey HSD, $p>0.05$). Concerning DIP, no significant differences were found in concentrations measured in outflowing water of different IMTA designs (Two-way ANOVA, $p>0.05$). Bioremediation efficiencies of 48-66% and 52-56% were observed for inflowing DIN and DIP, respectively.

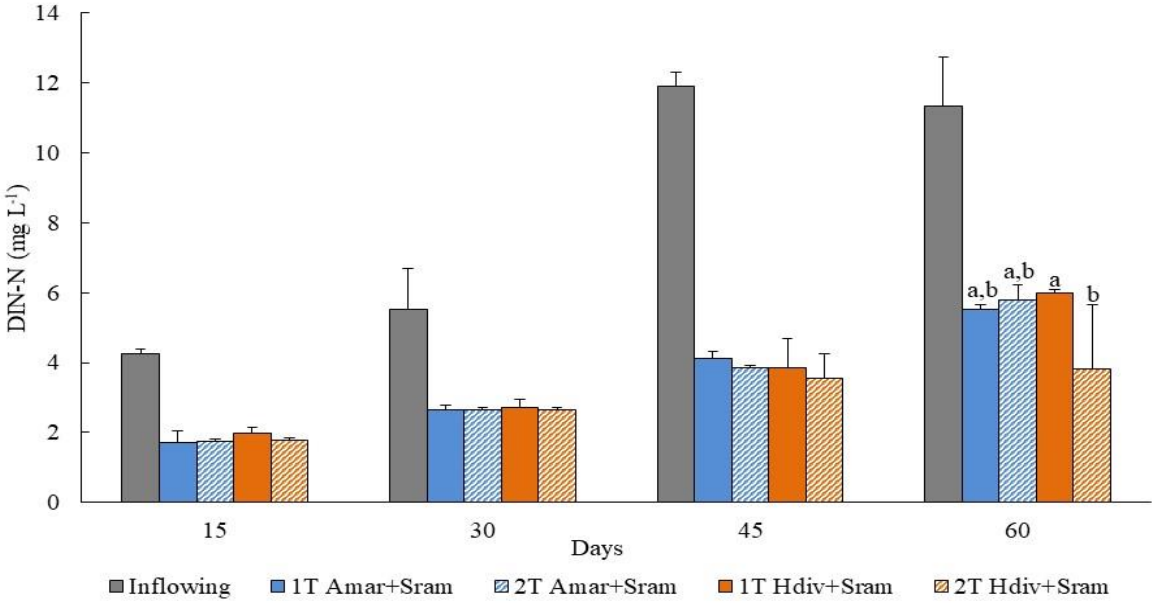


Figure 5.5. Dissolved inorganic nitrogen (DIN) quantified in the inflowing and outflowing effluent of IMTA designs tested in the present study using as extractive species polychaetes (*Arenicola marina* – Amar – and *Hediste diversicolor* – Hdiv) and halophytes (*Salicornia ramosissima* – Sram) cultured in the same tank (1T) or in two separate tanks (2T). Average values ± SD (n=5). Statistical analysis performed only for the period of 120 days when biomass was evaluated. Different letters indicate significant differences ($p<0.05$) between IMTA designs.

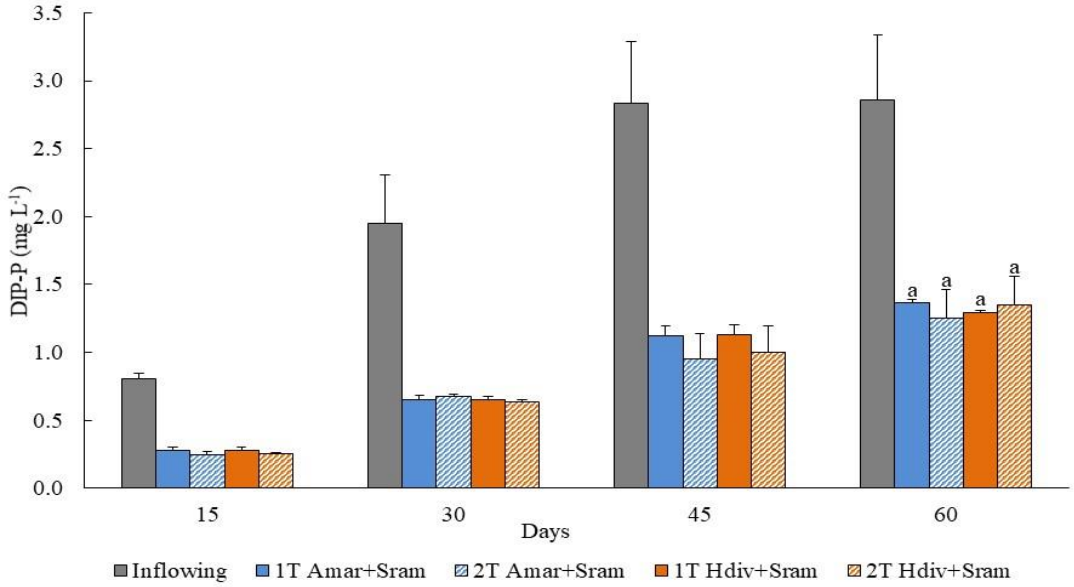


Figure 5.6. Dissolved inorganic phosphorus (DIP) quantified in the inflowing and outflowing effluent of IMTA designs tested using as extractive species polychaetes (*Arenicola marina* – Amar – and *Hediste diversicolor* – Hdiv) and halophytes (*Salicornia ramosissima* – Sram) cultured in the same tank (1T) or in two separate tanks (2T). Average values ±SD (n=5). Statistical analysis performed only at 120 days when the biomass of extractive species was evaluated. No significant differences (p<0.05) between IMTA designs were observed.

The average weight of *S. ramosissima* cultured under different IMTA designs stocked with Amar+Sram and Hdiv+Sram are displayed in Figure 5.7. At day 60, the plants grown on 1T designs revealed a significantly higher average weight (≈2-times higher) than the ones reported in 2T designs independently of stocked polychaete species (*Post-hoc* Tukey HSD, p<0.05).

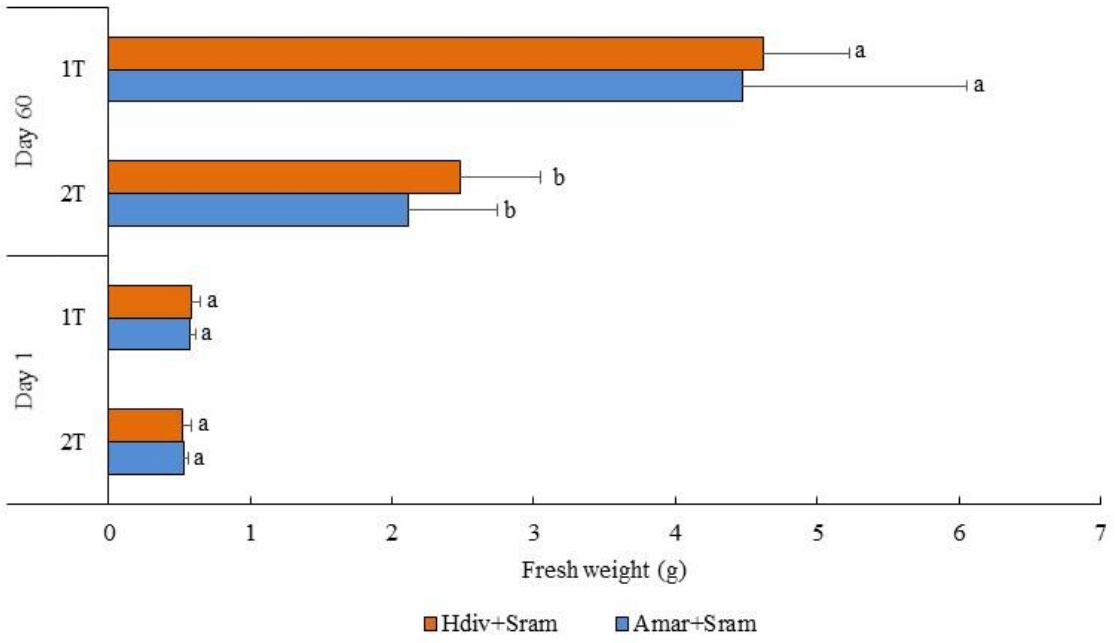


Figure 5.7. Fresh weight of *Salicornia ramosissima* (Sram) at day 1 and 60 cultured in the same tank (1T) or in two separate tanks (2T) than polychaetes (*Arenicola marina* – Amar – and *Hediste diversicolor* – Hdiv). Average ±SD (n=5). Different letters in each time-period indicates significant differences (p<0.05) between IMTA designs.

Table 5.3 summarizes the average values (±SD) of density and total biomass reported at day 60. In this period, 36-40% and 44-56% of the plants initially stocked in both 1T and 2T IMTA designs enter senescence and were considered not viable to biomass account, respectively.

Table 5.3. Final density, total plant fresh weight biomass, aboveground and belowground fresh weight biomass of halophytes (*Salicornia ramosissima* - Sram) cultured in the same tank (1T) or in separate tanks (2T) with polychaetes (*Arenicola marina* – Amar – and *Hediste diversicolor* – Hdiv) at day 60. Average values ±SD (n=5). Different letters indicate significant differences (p<0.05) between IMTA designs. The values between brackets indicate the dry weight biomass.

IMTA design	Density (Plants m^{-2})	Total plant ($g\ m^{-2}$)	Aboveground ($g\ m^{-2}$)	Belowground ($g\ m^{-2}$)
1T Amar+Sram	49 ± 6[a]	194.2 ± 80.5 [a,b] (15.8 ± 7.4)	148.6 ± 68.8 [a,b] (11.6 ± 5.4)	53.2 ± 25.4 [a] (4.3 ± 2.0)
2T Amar+Sram	37 ± 9[a]	84.9 ± 34.8 [c] (6.7 ± 2.7)	63.3 ± 26.3 [c] (4.9 ± 2.0)	21.8 ± 8.8 [b] (1.7 ± 0.7)
1T Hdiv+Sram	54 ± 9[a]	225.6 ± 54.4 [a] (17.7 ± 4.3)	171.1 ± 39.2 [a] (13.3 ± 3.1)	54.6 ± 15.3 [a] (4.4 ± 1.2)
2T Hdiv+Sram	47 ± 14[a]	114.1 ± 30.1 [b,c] (9.0 ± 2.3)	86.3 ± 22.2 [b,c] (6.7 ± 1.7)	27.8 ± 7.8 [a,b] (2.2 ± 0.6)

No significant differences were verified in final plant density reported in 1T and 2T designs (*Post-hoc* Tukey HSD, p<0.05). The total plant biomass and inherent aboveground biomass generated by both 1T designs were significantly higher than the ones reported in 2T (*Post-hoc* Tukey HSD, p<0.05), except between 2T Hdiv+Sram and 1T Amar+Sram (*Post-hoc* Tukey HSD, p>0.05). The belowground biomass produced was higher in 1T designs, with the values obtained in 1T Amar+Sram being significantly higher than the ones obtained in 2T Amar+Sram (*Post-hoc* Tukey HSD, p<0.05). The final biomass reported in 1T and 2T designs was ≈ 4.0 – 5.1 and 1.9 – 2.5 times higher than the initially stocked values, respectively. The aboveground fresh weight (FW) biomass represented 70-80% of total plant biomass produced, while their dry weight (DW) biomass corresponded to approximately 8% of the FW value. The plants acquired a yellowish coloration over the study (Fig. 5.8 a-c) with a large percentage of them going into senescence.

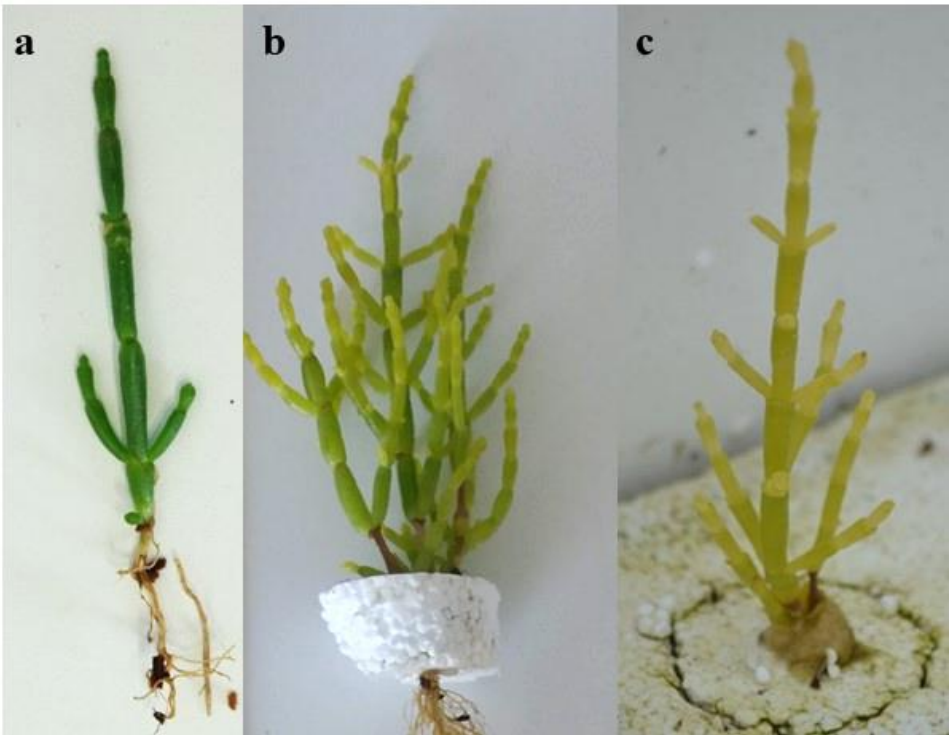


Figure 5.8. Evolution of coloration of *Salicornia ramosissima* over the experimental period: a) plants at day 1; b) plants at day 30 and c) plants at day 60.

5.1.3.4. Pigment profile of SALICORNIA RAMOSISSIMA *cultured under 1T and 2T IMTA designs*

The pigments recorded in *S. ramosissima* initially stocked, cultured and collected from the wild were the carotenoids 9'-*cis*-neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and β,β-carotene and the chlorophylls *a* and *b* (Chl *a* and Chl *b*) (Table 5.4). The average values (±SD) of pigment concentrations identified in *S. ramosissima* cultured under 1T and 2T IMTA designs, as well as the profile of initially stocked plants and wild conspecifics are displayed in Table 5.5. Concentrations of 9'-*cis*-neoxanthin, violaxanthin, lutein, β,β-carotene, Chls *a* and *b* were significantly higher in initially stocked and wild conspecifics compared to plants cultured under 1T and 2T (*Post-hoc* Tukey HSD, $p<0.05$). On the other hand, significant higher concentrations of zeaxanthin were observed in plants cultured under 1T and 2T when compared to initially stocked and wild plants (*Post-hoc* Tukey HSD, $p<0.05$). No significant differences in pigments profile was verified between plants cultured under 1T and 2T designs (*Post-hoc* Tukey HSD, $p>0.05$). The Chl *b*/Chl *a* ratio of cultured plants was lower to the ones exhibited by initially stocked and wild conspecifics (≈ 2-times lower), while total

carotenoids/chlorophyll and zeaxanthin/carotenoids ratios were higher (≈2.2 – 3.4 and 36 – 46 -times, respectively) (Fig. 5.9 a-c). Significant differences were found in the above-mentioned ratios between cultured and initially stocked and wild conspecifics plants (*Post-hoc* Tukey HSD, $p<0.05$).

Table 5.4. List of pigments detected in halophytes (*Salicornia ramosissima*) with average retention times and absorption maxima (λ max).

	Retention time	**λ max (nm)**
9'-*cis*-Neoxanthin	12.96	416, 438, 467
Violaxanthin	14.17	417, 441, 472
Anteraxanthin	16.10	424, 448, 477
Lutein	17.74	425, 448, 476
Zeaxanthin	17.99	430, 454, 481
Chlorophyll *b*	22.83	458, 596, 646
Chlorophyll *a*	24.46	430, 617, 663
β,β-Carotene	28.78	430, 454, 480

Table 5.5. Pigment concentrations (μg g^{-1} DW biomass) recorded in halophytes (*Salicornia ramosissima*) cultured cultured in the same tank (1T) or in separate tanks (2T) with polychaetes (*Arenicola marina* – Amar – and *Hediste diversicolor* – Hdiv), as well as initially stocked plants and conspecifics from the wild. Average values ± SD (n=5). Different letters indicate significant differences ($p<0.05$) between samples.

	1T	**2T**	**Initial**	**Wild**
9'-*cis*-Neoxanthin	2.8 ± 1.2^{a}	2.3 ± 0.5^{a}	73.7 ± 13.9^{b}	58.8 ± 7.8^{b}
Violaxanthin	7.4 ± 6.9^{a}	5.0 ± 1.1^{a}	182.7 ± 31.6^{b}	183.8 ± 29.7^{b}
Anteraxanthin	11.2 ± 3.6^{a}	9.6 ± 1.9^{a}	14.1 ± 2.9^{a}	15.6 ± 3.5^{a}
Lutein	31.0 ± 8.9^{a}	29.1 ± 4.4^{a}	353.7 ± 51.0^{b}	259.6 ± 37.8^{c}
Zeaxanthin	56.7 ± 14.7^{a}	59.5 ± 7.8^{a}	18.8 ± 4.1^{b}	12.6 ± 2.3^{b}
Chlorophyll *b*	25.5 ± 11.0^{a}	16.1 ± 5.9^{a}	661.4 ± 85.5^{b}	508.2 ± 74.3^{b}
Chlorophyll *a*	$16.5.9 \pm 33.5^{a}$	123.8 ± 34.3^{a}	1912.1 ± 231.9^{b}	1695.7 ± 262.8^{b}
β,β-Carotene	9.8 ± 1.4^{a}	9.0 ± 2.7^{a}	106.8 ± 13.0^{b}	95.1 ± 20.1^{b}

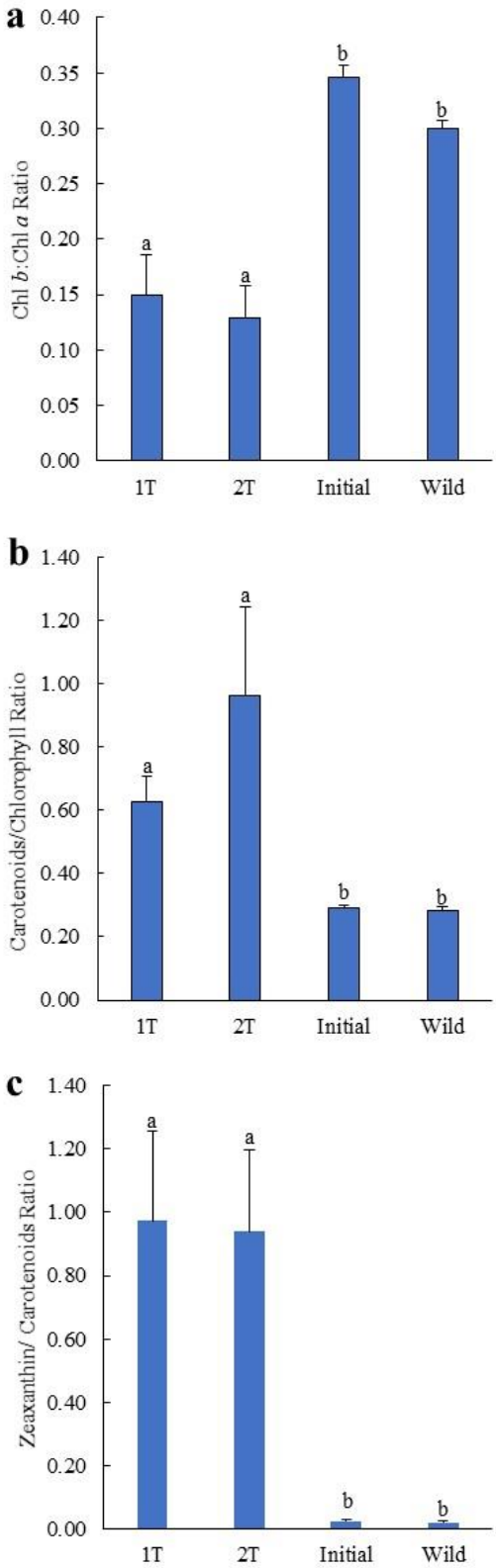


Figure 5.9. Chlorophyll *b*:chlorophyll *a* (Chl *b*/Chl *a*), total carotenoids/chlorophyll and zeaxanthin/carotenoids ratios measured in *Salicornia ramosissima* cultured in the same tank (1T) or in two separate tanks (2T) than polychaetes (*Arenicola marina* and *Hediste diversicolor*). Average values ±SD (n=5). Different letters indicate significant differences (p<0.05) between samples.

5.1.4. Discussion and conclusions

This work aimed to compare the bioremediation performance and biomass production through the combined culture of *A. marina* and *S. ramosissima* (Amar+Sram) and *H. diversicolor* and *S. ramosissima* (Hdiv+Sram) using a single polyculture tank (1T) and two trophic levels separated tanks (2T), IMTA designs with different operational areas (0.3 and 0.6 m^2, respectively). The experiment was performed outdoors, and great variability was verified for salinity and water temperature (*ca.* 12-20 g of salt per litre, 16-28 ºC, respectively), while for pH and dissolved oxygen (DO) more stable values were reported (8.1-8.4 and 6.8-9.5 mg L^{-1}, respectively). Despite the variability, all these parameters are within the range of values monitored in water and intertidal water pools of Aveiro coastal lagoon[63,64], place of collection of polychaetes used in the present study.

Bioremediation efficiencies of 74-87% POM, 56-64% DIN and 60-65% DIP of inflowing effluent, were reported in 1T and 2T IMTA designs stocked with Amar+Sram and Hdiv+Sram. The OM in the top 20 and 20 - 100 mm substratum depth was reported between 0.2 – 0.4 % LOI and no differences were verified in IMTA designs stocked with *A. marina* and *H. diversicolor*. Polychaete assisted sand filters are highly efficient in the retention of POM which is incorporated into valuable extractive worm biomass[11,41,65-67]. A very important role was played by the sand bed of PASFs by retaining and keeping OM available to sustain the growth of polychaetes, while these organisms promoted bioturbation (sediment re-working and bio-irrigation, which enables dissolved oxygen to reach deeper layers of the substratum), a feature which is paramount to avoid the clogging of the system and maintain the percolation of water through the sand bed[65]. This effect was clearly showed in the current study, as at the end of experimental period each of the designs tested stocked with polychaetes remained operational, while control tanks were clogged and water overflowed. The conversion of POM into valuable worm biomass is expected to occur in PASFs, although this was not the case for those stocked with *A. marina* which revealed mortalities above 90%. The biomass of this polychaete species was significantly lower than the one produced in designs stocked with *H. diversicolor*, however it is important to keep in mind that these differences result from the high mortality reported in 1T and 2T designs stocked with *A. marina*. When comparing the biomass of this polychaete (iteroparous species which reaches sexual maturity at 2-3 years of age) to the one produced by *H. diversicolor* which revealed a completely different life cycle (semelparous species which reaches sexual maturity at 1-2 years of age), we also

have to keep in mind that in a 120-day study, at best, we would only be able to compare the biomass gain of initially stocked individuals of *A. marina* with the biomass gain resulting from juveniles of *H. diversicolor* produced in the same period. Although temperatures above 20ºC are frequently monitored in intertidal water pools[63,64] next to wild stocks of *A. marina*, previous results showed that temperature above this threshold may have impaired the success culture of this polychaete species. For example, in a previous study carried out with temperatures between 12 - 20ºC which aimed to evaluate the viability of *A. marina* under IMTA conditions, the survival reported was higher than 90% at a density of ≈150 ind. m^{-2}; these worms were fed with fish waste and displayed a considerable increment of biomass (from 80 to 480 g m^{-2} during 55 days)[68]. In another study, the best growth performances of these worms were achieved when they were provided with a formulated fish feed and salmon faeces (growth rate ≈ 32% and 23% after 14 and 39 days, respectively), outgrowing conspecifics fed with other diets (e.g., fresh seaweed and organic matter in sediment without additional feed)[69]. The culture of this polychaete species was also evaluated using a substrate containing 25% of mud from aquaculture and 75% sand, with worms revealing an average growth of 106% after 39 days[70]. Mortalities reported in the two last studies referred were likely linked with water temperature also being recorded above optimal values, thus reinforcing the need to strictly control this parameter when aiming to culture *A. marina* outdoors.

Concerning *H. diversicolor*, the culture conditions employed proved to be adequate for their development, with the final densities recorded in 1T and 2T IMTA designs being approximately 15 times that of the initial stocking densities (final density ≈4000 – 5000 ind. m^{-2}). No significant differences were found between *H. diversicolor* FW biomass obtained in 1T and 2T designs, with the values obtained at the end being lower than the one present at the beginning of experiment (≈77 – 100 vs 130 g m^{-2}, respectively). Nonetheless, it is important to highlight that the specimens at the end of the experiment corresponded to a newly generated population of juveniles that was just starting to grow and yet to achieve commercial size. *Hediste diversicolor* is characterized by a single reproductive episode before its death (being a semelparous species) and in the literature it is possible to find studies performed during longer periods which evaluated the productivity in terms of juvenile biomass originated from initially stocked polychaetes. For example, in a study performed over a longer period (≈150 days), similar results to the ones recorded in the present study were obtained in terms of increment of polychaetes density, from ≈400 to 7000 ind. m^{-2}, in PASFs stocked with *H. diversicolor* being

supplied an organic-rich effluent generated by a super-intensive commercial RAS producing *Solea senegalensis*[11]. In this last-mentioned study, a combined culture of polychaetes and halophyte plants (*Halimione portulacoides*) was performed, with extractive species being cultured in separate tanks and contributing to remove 70% of POM and 65% of DIN, respectively. As mentioned before, the culture in separate tanks require a larger operational area, which is often pointed as one of the major constraints to successfully develop IMTA framework for new or ongoing operations[29]. On studies performed over shorter periods (less than 60 days), this polychaete species was tested under different culture densities (250 – 2000 ind. m^{-2}; 5 – 40 g m^{-2}) to bioremediate the solid waste generated from tanks stocked with rainbow trout (*Oncorhynchus mykiss*), with increments of polychaete biomass between 2.4 – 6-times the initial values reported (29.7 – 96.07 g m^{-2})[41]. This polychaete species was also tested during 8 weeks on the bioremediation of effluents generated by the farming of great sturgeon (*Huso huso*), with a decrease of density from 2.000 ind. m^{-2} to ≈1.510 ind. m^{-2} and an increase of biomass gain of ≈ 233 g m^{-2} (SGR≈3.4% d^{-1}) being reported[42].

Concerning the growth performance of halophytes, a significant higher average weight (≈2-times higher) was reported for plants cultured under 1T design, independently of the polychaete species being stocked. The total biomass reported in 1T and 2T designs after 60 days of experimental trial accounted for 5 - 5.7 and 1.7-2.4-times higher than the initially stocked biomass, representing productivities of edible aboveground biomass of ≈ 150 - 170 and 60 - 90 g m^{-2}, respectively. These values were lower than the ones obtained in previous works, such as for *S. bigelovii* cultured under hydroponic conditions at a density 3-times higher than the ones used in the present study (seedlings with ≈30 mm height planted at a density of ≈260 plants m^{-2}) featured a marketable yield ≈ 1.7 Kg m^{-2} after 28 days[71]. In another study, a productivity of ≈13 Kg (≈0.9 Kg m^{-2}) was reported for *S. dolichostachya* (at ≈38 plants m^{-2}) cultured in a zero-water-exchange RAS-IMTA (culture area - 4.8 m^2) during a 35-days trial[34]. In the present study, the culture conditions impaired *S. ramosissima* development. Very early on the trial, plants started to develop a yellowish coloration, with some even exhibiting evident signs of senescence. At the end of experimental period, approximately 40 to 60% of the plants were no longer viable, with this percentage being slightly higher in the 2T IMTA design. The development of the yellowish coloration was most likely related to a lack of iron, as all the saltwater used in the shrimp farming system and RAS-IMTA design was pumped from a borehole and pre-treated through chemical oxidation. This treatment promotes the precipitation and

removal of iron, along with other elements such as Mg, P, Ca and Mn[49,72]. These precipitates are then rapidly removed through the action of sand filters[36]. The development of a yellowish coloration was previously reported for *Tripolium pannonicum* cultured under aquaponic conditions in a zero-water-exchange RAS-IMTA[34] and for *S. dolichostachya* cultured at very low salinities (0-5 mM NaCl) under hydroponic conditions[73]. These results may reveal a lack of key elements (micronutrients) to promote plant growth, such as Fe, Zn and Ca, which may be biofortified through fertilizers[74]. Another explanation for the yellowish coloration and premature senescence may be associated with the fact of the nutrient rich water employed in this study being stored for 3 days in a reservoir tank without aeration (a submerged pump only mixed the water 5 min every hour). These conditions may have favoured the production of toxic gases, such as hydrogen sulfide (H_2S), which in plants has already proven to be a crucial player in the regulation of plant growth, development, and senescence[75]. However, it is important to note that plants of the genus *Salicornia* occur in wild predominantly at lower marshes where anoxic conditions were found[76] and the sulfide accumulation can be high[77]. Plants cultured in the 1T and 2T IMTA designs exhibited lower content of chlorophylls *a*, *b* and total carotenoids (≈124 – 166, 16 - 26 and 118 -128 µg g^{-1} DW biomass, respectively) than the ones recorded when they were initially stocked, as well as in conspecifics from the wild (≈1,912 – 1,695, 508 – 661 and 625 – 749 µg g^{-1} DW biomass, respectively). This decrease in pigment content may also have been caused by the use of borehole water pre-treated with chemical oxidation. In previous works it was found that the halophyte *T. pannonicum* displayed a significantly lower content of total chlorophyll and carotenoids when exposed to a media without iron supplementation (≈49 and 21 µg g^{-1} FW biomass, respectively) than conspecifics supplemented with this element (≈388 – 875 and 79 –159 µg g^{-1} FW biomass, respectively)[49]. The higher levels of zeaxanthin quantified in cultured plants are probably the result of the activation of the violaxanthin cycle, a two-step cycle in which violaxanthin is converted first to antheraxanthin and the latter pigment is converted to zeaxanthin. The activation of this cycle is photoprotective and activated by high light intensity, but it can also be triggered by other abiotic stressors (e.g., anoxia and high temperature)[78]. On the other hand, these high levels of zeaxanthin are worth further investigation, as this carotenoid plays a critical role in the prevention of age-related eye diseases[79]. Halophytes displaying enhanced levels of zeaxanthin may likely fetch higher values in the functional foods market.

In general, the experimental design 1T exhibited the best performance (i.e., similar bioremediation and polychaetes productivities and the best halophyte productivity). Moreover, it also allows to reduce by half the operational area required to implement an IMTA framework using these extractive species. The present study also revealed the significant limitations inherent to the culture of certain extractive species outdoors, namely when key abiotic conditions, such as water temperature, are difficult to control. In the present study, failing to control this parameter may have impaired the successful culture of *A. marina*. On the other hand, our study also showed that effluents from culture systems using brackish groundwater that has been treated to remove iron through chemical oxidation and rapid sand filtration may impair the use of some extractive species for IMTA. Indeed, the lack of iron (and eventually also other trace elements removed during chemical oxidation and rapid sand filtration) may be a bottleneck impairing the successful production of *S. ramosissima* and other valuable halophyte plants.

5.1.5. Chapter 5 - References

1. Chopin, T. *et al.* Multitrophic Integration for Sustainable Marine Aquaculture. *Encycl. Ecol.* **3**, 2463–2475 (2008).
2. Troell, M. *et al.* Ecological engineering in aquaculture - Potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. *Aquaculture* **297**, 1–9 (2009).
3. OECD & Chopin, T. Integrated Multi-Trophic Aquaculture. *in* Advancing the Aquaculture Agenda: Workshop Proceedings 195–217 (OECD publishing, 2010).
4. FAO, IFAD, UNICEF, W. and W. The State of Food Security and Nutrition in the World 2020. Transforming food systems for affordable healthy diets. ROME (2020).
5. FAO. The state of world fisheries and aquaculture: Sustainability in action. ROME (2020).
6. Tacon, A. G. J. & Metian, M. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture* **285**, 146–158 (2008).
7. Schneider, O., Sereti, V., Eding, E. H. & Verreth, J. A. J. Analysis of nutrient flows in integrated intensive aquaculture systems. *Aquac. Eng.* **32**, 379–401 (2005).
8. Boxman, S. E. *et al.* Performance evaluation of a commercial land-based integrated multi-trophic aquaculture system using constructed wetlands and geotextile bags for solids treatment. *Aquac. Eng.* **69**, 23–36 (2015).
9. Mirzoyan, N. *et al.* Quality of brackish aquaculture sludge and its suitability for anaerobic digestion and methane production in an upflow anaerobic sludge blanket (UASB) reactor. *Aquaculture* **279**, 35–41 (2008).
10. Worsfold, P. J. *et al.* Characterisation and quantification of organic phosphorus and organic nitrogen components in aquatic systems: A Review. *Anal. Chim. Acta* **624**, 37–58 (2008).
11. Marques, B., Calado, R. & Lillebø, A. I. New species for the biomitigation of a super-intensive marine fish farm effluent: Combined use of polychaete-assisted sand filters and halophyte aquaponics. *Sci. Total Environ.* **600**, 1922–1928 (2017).
12. Gunning, D., Maguire, J. & Burnell, G. The Development of Sustainable Saltwater-Based Food Production Systems: A Review of Established and Novel Concepts. *Water* **8**, 598 (2016).
13. Granada, L., Sousa, N., Lemos, M. F. L. & Lopes, S. Is integrated multitrophic aquaculture the solution to the sectors' major challenges? – a review. *Rev. Aquac.* **8**, 283–300 (2016).
14. Biswas, G., Kumar, P., Kailasam, M., Ghoshal, T. K. & Bera, A. Application of Integrated Multi Trophic Aquaculture (IMTA) Concept in Brackish water Ecosystem: The First Exploratory Trial in the Sundarban, India. *J. Coast. Res.* **86**, 49–55 (2019).
15. Chang, B. V. *et al.* Investigation of a farm-scale multitrophic recirculating aquaculture system with the addition of *Rhodovulum sulfidophilum* for milkfish (*Chanos chanos*) coastal aquaculture. *Sustainability* **11**, 1–15 (2019).

16. Jones, A. B., Preston, N. P. & Dennison, W. C. The efficiency and condition of oysters and macroalgae used as biological filters of shrimp pond effluent. *Aquac. Res.* **33**, 1–19 (2002).

17. Nardelli, A. E., Chiozzini, V. G., Braga, E. S. & Chow, F. Integrated multi-trophic farming system between the green seaweed Ulva lactuca, mussel, and fish: a production and bioremediation solution. *J. Appl. Phycol.* **31**, 847–856 (2019).

18. Shimoda, T., Suryati, E. & Ahmad, T. Evaluation in a shrimp aquaculture system using mangroves, oysters, and seaweed as biofilters based on the concentrations of nutrients and Chlorophyll *a*. *Japan Agric. Res. Q.* **40**, 189–193 (2006).

19. Shpigel, M., Neori, A., Popper, D. M. & Gordin, H. A proposed model for 'environmentally clean' land-based culture of fish, bivalves and seaweeds. *Aquaculture* **117**, 115–128 (1993).

20. Shpigel, M. & Neori, A. The Integrated Culture of Seaweed, Abalone, Fish and Clams in Modular Intensive Land-based Systems: I. Proportions of Size and Projected Revenues. *Aquac. Eng.* **15**, 313–326 (1996).

21. Song, X., Pang, S., Guo, P. & Sun, Y. Evaluation of carrying capacity for shrimp pond culture with integrated bioremediation techniques. *Aquac. Res.* **51**, 761–769 (2020).

22. Xianli, S. *et al.* Integrated bioremediation techniques in a shrimp farming environment under controlled conditions. *Acta Ocean. Sin.* **35**, 88–94 (2016).

23. Yeh, S., Dahms, H., Chiu, Y., Chang, S. & Wang, Y. Increased Production and Water Remediation by Land-Based Farm-Scale Sequentially Integrated Multi-Trophic Aquaculture Systems - An Example from Southern Taiwan. *Sustainability* **9**, 2–13 (2017).

24. Martínez-Córdova, L. R., López-Elías, J. A., Leyva-Miranda, G., Armenta-Ayón, L. & Martínez-Porchas, M. Bioremediation and reuse of shrimp aquaculture effluents to farm whiteleg shrimp, *Litopenaeus vannamei*: A first approach. *Aquac. Res.* **42**, 1415–1423 (2011).

25. Chen, X. *et al.* A novel combined recirculating treatment system for intensive marine aquaculture. *Aquac. Res.* **48**, 5062–5071 (2017).

26. Shpigel, M. *et al.* The sea urchin, *Paracentrotus lividus*, in an Integrated Multi-Trophic Aquaculture (IMTA) system with fish (*Sparus aurata*) and seaweed (*Ulva lactuca*): Nitrogen partitioning and proportional configurations. *Aquaculture* **490**, 260–269 (2018).

27. Bischoff, A. A., Fink, P. & Waller, U. The fatty acid composition of *Nereis diversicolor* cultured in an integrated recirculated system: Possible implications for aquaculture. *Aquaculture* **296**, 271–276 (2009).

28. Shpigel, M., Shauli, L., Odintsov, V., Ashkenazi, N. & Ben-ezra, D. *Ulva lactuca* biofilter from a land-based integrated multi trophic aquaculture (IMTA) system as a sole food source for the tropical sea urchin *Tripneustes gratilla elatensis*. *Aquaculture* **496**, 221–231 (2018).

29. Hughes, A. D. & Black, K. D. Going beyond the search for solutions: understanding trade-offs in European integrated multi-trophic aquaculture development. *Aquac. Environ. Interact.* **8**, 191–199 (2016).

30. Holdt, S. L. & Edwards, M. D. Cost-effective IMTA: a comparison of the production efficiencies of mussels and seaweed. *J. Appl. Phycol.* **26**, 1–13 (2014).

31. Reid, G. K. *et al.* Weight ratios of the kelps, *Alaria esculenta* and *Saccharina latissima*, required to sequester dissolved inorganic nutrients and supply oxygen for Atlantic salmon, *Salmo salar*, in Integrated Multi-Trophic Aquaculture systems. *Aquaculture* **408**, 34–46 (2013).

32. Bostock, J. *et al.* Aquaculture: global status and trends. *Philos. Trans. R. Soc. B Biol. Sci.* **365**, 2897–2912 (2010).

33. Shpigel, M. *et al.* Constructed wetland with *Salicornia* as a biofilter for mariculture effluents. *Aquaculture* **412–413**, 52–63 (2013).

34. Waller, U. *et al.* Integrated multi-trophic aquaculture in a zero-exchange recirculation aquaculture system for marine fish and hydroponic halophyte production. *Aquacult Int* **23**, 1473–1489 (2015).

35. Colt, J. Gas supersaturation - Impact on the design and operation of aquatic systems. *Aquac. Eng.* **5**, 49–85 (1986).

36. Timmons, M. B. & Ebelling, J. M. Recirculating Aquaculture. Northeastern Regional Aquaculture Center (2010).

37. Dashtgard, S. E. & Gingras, M. K. Marine Invertebrate Neoichnology. *Dev. Sedimentol.* **64**, 273–295 (2012).

38. Scaps, P. A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O. F. Müller) (Annelida: Polychaeta). *Hydrobiologia* **470**, 203–218 (2002).

39. Marques, B. *et al.* Adding value to ragworms (*Hediste diversicolor*) through the bioremediation of a super-intensive marine fish farm. *Aquac. Environ. Interact.* **10**, 79–88 (2018).

40. Santos, A. *et al.* Effect of three diets on the growth and fatty acid profile of the common ragworm *Hediste diversicolor* (O.F. Müller, 1776). *Aquaculture* **465**, 37–42 (2016).

41. Yousefi-Garakouei, M., Kamali, A. & Soltani, M. Effects of rearing density on growth, fatty acid profile and bioremediation ability of polychaete *Nereis diversicolor* in an integrated aquaculture system with rainbow trout (*Oncorhynchus mykiss*). *Aquac. Res.* **50**, 725–735 (2019).

42. Pajand, Z. O., Soltani, M., Bahmani, M. & Kamali, A. The role of polychaete *Nereis diversicolor* in bioremediation of wastewater and its growth performance and fatty acid composition in an integrated culture system with *Huso huso* (Linnaeus, 1758). Aquac. Res. 48, 5271–5279 (2017).

43. Pires, A. *et al.* Expansion of lugworms towards southern European habitats and their identification using combined ecological, morphological and genetic approaches. *Mar. Ecol. Prog. Ser.* **533**, 177–190 (2015).

44. Alvarez-Claudio, C. & Anadón, N. Reproductive biology of the polychaete *Arenicola marina* (L.) in the Villa viciosa estuary (North of Spain). *Bull. Soc. Zool. Fr.* **124**, 227–237 (1999).

45. Howie, D. I. D. The reproductive biology of the lugworm, *Arenicola marina* L. in Polychaete Reproduction (eds. Fischer, A. & Pfannenstiel, H. D.) 247–263 (G. Fischer, 1984).

46. Fish, J. & Fish, S. Annelida. in A student's guide to the seashore 141–189 (Cambridge University Press, 2011). Fish J.D., Fish S. (1989) Annelida. *In*: A Student's Guide to the Seashore. Springer, Dordrecht. https://doi.org/10.1007/978-94-011-5888-6_13Dordrecht

47. Rousselot, M. *et al. Arenicola marina* extracellular hemoglobin: A new promising blood substitute. *Biotechnol. J.* **1**, 333–345 (2006).

48. Thuillier, R. *et al.* Supplementation with a new therapeutic oxygen carrier reduces chronic fibrosis and organ dysfunction in kidney static preservation. Am. J. Transplant. 11, 1845–1860 (2011).

49. Buhmann, A. K., Waller, U., Wecker, B. & Papenbrock, J. Optimization of culturing conditions and selection of species for the use of halophytes as biofilter for nutrient-rich saline water. *Agric. Water Manag.* **149**, 102–114 (2015).

50. Koyro, H. W., Khan, M. A. & Lieth, H. Halophytic crops: A resource for the future to reduce the water crisis? *Emirates J. Food Agric.* **23**, 1–16 (2011).

51. Barreira, L. *et al.* Halophytes: Gourmet food with nutritional health benefits?. *J. Food Compos. Anal.* **59**, 35–42 (2017).

52. Custódio, M., Villasante, S., Cremades, J., Calado, R. & Lillebø, A. I. Unravelling the potential of halophytes for marine integrated multi-trophic aquaculture (IMTA)— a perspective on performance, opportunities, and challenges. *Aquac. Environ. Interact.* **9**, 445–460 (2017).

53. Maciel, E. *et al.* Polar lipidome profiling of *Salicornia ramosissima* and *Halimione portulacoides* and the relevance of lipidomics for the valorization of halophytes. *Phytochemistry* **153**, 94–101 (2018).

54. Isca, V. M. S., Seca, A. M. L., Pinto, D. C. G. A., Silva, H. & Silva, A. M. S. Lipophilic profile of the edible halophyte *Salicornia ramosissima*. *Food Chem.* **165**, 330–336 (2014).

55. Feng, L., Ji, B. & Su, B. Economic value and exploiting approaches of *sea asparagus*, a seawater-irrigated vegetable. *Agric. Sci.* **04**, 40–44 (2013).

56. Lopes, A. *et al.* Natural products from extreme marine environments: Searching for potential industrial uses within extremophile plants. *Ind. Crops Prod.* **94**, 299–307 (2016).

57. Glenn, E. P., Coates, W. E., Riley, J. J., Kuehl, R. O. & Swingle, R. S. *Salicornia bigelovii* Torr.: a seawater-irrigated forage for goats. *Anim. Feed Sci. Technol.* **40**, 21–30 (1992).

58. Eganathan, P., Subramanian, H. M., Latha, R. & Rao, C. S. Oil analysis in seeds of *Salicornia brachiata*. *Ind. Crops Prod.* **23**, 177–179 (2006).

59. Hosamani, K. M. & Sattigeri, R. M. Industrial utilization of *Rivea ornata* seed oil: A moderate source of vernolic acid. *Ind. Crops Prod.* **12**, 93–96 (2000).

60. Webb, J. M. *et al.* Halophyte filter beds for treatment of saline wastewater from aquaculture. *Water Res.* **46**, 5102–5114 (2012).

61. Webb, J. M. *et al.* The effect of halophyte planting density on the efficiency of constructed wetlands for the treatment of wastewater from marine aquaculture. *Ecol. Eng.* **61**, 145–153 (2013).

62. Mendes, C. R., Cartaxana, P. & Brotas, V. HPLC determination of phytoplankton and microphytobenthos pigments: Comparing resolution and sensitivity of a C18 and a C8 method. *Limnol. Oceanogr. Meth.* **5**, 363–370 (2007).

63. Nunes, M. *et al.* The macrobenthic community along a mercury contamination in a temperate estuarine system (Ria de Aveiro, Portugal). *Sci. Total Environ.* **405**, 186–194 (2008).

64. Rebelo, J. E. The ichthyofauna and abiotic hydrological environment of the Ria de Aveiro, Portugal. *Estuaries* **15**, 403–413 (1992).

65. Palmer, P. J. Polychaete-assisted sand filters. *Aquaculture* **306**, 369–377 (2010).

66. Palmer, P. J., Wang, S., Houlihan, A. & Brock, I. Nutritional status of a nereidid polychaete cultured in sand filters of mariculture wastewater. *Aquac. Nutr.* **20**, 675–691 (2014).

67. Wang, H. *et al.* Growth and nutritional composition of the polychaete *Hediste diversicolor* (OF Müller, 1776) cultivated on waste from land-based salmon smolt aquaculture. *Aquaculture* **502**, 232–241 (2019).

68. Olive, P. J. W., Craig, S. & Cowin, P. B. D. Aquaculture of marine worms. US 7004109 B2. 1-18 (2006).

69. Lamprianidou, F. Development of a model for evaluating and optimizing the performance of Integrated Multitrophic Aquaculture (IMTA) systems. (University of Stirling, 2015). http://hdl.handle.net/1893/22916

70. Guerrero, S. & Creamades, J. Integrated Multi-trophic Aquaculture (IMTA): A sustainable, pioneering alternative for marine cultures in Galicia. (XUNTA DE GALICIA 2012).

71. Kong, Y. & Zheng, Y. Potential of producing *Salicornia bigelovii* hydroponically as a vegetable at moderate NaCl salinity. *HortScience* **49**, 1154–1157 (2014).

72. Colombo, C., Palumbo, G., He, J., Pinton, R. & Cesco, S. Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. *J Soils Sediments* **14**, 538–548 (2014).

73. Katschnig, D., Broekman, R. & Rozema, J. Salt tolerance in the halophyte *Salicornia dolichostachya* Moss: Growth, morphology and physiology. *Environ. Exp. Bot.* **92**, 32–42 (2013).

74. Frossard, E., Bucher, M., Ma, F., Mozafar, A. & Hurrell, R. Review Potential for increasing the content and bioavailability of Fe, Zn and Ca in plants for human nutrition. *J. Sci. Food Agric.* **80**, 861–879 (2000).

75. Jin, Z. & Pei, Y. Physiological implications of hydrogen sulfide in plants: Pleasant exploration behind its unpleasant odour. *Oxid. Med. Cell. Longev.* 2015, 397502 (2015).

76. Davy, A. J., Brown, M. J. H., Mossman, H. L. & Grant, A. Colonization of a newly developing salt marsh: Disentangling independent effects of elevation and redox potential on halophytes. *J. Ecol.* **99**, 1350–1357 (2011).

77. Lamers, L. P. M. *et al.* Sulfide as a soil phytotoxin-a review. *Front. Plant Sci.* **4**, 268 (2013).

78. Fernández-Marín, B., Míguez, F., Becerril, J. M. & García-Plazaola, J. I. Activation of violaxanthin cycle in darkness is a common response to different abiotic stresses: A case study in *Pelvetia canaliculata*. *BMC Plant Biol.* **11**, 181 (2011).

79. Bovier, E. R. & Hammond, B. R. A randomized placebo-controlled study on the effects of lutein and zeaxanthin on visual processing speed in young healthy subjects. *Arch. Biochem. Biophys.* **572**, 54–57 (2015).

5.1.6. Chapter 5 - Supporting Information

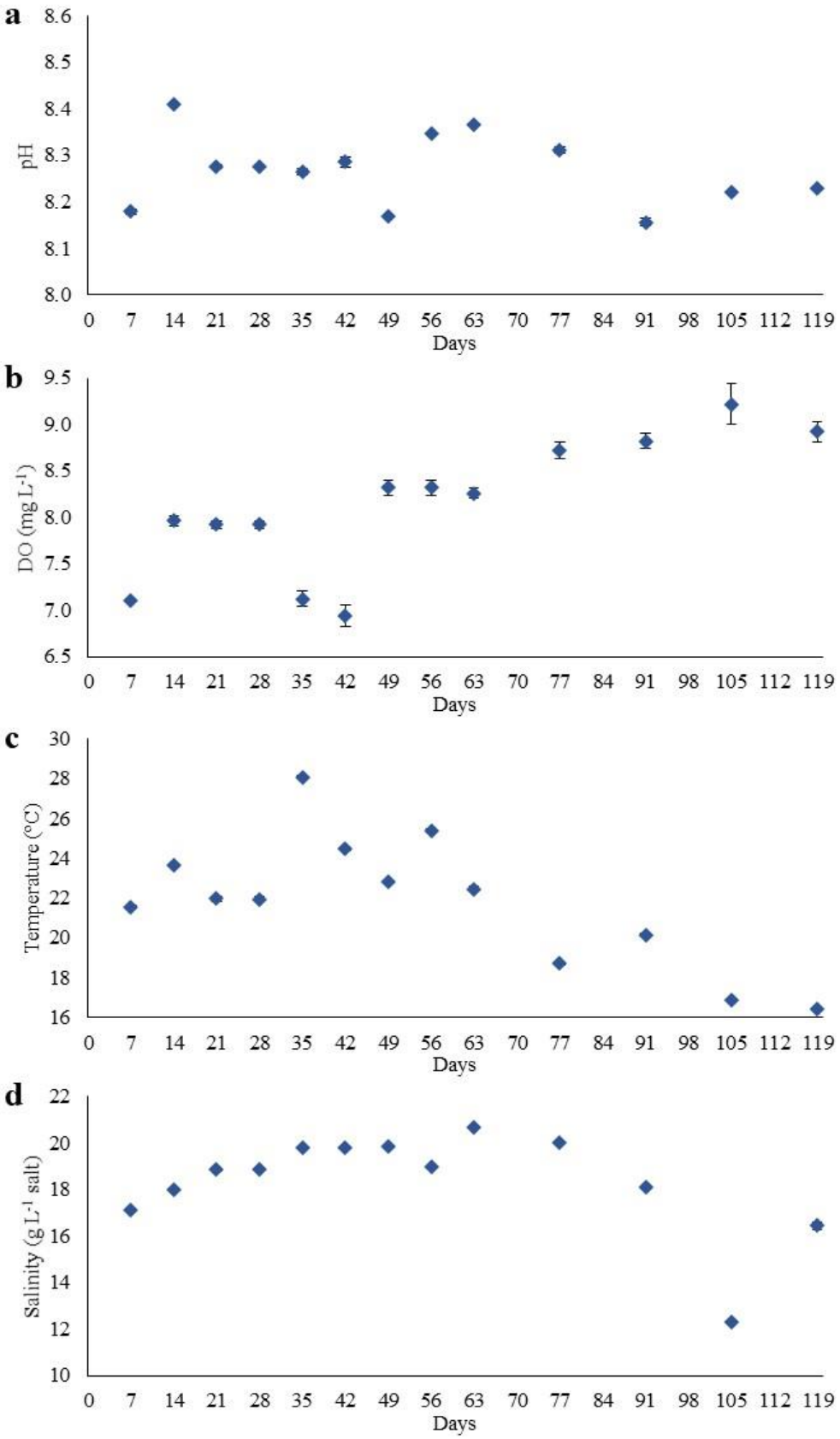


Figure S5.1. pH (S5.1a), dissolved oxygen (S5.1b), temperature (S5.1c) and salinity (S5.1d) measured in the inflowing water supplied to IMTA designs over the study period. Average values (±SD) (n=5).

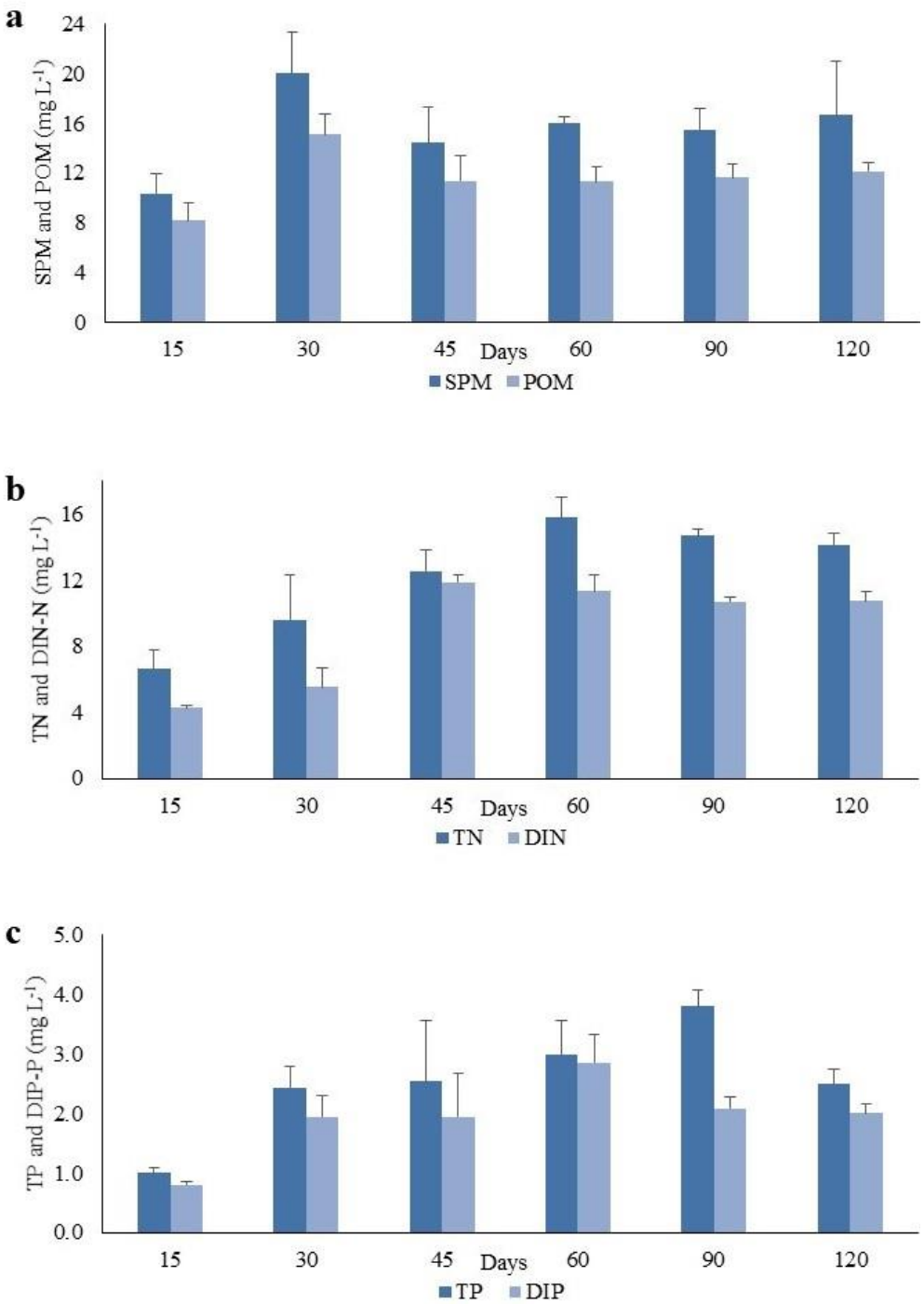


Figure S5.2. Suspended particulate matter (SPM) and particulate organic matter (POM) (S5.2a), total nitrogen (TN) and dissolved inorganic nitrogen (DIN-N) (S5.2b) and total phosphorus (TP) and dissolved inorganic phosphorus (DIP-P) (S5.2c) measured in the inflowing water supplied to IMTA designs over the study period. Average values (±SD) (n=5).

Table S5.1. Total water supplied to RAS-IMTA (sum of the whole outflowing and inflowing water volume of culture tanks) and estimation of the water volume entering each tank and associated particulate organic matter (POM), total nitrogen and phosphorus (TN and TP) and dissolved inorganic nitrogen and phosphorus (DIN-N and DIP-P). Estimate of water composition is supported by data displayed on Table 5.1.

Period (Days)	Total water supplied RAS-IMTA (L)	Total water supplied per tank (L)	POM (g)	TN (g)	DIN-N (g)	TP (g)	DIP-P (g)
0 - 60	12240	490	5.6	5.5	4	1.1	0.93
60 - 120	11560	462	5.4	6.9	5	1.4	1.1

Table S5.2. Results of two-way ANOVAs performed to evaluate the existence of significant differences in the bioremediation (POM, DIN-N and DIP-P concentration in outflowing water and OM present in sand filter substratum) of different IMTA designs tested in the present study using as extractive species polychaetes (*Arenicola marina* – Amar and *Hediste diversicolor* - Hdiv) and halophyte plants (*Salicornia ramosissima* - Sram) cultured in the same tank (1T) or in two separate tanks. Significant differences were considered at $p<0,05$.

Factor	*F - value*	*p - value*	*R_2 (%)*
Figure 2: POM monitored in outflowing water between different IMTA designs			
Polychaete species	0.04	0.853	
IMTA design	0.22	0.647	14.28
Polychaete species x IMTA design	2.41	0.140	
Figures 3a: OM monitored in 0-20 mm substratum depth between different IMTA designs			
Polychaete species	5.53	0.032	
IMTA design	0.01	0.931	32.61
Polychaete species x IMTA design	2.25	0.153	
Figures 3b: OM monitored in 20-100 mm substratum depth between different IMTA designs			
Polychaete species	0.28	0.607	
IMTA design	0.19	0.666	3.13
Polychaete species x IMTA design	0.04	0.853	
Figure 5: DIN-N monitored in outflowing water between different IMTA designs			
Polychaete species	0.62	0.441	
IMTA design	2.79	0.114	46.41
Polychaete species x IMTA design	12.92	0.002	
Figure 6: DIP-P monitored in outflowing water between different IMTA designs			
Polychaete species	0.00	0.991	
IMTA design	0.43	0.522	12.63
Polychaete species x IMTA design	1.47	0.243	

Table S5.3. *Post-hoc* Tukey HSD tests performed to evaluate the existence of significant differences in the bioremediation (POM, DIN-N and DIP-P concentration in outflowing water and OM present in sand filter substratum) of different IMTA designs tested in the present study using as extractive species polychaetes (*Arenicola marina* – Amar and *Hediste diversicolor* - Hdiv) and halophyte plants (*Salicornia ramosissima* - Sram) cultured in the same tank (1T) or in two separate tanks. Significant differences were considered at $p<0,05$.

Pair wise test	OM in 0-20 mm substratum depth		DIN-N monitored in outflowing water	
	T-value	*p*	*T-value*	*p*
$2T^{Amar+Sram} – 1T^{amar+Sram}$	0.78	0.863	1.87	0.279
$1T^{Hdiv+Sram} – 1T^{amar+Sram}$	-0.57	0.938	2.42	0.113
$2T^{Hdiv+Sram} – 1T^{amar+Sram}$	-1.91	0.261	-0.79	0.856
$1T^{Hdiv+Sram} – 2T^{amar+Sram}$	-1.35	0.544	0.55	0.945
$2T^{Hdiv+Sram} – 2T^{amar+Sram}$	-2.69	0.069	-2.66	0.073
$2T^{Hdiv+Sram} – 1T^{Hdiv+Sram}$	-1.34	0.553	-3.21	0.025

Table S5.4. Results of two-way ANOVAs performed to evaluate the existence of significant differences in the productivity of polychaetes (*Arenicola marina* – Amar and *Hediste diversicolor* – Hdiv) and halophytes (*Salicornia ramosissima* - Sram) cultured under IMTA designs with extractive species in the same tank (1T) or in two separate tanks (2T). Significant differences were considered at $p<0,05$.

Factor	*F - value*	*p - value*	*R_2 (%)*
Table 3: Biomass of polychaetes between different IMTA designs			
Polychaete species	54.20	0.000	
IMTA design	0.09	0.772	78.5
Polychaete species x IMTA design	4.14	0.059	
Table 4: Density of halophyte plants between different IMTA designs			
Polychaete species	2.73	0.118	
IMTA design	5.08	0.039	33.83
Polychaete species x IMTA design	0.36	0.556	
Table 4: Total plant biomass of halophyte plants between different IMTA designs			
Polychaete species	1.59	0.225	
IMTA design	21.11	0.000	58.66
Polychaete species x IMTA design	0.00	0.964	
Table 4: Aboveground biomass of halophyte plants between different IMTA designs			
Polychaete species	1.39	0.255	
IMTA design	19.43	0.000	56.56
Polychaete species x IMTA design	0.00	0.992	
Table 4: Belowground biomass of halophyte plants between different IMTA designs			
Polychaete species	0.27	0.613	
IMTA design	16.69	0.001	51.60
Polychaete species x IMTA design	0.10	0.751	
Figure 7: Plant average weight at day 0 between different IMTA designs			
Polychaete species	0	1	
IMTA design	4.42	0.052	22.04
Polychaete species x IMTA design	0.1	0.751	
Figure 7: Plant average weight at day 60 between different IMTA designs			
Polychaete species	1.07	0.316	
IMTA design	32.6	0.000	67.87
Polychaete species x IMTA design	0.12	0.729	

Table S5.5. *Post-hoc* Tukey HSD tests performed to evaluate the existence of significant differences in the productivity of polychaetes (*Arenicola marina* – Amar and *Hediste diversicolor* – Hdiv) and halophytes (*Salicornia ramosissima* - Sram) cultured under IMTA designs with extractive species cultured in the same tank (1T) or in two separate tanks (2T). Significant differences were considered at $p<0,05$.

Pair wise test	Total polychaetes biomass		Plant density		Total plant biomass		Plant aboveground biomass		Plant belowground biomass		Average weight day 60	
	T-value	*p*	*T-value*	*p*	*T-value*	*p*	*T-value*	*p*	*T-value*	*p*	*T-value*	*p*
$2T^{Amar+Sram}$ – $1T^{amar+Sram}$	1.65	0.382	-2.02	0.222	-3.22	0.025	-3.12	0.030	-3.12	0.030	-3.92	0.006
$1T^{Hdiv+Sram}$ – $1T^{amar+Sram}$	6.64	0,000	0.74	0.878	0.93	0.792	0.83	0.841	0.14	0.999	0.26	0.994
$2T^{Hdiv+Sram}$ – $1T^{amar+Sram}$	5.41	0,000	-0.43	0.973	-2.36	0.127	-2.28	0.144	-2.52	0.094	-3.32	0.02
$1T^{Hdiv+Sram}$ – $2T^{amar+Sram}$	5,00	0.001	2.76	0.060	4.14	0.004	3.95	0.006	-3.25	0.023	4.17	0.004
$2T^{Hdiv+Sram}$ – $2T^{amar+Sram}$	3.77	0.008	1.59	0.409	0.86	0.825	0.84	0.834	0.59	0.933	0.59	0.932
$2T^{Hdiv+Sram}$ – $1T^{Hdiv+Sram}$	-1.23	0.618	-1.17	0.654	-3.28	0.022	-3.11	0.031	-2.66	0.073	-3.58	0.012

Chapter 6

6.1. Growth and bioremediation performances of *Salicornia ramosissima* under different salinities and iron concentrations - implications for Integrated Multi-Trophic Aquaculture (IMTA)

Submitted for publication

6.1. Growth and bioremediation performances of *Salicornia ramosissima* under different salinities and iron concentrations - implications for Integrated Multi-Trophic Aquaculture (IMTA)

Abstract

Salicornia ramosissima J. Woods is a candidate species with great potential for the development of production models valuing saline resources that are usually unexplored (e.g., aquaculture effluents, salinized soils). Growth, elemental composition and bioremediation performance of this halophyte plant were evaluated under different brackish water salinities within the species tolerance range (Sal.15, Sal.20 and Sal.25 ≈257, 342 and 428 mM NaCl, respectively) and under different concentrations of iron (Fe) (FeDeficiency, FeNormal and FeEnriched ≈5 - 10, 10 - 30 and 250 - 500 µg Fe^{2+} L^{-1}, respectively). Under different salinities, similar biomass generation was determined for all treatments, with Relative Growth Rates (RGR) ranging between 6.3 and 6.9 % day^{-1} for period of 1-30 days and from 1.6 to 2.6 % day^{-1} for period 31-60 days. Plants grown under Sal.20 exhibited a slightly higher final biomass, but no significant differences from other salinity conditions were recorded (edible aboveground biomass: 23 – 30 g FW $Plant^{-1}$). Bioremediation efficiencies of dissolved inorganic nitrogen and phosphorus (DIN-N and DIP-P, respectively) of ≈2.4 - 4 mg and 0.23 – 0.25 mg $plant^{-1}$, respectively, were reported for different salinity conditions throughout the study. During the 60-days trial, plants from different salinity treatments incorporated in its edible biomass 50 - 63 mg of nitrogen, 4.2 - 5.5 mg of phosphorus and 296 - 368 mg of carbon. A positive correlation was recorded between growth and the increment of Fe concentration in hydroponic media. FeDeficiency plants generated a significant lower biomass (total, edible aboveground and belowground) than the plants cultured under the other conditions tested in the present work. FeDeficiency treatment affected the pigment profile and photosystems efficiency of plants (with significantly lower values of chlorophyll and carotenoids being recorded, as well as lower maximum quantum efficiency of PSII photochemistry [*Fv/Fm*]). A positive correlation between the increment of Fe concentration in hydroponic media and the increment of C, H, Mn, Fe, Cu, Zn and Mo in plant edible aboveground biomass was recorded. Concerning bioremediation efficiency, FeDeficiency plants also exhibited a lower performance (1.8 – 2.3 mg DIN-N $plant^{-1}$ and

0.06 – 0.22 mg DIP-P plant^{-1}) than FeNormal and FeEnriched plants (2.4 – 4.0 and 0.16 – 0.24 mg of DIN-N and DIP-P plant^{-1}, respectively). During the 60 days of the experimental period FeEnriched plants incorporated a significantly higher amount of N, P and C into edible aboveground biomass (≈63, 5.5 and 369 mg plant^{-1}, respectively) than FeDeficiency plants (≈28, 3.7 and 161 mg plant^{-1}, respectively). This study draws attention to the possible effects that may result from the integration of these plants as extractive species in recirculating aquaculture systems (RAS) that use water treatments that promote the precipitation and oxidative elimination of Fe (among other essential micro and macronutrients) (e.g., ozonation, chemical oxidation). It further demonstrates that under controlled conditions, it is possible to produce iron enriched salty vegetables using an environmentally friendly approach, and therefore highlighting the potential of halophyte plants for integrated multi-trophic aquaculture (IMTA).

6.1.1. Introduction

Halophytes are naturally evolved salt-resistant plants adapted to grow in saline environments and, in some cases, require an exposure to salinity to thrive[1-4]. The development of production models for these plants emerged as a potential answer to various interconnected scenarios, namely: 1) a growing undernourished population[5,6]; 2) a decrease in freshwater resources and an increase in soils salinization in many parts of the world[7-10]; and 3) the need to valorise resources unsuitable for conventional crop production, such as saline irrigation [3,10-13]. Halophytes have already gained their space as new vegetable products with several applications (e.g., food, fodder, nutraceuticals, pharmaceuticals, biodiesel)[14,15]. Previous research has shown halophyte plants as premium candidates to be integrated as inorganic extractive species under integrated multi-trophic aquaculture (IMTA) conditions[16-28]. This ecosystem approach that integrates extractive species from different trophic levels to recover unused nutrients from fed aquaculture (e.g., finfish or shrimp) and convert them into valuable extractive species biomass[29-32]. This concept has been the subject of numerous reviews in recent years and allows to minimize some of the negative impacts inherent to aquaculture, such as effluent discharge and the wasting of unused nutrients (e.g., nitrogen [N] and phosphorus [P]), among others[2,33-35]. In these systems, halophytes can play a key

role in the recovery of DIN-N and DIP-P. Previous research assessing the bioremediation and growth performances of these plants were performed using constructed wetlands or drainage lysimeters[23,27,28,36], as well as soilless systems, such as hydroponics/aquaponics [16,20,25,37,38]. The last mentioned systems allow: the production in areas where soil is unavailable or unsuitable, the reduction of intensive labour (which is inherent to traditional crop methods), the conservation of water and nutrients, an easier eradication of plant diseases in closed systems, and the possibility of operation at maximum production yields[2,39]. More than 50% of all IMTA studies performed with halophytes included *Salicornia* spp.[17-19,23,25-28], as these halophyte plants are already used for human consumption as uncooked vegetables or pickles[3,40]. Their valuable biomass is rich in antioxidants (*β*-carotene, phenolic compounds and ureides), lipids (e.g., approx. 241 mg 100 g^{-1} FW) which include a considerable omega-3 fraction (47.6%) and a high protein content (253 mg $100g^{-1}$ FW)[41]. In addition, their seeds contain considerable levels of oil (22-33%) and protein (31%)[42,43]. All these features highlight the relevance of developing suitable production models for these plants.

To date, few research studies tested *Salicornia* spp. under deep water culture technique, also named as raft or float systems, under IMTA conditions[25]. The effect of salinity on the performance of several *Salicornia* spp. has already been evaluated, with best growth and bioremediation performances being achieved under brackish conditions (salinity 10 – 25) [17,25,41,44,45]. However, in some production systems (e.g., outdoor systems) it may not be easy to control fluctuations of salinity levels, as salts can either be diluted or concentrated (e.g., through rainfall and evaporation, respectively). Therefore, it is important to know if these plants maintain a similar biomass production and bioremediation efficiency under different brackish water conditions (e.g., salinities ranging between 15 and 25). There is also a lack of knowledge on how the limitation of certain micronutrients, such as Fe, may impact these plants. An adequate concentration of Fe (≈ 1.1 mg L^{-1}) is paramount to ensure a healthy plant growth, with supplementation sometimes being required to ensure an optimal performance [37]. A side effect of some aquaculture treatments which promote water oxidation in recirculating aquaculture systems (RAS) (e.g., ozonation and chemical oxidation) is the formation and precipitation of Fe and manganese (Mn). The oxidative elimination of these elements may limit plant growth in RAS–IMTA systems (as fed species continue to receive these micronutrients through aquafeeds)[25].

For halophyte plant production models to become a reality, it is essential to acquire knowledge of their growth and bioremediation performances, along with and elemental composition, under different culture conditions, such as different ranges of salinity and Fe. To shed light over these issues two independent experiments were performed simultaneously: 1) the evaluation of *S. ramosissima* performance species under different brackish water conditions (Salinity experiment) (Sal.15, Sal.20 and Sal.25); and 2) the evaluation of *S. ramosissima* performance under different concentrations of Fe (Iron experiment), which evaluated the impact of a deficiency scenario promoted by aquaculture treatments (e.g. ozonation, chemical oxidation) (FeDeficiency), a scenario with iron concentrations equal to natural brackish water (FeNormal), and a scenario in which this element was enriched (FeEnriched).

6.1.2. Material and methods

6.1.2.1. Plant material

The present study was performed in the Advanced Scientific Research Centre (CICA) – University of A Coruña, Spain (43° 19' 57.36" N 8° 24' 30.275" W) from May to July 2019. The *Salicornia ramosissima* plants used in the experimental trials were germinated from seeds (February 2019; seeds originated from wild plants and harvested at Murtosa - Portugal [40 ° 46'06.3 "N 8 ° 39'29.4" W] in 2018) in sand trays after a cold-stratification period at 4 ºC, over 30 days, as described by Gunning (2016b)[44]. After this period, seeded trays were maintained indoors and near windows under natural conditions of photoperiod (≈10L:14D) for 1.5 months. The sand was maintained wet through bottom irrigation using freshwater and, after germination, was nutritionally enriched by using a modified Hoagland's solution, whose elemental nutrient concentration was as follows: 60 mg K L^{-1}, 56 mg N L^{-1}, 40 mg Ca L^{-1}, 16 mg Mg L^{-1}, 16 mg P L^{-1}, 1.12 mg Fe L^{-1}, 0.34 mg Mo L^{-1}, 0.28 mg B L^{-1}, 0.13 mg Zn L^{-1}, 0.11 mg Mn L^{-1}, 0.03 mg Cu L^{-1}. After this period (plants with ≈15 mm), trays were transferred outdoors and the development of plants took place under natural temperature and photoperiod conditions for 2.5 more months, being irrigated with the modified Hoagland's solution at a salinity of 20 (342 mM NaCl). Plants with a similar weight (average initial weight between 2.3 – 2.5 g) were then selected and randomly distributed over experimental

hydroponic units. A two-weeks acclimation period was used prior beginning experimental trials.

6.1.2.2. Salinity and Iron experimental setups

Both, Salinity and Iron experiments were performed simultaneously in a culture chamber for a period of 60 days (June to August 2019) under controlled photoperiod (16L:8D) and temperature (20 ºC). Hydroponic units were illuminated from above with 4 Samsung LED surface panel (6000 K), delivering an average photosynthetically active radiation (PAR) to plants ranging between 200-350 µmol m^{-2} s^{-1}, with this parameter being monitored weekly using an Original Quantum Sensor (apogee instruments). Temperature, pH and dissolved oxygen (DO) of hydroponic media were monitored every 15 days using a Delta OHM HD 2105.2 pH/mV meter, with oxygen being monitored using a Eutech DO 6+ probe. From day 0 to 25, the hydroponic units contained 90 mL of media that was changed every 3 days and, from day 26 to day 60 hydroponic units contained 260 mL of media that was changed every day due to root development. Roots were completely immersed, thus this experiment being termed as deep-water culture. Plastic disks were used as plant support and were perched on the top of hydroponic units.

In the experimental design of the Salinity experiment, the performances of *S. ramosissima* were evaluated under 3 different salinity conditions: Sal.15, Sal.20 and Sal.25. Salinities were adjusted by mixing fresh tap water with filtered natural saltwater and using a hydrotherm densimeter (Nahita 1000-1100 Kg m^{-3}). In the Iron experiment, the performances of *S. ramossissima* were evaluated under different Fe concentrations present in the hydroponic media: FeDeficiency = 5 - 10 µg L^{-1}; FeNormal = 10 - 30 µg L^{-1} and FeEnriched = 250 - 500 µg L^{-1}. The saltwater used in each of the above-mentioned treatments was previously cartridge filtered (100 µm) and, in the FeDeficiency treatment, the following procedure to promote Fe oxidation and precipitation was employed: saltwater was strongly aerated in a tank during 24 h and then filtered with a 20 µm mesh filter. This procedure mimicked the Fe precipitation and removal promoted in aquaculture production units when using iron-rich borewater[46]. A constant salinity of 20 was used for all treatments in the Iron experiment.

For each treatment in the Salinity and Iron experiments, eight replicates were used, each one of them being a single plant. Forty plants were randomly selected and distributed over

the experimental units of each treatment, with one of them being common to both experiments (FeEnriched and Sal.20) (thus five treatments x eight replicates = 40 plants). The replicates of each treatment were distributed in rows, and every day a line was moved longitudinally and each column laterally in order to eliminate any potential effect caused by plant's position on the culture tray.

To maintain a constant movement of hydroponic media over plant roots, experimental units were maintained in an orbital shaker Infors HT – Labotron (50 rpm).

For both Salinity and Iron experiments, concentrated solutions of $NaNO_3$ (10M) and NaH_2PO_4. $2H_2O$ (1M) were used as a source of N and P, respectively. Both solutions were always added at a concentration of 20 mg L^{-1} and 1 mg L^{-1}, respectively, with these values being in line with those recorded in intensive aquaculture production. Other macro and micro elements were also supplied by adding 0.5 ml L^{-1} of a concentrated trace metals solution with the following composition: Na_2 EDTA.$2H_2O$ (14 g L^{-1}), $Fe(NH_4)_2(SO_4)_2.6H_2O$ (14 g L^{-1}), $MnSO_4.4H_2O$ (1.6 g L^{-1}), $FeCl_3.6H_2O$ (0.5 g L^{-1}), $ZnSO_4.7H_2O$ (0.2 g L^{-1}), $CoSO_4.7H_2O$ (0.05 g L^{-1}). For FeDeficiency and FeNormal treatments, the trace metals solution was also used but Fe salts were not employed.

6.1.2.3. Growth performance and characterisation of photosynthetic pigments

At the beginning of the Salinity and Iron experiments plants were randomly selected, weighed and distributed over each hydroponic unit. Growth was evaluated at day 30 and 60, through the quantification of the whole plant biomass, as well as aboveground (apical shoots) and belowground (roots) biomass in terms of fresh weight (FW) and dry weight (DW) at day 60. The DW biomass was estimated after weight stabilization in oven at 45ºC. The relative growth rate (RGR) of plants was determined by the formula:

1) RGR $(\%day^{-1}) = \frac{Ln\ Fw - Ln\ Iw}{T} * 100$

Where:

Fw=Final fresh weight

Iw=Initial fresh weight

T= Days in culture

After 60 days, the apical aboveground portions of plants from the different treatments of the Iron experiment were immediately frozen in liquid nitrogen (-80 ºC) for the determination of chlorophylls (*a*, *b* and *total*) using the Acetone method and equations 2 - 4

which (see below), as described in Sudhakar *et al*. (2016)[47]. Total carotenoids were estimated by UV–VIS Spectrophotometer Method and equation 5 (Price and Hendry, 1991 *in* Sudhakar *et al.*, 2016)[47].

2) mg chlorophyll *a* per g tissue $= 12.7\text{x}(A663) - 2.69\text{x}(A645)\text{x}\frac{\text{V}}{1000\text{xW}}$

3) mg chlorophyll *b* per g tissue $= 22.9\text{x}(A645) - 4.68\text{x}(A663)\text{x}\frac{\text{V}}{1000\text{xW}}$

4) mg total chlorophyll per g tissue $= 20.2\text{x}(A645) + 8.02\text{x}(A663)\text{x}\frac{\text{V}}{1000\text{xW}}$

5) Total carotenoids (mg per g FW) $= [A480 + (0.114\text{x}A663) - (0.638 - A645)]\text{x}\frac{\text{V}}{1000}\text{xW}$

Where:

A= absorbance at specific wavelength

V= final volume of chlorophyll extract

W= fresh weight of tissue extracted

Maximum quantum efficiency of PSII photochemistry (*Fv/Fm*) was determined by using a portable pulse modulated fluorescence measurer (Junior PAM Chlorophyll Fluorometer, Waltz, Germany) after adapting plants to the dark during 20 minutes, and following the procedures described in Murchie and Lawson (2013)[48].

6.1.2.4. Hydroponics media analysis

Retention times (RT - length of time wastewater remains in hydroponic units) and values of initial DIN-N (NO_3-N) and DIP-P (PO_4-P) used over the study period are summarized in supplementary Table S1. For both Salinity and Iron experiments, it was identified at day 25 the need to increase the volume and frequency of hydroponic media exchange, due to root development and in order to avoid limiting the plants nutritionally. Samples from the initial hydroponic media were collected after preparation to certify the presence of all nutrients previously defined. Furthermore, samples of the final hydroponic media were also collected after RT at day 30 and 60 to ensure that hydroponic solutions were nutrient-rich over the 24-hour period. Samples were filtered (Puradisc 25 PP serynge filter - 0.45µm Whatman™) and immediately frozen at -20 ºC prior to analysis. To determine the concentrations of DIN-N, DIP-P and Fe (the latter only in the Iron experiment) a UV spectrophotometer (Hewlett Packard 8453) was used. The determination of DIN-N was performed according to method 4500-NO3.B, described in APHA (1992)[49] and samples were 1:10 diluted (hydroponic media: distilled water), as the method is only linear until concentrations ≈10 mg N L^{-1}. The

analytical quality control was ensured by using calibration curves (0.2, 0.4, 0.8, 1, 2, 4, 5, 7 mg N L^{-1}) determined after running standard solutions at the beginning and in parallel with blanks and samples. The determination of DIP-P was performed following the method described by Grasshoff *et al*. (1999)[50] with analytical quality control being ensured as described above for the determination of DIN-N (calibration curves - 0.1, 0.2, 0.5, 1 mg P L^{-1}). Determination of Fe concentration in the Iron experiment was performed following the procedures described by Koroleff & Kremling *in* Grasshoff *et al.* (1999)[50]. Samples of FeEnriched treatment were 1:10 diluted due to values above 100 µg L^{-1} Fe^{2+}. The analytical quality control was ensured by using calibration curves (5, 20, 50, 75 and 100 µg Fe^{2+} L^{-1}), that were prepared after running standard solutions at the beginning and in parallel with blanks and samples.

6.1.2.5. Elemental characterisation and bioremediation of SALICORNIA RAMOSISSIMA

The DW biomass (oven weight stabilisation at 45 ºC) of *S. ramosissima* was processed to determine its elemental composition. The elemental analysis was performed by a certified laboratory at the University of A Coruña (SAI-UTIA – UDC Research Support Services) and according to the procedures described below. Three samples of edible aboveground biomass were randomly collected in plants from different treatments from both the Salinity and Iron experiments for determination of carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) content using an elemental analyzer FlashEA112 (ThermoFinnigan).
Additionally, three samples from different treatments of the Iron experiment (aboveground biomass) were also randomly collected to determine sodium (Na), potassium (K), magnesium (Mg), phosphorus (P), calcium (Ca), boron (B), manganese (Mn), iron (Fe), nickel (Ni), copper (Cu), zinc (Zn), molybdenum (Mo) and selenium (Se) content using a Thermo Finnigan ELEMENTXR Magnetic Sector ICP-MS team.

The 24-hour bioremediation of DIN-N and DIP-P in hydroponic media was evaluated by the difference between initial and final concentrations in culture media. The incorporation of N, P and C in edible aboveground plant biomass was determined by the differences in initial and final DW biomass and the inherent plant composition in these elements in both periods (initial composition presented in supplementary table S2). Wild plants biomass for

comparison with cultured conspecifics was harvested in Murtosa - Portugal (40 ° 46'06.3 "N 8 ° 39'29.4" W) in August 2019.

6.1.2.6. Statistical analysis

To evaluate the existence of differences in productivity (RGR and total plant biomass at day 30 and 60 of the experiment, final aboveground and belowground biomass) data from both the Salinity and Iron experiments were analysed using one-way ANOVAs with *Post-hoc* Tukey's HSD test for comparison of individual means being performed whenever significance was observed. Data were previously checked for normality and homogeneity of variances through Anderson-Darling, Bartlett´s and Levene´s tests. When the assumption of normality was not verified, a Kruskal-Wallis test was performed between each pair of treatments from the same experiment. Significant differences were always considered at $p < 0.05$. The same statistical procedure was performed to ascertain the existence of differences in bioremediation performances (DIN-N and DIP-P at day 30 and 60), photosynthetic pigments (chlorophyll *a*, *b* and *total*, carotenoids, carotenoids/chlorophyll) and efficiency of photosystems performance (*Fv/Fm*), as well as the incorporation of N, P and C into edible aboveground biomass. All the above-mentioned statistical analysis were performed using Minitab 18 Statistical Software (State College, PA).

To evaluate differences in elemental composition (C, H, N, S, Na, K, Mg, P, Ca, B, Mn, Fe, Ni, Cu, Zn, Mo and Se) between Iron treatments, as well as when comparing cultured plants and conspecifics collected from the wild, a one-factor PERMANOVA+ was performed with "Fe level *vs.* wild" being used as a fixed factor with four levels (FeDeficiency, FeNormal, FeEnriched, Wild). All analysis were performed using a resemblance matrix´s produced using Bray Curtis similarity coefficient of data, previously transformed using the formula log (x+1). Significant differences were considered at $p < 0.05$. A SIMPER analysis was also performed to evaluate which elements (from both cultured and wild plants) contributed the most to the dissimilarities recorded between treatments until a total of 50% of dissimilarity was achieved. All multivariate statistical analysis were performed using PRIMER v6 with the PERMANOVA+ add-on. For a detailed description of all the multivariate statistical analysis described above please refer to Anderson *et al*. (2008)[51]. The statistical results of the tests mentioned above are summarised in supplementary material (Tables S4 – S7).

6.1.3. Results

Images from day 0 and 60 of *S. ramosissima* cultured in both the Salinity and Iron experiments (performed simultaneously) are displayed in supplementary Figures S6.1A and S6.1B. The average values (±SD) of atmospheric temperature and photosynthetically active radiation (PAR) inside the culture chamber varied between 19 – 21 ºC and 230 – 270 µmol m^{-2} s^{-1} throughout experiments, respectively (Supplementary Figures S6.2 and S6.3, respectively).

The average values (±SD) of temperature, pH and DO monitored in the hydroponic media varied between 21.2 – 21.6 ºC, 6.4 – 6.7 and 3.9 – 4.6 mg L^{-1}, respectively (Supplementary Table S6.3).

6.1.3.1. Salinity Experiment

6.1.3.1.1. Plants growth performance

The images of *S. ramosissima* after 60 days of culture under Sal.25, Sal.20 and Sal.15 are displayed in Figure 6.1, with all plants exhibiting a dark green coloration. The average values (±SD) of total plant biomass, edible aboveground and belowground biomass and RGR determined over the experiment are summarized in Table 6.1. No significant differences were recorded in biomass generation (total, aboveground and belowground) between the plants cultured under different salinities (ANOVA; $p>0.05$). The edible FW aboveground biomass corresponded to 75-78% of total plant biomass (the DW biomass corresponded to 7.3-7.8% of FW biomass). No significant differences were identified in aboveground/belowground biomass ratio between different salinity conditions (ANOVA; $p>0.05$). In the period between days 31 and 60, a significantly higher RGR was determined for plants produced in Sal.20 and Sal.25 when compared to plants grown at Sal.15 plants (*Post-hoc* Tukey HSD; $p<0.05$).

Figure 6.1. *Salicornia ramosissima* in the Salinity experiment after 60 days of culture under salinities of 25, 20 and 15 (Sal.25, Sal.20 and Sal.15, respectively).

Table 6.1. Productivities obtained from *Salicornia ramosissima* cultured in hydroponic media with under different salinities (Sal.15, Sal.20 and Sal.25). Values are averages (±SD) of 8 replicates. Values in fresh weigh (FW) and dry weight (DW).

	Sal.15	Sal.20	Sal.25
Total plant biomass (g plant^{-1})			
Start (FW)	2.47 ± 1.18 [a]	2.47 ± 1.09 [a]	2.32 ± 0.85 [a]
30º day (FW)	18.62 ± 5.83 [a]	18.16 ± 4.34 [a]	14.94 ± 3.55 [a]
60º day (FW)	30.65 ± 9.72 [a]	38.86 ± 6.74 [a]	31.42 ± 6.09 [a]
Final aboveground and belowground biomass (g plant^{-1})			
Aboveground (FW)	22.91 ± 7.19 [a]	29.59 ± 5.42 [a]	24.49 ± 4.83 [a]
Aboveground (DW)	1.69 ± 0.50 [a]	2.16 ± 0.42 [a]	1.92 ± 0.31 [a]
Belowground (FW)	7.75 ± 2.82 [a]	9.27 ± 1.65 [a]	6.93 ± 1.48 [a]
Belowground (DW)	0.58 ± 0.19 [a]	0.63± 0.09 [a]	0.57 ± 0.09 [a]
Above/Below (FW)	3.02 ± 0.58 [a]	3.21 ± 0.40 [a]	3.57 ± 0.51 [a]
Relative Growth Rate (% day^{-1})			
0-30º day	6.92 ± 1.10 [a]	6.84 ± 1.03 [a]	6.31 ± 1.04 [a]
30-60º day	1.63 ± 0.36 [a]	2.58 ± 0.34 [b]	2.50 ± 0.38 [b]

Means within a row followed by different letters indicate significant differences (p<0.05) (statistical analysis presented in Supplementary Table S6.1 and S6.2)

6.1.3.1.2. Bioremediation of dissolved inorganic nitrogen (DIN-N) and phosphorus (DIP-P) and incorporation of N, P and C into edible biomass.

The bioremediation efficiencies of DIN-N and DIP-P present in the hydroponic media are displayed in figures 6.2A and 6.2B, respectively. For both monitoring periods, no significant differences were recorded between the different salinity treatments concerning their bioremediation efficiency of DIN-N (≈ 62 -78% and 47-52% reduction of initial concentration, respectively) and DIP-P (≈ 88-96% and 89-95% reduction of initial concentration, respectively) (ANOVA; $p>0.05$). After 60 days, N, P and C incorporation into edible aboveground biomass of plants at Sal.20 was slightly higher than that reported for plats in other salinity treatments, but no significant differences were recorded (ANOVA; $p>0.05$) (Fig. 6.3A – 6.3C).

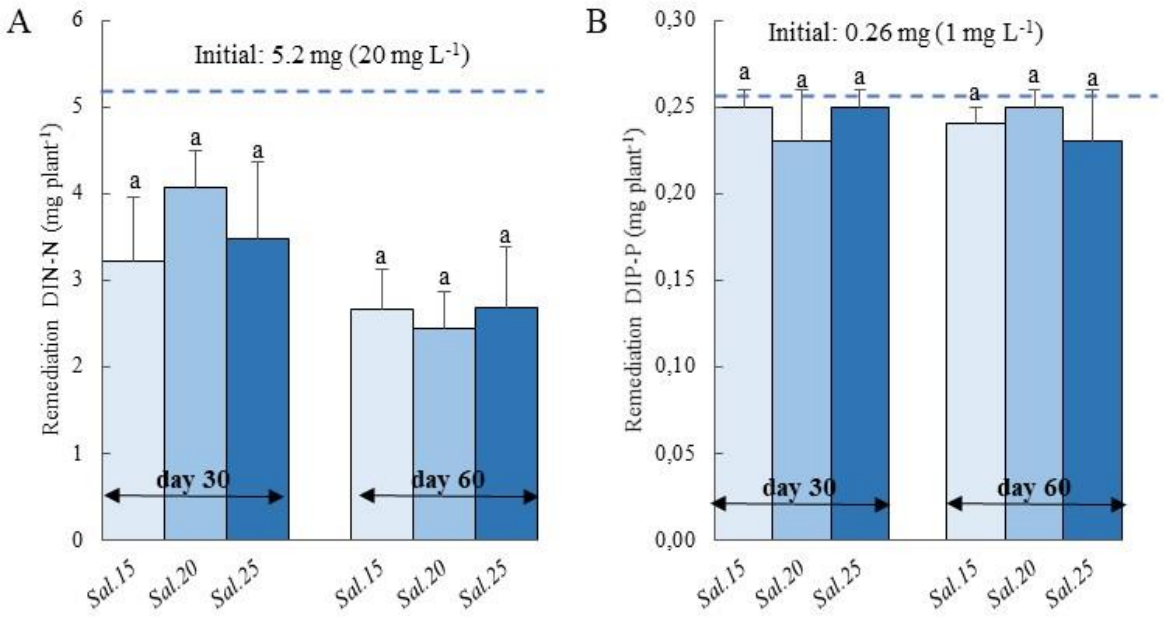


Figure 6.2. Bioremediation of dissolved inorganic nitrogen (DIN-N) (6.2A) and phosphorus (6.2B) (mg plant^{-1}; 24-hour period) displayed by *Salicornia ramosissima* cultured in hydroponic media with different brackish water salinities (Sal.15, Sal.20 and Sal.25). Values are averages (±SD) of 8 replicates. The blue line represented in graphic 6.2A and 6.2B marks the initial concentration of DIN-N and DIP-P, respectively. Means in each time-period followed by different letters indicate significant differences ($p<0.05$) (statistical analysis presented in Supplementary Table S6.4.1 and S6.4.2).

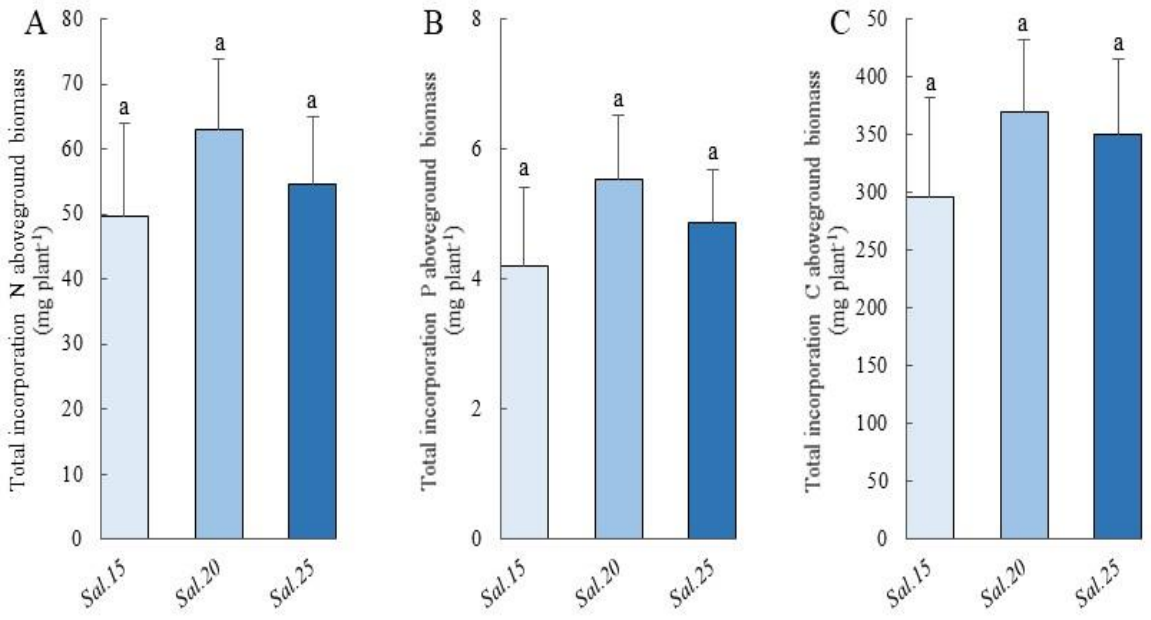


Figure 6.3. Total incorporation (mg plant^{-1}) of nitrogen (6.3A), phosphorus (6.3B) and carbon (6.3C) into edible aboveground biomass of *Salicornia ramosissima* cultured in hydroponic media during 60 days under different brackish water salinities (Sal.15, Sal.20 and Sal.25). Values are averages (±SD) of 8 replicates. Means followed by different letters indicate significant differences ($p<0.05$) (statistical analysis presented in Supplementary Table S6.4.1 and S6.4.2).

6.1.3.2. Iron Experiment

The average value (±SD) of Fe concentration determined in FeDeficiency, FeNormal and FeEnriched hydroponic media was 5.2 ± 2.2 µg L^{-1}, 18.8 ± 6.9 µg L^{-1} and 357.0 ± 79.8 µg L^{-1}, respectively (Supplementary Figure S6.4).

6.1.3.2.1. Plants growth performance

The images of *S. ramosissima* after 60 days of culture under different Iron treatments are displayed in Figure 6.4. A greenish yellow coloration was verified in FeDeficiency plants, while FeNormal and FeEnriched plants exhibited a green and dark green coloration, respectively. The average values (±SD) of total plant biomass, edible aboveground biomass and belowground biomass, and RGR determined over the experiment are summarized in Table 6.2. A positive correlation between plant growth and the increment of Fe concentration in the hydroponic media was verified for all tested conditions, and at the end of the experiment, significant differences were found in the total FW biomass exhibited by plants from different treatments (*Post-hoc* Tukey HSD; $p<0.05$). However, for plants of treatments

FeNormal and FeEnriched these differences were only explained by the significant differences in belowground FW biomass (*Post-hoc* Tukey HSD $p<0.05$), with these being higher in the last-mentioned treatment. The edible aboveground FW biomass corresponded to 86%, 76% and 78% of all plant biomass for FeDeficiency, FeNormal and FeEnriched treatments, respectively (the DW biomass corresponded to 7.3% - 8.4% of FW biomass).

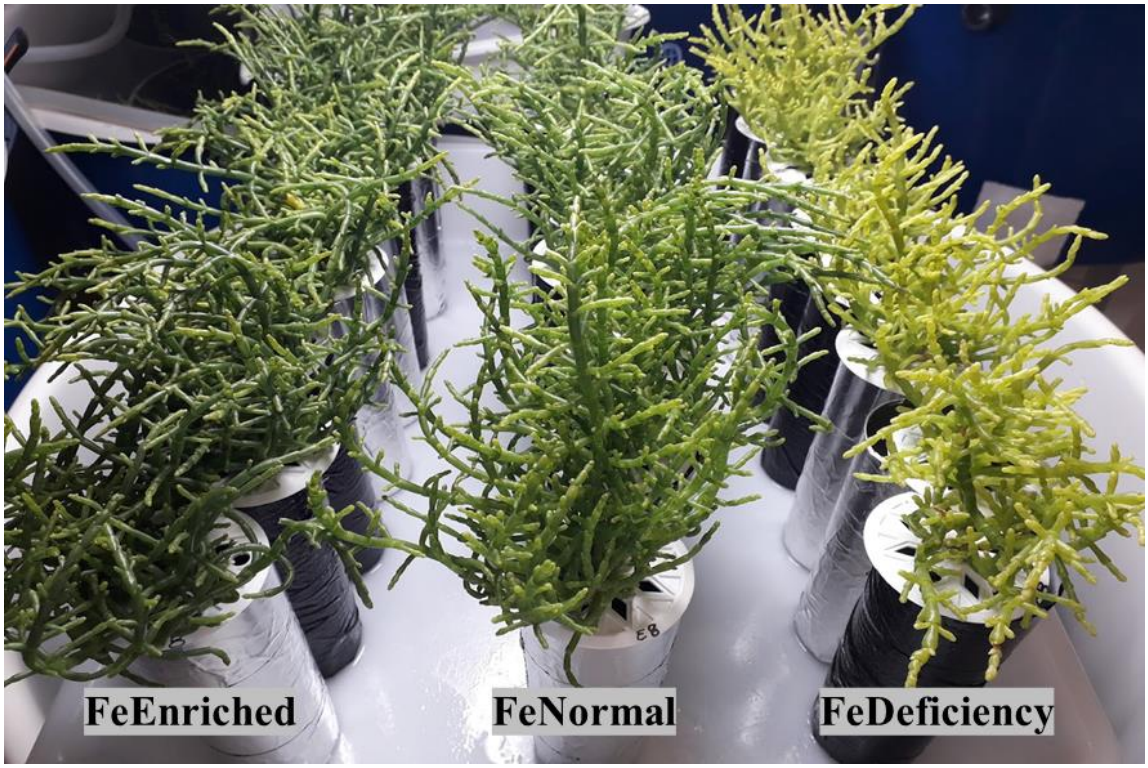


Figure 6.4. *Salicornia ramosissima* cultured during 60 days in hydroponic media with a deficiency, normal and enriched concentration of Fe (FeDeficiency, FeNormal and FeEnriched, respectively).

Table 6.2. Productivities obtained from *Salicornia ramosissima* cultured in hydroponic media with a deficiency, normal and enriched concentration in Fe (FeDeficiency, FeNormal and FeEnriched, respectively). Values are averages (±SD) of 8 replicates. Values in fresh weigh (FW) and dry weight (DW).

	FeDeficiency	FeNormal	FeEnriched
Total plant biomass (g plant^{-1})			
Start (FW)	2.41 ± 0.88 ^{a}	2.25 ± 0.94 ^{a}	2.47 ± 1.09 ^{a}
30º day (FW)	12.44 ± 2.23 ^{a}	15.62 ± 5.82 $^{a; b}$	18.16 ± 4.34 ^{b}
60º day (FW)	18.38 ± 3.64 ^{a}	30.02 ± 8.59 ^{b}	38.86 ± 6.74 ^{c}
Final aboveground and belowground biomass (g plant^{-1})			
Aboveground (FW)	15.82 ± 3.16 ^{a}	23.55 ± 6.67 ^{b}	29.59 ± 5.42 ^{b}
Aboveground (DW)	1.34 ± 0.31 ^{a}	1.88 ± 0.53 $^{a; b}$	2.16 ± 0.42 ^{b}
Belowground (FW)	2.56 ± 0.93 ^{a}	6.47 ± 2.32 ^{b}	9.27 ± 1.65 ^{c}
Belowground (DW)	0.28 ± 0.08 ^{a}	0.56 ± 0.19 ^{b}	0.63± 0.09 ^{b}
Above/Below (FW)	6.66 ± 1.90 ^{a}	3.83 ± 1.19 ^{b}	3.22 ± 0.41^{b}
Relative Growth Rate (% day^{-1})			
0 - 30º day	5.64 ± 0.95 ^{a}	6.49 ± 0.64 $^{a; b}$	6.85 ± 1.03 ^{b}
30 - 60º day	1.29 ± 0.32 ^{a}	2.27 ± 0.52 ^{b}	2.58 ± 0.34 ^{b}

Means within a row followed by different letters indicate significant differences (p<0.05). (Statistical analysis in Supplementary Table S6.5.1 and S6.5.2)

6.1.3.2.2. Pigments and efficiency of photosystems

The average values (±SD) of pigments (clorophylls and carotenoids) and maximum quantum efficiency of PSII photochemistry (*Fv/Fm*) determined in plants cultured under different Fe concentrations are displayed in Table 6.3. A strong positive correlation between the increase of Fe concentration in hydroponic media and the increment of concentration of chlorophyll and carotenoid was verified. Plants from FeDeficiency exhibited a significantly lower concentration of total chlorophyll and carotenoids when compared to the ones from other treatments (*Post-hoc* Tukey HSD*; p*<0.05). Chlorophyll *b* was not detected in plants from FeDeficiency, while very low concentrations of this pigment were determined in plants from other treatments (≈15 - 20% of total chlorophyll). Similar carotenoids/chlorophyll ratios and *Fv/Fm* values were determined in FeNormal and FeEnriched plants, while significant differences were identified in the values exhibited by FeDeficiency plants (which exhibited higher carotenoids/chlorophyll ratios and lower *Fv/Fm* values) (Kruskal-Wallis*; p*<0.05).

Table 6.3. Pigments characterization and photosystems efficiency of *Salicornia ramosissima* cultured in hydroponic media with a deficiency, normal and enriched concentration in Fe (FeDeficiency, FeNormal and FeEnriched, respectively). Values are averages (±SD) of 8 replicates.

	FeDeficiency	FeNormal	FeEnriched
Plant pigments (µg g^{-1} DW)			
Chlorophyll *a*	60 ± 10 [a]	190 ± 50 [b]	270 ± 90 [c]
Chlorophyll *b*	ND	30 ± 20 [a]	50 ± 20 [a]
Chlorophyll *total*	60 ± 10 [a]	220 ± 60 [b]	320 ± 110 [c]
Carotenoids	50 ± 20 [a]	120 ± 40 [b]	150 ± 40 [b]
Carotenoids:Chlorophyll	0.89 ± 0.40 [a]	0.53 ± 0.11 [b]	0.48 ± 0.11 [b]
Fv/Fm			
Day 30	0.70 ± 0.06 [a]	0.83 ± 0.05 [b]	0.85 ± 0.01 [b]
Day 60	0.68 ± 0.08 [a]	0.82 ± 0.04 [b]	0.83 ± 0.03 [b]

Means within a row followed by different letters indicate significant differences (p<0.05). (Statistical analysis in Supplementary Table S6.5.1 and S6.5.2)

6.1.3.2.3. Comparison of elemental composition of cultured plants under different concentrations of Fe and wild conspecifics

The elemental composition of *S. ramosissima* cultured under different concentrations of Fe are summarized in Table 6.4. A positive correlation between the increment of Fe concentration in hydroponic media and the increment of C, H, Mn, Fe, Cu, Zn and Mo in plant biomass was recorded. Significant differences were found in the elemental composition exhibited by the plants under different Fe concentrations (PERMANOVA; p<0.05; Supplementary Table S6.6). The SIMPER analysis (cut-off 50%) revealed dissimilarities of ≈ 5.0%, 6.7% and 9.8% between FeDeficiency-FeNormal, FeNormal-FeEnriched and FeDeficiency-FeEnriched plants, respectively (macro and micronutrients that contributed most to these differences are summarized in supplementary Table S6.7). Significant differences were identified between the elemental compositions of cultured plants (all tested conditions) and wild conspecifics of *S. ramosissima* (PERMANOVA; p<0.05), with SIMPER analysis (cut-off 50%) revealing dissimilarities between wild and FeDeficiency, FeNormal and FeEnriched plants of 17.0%, 14.4% and 9.8%, respectively (supplementary Table S7). Wild conspecifics exhibited the highest concentrations of Fe and Mn and the lowest concentrations of N, K when compared to cultured plants.

Table 6.4. Elemental composition (mg g^{-1} DW) of *Salicornia ramosissima* cultured in hydroponic media with a deficiency, normal and enriched concentration of Fe (FeDeficiency, FeNormal and FeEnriched, respectively). Values are averages (±SD) of 3 replicates.

	FeDeficiency	FeNormal	FeEnriched	Wild
Macronutrients				
C (mg g^{-1})	186.49 ± 17.06	215.61 ± 6.16	222.69 ± 18.16	231.35 ± 6.75
H (mg g^{-1})	21.60 ± 1.37	26.14 ± 1.01	26.27 ± 2.85	30.31 ± 1.12
N (mg g^{-1})	32.41 ± 2.44	31.29 ± 3.67	37.59 ± 2.83	13.42 ± 0.61
S (mg g^{-1})	3.15 ± 1.42	2.17 ± 0.35	2.98 ± 0.24	4.14 ± 0.31
Na (mg g^{-1})	130.57 ± 14.42	111.32 ± 1.23	111.48 ± 4.89	112.86 ± 2.74
K (mg g^{-1})	25.82 ± 3.90	28.80 ± 1.22	23.40 ± 5.32	8.25 ± 0.23
Mg (mg g^{-1})	12.22 ± 0.11	11.45 ± 0.85	7.76 ± 0.19	8.08 ± 0.49
P (mg g^{-1})	3.06 ± 0.38	2.75 ± 0.17	2.77 ± 0.10	1.42 ± 0.09
Ca (mg g^{-1})	3.02 ± 0.11	2.62 ± 0.28	2.76 ± 0.24	3.46 ± 0.32
Micronutrients				
B (µg g^{-1})	73.45 ± 4.80	63.98 ± 6.87	67.49 ± 12.03	48.73 ± 0.80
Mn (µg g^{-1})	3.69 ± 0.97	6.36 ± 0.48	7.47 ± 0.75	32.02 ± 1.33
Fe (µg g^{-1})	8.04 ± 0.46	22.42 ± 1.92	107.33 ± 37.64	947.12 ± 114.58
Ni (µg g^{-1})	9.17 ± 3.30	9.64 ± 1.27	3.74 ± 0.96	0.74 ± 0.06
Cu (µg g^{-1})	0.77 ± 0.24	1.29 ± 0.06	6.18 ± 0.20	5.13 ± 0.20
Zn (µg g^{-1})	14.87 ± 6.89	20.77 ± 3.70	41.54 ± 5.32	31.13 ± 2.42
Mo (µg g^{-1})	1.54 ± 0.30	3.04 ± 1.46	3.12 ± 0.33	1.26 ± 0.07
Se (µg g^{-1})	<0.50	<0.50	<0.50	<0.50

Statistical analysis in Supplementary Table S6 and S7.

6.1.3.2.4. Bioremediation of dissolved inorganic nitrogen (DIN-N) and phosphorus (DIP-P) and incorporation of N, P and C into edible biomass

The bioremediation efficiencies of DIN-N and DIP-P present in the hydroponic media are displayed in Figures 6.5A and 6.5B, respectively. At day 30, a similar bioremediation efficiency of DIN-N was reported for FeNormal and FeEnriched treatments (≈65 - 78% reduction of initial concentration), while a significant lower efficiency was reported for FeDeficiency plants (≈35% reduction of initial concentration) (*Post-hoc* Tukey HSD*;* $p<0.05$). In this period, no significant differences were detected in bioremediation efficiencies of DIP-P (≈85 - 91% reduction of initial concentration) (Kruskal-Wallis, $p>0.05$). At day 60, while no significant differences were found in the bioremediation efficiency of DIN-N exhibited by plants under different Fe concentrations (≈ 44 - 53%

reduction of initial concentration) (ANOVA; $p>0.05$), a significantly lower bioremediation of DIP-P was recorded in plants from FeDeficiency. Indeed, there was a ≈ 23% reduction of initial concentration of DIP-P in plants from FeDeficiency, while other treatments displayed a ≈ 63 – 94% reduction of initial concentration (Kruskal-Wallis; $p<0.05$).

At the end of the experimental trial (60 days), significant differences were found in total incorporation of N, P and C into edible aboveground biomass in plants from FeEnriched and FeDeficiency (*Post-hoc* Tukey HSD; $p<0.05$), while those under higher Fe concentration in hydroponic media exhibited higher values of incorporation (Fig. 6.6A - 6.6C). Significant differences were also detected between FeEnriched and FeNormal plants, but only for the incorporation of N into edible aboveground biomass (*Post-hoc* Tukey HSD; $p<0.05$).

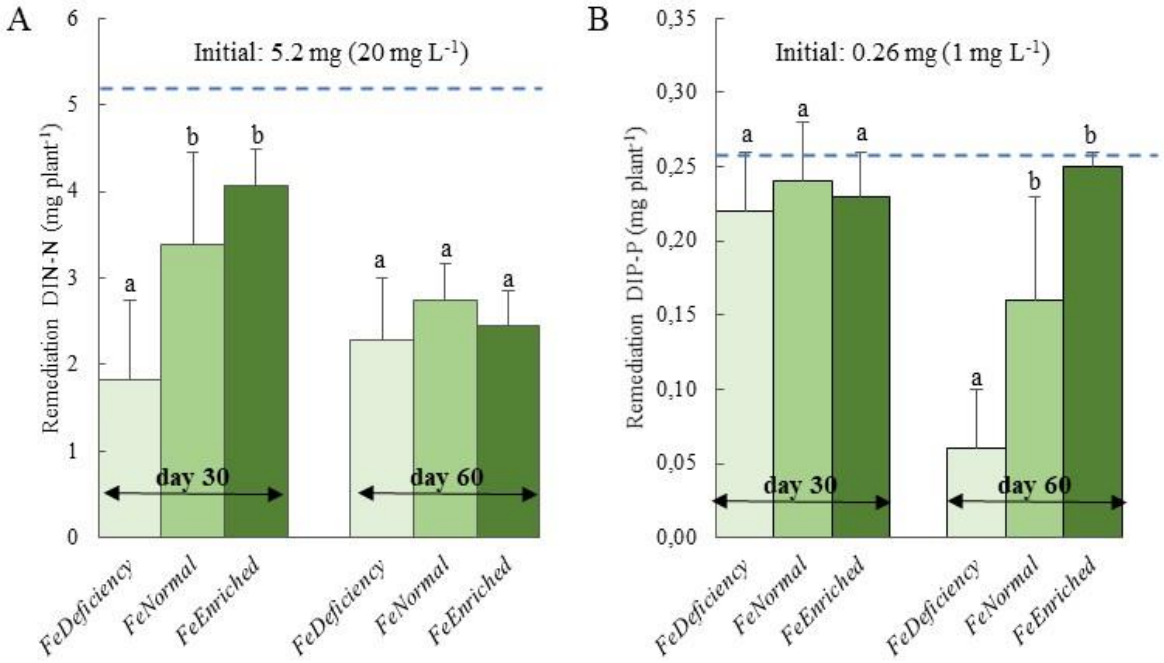


Figure 6.5. Bioremediation of dissolved inorganic nitrogen (DIN-N) (6.5A) and phosphorus (DIP-P) (6.5B) (mg plant^{-1}; 24-hour period) exhibited by *Salicornia ramosissima* cultured in hydroponic media with a deficiency, normal and enriched concentration of Fe (FeDeficiency, FeNormal and FeEnriched, respectively). Values are averages (±SD) of 8 replicates. The blue line represented in graphic 6.5A and 6.5B marks the initial concentration of DIN-N and DIP-P, respectively. Means in each time-period followed by different letters indicate significant differences ($p<0.05$) (statistical analysis presented in Supplementary Table S6.5.1 and S6.5.2.).

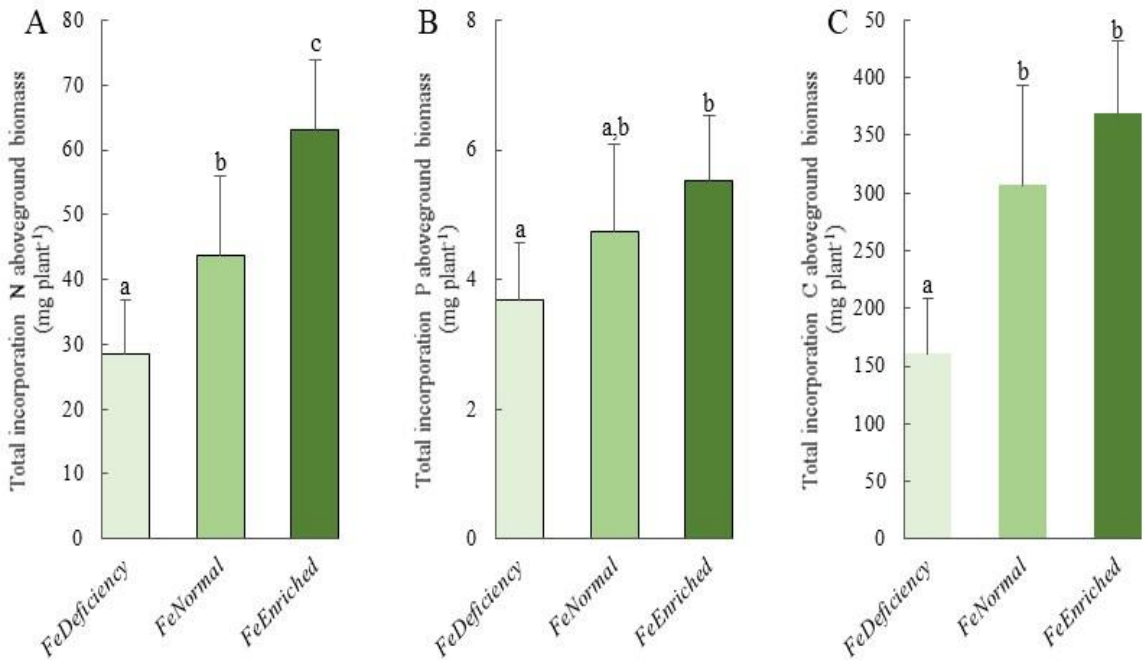


Figure 6.6. Total incorporation (mg plant^{-1}) of nitrogen (6.6A), phosphorus (6.6B) and carbon (6.6C) into edible aboveground biomass of *Salicornia ramosissima* cultured in hydroponic media during 60 days with a deficiency, normal and enriched concentration of Fe (FeDeficiency, FeNormal and FeEnriched, respectively). Values are averages (±SD) of 8 replicates. Means in each time-period followed by different letters indicate significant differences ($p<0.05$) (statistical analysis presented in Supplementary Table S6.5.1 and S6.5.2.).

6.1.4. Discussion and conclusions

6.1.4.1. Salinity Experiment

After 60 days of culture under different salinities no morphological differences were displayed by plants of *S. ramosissima*, thus confirming the potential to produce this halophyte plant under a range of brackish-water salinities. Previously, Singh *et al.* (2014)[1] described *S. ramosissima* as presenting a more compact growth with many branches of steam and more biomass per unit volume than *S. dolichostachya*, with these traits being advantageous for commercial production. The salinity range tested during this experiment is within 100 – 340 mM NaCl usually indicated as optimal for *Salicornia* spp.[1,45,52-54]. To better understand what impact a non-optimal salinity can have on the morphology and growth of these plants it is important to look at results from previous studies. For example, Singh *et al.* (2014)[1] showed that when cultured at salinities 100 mM NaCl *S. dolichostachya* produced more branches and was taller than plants cultured under 0 and 200 mM NaCl. These authors

also verified that *S. ramosissima* cultured at 257 mM NaCl produced more harvestable biomass than *S. dolichostachya*, with the gain in biomass being twice higher than that obtained under a salinity of 513mM NaCl. The performances under different salinities will always be species-dependent and differences can be found in distinct species ecotypes[41]. In the present study, only after 30 to 60 days of culture was it possible to identify a significant difference in RGR exhibited by plants under Sal.20 and Sal.25 plants (≈2.5 – 2.6 % day^{-1}) when compared to that for plants at Sal.15 (≈1.6 % day^{-1}). The better growth performances of plants cultured under higher salinity treatments in this last experimental period were not, however, sufficient to translate into significant differences in final FW and DW biomass produced (total, aboveground and belowground). However, at the end of 60 days, plants cultured under Sal.20 exhibited a slightly higher growth performance and biomass production and, most likely, if the study was extended over time it is possible that significant differences could have been detected. An extrapolation between produced biomass *vs.* area occupied by each treatment allow us to predict that the culture of ≈490 plants m^{-2} will originate productivities of edible aboveground biomass of 5.7, 7.3 and 6.0 kg FW m^{-2} $month^{-1}$ (0.50, 0.53 and 0.47 kg DW m^{-2} $month^{-1}$) for Sal.15, Sal.20 and Sal.25 plants, respectively (based on the surface area of 0.0162 m^2 used in the cultivation of the 8 plants and the growth in 60 days). This edible aboveground biomass represented ≈76% of all biomass produced, with DW representing ≈7.5% of FW biomass. If an intensive production was implemented with plants being permanently replaced by new ones, the aboveground production mentioned above could account for productivities between 64 - 82 Kg FW m^{-2} yr^{-1} (4.8 – 6.2 kg DW m^{-2} yr^{-1}). The productivities here reported were estimated based on the growth obtained during 60 days and without applying any intermediate harvest during this period. These productivities by time scale were superior to most values reported to date in previous studies addressing *Salicornia* spp. (see Table 6.5). The best performances were always achieved under values of salinity in line with the ones tested in the present study, such as the productivity of ≈60 g FW m^{-2} day^{-1} of edible aboveground biomass and growth rate of ≈9.9% day^{-1} obtained for *S. dolichostachya* (40 plants m^{-2}) cultured at a salinity of 15 - 16[25] and the productivities between 10 - 12 Kg FW m^{-2} (0.7 - 0.8 Kg DW m^{-2}) determined for *S. persica* (1000 plants m^{-2}) cultured during 6 months under relative seawater concentrations (RSW) of 50 and 75% (≈299 - 449 mM NaCl)[41]. Under RSW of 100% (≈598 mM NaCl) the last mentioned authors reported a decrease in performance with values of ≈9 Kg FW m^{-2} (0.65 -

0.75 Kg DW m^{-2})[41]. In another study, Ahmad *et al.* (2013)[45] tested *S. persica* under a salinity range between 0 - 40 (0 - 700 mM NaCl), during 60 days, and concluded that the best growth performances were achieved at a salinity of 12 (≈200 mM NaCl) and reported the production of an edible biomass of ≈36 g FW plant $^{-1}$ (≈3.1 g DW $plant^{-1}$), values which are higher than the ones obtained in the present study. These authors also reported a positive correlation between the increment of salinity and growth, in ranges between 0 – 12 (0 - 200 mM NaCl), and a negative correlation between 12 – 40 (200 - 700 mM NaCl). In this last-mentioned study, the plants grew in pots with sandy mixture, and the belowground biomass contributed to ≈0.9 g FW $plant^{-1}$ (≈0.25 g DW $plant^{-1}$), values which are approximately 10 times lower than the ones reported in the present study, in which the plants were grown using the deep-water culture technique. In another study, Brown *et al.* (1999)[17] tested *S. persica* under different salinities (0.5, 10 and 35 ≈8.6, 171 and 598 mM NaCl, respectively) and the best performances were achieved in plants irrigated with salinity 10, being produced edible aboveground biomass of ≈40.97 g DW $plant^{-1}$ and RGR rates of 4.3% day^{-1} at the end of 12 weeks experimental period. According to these authors, under lower salinity, the values were slightly inferior (34.8 g DW $plant^{-1}$; RGR: 4.1% day^{-1}) and at a higher salinity the values decreased drastically (2.6 g DW $plant^{-1}$; RGR: 3% day^{-1}).

Table 6.5. Productivities reported for *Salicornia* spp. Values in fresh weight (FW) and dry weight (DW) biomass.

Species	Productivity	Period	Plant density (plants m^{-2})	Reference
S. europaea	7 - 10.1 Kg FW m^{-2}	3 months	200 - 10.000	Webb *et al.* 2013[28]
S. persica	9 - 12 Kg FW m^{-2} (0.6 - 0.8 Kg DW m^{-2})	6 months	1000	Ventura *et al.* 2011[41]
	23 - 26 Kg m^{-2}	1 year	100	Shpigel *et al.* 2013[23]
S. dolichostachya	60 g FW m^{-2}	1 day	40	Waller *et al.* 2015[25]
	1.1 Kg DW m^{-2}	1 year	38	Singh *et al.* 2014[1]
S. brachiata	1.1 - 4.2 Kg FW m^{-2} (0.13 - 0.51 Kg DW m^{-2})	-	-	Singh *et al.* 2015[55]

Concerning bioremediation efficiencies of DIN-N and DIP-P determined in hydroponic media, similar performances were recorded between the different salinity treatments evaluated (≈61 – 78% and ≈88 - 96% reduction of initial concentrations for DIN-N and DIP-P, respectively). During this experiment it was estimated that each plant from the different salinity treatments incorporated into edible aboveground biomass ≈50 - 63 mg of N, 4.2 – 5.5 mg of P and 296 - 368 mg of C. At this level, it is important to have in mind that the uptake of carbon mostly occurs via photosynthesis and not through the uptake of dissolved inorganic carbon. The values of N and P incorporation into edible FW biomass (≈75% of total plant biomass; total plant biomass: 30 - 39 g $plant^{-1}$) reported in present study after 60 days of culture were not in agreement with those reported by Waller *et al.* (2015)[25] for smaller plants of *S. dolichostachya* (≈ 5.2 g $plant^{-1}$). In this last study, the authors reported a higher incorporation of N and P per plant (167 mg N and 23 mg P) during a 35-days period under aquaponic conditions. It is important to bear in mind that, contrary to what was done in our study, in this last mentioned work the incorporation was estimated for all plant biomass (including both aboveground and belowground biomass). Marques *et al.* (2017)[20], determined the incorporation of these key elements in the tissues of *Halimione portulacoides* cultured in aquaponics using a fish farm effluent (salinity 20 ≈342 mM NaCl). These authors verified that biomass increased from 1.4 Kg FW m^{-2} to 18.6 Kg FW m^{-2} after 5 months and estimated that ≈1.3 Kg C m^{-2} , ≈15 g N m^{-2} and ≈8 g P m^{-2} were incorporated in aerial tissues (76% of total biomass), with ≈0.5 Kg C m^{-2}, ≈3 g N m^{-2} and ≈2 g P m^{-2} being incorporated in roots (24% of total biomass). One must highlight that *H. portulacoides* displays a different elemental composition from *S. ramosissima* and therefore values must be compared with caution; nonetheless, it is possible to estimate that ≈0.15 – 0.18 Kg C m^{-2}, ≈24.5 – 31.8 g N m^{-2} and ≈2.1 – 2.7g P m^{-2} (considering 490 plants m^{-2}) will be incorporated into edible aboveground biomass of *S. ramosissima* during the 60 days of culture.

While the present study has not evaluated the potential of salinity to promote biofortification in certain target elements, this effect has been documented in previous studies (e.g., increment of salinity exhibited a positive correlation in the increment of Na content and a negative correlation in the content of K, Mg and Ca)[45,52,56,57]. These plants responded to salt stress by synthesizing organic compounds due to the active transport of toxic ions through vacuolar membranes, a process which presents considerable energy costs and results in delayed growth and productivity[58,59]. Osmotically active molecules, such as

sucrose and non-enzymatic compounds with antioxidant properties (e.g., ascorbate, carotenoids, polyphenols and ureides), are accumulated in plant cells to compensate for the oxidative stress caused by salinity and contribute to its enrichment from a nutritional point of view[41,56,60-62]. In halophyte plants such as *Salicornia persica* and *Crithmum maritimum*, a nutritional profile rich in lipids, including omega-3 fatty acids, has been described[41,58], with this content being biofortified with the increase of salinity[58]. Maciel *et al.* (2018)[63] characterized the fatty acid profile of *S. ramosissima* and verified that ≈40.4 % and 20.7% corresponded to omega-3 and omega-6 FA, respectively.

6.1.4.2. Iron Experiment

The FeDeficiency scenario addressed in the present work can occur, for example, if halophyte plants are grown in a RAS–IMTA system that use water treatments that promote precipitation and oxidative elimination of Fe (among other essential micro and macronutrients) (e.g., ozonation, chemical oxidation). The best RGR for all tested conditions were achieved during the first monitoring period (from day 1 to 30), with values recorded for the second period monitored (from day 31 to 60) being 3-4-times lower (≈5.6 – 6.9 and 1.3 - 2.6 % day^{-1}, respectively). Growth under different Fe concentrations resulted in significant differences in total plant FW biomass, and increases of ≈8-, 13- and 16-times to that of the initial value (2.3 – 2.4 g $plant^{-1}$) were reported for FeDeficiency, FeNormal and FeEnriched plants, respectively. A positive correlation between the increase in Fe concentration and the increase of growth was verified, and it is possible that the requirement of *S. ramosissima*, to reach its best growth performance could be slightly above the 30 µg L^{-1} usually present in normal brackish water (used in the FeNormal scenario). The growth here reported could represent the generation of edible aboveground biomass of ≈3.9, 5.8 and 7.3 kg FW m^{-2} $month^{-1}$ (≈0.33, 0.46 and 0.53 kg DW m^{-2} $month^{-1}$), respectively, assuming a plant density of ≈490 plants m^{-2} (based on the surface area of 0.0162 m^2 used in the cultivation of the 8 plants and considering the growth obtained in 60 days). A decrease in growth associated with Fe deficiency had already been reported in previous studies, with Buhmann *et al.* (2015)[37] referring that *Tripolium pannonicum* cultured in media without Fe exhibited a lower biomass gain (20 g FW; 4 g DW) when compared to conspecifics grown with the addition of different Fe chelating agents (114 - 214 g FW; 11 - 18 g DW). Productivities obtained in the present study mainly for FeNormal and FeEnriched scenarios

were, in most cases, superior to the ones previously reported for *Salicornia* spp. (see Table 6.5). Iron deficiency also caused a significant lower production in belowground biomass, with this accounting for ≈14% of the total plant biomass in FeDeficiency plants and ≈21 - 23% in FeNormal and FeEnriched plants. These differences are likely due to the fact that plants under FeNormal and FeEnriched exhibited a healthier physiological condition and were able to more promptly respond to any stressing condition through root development[53]. Here, it is important to remember that P was close to being fully consumed during the experiment, mainly in FeNormal and FeEnriched treatments, with this being considered the second most important element for plant nutrition (after N) and significantly contributing to the reduction of plant growth if not supplied in suitable levels[64]. Plants usually respond to the availability of this element by adjusting belowground biomass to maintain adequate incorporation and productivity[64]. Thus, it is very likely that the higher root development recorded in plants grown under FeNormal and FeEnriched conditions may have been a stress response to the low concentration of P in culture media at the end of each cycle of hydroponic media. The proportion of ≈70 - 80% edible aboveground biomass produced in FeNormal and FeEnriched conditions was in agreement with what was verified in previous studies, where halophyte plants were tested in soilless systems[20,25].

Iron is not easily available in neutral to alkaline environments, often rendering plants Fe-deficient despite its abundance[65]. The redox potential (i.e., oxidizing or reducing conditions) and pH governing the behaviour of Fe^{2+} and neutral pH conditions promote the precipitation of poorly ordered Fe minerals (ferrihydrite), whereas reduced and acid conditions promote the mobilization of Fe minerals[66]. In the present study, oxygen levels in hydroponic media were kept between 3.8 – 4.6 mg L^{-1} and pH has always remained slightly acidic, between 6.4 – 6.6, which may have favoured that Fe, and elements such as Mg, P, Ca and Mn were maintained bioavailable to plants. Buhmann *et al.* (2015)[37] showed the importance of adding Fe in chelated form to promote the growth of healthy plant biomass and the importance of using a chelated agent stable in alkaline water, with plants treated with Fe-EDDHA showing significantly higher chlorophyll and carotenoids than plants treated with a less stable agent, such as Fe-EDTA. Moreover, in seawater pH always tends to be slightly alkaline, and treatments commonly used in aquaculture production systems (e.g., water ozonation, chemical oxidation) also promote media alkalinisation, thus rendering some essential nutrients (such as Fe) less bioavailable or even unavailable for plants[25,46]. Singh *et al.* (2014)[1]

found that plants produced in hydroponics at pH 8.0 - 8.4 became chlorotic, while those grown in sand did not show any signs of discoloration. The dark green coloration in leaves may be indicative of Fe overdose; a level of approximately 1.2 mg L^{-1} can cause this condition, with better growth performances and coloration being obtained with half of the mentioned concentration[37]. In the present work, Fe concentration used in FeEnriched treatment was approximately half of the overdose value mentioned above and there is no evidence that these plants have been subjected to this condition.

Concerning pigments and efficiency of photosystems, it was shown that plants under FeDeficiency exhibited signs of chlorosis, having a yellowish green coloration at day 60, while conspecifics from FeNormal and FeEnriched exhibited the typical coloration of unstressed plants. Moreover, the most obvious characteristic of the leaves from Fe deficient plants is chlorosis, due to the low concentration per area of photosynthetic pigments (e.g., chlorophylls and carotenoids)[67,68]. Plants under FeDeficiency exhibited 3 and 2-times lower total chlorophyll and carotenoids content, respectively, to the ones recorded in plants cultured under FeNormal. Still, plants under FeDeficiency condition exhibited a significantly higher carotenoid/chlorophylls ratio than the ones verified in other tested conditions. These results are in agreement with the ones reported by Buhmann *et al.* (2015)[37], which verified lower levels of chlorophyll and carotenoids in *T. pannonicum* grown in an Fe-free media compared to those grown with supplementation of this element through the addition of most stable chelating agent at a pH above 7 (chlorophyll: 49 and 875 µg g^{-1} FW; carotenoids: 21 and 150 µg g^{-1} FW, respectively). According to Waller *et al.* (2015)[25], pigment loss can be an indicator of nutrient deficiency.

The determination of maximum quantum efficiency of photosystem II (PSII) photochemistry (*Fv/Fm*) is one of the most common techniques for measuring "stress" in leaves[48]. The values determined in FeNormal and FeEnriched plants were consistent to be in line with a state very close to the maximum quantum yield of photosynthesis exhibited by unstressed plants (*Fv/Fm* ≈0.83)[48,69]. Conversely, the values measured in FeDeficiency plants were indicative that the lack of Fe in hydroponic media compromised the efficiency of PSII (often referred as photoinibition) or the induction of sustained quenching[48,70,71]. These results are in agreement with the ones verified by Buhmann *et al.* (2015)[37] that reported superior values of *Fv/Fm* in *T. pannonicum* supplemented with Fe (cultured without Fe: 0.57; cultured with Fe: 0.79 - 0.84). According to Morales *et al.* (2000)[68], extremely Fe

deficient leaves of pear trees showed a decrease in PSII efficiency, reporting a decrease in the amounts of excess light that can be used in photosynthesis by reducing absorbance, but also by increasing the relative amount of light dissipated thermally by the PSII antenna (>20% in iron deficit pear leaves than control plants). Deficiency in Fe acts specifically on the chloroplast structure and function, as Fe ions are mainly (up to 60 - 80%) localized in photosynthetic membranes[72,73]. Ladygin (2004)[73] verified that Fe deficiency inhibited photosynthesis with chlorophyll content decreasing faster than the number of reaction centres of PSI and PSII.

In the present study, the culture under different concentrations of Fe originated plants with significant differences in terms of elemental composition and a positive correlation was recorded between the increment of Fe concentration in hydroponic media and the increment of C, H, Mn, Fe, Cu, Zn and Mo in plant biomass. Besides these elements, plants from FeEnriched treatment exhibited the highest content of C, and N, while those form FeDeficiency exhibited the highest content of S, Na, Mg, P, Ca and B. These results are in agreement with the ones reported by Giordano *et al.* (2019)[74], which revealed that the addition of 2mM of Fe in the nutrient solution resulted in increased Fe content in the leaves of lettuce. These authors have also reported a negative effect on the concentration of Ca, K and Mg, with competition between cations, root damage and oxidative stress, to be pointed out as the most likely causes for these differences. These biochemical changes can be important if the biofortification of plants with a certain level of Fe is intended. It should also be noted that when compared to wild plants, *S. ramosissima* from FeEnriched exhibited the highest values of similarity. Indeed, wild plants featured the highest content of Fe, Mn, C, H, S and Ca and the lowest content of N, K, P, B, Ni and Mo in respect to the plants culture under the different experimental treatments. The results achieved in this study allowed to verify that it is possible to produce iron enriched salt vegetables using an environmentally friendly method (with no use of arable land, no use of freshwater resources, reusing water from aquaculture effluents or other sources). Moreover, this ability is particularly relevant if one considers that 30% of global cultivated soils are calcareous with low Fe availability, as this element is present in insoluble oxidized forms[65,75,76]. The human recommended oral dose for Fe is 0.7 mg Kg^{-1} day^{-1} [77,78], which means that it would be required to ingest ≈6.5 grams DW biomass of FeEnriched plant biomass (Fe content: 0.11 mg g^{-1} DW) to reach this dose. As the DW corresponds to about 7% of the FW, this dose would be reached by ingesting

about 93 grams of FW biomass from FeEnriched plants. To achieve this same dose using plants from FeDeficiency, it would be necessary to ingest ≈87.5 g DW biomass, the equivalent to 1.3 Kg FW biomass.

The concentrations of DIN-N and DIP-P used in the present study in hydroponic media (DIN-N: 20 mg L^{-1} and DIP-P: 1 mg L^{-1}) were similar to the ones found in intensive aquaculture effluents[20,25]. The bioremediation efficiencies recorded showed that these were adequate concentrations for the cultivation of *S. ramosissima*, and only DIP-P was close to be fully consumed at the end of each hydroponic media cycle (24-hour). Two distinct trends of bioremediation performances of DIN-N and DIP-P were determined at day 30 and 60, most likely due to different growth efficiencies observed between both periods. At day 30, the consequences that these plants may face when grown in media lacking Fe were evident, with plants from FeDeficiency exhibiting a significantly lower bioremediation of DIN-N than the ones from other treatments (≈35% reduction of initial concentration for FeDeficiency and 65-78% reduction for FeNormal and FeEnriched). The bioremediation of DIP-P at day 30 was very similar between all tested conditions (≈81 – 91% reduction of initial concentration), and an almost complete depletion of this element was verified. Conversely at day 60, it was possible to record similar bioremediation efficiencies for DIN-N between treatments (≈43.8 - 57.7% reduction of initial concentration), and a significantly higher bioremediation efficiency for DIP-P being displayed by FeEnriched plants when compared to the ones from FeDeficiency (≈23 and 95% reduction of initial concentration, respectively). The lower bioremediation efficiencies of DIN-N and DIP-P (except DIP-P for FeEnriched treatment) determined at day 60 may have been a consequence of an excess of biomass per unit of production area (due to the biomass gain during experiment) and the inherent difficulty of some apical portions to receive suitable light levels for photosynthesis (see image S6.1B). It is also possible, that in this last period (31- 60 days), a deficiency in the N:P:C ratio that is essential to maintain a maximum growth efficiency may have occurred[64,79,80], as plant biomass had already peaked at 10-20-times higher values than those recorded at the beginning of the experimental trial. Here it is important to note that while plants were not limited in N and P, atmospheric C inside the growth chamber was not monitored. Another possibility to justify the results of lower bioremediation efficiencies (and growth performance) exhibited by plants from FeNormal and FeEnriched treatments in the second period (31-60 days) is that these plants were exhibited a very fast growth at an early

stage of the study (0-30 days) and, inherently, incorporated larger amounts of nutrients and then, when they had reached a certain average weight, their growth potential had been reduced and their bioremediation effect also drastically affected. In its turn, plants from FeDeficiency exhibited a lower and more constant growth and a rather more stable bioremediation efficiency all over the study. Although bioremediation efficiencies determined in the hydroponic media in both monitored periods have not maintained a stable trend during the experiment, at the end of the trial it was possible to determine that plants that were FeEnriched incorporated a significanly higher amount of N, P and C into their edible aboveground biomass (≈63, 5.5 and 369 mg, respectively) than plants under FeDeficiency (≈28, 3.7 and 161 mg, respectively). Between plants from FeEnriched and FeNormal, these differences were less pronounced and only a significantly higher incorporation of N was reported for plants that were FeEnriched (≈43 and 63 mg for FeNormal and FeEnriched plants, respectively). The results of bioremediation and nutrient incorporation here reported demonstrate the great potential of these plants as extractive species for recovering inorganic nutrients from aquaculture effluents[20,23,25,27,28,37]. The values of N and P incorporation here reported were lower than the ones determined by Waller *et al.* (2015)[25] for *S. dolichostachya* grown hydroponically, which assimilated 167 and 23 mg, respectively, during 35 days. These authors estimated that 14.4 m^2 of hydroponic area with 1128 plant of *S. dolichostachya* (≈78 plants m^2) would have been necessary to remove 189 g N excreted by fish during 35 days (248 *Dicentrarchus labrax* juveniles with initial average weight of 38.1 g), resulting in 84 kg FW biomass (64 Kg marketable leafy vegetable). The results obtained in the present study are in agreement to the ones reported by Buhmann *et al.* (2015)[37], which verified that plants cultured without the supplementation of Fe showed significantly lower gains of biomass and lower uptakes of N and P than conspecifics supplemented with this element.

The present work confirmed the potential of *S. ramosissima* cultured under hydroponics conditions to recover dissolved nutrients into valuable saline vegetable biomass. In the "Salinity experiment" it was shown that this halophyte plant can be successfully cultured in a range of salinities between 15 - 25, with the growth performance achieved at a salinity of 20 being slightly higher than the ones obtained in the other conditions tested. The "Iron experiment" showed that it is essential to optimize culture conditions, namely when certain treatments (e.g., ozonation, chemical oxidation) are applied to hydroponic/aquaponic media

and promote the precipitation to an insoluble condition and make unavailable salts of certain essential elements (e.g., Fe, Mg, P, Ca and Mn). Plants grown under FeDeficiency assumed a greenish yellow coloration and displayed an inferior growth and bioremediation performances. Significant differences were recorded in the elemental composition of plants cultured under different Fe concentrations, with FeEnriched plants exhibiting concentrations of this element ≈13- and 5- times higher than the ones recorded in conspecifics from FeDeficiency and FeNormal, respectively. These findings demonstrate that biofortification in Fe can be successfully performed for *S. ramosissima* cultured in hydroponics.

6.1.5. Chapter 6 – References

1. Singh, D., Buhmann, A. K., Flowers, T. J., Seal, C. E. & Papenbrock, J. Salicornia as a crop plant in temperate regions: Selection of genetically characterized ecotypes and optimization of their cultivation conditions. *AoB Plants* **6**, (2014).
2. Gunning, D., Maguire, J. & Burnell, G. The development of sustainable saltwater-based food production systems: A review of established and novel concepts. *Water* **8, 598**, 1-37 (2016).
3. Panta, S. *et al.* Halophyte agriculture: Success stories. *Environ. Exp. Bot.* **107**, 71–83 (2014).
4. Flowers, T. J. & Colmer, T. D. Plant salt tolerance: adaptations in halophytes. *Ann. Bot.* **115**, 327–331 (2015).
5. Flett, I. How aquaponics can improve aquaculture and help feed a hungry world. MSc Thesis – University of Akureyri (2017). http://hdl.handle.net/1946/28671
6. United Nations. The future we want - Outcome document of the United Nations Conference on Sustainable Development. 41 (2012)
7. Turcios, A. E. & Papenbrock, J. Sustainable treatment of aquaculture effluents-What can we learn from the past for the future? *Sustain.* **6**, 836–856 (2014).
8. Shahid, M., Jaradat, A. A. & Rao, N. K. Use of Marginal Water for *Salicornia bigelovii* Torr, Planting in the United Arab Emirates. *Develop. in soil salinity assessment and reclamation* **31**, 451-808 (2013).
9. Gilliham, M. Salinity tolerance of crops - what is the cost ?. New Phytol. 208, 668-673 (2015).
10. Scagel, C. F., Lee, J. & Mitchell, J. N. Salinity from NaCl changes the nutrient and polyphenolic composition of basil leaves. *Ind. Crops Prod.* **127**, 119–128 (2019).
11. Ventura, Y., Eshel, A., Pasternak, D. & Sagi, M. The development of halophyte-based agriculture: Past and present. *Ann. Bot.* **115**, 529–540 (2015).
12. Buhmann, A. & Papenbrock, J. An economic point of view of secondary compounds in halophytes. *Funct. Plant Biol.* **40**, 952–967 (2013).
13. Ventura, Y. & Sagi, M. Halophyte crop cultivation: The case for Salicornia and Sarcocornia. *Environ. Exp. Bot.* **92**, 144–153 (2013).
14. Ksouri, R. *et al.* Medicinal halophytes: potent source of health promoting biomolecules with medical, nutraceutical and food applications. *Crit. Rev. Biotechnol.* **32**, 1–38 (2011).
15. Lopes, A. *et al.* Natural products from extreme marine environments: Searching for potential industrial uses within extremophile plants. *Ind. Crops Prod.* **94**, 299–307 (2016).
16. Boxman, S. E. *et al.* Effect of support medium, hydraulic loading rate and plant density on water quality and growth of halophytes in marine aquaponic systems. *Aquac. Res.* 1–15 (2016)
17. Brown, J. J., Glenn, E. P., Fitzsimmons, K. M. & Smith, S. E. Halophytes for the treatment of saline aquaculture effluent. *Aquaculture* **175**, 255–268 (1999).
18. Chen, X. *et al.* A novel combined recirculating treatment system for intensive marine aquaculture. *Aquac. Res.* **48**, 5062–5071 (2017).

19. Ma, X. *et al.* Characterization of microbial communities in pilot-scale constructed wetlands with Salicornia for treatment of marine aquaculture effluents. *Hindawi archaea* **2018**, 1–12 (2018).

20. Marques, B., Calado, R. & Lillebø, A. I. New species for the biomitigation of a super-intensive marine fish farm effluent: Combined use of polychaete-assisted sand filters and halophyte aquaponics. *Sci. Total Environ.* **599-600**, 1922–1928 (2017).

21. Pinheiro, I. *et al.* Production of the halophyte *Sarcocornia ambigua* and Pacific white shrimp in an aquaponic system with biofloc technology. *Ecol. Eng.* **100**, 261–267 (2017).

22. Poli, M. A. *et al.* Integrated multitrophic aquaculture applied to shrimp rearing in a biofloc system. *Aquaculture* **511**, 1–6 (2019).

23. Shpigel, M. *et al.* Constructed wetland with Salicornia as a biofilter for mariculture effluents. *Aquaculture* **412–413**, 52–63 (2013).

24. Vlahos, N. *et al.* An experimental brackish aquaponic system using juvenile gilthead sea bream (*Sparus aurata*) and rock samphire (*Crithmum maritimum*). *Sustain.* **11**, (2019).

25. Waller, U. *et al.* Integrated multi-trophic aquaculture in a zero-exchange recirculation aquaculture system for marine fish and hydroponic halophyte production. *Aquacult Int* **23**, 1473–1489 (2015).

26. Watanabe, W. O. & Farnell, R. D. Experimental evaluation of the Halophyte, *Salicornia virginica*, for biomitigation of dissolved nutrients in effluent from a recirculating aquaculture system for marine finfish. *J. World Aquac. Soc.* (2017).

27. Webb, J. M. *et al.* Halophyte filter beds for treatment of saline wastewater from aquaculture. *Water Res.* **46**, 5102–5114 (2012).

28. Webb, J. M. *et al.* The effect of halophyte planting density on the efficiency of constructed wetlands for the treatment of wastewater from marine aquaculture. *Ecol. Eng.* **61**, 145–153 (2013).

29. Chopin, T. Integrated multi-Trophic aquaculture. In Advancing the aquaculture agenda: OECD Workshop, 195–217 (OECD publishing, 2010).

30. Chopin, T. *et al.* Multitrophic integration for sustainable marine aquaculture. *Encycl. Ecol.* **3**, 2463–2475 (2008).

31. Barrington, K., Chopin, T. & Robinson, S. Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. Integr. Maric. - A Glob. Rev. - *FAO Fish. Aquac.* **529,** 7–46 (2009).

32. Troell, M. *et al.* Ecological engineering in aquaculture - Potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. *Aquaculture* **297**, 1–9 (2009).

33. Custódio, M., Villasante, S., Cremades, J., Calado, R. & Lillebø, A. I. Unravelling the potential of halophytes for marine integrated multi-trophic aquaculture (IMTA) - a perspective on performance, opportunities and challenges. *Aquac. Environ. Interact*. **9**, 445–460 (2017).

34. Granada, L., Sousa, N., Lemos, M. F. L. & Lopes, S. Is integrated multitrophic aquaculture the solution to the sectors' major challenges? – a review. *Rev. Aquac.* **8**, 283–300 (2016).

35. Hughes, A. D. & Black, K. D. Going beyond the search for solutions: understanding trade-offs in European integrated multi-trophic aquaculture development. *Aquac. Environ. Interact.* **8**, 191–199 (2016).

36. Buhmann, A. & Papenbrock, J. Biofiltering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives. *Environ. Exp. Bot.* **92**, 122–133 (2013).

37. Buhmann, A. K., Waller, U., Wecker, B. & Papenbrock, J. Optimization of culturing conditions and selection of species for the use of halophytes as biofilter for nutrient-rich saline water. *Agric. Water Manag.* **149**, 102–114 (2015).

38. Boxman, S. E., Nystrom, M., Ergas, S. J., Main, K. L. & Trotz, M. A. Evaluation of water treatment capacity, nutrient cycling, and biomass production in a marine aquaponic system. *Ecol. Eng.* **120**, 299–310 (2018).

39. Jones, J. B. Hydroponics: A Practical Guide for the Soilless Grower -2nd ed. (CRC Press, 2016).

40. Feng, L., Ji, B. & Su, B. Economic value and exploiting approaches of sea asparagus, a seawater-irrigated vegetable. *Agric. Sci.* **04**, 40–44 (2013).

41. Ventura, Y. *et al.* Effect of seawater concentration on the productivity and nutritional value of annual Salicornia and perennial Sarcocornia halophytes as leafy vegetable crops. *Sci. Hortic*. **128**, 189–196 (2011).

42. Glenn, E. P., Coates, W. E., Riley, J. J., Kuehl, R. O. & Swingle, R. S. *Salicornia bigelovii* Torr.: a seawater-irrigated forage for goats. *Anim. Feed Sci. Technol.* **40**, 21–30 (1992).

43. Eganathan, P., Subramanian, H. M., Latha, R. & Rao, C. S. Oil analysis in seeds of *Salicornia brachiata*. *Ind. Crops Prod.* **23**, 177–179 (2006).

44. Gunning, D. Cultivating *Salicornia europaea* (Marsh Samphire). (University College Cork, Ireland,2016).**;** www.bim.ie/media/bim/content/news,and,events/BIM,Cultivating,,Salicornia,europaea,-,Marsh,Samphire.pdf

45 . Ahmad, S. T., Sima, N. A. K. K. & Mirzaei, H. H. Effects of sodium chloride on physiological aspects of *Salicornia persica* growth. *J. Plant Nutr.* **36**, 401–414 (2013).

46. Mosing, J. and Fallu, R.. Australian Fish Farmer: A practical guide. (Landlinks Press – 2004)

47. Sudhakar, P., Latha, P. & Reddy, P. V. Phenotypic crop plants for physiological and biochemical traits. Academic Press, 1-194 (2016).

48. Murchie, E. H. & Lawson, T. Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. *J. Exp. Bot.* **64**, 3983–3998 (2013).

49. APHA/AWWA/WEF. Method 4500-NO_3 - Standard Methods for the Examination of Water and Wastewater. Stand. *American Public Health association* (2012).

50. Grasshoff, K., Kremling, K. & Ehrhardt, M. Methods of Seawater Analysis – 3rd ed. Wiley-VCH, 1-634. (1999).

51. Anderson, M., Gorley, R. N. & Clarke, K. R. PERMANOVA + for PRIMER: Guide to software and statistical methods. Plymouth, 1:214 (2008).

52. Cooper, A. The effects of salinity and waterlogging on the growth and cation uptake of salt marsh plants. *New Phytol.* **90**, 263–275 (1982).

53. Katschnig, D., Broekman, R. & Rozema, J. Salt tolerance in the halophyte *Salicornia dolichostachya* moss: Growth, morphology and physiology. *Environ. Exp. Bot.* **92**, 32–42 (2013).

54. Parida, A. K. & Jha, B. Physiological and biochemical responses reveal the drought tolerance efficacy of the halophyte *Salicornia brachiata*. *J. Plant Growth Regul.* **32**, 342–352 (2013).

55. Singh, N., Tasung, A., Tripathi, S., Patel, P.B., Bafna, A.M., Patil, R.G., 2015. Aquaculture Effluent: Effect on Yield, Nutrient Content and Uptake in *Salicornia bachiata* Roxb. Journal of Aquaculture Research & Development 6 (11), 6–13 (2015)

56. Flowers, T. J. & Colmer, T. D. Salinity tolerance in halophytes. *New Phytol*. **179**, 945–963 (2008).

57. Koyro, H. W. Effect of salinity on growth, photosynthesis, water relations and solute composition of the potential cash crop halophyte *Plantago coronopus* (L.). *Environ. Exp. Bot.* **56**, 136–146 (2006).

58. Ventura, Y. *et al.* Effects of salinity on flowering, morphology, biomass accumulation and leaf metabolites in an edible halophyte. *AoB Plants* (2014)

59. Zhu, J. K. Plant salt tolerance. *Trends Plant Sci.* **6**, 66–71 (2001).

60. Rodrigues, M. J. *et al.* Growth performance, in vitro antioxidant properties and chemical composition of the halophyte *Limonium algarvense* Erben are strongly influenced by the irrigation salinity. *Ind. Crops Prod.* **143** (2020).

61. Rodrigues, M. J. *et al.* Unravelling the antioxidant potential and the phenolic composition of different anatomical organs of the marine halophyte *Limonium algarvense*. *Ind. Crops Prod.* **77**, 315–322 (2015).

62. Cieślik, E., Gręda, A. & Adamus, W. Contents of polyphenols in fruit and vegetables. *Food Chem.* **94**, 135–142 (2006).

63. Maciel, E. *et al.* Polar lipidome profiling of *Salicornia ramosissima* and *Halimione portulacoides* and the relevance of lipidomics for the valorization of halophytes. *Phytochemistry* **153**, 94–101 (2018).

64. Kvakić, M. *et al.* Carbon and phosphorus allocation in annual plants: An optimal functioning approach. *Front. Plant Sci.* **11**, 1–14 (2020).

65. Rout, G. R. Role of iron in plant growth and metabolism. *Rev. in Agricul. Sci.* **3**, 1-24 (2015)

66. Colombo, C., Palumbo, G., He, J., Pinton, R. & Cesco, S. Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. *J Soils Sediments* **14**, 538–548 (2014).

67. Morales, F., Abadía, A. & Abadía, J. Characterization of the xanthophyll cycle and other photosynthetic pigment changes induced by iron deficiency in sugar beet (*Beta vulgaris* L.). *Plant Physiol.* **94**, 607–613 (1990).

68. Morales, F., Belkhodja, R., Abadía, A. & Abadía, J. Photosystem II efficiency and mechanisms of energy dissipation in iron-deficient, field-grown pear trees (*Pyrus communis* L.). *Photosynth. Res.* **63**, 9–21 (2000).

69. Björkman, O. & Demmig, B. Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* **170**, 489–504 (1987).

70. Long, S. P., Humphries, S. & Falkowski, P. G. Photoinhibition of photosynthesis in nature. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45**, 633–662 (1994).

71. Demmig-Adams, B. & Adams, W. W. Photoprotection in an ecological context: The remarkable complexity of thermal energy dissipation. *New Phytol.* **172**, 11–21 (2006).

72. Terry, N. & Abadia, J. Function of iron in chloroplasts. *J. Plant Nutr.* **9**, 609–646 (1986).

73. Ladygin, V. G. The effect of root hypoxia and iron deficiency on the photosynthesis, biochemical composition, and structure of pea chloroplasts. *Russian J. of plant Physiol.* **51**, 28–40 (2004).

74. Giordano, M. *et al.* Iron biofortification of red and green pigmented lettuce in closed soilless cultivation impacts crop performance and modulates mineral and bioactive composition. *Agronomy* **9**, 1-21 (2019).

75. Guerinot, M. L. & Yi Ying. Iron: Nutritious, noxious, and not readily available. *Plant Physiol.* **104**, 815–820 (1994).

76. Mori, S. Iron acquisition by plants. *Curr. Opin. Plant Biol.* **2**, 250–253 (1999).

77. Sharma, A., Katnoria, J.K., Nagpal, A.K. Heavy metals in vegetables: screening health risks involved in cultivation along wastewater drain and irrigating with wastewater. *Springerplus* **5**, 1–16 (2016).

78. US Environmental Protection Agency (US EPA) (2015) Human health risk assessment: risk-based concentration table. http://www.epa.gov/reg3hwmd/risk/human/rb-concentration_table/Generic_Tables/index.htm

79. Jin, J., Tang, C. & Sale, P. The impact of elevated carbon dioxide on the phosphorus nutrition of plants: A review. *Ann. Bot.* **116**, 987–999 (2015).

80. Yi, Y., Sugiura, D. & Yano, K. Nitrogen and water demands for maximum growth of *Solanum tuberosum* under doubled CO_2: Interaction with phosphorus based on the demands. *Environ. Exp. Bot.* **176** (2020).

6.1.6. Chapter 6 – Supporting information

Table S6.1. Retention time period (RT), volume and initial DIN-N (NO_3-N) and DIP-P (PO_4-P) concentration present in hydroponic media.

Experiment	Period (Days)	Hydroponic media (mL)	Retention times (Days)	Initial DIN-N (mg L^{-1})	Initial DIP-P (mg L^{-1})
Salinity and Iron experiments	0 - 25	90	3	20	1
	26 - 60	260	1	20	1

Table S6.2. Carbon (C), nitrogen (N) and phosphorus (P) determined in the initial biomass of *Salicornia ramosissima* used in Salinity and Iron experiments. Values are averages (±SD) of 3 replicates (mg g^{-1} DW).

	Initial biomass
C (mg g^{-1})	232.19 ± 8.82
N (mg g^{-1})	37.05 ± 1.64
P (mg g^{-1})	1.46 ± 0.18

Figure S6.1. *Salicornia ramosissima* in Salinity and Iron experiments at day 0 (S1A) and day 60 (S1B) in a photoperiod and temperature-controlled growth chamber.

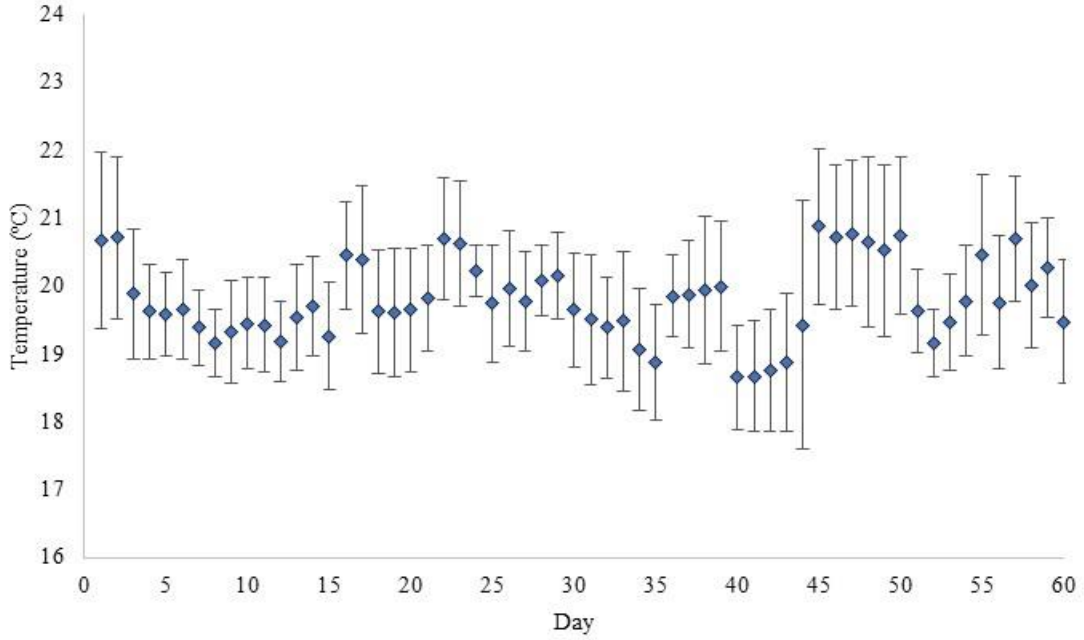


Figure S6.2. Daily temperature inside climatic chamber during Salinity and Iron experiments. Values are averages (±SD) of 3 replicates.

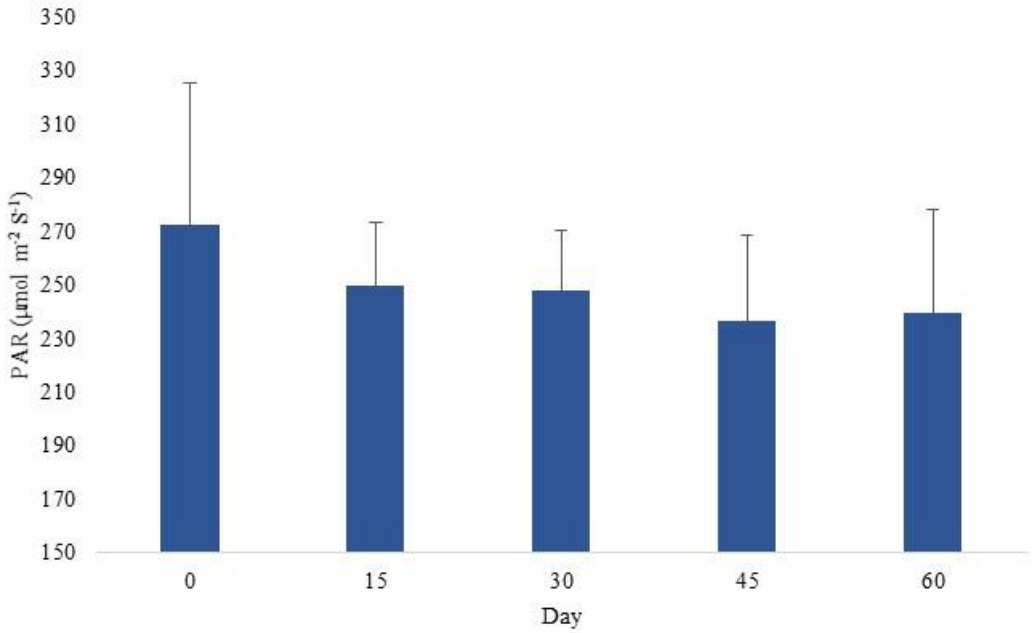


Figure S6.3. Photosynthetically active radiation (PAR) weekly measured inside climatic chamber during Salinity and Iron experiments. Values are averages (±SD) of 7 replicates.

Table S6.3. pH, dissolved oxygen (DO) (mg L^{-1}) and water temperature (ºC) measured fortnightly in the hydroponic media used to grow *Salicornia ramosissima* during Salinity and Iron experiments. Values are averages (±SD) of 8 replicates.

	Iron experiment			Salinity experiment		
	FeDeficiency	FeNormal	FeEnriched	Sal.15	Sal.20	Sal.25
pH	6.57 ± 0.48	6.40 ± 0.41	6.59 ± 0.33	6.53 ± 0.43	6.59 ± 0.33	6.65 ± 0.45
DO (mg L^{-1})	4.56 ± 1.55	3.86 ± 1.27	4.17 ± 1.72	4.58 ± 1.96	4.17 ± 1.72	4.62 ± 2.09
Water Temp. (ºC)	21.16 ± 1.81	21.35 ± 1.87	21.39 ± 1.89	21.58 ± 2.02	21.39 ± 1.89	21.32 ± 1.80

Table S6.4.1. One-Way ANOVA to evaluate variations in growth performance, pigments/ efficiency of photosystems and bioremediation performance exhibited by *Salicornia ramosissima* cultured under different treatments of salinity: Sal.15, Sal.20 and Sal.25.

Statistical test	*F - value*	*p - value*	*R_2 (%)*
Average fresh weight			
Average weight Day 0	0.06	0.945	0.53
Average weight Day 30	1.47	0.252	12.30
Average weight Day 60	2.79	0.084	20.98
Regular Growth Rate			
RGR 0 - 30 Day	0.78	0.471	6.92
RGR 30 - 60 Day	16.95	0.000	61.75
Final aboveground and belowground biomass			
Aboveground FW	2.81	0.083	21.09
Aboveground DW	2.53	0.104	19.40
Belowground FW	2.63	0.095	20.05
Belowground DW	0.43	0.658	3.91
Above/Below	2.41	0.114	18.69
Bioremediation in hydroponic media			
DIN-N Day 30		0.119*	
DIN-N Day 60	0.36	0.700	3.34
DIP-P Day 30		0.084*	
DIP-P Day 60		0.403*	
Incorporation of N, P and C in edible biomass			
Nitrogen	2.58	0.100	19.70
Phosphorus	3.33	0.055	24.08
Carbon	2.16	0.141	17.04

*Kruskal-Wallis test; Significant differences when $p<0.05$.

Table S6.4.2. *Post-hoc* Tukey HSD test to evaluate variations in growth and bioremediation performances exhibited by *Salicornia ramosissima* cultured under different treatments of salinity: Sal.15, Sal.20 and Sal.25

	Sal.20 - Sal.15		Sal.25 - Sal.15		Sal.25 - Sal.20	
	T-value	*p*	*T-value*	*p*	*T-value*	*p*
Regular Growth Rate						
RGR 30 - 60 Day	5.24	0.000	4.82	0.000	-0.42	0.909

Significant differences when $p<0.05$.

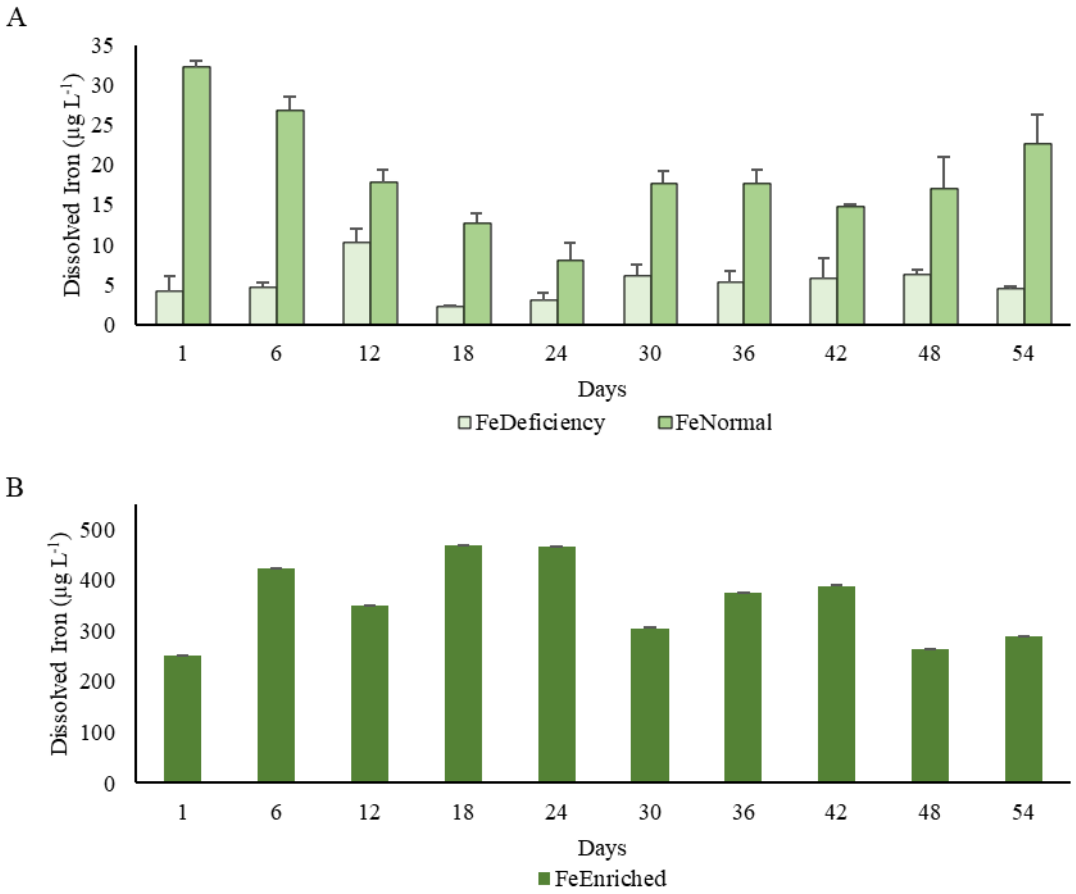


Figure S6.4. Concentration of iron (Fe) measured in hydroponic media with a deficiency and normal (S6.4A), and enriched (S6.4B) concentration of Fe (FeDeficiency, FeNormal and FeEnriched, respectively).

Table S6.5.1. Results of One-Way ANOVA to evaluate variations in growth performance, pigments and efficiency of photosystems and bioremediation performance of *Salicornia ramosissima* cultured in hydroponic media with a deficiency, normal and enriched concentration of Fe (FeDeficiency, FeNormal and FeEnriched, respectively).

Statistical test	*F - value*	*p - value*	*R_2 (%)*
Average fresh weight			
Average weight Day 0	0.11	0.897	1.03
Average weight Day 30	3.42	0.049	24.58
Average weight Day 60	19.13	0.000	64.56
Regular Growth Rate			
RGR 0 - 30 Day	3.90	0.036	27.09
RGR 30 - 60 Day	22.12	0.000	67.81
Final aboveground and belowground biomass			
Aboveground FW	13.66	0.000	56.54
Aboveground DW	7.51	0.003	41.69
Belowground FW	30.37	0.000	74.31
Belowground DW	16.36	0.000	60.90
Above/Below		0.002*	
Pigments and efficiency of photosystems			
Chlorophyll *a*	96.47	0.000	90.18
Chlorophyll *b*	4.50	0.055	24.34
Chlorophyll *Total*	27.02	0.000	72.01
Carotenoids	17.83	0.000	62.93
Carot:chlor		0.005*	
Fv/Fm Day 30		0.001*	
Fv/Fm Day 60		0.001*	
Bioremediation in hydroponic media			
DIN-N Day 30	14.46	0.000	57.93
DIN-N Day 60	1.51	0.243	12.60
DIP-P Day 30		0.188*	
DIP-P Day 60		0.005*	
Incorporation of N, P and C in edible biomass			
Nitrogen	21.30	0.000	66.98
Phosphorus	5.86	0.010	35.82
Carbon	19.93	0.000	65.50

*Kruskal-Wallis test; Significant differences when $p<0,05$;

Table S6.5.2. *Post-hoc* Tukey HSD test to evaluate variations in growth performance, pigments and efficiency of photosystems and bioremediation performance of *Salicornia ramosissima* cultured in hydroponic media with a deficiency, normal and enriched concentration of Fe (FeDeficiency, FeNormal and FeEnriched, respectively)

	FeEnriched -FeDeficiency		*FeNormal -FeDe*ficiency		*FeNorm*al *-FeEnrich*ed	
	T-value	*p*	*T-value*	*p*	*T-value*	*p*
Average fresh weight						
Average weight Day 30	2.61	0.041	1.45	0.333	-1.10	0.490
Average weight Day 60	6.17	0.000	3.50	0.006	-2.66	0.037
Regular Growth Rate						
RGR 0 - 30 Day	2.72	0.033	1.91	0.161	0.81	0.701
RGR 30 - 60 Day	6.37	0.000	4.85	0.000	-1.52	0.302
Final aboveground and belowground biomass						
Shoots FW	5.21	0.000	2.93	0.021	-2.29	0.080
Shoots DW	7.51	0.003	2.49	0.055	-1.33	0.397
Roots FW	7.76	0.000	4.52	0.001	-3.24	0.011
Roots DW	5.41	0.000	4.32	0.001	-1.08	0.535
Above/Below		0.002*		0.005*		0.248*
Pigments and efficiency of photosystems						
Chlorophyll *a*	-12.96	0.000	-10.82	0.000	2.14	0.106
Chlorophyll *Total*	7.29	0.000	4.49	0.001	-2.79	0.028
Carotenoids	5.88	0.000	3.83	0.003	-2.05	0.125
Carot:chlor		0.005*		0.009*		0.401*
Fv/Fm Day 30		0.001*		0.002*		0.317*
Fv/Fm Day 60		0.001*		0.001*		0.637*
Bioremediation in hydroponic media						
DIN-N Day 30	5.24	0.000	3.65	0.004	-1.59	0.272
DIP-P Day 60		0.005*		0.021*		0.115*
Incorporation of N. P and C in edible biomass						
Nitrogen	6.51	0.000	2.85	0.025	-3.66	0.004
Phosphorus	3.41	0.007	1.97	0.144	-1.44	0.341
Carbon	6.15	0.000	4.32	0.001	-1.83	0.185

*Kruskal-Wallis test; Significant differences when $p<0.05$.

Table S6.6. PERMANOVA test to evaluate variations in elemental composition exhibited by *Salicornia ramosissima cultured* in hydroponic media with a deficiency, normal and enriched concentration of Fe (FeDeficiency, FeNormal and FeEnriched, respectively).

	T-value	*p (Mc)*
Between treatments		
FeEnriched - FeDeficiency	5.31	0.002
FeNormal - FeDeficiency	2.60	0.021
FeNormal - FeEnriched	4.72	0.004
Between treatments and wild		
FeDeficiency - Wild	10.92	0.001
FeNormal - Wild	8.92	0.002
FeEnriched - Wild	14.20	0.001

Significant differences when $p<0.05$.

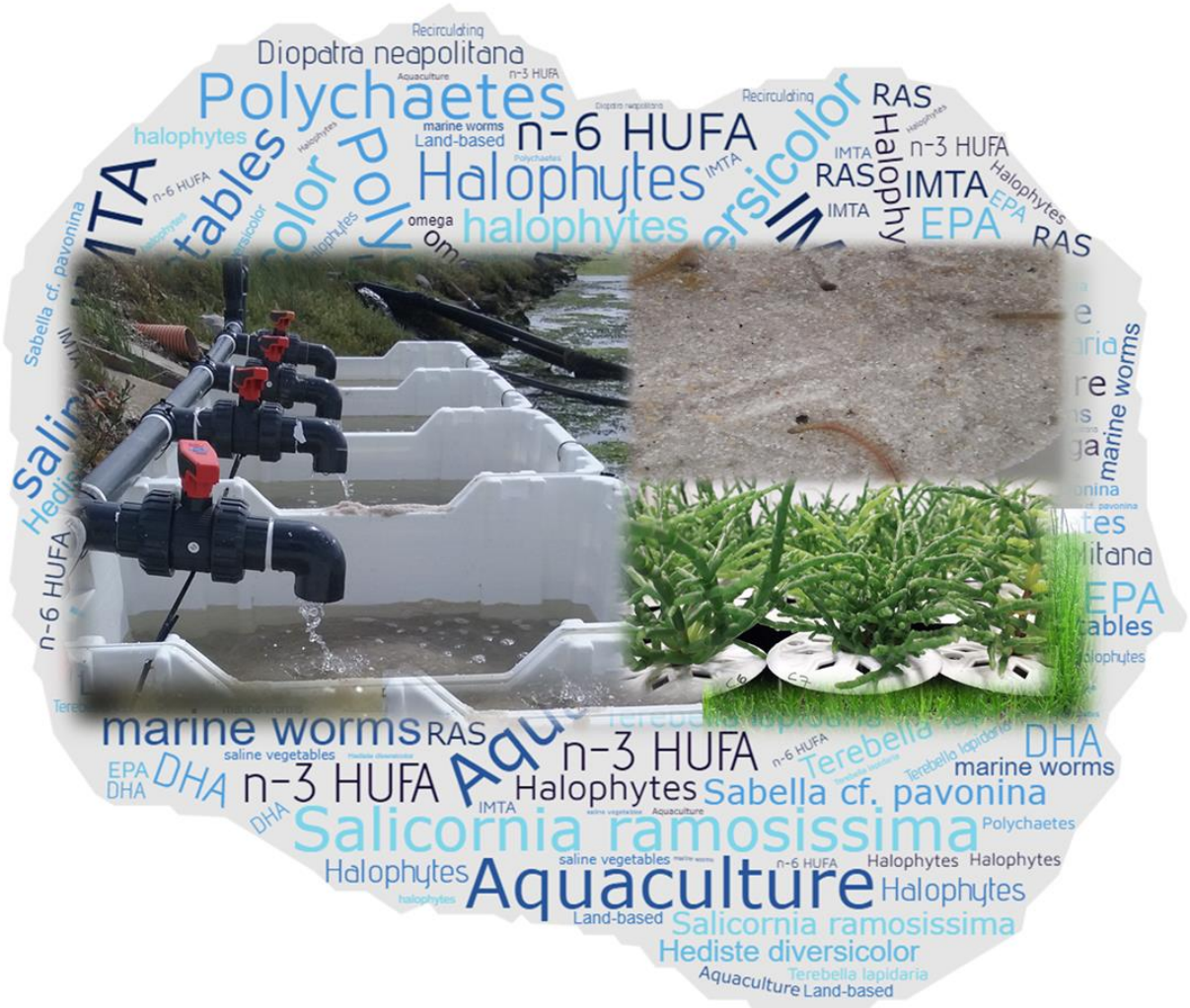
Table S6.7. SIMPER analysis (Cut-off 50%) to evaluate dissimilarities of elemental composition exhibited by *Salicornia ramosissima cultured* in hydroponic media with a deficiency, normal and enriched concentration of Fe (FeDeficiency, FeNormal and FeEnriched, respectively).

FeDeficiency & FeNormal			FeNormal & FeEnriched			FeDeficiency & FeEnriched		
Avg. dissimilarity: 4.98%			Avg. dissimilarity: 6.66%			Avg. dissimilarity: 9.82%		
Element	Contrib. %	Cum. %	Element	Contrib. %	Cum. %	Element	Contrib. %	Cum. %
Fe	22.53	22.53	Fe	25.01	25.01	Fe	28.47	28.47
Mn	11.02	33.55	Cu	19.15	44.15	Cu	16.42	44.89
Zn	10.53	44.08	Ni	13.72	57.88	Zn	12.44	57.33
Mo	10.39	54.46						

FeEnriched & Wild			FeNormal & Wild			FeDeficiency & Wild		
Avg. Dissimilarity: 9.81%			Avg. Dissimilarity: 14.42%			Avg. Dissimilarity: 17.04%		
Element	Contrib. %	Cum. %	Element	Contrib. %	Cum. %	Element	Contrib. %	Cum. %
Fe	24.50	24.50	Fe	28.79	28.79	Fe	31.41	31.41
Mn	15.12	39.62	Ni	42.84	42.84	Mn	13.29	44.69
Ni	10.94	50.56	Mn	54.53	54.53	Ni	11.61	56.31

Chapter 7

7.1. Final Considerations and Future Perspectives



7.1. Final considerations and future perspectives

The aquaculture industry plays a key role in world food security, and modern recirculating production systems (RAS) are considered one of the great paradigms of the Blue Revolution, as they allow "growing fish anywhere". Effluents derived from these systems are rich in nutrients that have not been incorporated into biomass of target species being supplied aquafeeds. In recent years, there has been an increasing number of efforts to align aquaculture production with the principles of a Blue Growth strategy (and the inherent Sustainable Development Goals for the current decade advocated by the United Nations). Responsible and sustainable aquaculture production is of utmost importance, as it allows to reconcile economic growth of marine sectors with the conservation of natural marine resources. Integrated multi-trophic aquaculture (IMTA) is well aligned with these standards and underlies all the studies developed during this thesis. The present work conceptualized and demonstrated the recovery of nutrients present in aquaculture effluents and their incorporation into valuable extractive species biomass, namely of polychaetes (e.g., mostly *Hediste diversicolor*) and halophyte plants (e.g., *Salicornia ramosissima*). These two groups of organisms played a key role in the recovery of nutrients present in POM and DIM (included DIN and DIP). This thesis aimed to clarify whether the extractive species mentioned above are viable options for IMTA frameworks, thus contributing to the bioremediation of aquaculture effluents, a more efficient use of nutrients supplied by aquafeeds to fed species and the production of value-added biomass of extractive species. The objectives outlined in this thesis were all successfully achieved, being summarized as follows:

1) In the first chapter of this thesis the review performed on the state of the art of world aquaculture evidenced the key role played by this agro-sector in world food security. It was also highlighted that it is essential to continue to screen for alternative sources of nutrients (e.g., proteins, lipids, essential fatty acids), prioritizing those that may help to decrease the dependence on two increasingly scarcer and costly marine ingredients, fishmeal and fish oil, that are still paramount in aquafeed formulations (mainly for marine carnivorous species). The role of extractive species included in marine land-based IMTA designs was also addressed, and a systematic review concluded that

polychaetes and halophyte plants were included in 11% (each) of all scientific studies on this topic performed until 2020. In this review it was also possible to demonstrate that IMTA designs which integrated extractive species from different trophic levels to recover unused nutrients present in POM and DIM were rarely employed on marine land-based aquaculture production.

2) The work described in Chapter 2 allowed to conclude that the reproductive success of *H. diversicolor* was achieved only in the two PASFs which received effluent water with a higher concentration of POM. This study highlighted some limitations inherent to works performed using semi-intensive systems, as larvae of other marine invertebrate species naturally colonized the PASFs. This was the case of several polychaete species, such as *D. neapolitana*, *S.* cf. *pavonina* and *T. lapidaria*, which adapted to IMTA culture conditions and showed potential to integrate such frameworks in future studies. Future research perspectives also emerged from this study, such as the need to develop PASFs with the ability to filter higher volumes of effluent water to make this solution more appealing for the aquaculture industry. At this level, for example, shallow raceway tank designs, where the water flows through the entire length of the tank can promote the settlement of POM particles throughout the substrate bed, a feature that may allow filtering larger volumes of water by not requiring that 100% of the water has to percolate through the substrate. On the other hand, the results achieved in this study also allowed to verify that the PASFs designs tested should preferably be integrated in closed intensive production systems, which usually display effluents free of other organisms (e.g., larvae of other polychaete species) that may jeopardize a controlled production of target extractive species.

3) The work described in Chapter 3 allowed to show that the four polychaete species mentioned above displayed a biochemical profile enriched with *n*-3 HUFA (including EPA and DHA), with the FA profile of *D. neapolitana*, *S.* cf. *pavonina* and *T. lapidaria* cultured under IMTA conditions being described for the first time ever in the present work. It was also concluded that the FA profiles exhibited by *H. diversicolor* and *T. lapidaria* were similar in terms of composition/total FA concentration, with both of them also overlapping with the FA profile exhibited by aquafeeds provided to fish located upstream in the IMTA design. Based on these findings, as a future perspective, it is important to continue to study the performance (namely bioremediation and biomass

generation) of each polychaete species under controlled conditions to determine their true potential for IMTA applications. Moreover, it is also important to highlight the potential of *T. lapidaria* as a future extractive species for IMTA designs (due to its high similarity in FA profile with the most-well studied polychaete species under IMTA conditions – *H. diversicolor*).

4) The third work described in Chapter 4 allowed to conclude that the different conditions tested (temperature x salinity) did not contribute to significantly modify the FA profile of cultured *H. diversicolor*. A progressive increment in total FA concentration was observed over time, and no plateau was achieved during the 40 days of feeding with a commercial aquafeed, either in terms of total FA concentration, or in terms of *n*-3 and *n*-6 FA concentration. Still, it was possible to show that polychaetes supplied with a commercial aquafeed for 40 days display a FA profile with a greater similarity to that of aquafeeds than the ones exhibited by initially stocked conspecifics, or ragworms in the wild. The identification of *de novo* biosynthesis pathways, whose activation may be interconnected with the development of maturation states in *H. diversicolor* (mainly males), is also worth highlighting. As future perspectives for the results of this work, it is important to further explore the enrichment of polychaetes biomass in EFA in such a short time-frame (40 days); this biomass can be directed towards the formulation of premium aquafeeds (e.g., finishing and reproduction diets). The results here obtained confirmed the huge potential of *H. diversicolor* to filter effluents from intensive units that usually contain large amounts of uneaten aquafeeds.

5) The fourth work described in Chapter 5 allowed to conclude that polychaetes and halophyte plants cultivated using a single polyculture tank (1T) display similar bioremediation values when compared to those achieved when culturing these extractive two species in two separated tanks (2T). This finding makes possible to reduce by half the operational area required to implement this IMTA framework. It was also possible to show that the polychaete *H. diversicolor* is much better fitted to be stocked in the PASFs tested than *Arenicola marina*, which largely failed to cope with the culture conditions tested (most likely due to the species thermal limit having been exceeded) and exhibiting a very low survival (<10%). The productivity of the halophyte *S. ramosissima* obtained in the 1T design was approximately twice the one achieved under the 2T design. The development of a yellowish coloration in farmed halophyte plants, was most likely

due to the water treatment applied in the RAS system (chemical oxidation and subsequent filtration), which removed iron (and most probably other essential elements) and, as such, highlighted some of the limitations of using the IMTA design tested in this system. As future perspectives, the results achieved demonstrate that it is advisable to develop vertical IMTA designs (e.g., overlapped tanks) that integrate species from different trophic levels. This approach will allow to answer one of the major limitations commonly pointed out to successfully implement IMTA designs, i.e., the operational area required for their operation.

6) The fifth work described in Chapter 6 allowed to confirm the potential of *S. ramosissima* cultured under hydroponics conditions to recover dissolved nutrients into valuable saline vegetable biomass. In the salinity experiment it was shown that this halophyte plant can be successfully cultured in a range of salinities between 15 - 25, with the growth performance achieved at a salinity of 20 being slightly higher than those obtained under the other conditions tested. The iron experiment showed that it is essential to optimize culture conditions, namely when certain treatments (e.g., ozonation and chemical oxidation) are applied to hydroponic/aquaponic media and promote the precipitation to an insoluble condition and make unavailable salts of certain essential elements (e.g., Fe, Mg, P, Ca and Mn). Plants grown under FeDeficiency assumed a greenish-yellow coloration and displayed inferior growth and bioremediation performances. Significant differences were recorded in the elemental composition of plants cultured under different Fe concentrations, with FeEnriched plants exhibiting concentrations of this element ≈13- and 5- times higher than the ones recorded in conspecifics from FeDeficiency and FeNormal, respectively. These findings demonstrate that biofortification in Fe can be successfully performed for *S. ramosissima* cultured in hydroponics. This study allowed to understand the origin of the main limitations for the cultivation of this plant in real production conditions reported in chapter 5. As future perspectives, the results here reported demonstrate that to make possible the integration of these plants as extractive species in IMTA designs using RAS treated water, it most likely required to perform a supplementation of culture media with lacking micro and macronutrients, thus allowing to maximize growth, bioremediation and richness in key elements. A previous characterisation of the nutrient profile of culture media is therefore strongly recommended before halophyte plats are used as extractive species in IMTA designs.

The two main extractive species included in this thesis (the polychaete *H. diversicolor* and the halophyte *S. ramosissima*) already have well-established market destinations. Considering that enriched biomass of *H. diversicolor* produced under an IMTA framework can integrate premium aquafeed formulations, and that *S. ramosissima* shoots and its co-products can be targeted for human and animal nutrition, it is essential that both comply with the strictest quality and food-safety standards. At this level it is important to consider that IMTA products can potentially bioaccumulate some noxious substances that can be present in aquaculture effluents (e.g., chemicals, metals) and, therefore, future experiments should survey those products to ensure that the biomass produced is free of contaminants. The controlled production will ensure traceability and high-quality standards, resulting in a nutritionally enriched biomass compared to the one displayed by wild conspecifics that grow under uncontrolled conditions and may exhibit contaminants.

In the current scenario of resource scarcity (e.g., arable land and freshwater resources), the development of halophyte crops using resources such as salinized soils and marine aquaculture effluents are in line with Blue Growth policies and SDGs. The achievements described in this thesis are relevant contributions to further develop sustainable aquaculture practices, as they foster the recycling, production and valorisation of marine biomass and are clearly aligned with SDGs 2, 6, 12 and 14. Food security, nutrition, poverty alleviation, efficient use of resources, waste reduction and protection of marine coastal areas and ecosystems are all goals that can be pursued through eco-friendly aquaculture practices.

Although the IMTA is being framed and encouraged by EU policies (including the Blue Growth strategy), socioeconomic, administrative, and regulatory bottlenecks still constrain the transfer of known-how to industrial scale applications. To foster the development of intensive marine land-based aquaculture (already applying industry 4.0 principles), it will be required that IMTA applications undergo the same level of precision. The future of an intensive IMTA industry may result in the development of independent production units with water flows recirculating between them with prior optimization before entering each unit (e.g., nutrient and mineral supplementation, fine tuning of optimal abiotic conditions). The level of complexity that results from a production system with these characteristics is an enormous challenge that must engage all those interested in developing a more eco-friendly, efficient and sustainable aquaculture industry.

8.1. Acknowledgements

The works performed in this thesis were supported by the Integrated Programme of SR&TD "SmartBioR - Smart Valorization of Endogenous Marine Biological Resources Under a Changing Climate" (Chapter 2, 3, 4 and 5) (Centro-01-0145-FEDER-000018), co-funded by Centro 2020 program, Portugal 2020, European Union, through the European Regional Development Fund, by the project "AquaMMIn - Development and validation of a modular integrated multitrophic aquaculture system for marine and brackish water species" (Cahpter 2, 3, 4 and 5) (MAR-02.01.01-FEAMP-0038) co-funded by Portugal 2020 and the European Union through Mar2020, the Operational Programme (OP) for the European Maritime and Fisheries Fund (EMFF) in Portugal and by the project "VALORMAR – Full valorisation of marine resources: potential, technological innovation and new applications" (Chapter 4 and 5) (24517, 10/SI/2016 - Mobilizing R&TD Programs) co-funded by Compete 2020, Lisboa 2020, Algarve 2020, Portugal 2020 and European Union, through the European Regional Development Fund (ERDF). Daniel Jerónimo acknowledges FCT (Portuguese Foundation for Science and Technology) by financially supporting his PhD grant (PD/BD/127989/2016). Thanks for financial support are also due to CESAM (UIDB/50017/2020+UIDP/50017/2020), to FCT/MEC through national funds, and the cofounding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. BioCost Research Group acknowledges funding from Xunta de Galicia (grants ED431B 2018/49 and ED431B 2021/32) by program "Consolidation and structuring of competitive research units of Galician University System (SUG)". A special thank is addressed for Rui Pereira and Helena Abreu for providing the conditions to perform this study at AlgaPlus and Ascensão A. Ravara, Luísa Marques and Andreia Santos for the technical support provided in the identification of polychaetes described in Chapter 2 and 3. Thanks are also due to Luísa Marques and Elisabete da Costa for FA profile characterization performed in the aquafeed described in Chapter 3. Regarding Chapter 4, a special thanks is addressed to Felisa Rey, Tiago Conde and Daniela Couto for all the assistance provided in the lipid extraction and fatty acid analysis. Finally, in respect to Chapter 5, a special thank is addressed to Renata Serradeiro for providing the conditions to perform this study at RiaSearch Lda and also to Paulo Cartaxana for all the assistance provided in the determination of pigment profile of halophyte *S. ramosissima*.