

Dissertation for the Master's degree in Aquaculture

**FATTY ACIDS AS DIETARY TRACERS IN
INTEGRATED PRODUCTION OF
FISH/OYSTERS/MACROALGAE IN EARTHEN
PONDS**

Gonçalo Cardoso de Menezes Villa de Brito

Faro, janeiro 2018

FATTY ACIDS AS DIETARY TRACERS IN INTEGRATED PRODUCTION OF FISH/OYSTERS/MACROALGAE IN EARTHEN PONDS

Gonçalo Cardoso de Menezes Villa de Brito

Dissertation for the Master's degree in Aquaculture

Work elaborated under the supervision of:

Dra. Maria Emília Cunha (Instituto Português do Mar e da Atmosfera, EPPO)

Dra. Sarah Nahon (Institut National de la Recherche Agronomique, NúMeA)

Dr. João Silva (Faculty of Science and Technology, University of Algarve)



Work integrated in the European Project IMTA-EFFECT



2017

**USE OF FATTY ACIDS AS DIETARY TRACERS IN INTEGRATED
PRODUCTION OF FISH/OYSTERS/MACROALGAE IN EARTHEN PONDS**

Declaração de autoria de trabalho

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

Universidade do Algarve, 11 de janeiro de 2018

Assinatura: _____

© 2017 Gonçalo Cardoso de Menezes Villa de Brito

“A Universidade do Algarve tem o direito, perpétuo e sem limites geográficos, de arquivar e publicitar este trabalho através de exemplares impressos reproduzidos em papel ou de forma digital, ou por qualquer outro meio conhecido ou que venha a ser inventado, de o divulgar através de repositórios científicos e de admitir a sua cópia e distribuição com objetivos educacionais ou de investigação, não comerciais, desde que seja dado crédito ao autor e editor.”

Acknowledgments

I would like to thank my formal supervisor Dra. Maria Emília Cunha, from the Aquaculture Research Centre, National Institute for the Sea and Atmosphere (IPMA), for the opportunity of developing this work and all the patience, scientific formation and support provided. I also want to thank my other formal supervisor, Dr. João Silva from the Faculty of Science and Technology of Algarve University. A special thank to Dra. Sarah Nahon, from the research unity Nutrition and Metabolism in Aquaculture, at National Institute of Agronomic Research (INRA) in France, for receiving me and providing scientific formation on fatty acids extraction and analysis, as well as giving me the opportunity to join a team of researchers with whom I exchanged ideas and learned a lot. I want to thank all the researchers, staff and friends at INRA who helped and supported me during the 5 months of laboratorial analysis, especially Sandrine Skiba-Cassy, Geneviève Corraze, Laurence Larroquet, Karine Dias, Gaïax Pégouriex, Luis Paulo and Manuelle Vandermeeren. Also, a special thanks to all the researchers, staff and friends in EPPO-IPMA, especially to Hugo Ferreira, for all the support and valuable formation regarding the culture of several species in earthen ponds, as well for being a great friend, bringing joy and happiness to work every day. Also from EPPO-IPMA, I would like to thank Dr. Pedro Pousão, Dra. Florbela Soares, Dra. Laura Ribeiro, Márcio Moreira, Ivo Monteiro, Ravi Luna, Valeriy Afilov, Glauco Favot, Nuno Caturra, Diogo Teixeira, and many others for all the support and help during the culture and sampling of all species. I would also like to thank Dra. Sofia Gamito from CCMAR for the hints provided for statistical analysis with PRIMER software. Finally, I would like to give a special thank to my Master's director Dra. Elsa Cabrita, for all the contacts made previously which enabled me to work on this project., as well for all the support and advices and for being an excellent director and teacher along the two years of my master studies, providing me a broader vision about many important topics in aquaculture.

I dedicate this thesis to my parents and brother, for always believing in me and supporting my decisions. I also wish to dedicate this thesis to all my closest friends who gave me important motivation but especially to my friend Pedro Durão who showed me that determination and perseverance are essential, no matter how big are the obstacles we might face in our way.

Abstract

Semi-intensive fish culture developed in earthen ponds is the main type of production system used nowadays in Portugal. One way to decrease its environmental footprint, by reducing waste effluents, is to integrate the production of organic and inorganic extractive species such as bivalves and seaweeds directly in the ponds, under the principles of IMTA. Furthermore, the total biomass of product at one site is increased and diversification of products is accomplished, promoting higher profitability. There are needs for integrated knowledge concerning the efficiency of nutrient use, such as fatty acids. The present study refers to a pond where the only input was the feed, for the culture of three fish species (*Argyrosomus regius*, *Diplodus sargus*, *Mugil cephalus*) along with oysters (*Crassostrea gigas*) and sea lettuce (*Ulva flexuosa*). Additionally, natural occurring organisms in the ponds were sampled, such as phytoplankton, suspended particulate organic matter, zooplankton, polychaetas and other two macroalgal species, *Ulva intestinalis* and *Rhizoclonium riparium*. The main objective of the work was to identify food sources used by heterotrophic species present in the ponds using fatty acids as trophic markers. Three configurations (treatments) were tested, one comprised only the culture of fish and oysters, another comprised only fish and algae, while the last comprised all species cultured together. Profiles from each heterotrophic species across treatments were compared, and their differences evaluated to identify possible variations in feeding sources across treatments. Polychaetes showed dietary tracers of the commercial feed, macroalgae, sediment and additionally is suggested the ability to biosynthesize C20:2 ω 6 from Linoleic Acid. In zooplankton, the dietary tracers found reflect a consumption of phytoplankton, suspended matter and bacteria, while the higher levels of DHA and EPA could be linked with a higher accumulation of PUFA with decreasing temperatures. Oysters reflected trophic markers of phytoplankton, suspended matter, zooplankton and bacteria, while higher levels of ARA, DHA and EPA than those found in their food sources confirm that these fatty acids accumulate in oyster tissues. In addition, ARA and EPA could be a product of biosynthesis form shorter PUFA ω 6 and PUFA ω 3, respectively. All three fish species reflected the feed dietary tracers to a great extent. While meagre and white seabream consumed the commercial feed predominantly, the higher dispersion of mullet samples might suggest the consumption of additional dietary items (such as phytoplankton, suspended matter, zooplankton, sediment and polychaetas. Additionally,

all species reflected higher percentages of ARA and EPA, but especially DHA, than those found in the feed, indicating their ability to accumulate these fatty acids in their tissue. In mullet, might also exist an ability to synthesize PUFA from shorter fatty acids. Overall, this study permitted a characterization of trophic interconnections of nutrients, such as fatty acids, from the feed and lower trophic level organisms to all potential consumers present in the culture ponds.

Keywords: meagre, white seabream, mullet, sea lettuce, oyster, fatty acid, diet

Resumo

A produção semi-intensiva de peixe em tanques de terra é o principal tipo de cultivo praticado em Portugal. Uma das maneiras de reduzir a pegada ecológica do sistema, através da redução dos seus efluentes, passa pela introdução de espécies extrativas orgânicas e inorgânicas, tais como bivalves e algas, diretamente nos tanques de terra, seguindo as práticas da aquacultura multi-trófica integrada. Adicionalmente, o total de biomassa produzido no mesmo local é aumentado e consegue-se uma diversificação de produtos, promovendo maiores retornos económicos. Existe, no entanto, a necessidade de detalhar o conhecimento referente a eficiência do uso de nutrientes, tais como os ácidos gordos. Este estudo refere-se por isso a uma produção em tanques de terra, no qual o único input foi uma ração comercial, para o cultivo de três espécies de peixe (*Argyrosomus regius*, *Diplodus sargus*, *Mugil cephalus*) em conjunto com ostras (*Crassostrea gigas*) e alface-do-mar (*Ulva flexuosa*). Foram também amostrados organismos presentes naturalmente nos tanques de terra, tais como fitoplâncton, material particulado em suspensão, zooplâncton, poliquetas e outras duas espécies de algas, *Ulva intestinalis* e *Rhizoclonium riparium*. O objectivo principal do trabalho foi identificar as fontes de alimento das espécies heterotróficas presentes nos tanques de terra utilizando ácidos gordos para rastreabilidade das suas dietas. Três configurações (tratamentos) foram testadas, no primeiro apenas se procedeu ao cultivo de peixe e ostras, no segundo apenas peixe e algas, enquanto no terceiro todas as espécies foram cultivadas em conjunto. Os perfis de cada espécie heterotrófica nos diferentes tratamentos foram comparados, e as suas

diferenças avaliadas de modo a identificar possíveis variações nas fontes de alimento entre tratamentos. Os poliquetas apresentaram ácidos gordos característicos da ração comercial, das algas e do sedimento. Os resultados sugerem também uma possível habilidade dos poliquetas para biossíntese de C20:2 ω 6 através do ácido Linoleico. O zooplâncton refletiu ácidos gordos característicos do fitoplâncton, do material particulado em suspensão e de bactérias, enquanto os níveis elevados de DHA e EPA sugerem uma maior acumulação de ácidos gordos polinsaturados com a descida das temperaturas. As ostras refletiram ácidos gordos característicos do fitoplâncton, do material particulado em suspensão, do zooplâncton e de bactérias, enquanto os níveis mais elevados de ARA, DHA e EPA comparativamente com essas possíveis fontes de alimento confirma a sua favorável acumulação nos tecidos das ostras. Além disso, ARA e EPA podem ser um produto da biossíntese de ácidos gordos polinsaturados ω 6 e ω 3. As três espécies de peixe refletiram os ácidos gordos característicos da ração em grande medida. Enquanto a corvina e o sargo consumiram apenas a ração, a maior dispersão das amostras na tainha sugere o consumo adicional de outras fontes de alimento, tais como o fitoplâncton, o material particulado em suspensão, o zooplâncton, o sedimento e os poliquetas. As três espécies de peixe refletiram maiores percentagens de ARA, EPA e especialmente DHA, em comparação com a ração, indicando a habilidade para acumular estes ácidos gordos nos seus músculos. Na tainha, pode também existir a habilidade para biossíntese de ácidos gordos polinsaturados através de ácidos gordos de menor cadeia. Em termos gerais, este estudo permitiu a caracterização das interconexões de nutrientes, tais como os ácidos gordos, desde a ração e dos organismos de baixo nível trófico até a todos os potenciais consumidores presentes no sistema de cultivo em tanques de terra.

Palavras-chave: corvina, sargo, tainha, ostra, alface-do-mar, ácidos gordos, dieta

Abbreviations

µm – micrometres

ω3 – omega three

ω6 – omega six

ALA – alpha-linolenic acid

ANOVA – analysis of variance

ANOSIM – analysis of similarities

ARA – arachidonic acid

BHT – butylated hydroxytoluene

cm – centimeter

°C – degrees celsius

DHA – docosahexaenoic acid

DIN – dissolved inorganic nutrients

DO – dissolved oxygen

EFA – essential fatty acid

EPA – eucosapentaenoic acid

EPPO – estação piloto piscicultura em olhão – aquaculture research station in olhão

FA – fatty acid

FAME – fatty acid methyl ester

FATM – fatty acid trophic marker

F+O+A – fish, oyster and algae

F+O – fish and oyster

F+A – fish and algae

g – gram

GC-FID – gas chromatography with flame ionization detector

IMTA – integrated multi-trophic aquaculture

IPMA – instituto português do mar e atmosfera

kg – kilogram

kg.m⁻³ – kilograms per cubic meter

LA – linoleic acid

LC-PUFA – long chain polyunsaturated fatty acid

m – meter

mL – millilitre

MDS – multidimensional scaling

MUFA – monounsaturated fatty acid

OA – oleic acid

PERMANOVA – permutational analysis of variance

POM – particulate organic matter

PRIMER – Plymouth routines in multivariate ecological research

PUFA – polyunsaturated fatty acid

rpm – rotations per minute

s.d. – standard deviation

SFA – saturated fatty acid

SIMPER – similarity percentages

Sq. Dist./SD – square distance over standard deviation

T1 – tank 1

T2 – tank 2

T3 – tank 3

T4 – tank 4

T5 – tank 5

T6 – tank 6

TFA – total fatty acid

Contents

Objectives.....	1
1. Introduction.....	2
1.1 Integrated Multi-Trophic Aquaculture.....	2
1.2 Integrated production in Earthen ponds	4
1.3 Farmed species characterization	6
1.3.1 Meagre – <i>Argyrosomus regius</i> (Asso, 1801)	6
1.3.2 White Sea Bream – <i>Diplodus sargus sargus</i> (Linnaeus, 1758)	7
1.3.3 Flathead Grey Mullet – <i>Mugil cephalus</i> (Linnaeus, 1758).....	8
1.3.4 Pacific Oyster – <i>Crassostrea gigas</i> (Thunberg, 1793).....	10
1.3.5 Sea Lettuce – <i>Ulva flexuosa</i> (Roth, 1800)	12
1.4 Fatty Acids.....	14
1.4.1 Definition and nomenclature	14
1.4.2 Essential fatty acids	15
1.4.3 Fatty acid trophic marker concept.....	16
1.5 Expected fatty acids.....	17
1.5.1 Phytoplankton	17
1.5.2 Green Macroalgae	18
1.5.3 Zooplankton.....	19
1.5.4 Polychaeta	19
1.5.5 Oyster.....	20
1.5.6 Fish.....	21
2 Material and Methods	22
2.1 Experimental design	22
2.2 Earthen ponds and grow-out management	24
2.3 Sampling.....	28
2.4 Fatty acids extraction	29
2.5 Analysis with GC-FID	30
2.6 Data interpretation and statistical analysis	30
3 Results.....	33
3.1 Environmental Parameters.....	33
3.1.1 Temperature.....	33

3.1.2	Salinity	34
3.1.3	Dissolved oxygen	35
3.1.4	pH.....	36
3.1.5	Turbidity.....	37
3.2	Fatty acids profiles.....	38
3.2.1	Feed	38
3.2.2	Sediment.....	39
3.2.3	Polychaeta	40
3.2.4	Phytoplankton	41
3.2.5	Suspended particulate organic matter	42
3.2.6	Zooplankton	43
3.2.7	Rhizoclonium riparium	44
3.2.8	Ulva intestinalis.....	45
3.2.9	Ulva flexuosa (cultured algae)	46
3.2.10	Crassostrea gigas	47
3.2.11	Meagre.....	48
3.2.12	White seabream.....	49
3.2.14	Comparison between all species.....	51
4.	Discussion.....	52
4.1	Environmental Parameters.....	52
4.2	Fatty acids.....	54
5.	Conclusions	62
	References	63
	ANNEXES.....	69

List of Figures

Figure 1. Representation of <i>Argyrosomus regius</i> (Adapted from FAO).....	6
Figure 2. Representation of <i>Diplodus sargus sargus</i> (Adapted from FAO)	7
Figure 3. Representation of <i>Mugil chepalus</i> (Adapted from FAO).....	8
Figure 4. Representation of <i>Crassostrea gigas</i> (Adapted from FAO).....	10
Figure 5. Representation of <i>Ulva spp.</i> (Adapted from “Sowerby’s Botany”, 1814)	12
Figure 6. Molecular structure of stearic acid, oleic acid, linoleic acid and alpha-linolenic acid.	14
Figure 7. Aerial view of Estação Piloto de Piscicultura de Olhão – EPPO.....	22
Figure 8. Diagram of tanks 1 to 6 with respective treatment.....	23
Figure 9. Long-lines of oyster bags floating at the surface.	25
Figure 10. Oyster bags being submerged in water.	26
Figure 11. a) Collection of new <i>Ulva flexuosa</i> form the station’s settling tank. b) Manual fixation on the net. c) Structure overview	27
Figure 12. Temperature mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.	33
Figure 13. Salinity mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.	34
Figure 14. Dissolved oxygen mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.	35
Figure 15. pH mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.	36
Figure 16. Turbidity mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.	37
Figure 17. Fatty acid profile of the feed.....	38
Figure 18. Fatty acid profile of the sediment.....	39

Figure 19. Fatty acid profile of polychaete	40
Figure 20. Fatty acid profile of the Phytoplankton in the three treatments.....	41
Figure 21. Fatty acid profile of suspended particulate organic matter in the three treatments.....	42
Figure 22. Fatty acid profile of the zooplankton in the three treatments.	43
Figure 23. Fatty acid profile of the <i>Rhizoclonium riparium</i> in the two treatments.....	44
Figure 24. Fatty acid profile of the <i>Ulva intestinalis</i> in the two treatments	45
Figure 25. Fatty acid profile of the <i>Ulva flexuosa</i> in the two treatments.....	46
Figure 26. Fatty acid profile of <i>Crassostrea gigas</i> in the two treatments.....	47
Figure 27. Fatty acid profile of the Meagre in the three treatments.....	48
Figure 28. Fatty acid profile of white seabream in the three treatments.....	49
Figure 29. Fatty acid profile of Mullet in the three treatments.....	50
Figure 30. MDS plot of samples of all organisms sampled in the final period	51

List of Tables

Table 1: Initial quantity, average weight (g), biomass (kg) and density (kg.m ⁻³) of each fish species introduced per tank.....	23
Table 2: Initial quantity, average weight (g), biomass (kg) and density (kg.m ⁻³) of oysters introduced per tank in tanks 1, 2, 4 and 6, and algae introduced per tank in tanks 1, 3, 5 and 6.	24
Table 3: Feed description (analytic constituents and additives per kilogram of feed), along with raw materials of the feed. Information provided by the manufacturer.....	24
Table 4: Number of samples collected.....	28
Table 5: Left table – Temperature mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). Table on the right – P-values obtained in statistical analysis (“bold”: $p < 0.05$).....	33
Table 6: Left table – Salinity mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). Table on the right – P-values obtained in statistical analysis (“bold”: $p < 0.05$).....	34
Table 7: Left table – Dissolved oxygen mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). Table on the right – P-values obtained in statistical analysis (“bold”: $p < 0.05$).....	35
Table 8: Left table – pH mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). Table on the right – P-values obtained in statistical analysis (“bold”: $p < 0.05$).....	36
Table 9: Left table – Turbidity mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). Table on the right – P-values obtained in statistical analysis (“bold”: $p < 0.05$).....	37

Objectives

The system presented in this work is the continuity of the research about integrated multi-trophic aquaculture developed at EPO (IPMA's Aquaculture Research Station in Olhão). The system forms a part of the European project IMTA-EFFECT (IMTA for EFFiciency and Environmental ConservaTion), which considers several production systems developed in fresh and salt waters in different countries and climates. Overall, the project will pay special attention on food-web characterization but also on acquiring theoretical bases for modelling, life cycle assessment, economic and social evaluation of IMTA systems. By agreeing on making use of the same methodology, future comparisons between systems will be more easily achieved.

The system presented in this work consists in the integrated culture in earthen ponds of three fish species, such as meagre (*Argyrosomus regius*), white seabream (*Diplodus sargus*) and mullet (*Mugil cephalus*) along with pacific oyster (*Crassostrea gigas*) and autochthonous sea lettuce (*Ulva flexuosa*).

The main objective of the work is to identify food sources used by heterotrophic species present in the IMTA ponds using fatty acids as trophic markers. Three configurations (treatments) were tested, one comprised only the culture of fish and oysters, another comprised only fish and algae, while the last comprised all species cultured together. To identify food sources, the fatty acid profile was first determined for each heterotrophic species and afterwards determined for all potential food sources (commercial feed and natural sources occurring in the ponds). Finally, by comparing fatty acid profiles, and knowing that fatty acids are in many circumstances incorporated into consumers in a conservative manner, the food sources of heterotrophic species were identified. Profiles from each heterotrophic species across treatments were compared, and their differences studied to evaluate possible variations in feeding sources across systems.

1. Introduction

1.1 Integrated Multi-Trophic Aquaculture

The intensive monoculture that has been done nowadays in fish production, is a practice that has negative impacts on the environment, much like agriculture, due to the excess of nutrients released to the ecosystems, disrupting ecological balances. The actual challenge is to manage the sector's growth in a sustainable way without putting at risk the environment. One way to achieve this, is by integrating the production of organisms from different trophic levels under the principles of IMTA - Integrated Multi-Trophic Aquaculture (Soto, 2009). As the name suggests, it is the co-culture of harvestable species at multiple trophic or nutritional levels within the same or interrelated compartments (Chopin, 2006). It is an approach with application to numerous systems – land-based or open-water, marine or freshwater, comprising limitless species combinations (Neori *et al.*, 2004). The practice is much related to the concept of circular economy – where waste streams from one industry provide the raw materials for another (Hughes and Black, 2016). In this case, the waste streams originated from fed aquaculture species are being recycled to become inputs (fertilizer, food and energy) for organic and inorganic extractive species (Barrington *et al.*, 2009). This way, IMTA is recreating a simplified, cultivated ecosystem in balance with its surroundings instead of introducing a biomass of a certain type expecting this can be cultivated in isolation of everything else (Barrington *et al.*, 2009).

An IMTA system typically includes two main productive compartments, a fed aquaculture species (e.g. finfish or shrimp) cultured along with extractive species (e.g. shellfish and seaweeds). Extractive species are the harvestable low trophic level organisms introduced in the IMTA system which rather than being raised with supplemental feed, actively feed on natural plankton communities and uptake the excess of dissolved inorganic nutrients (DIN) and particulate organic matter (POM) present in the water deriving out of the fish or shrimp production, thus improving its quality. The presence of low trophic organisms in fish ponds may also help control the development of fish parasites (dinoflagellates like *Amyloodinium spp.*), microorganisms and microalgae blooms within the system (Cunha, 2011).

These extractive organisms are either named organic extractive (e.g. suspension feeders, deposit feeders and herbivorous fish) or inorganic extractive (e.g. seaweeds and halophyte plants). Those of interest and with the highest potential for development in IMTA systems in marine temperate waters include molluscs (*Haliotis*, *Crassostrea*, *Pecten*, *Argopecten*, *Placopecten*, *Mytilus*, *Choromytilus* and *Tapes*), polychaetes (*Nereis*, *Arenicola*, *Glycera* and *Sabella*), echinoderms (*Strongylocentrotus*, *Paracentrotus*, *Psammechinus*, *Loxechinus*, *Cucumaria*, *Holothuria*, *Stichopus*, *Parastichopus*, *Apostichopus* and *Athyonidium*), and seaweeds (*Laminaria*, *Saccharina*, *Sacchoriza*, *Undaria*, *Alaria*, *Ecklonia*, *Lessonia*, *Durvillaea*, *Macrocystis*, *Gigartina*, *Sarcothalia*, *Chondracanthus*, *Callophyllis*, *Gracilaria*, *Gracilariopsis*, *Porphyra*, *Chondrus*, *Palmaria*, *Asparagopsis* and *Ulva*) (Barrington *et al.*, 2009). Their selection is due to species established husbandry practices, habitat appropriateness and bio mitigation ability. Biological and chemical processes within the system are thus balanced through the careful selection and proportions of extractive species providing different known ecosystem functions (Barrington *et al.*, 2009).

This way, IMTA represents a more ecologically friendly production system when compared to conventional aquaculture, by being able to mitigate environmental impacts in-situ through bioremediation of the extractive species. In terms of volume, half of the world aquaculture production in 2014, including seaweeds and microalgae (27%) and filter-feeding animal species (22.5%), was realized without feeding (FAO, 2016). By supporting this trend, IMTA represents one of the most promising pathways in the evolution of aquaculture systems (Troell *et al.*, 2003). The direct use of nitrogen and phosphorous loads originated from fed-species by the reproduction of natural nutrient cycles on small scale appears to be the logical way for sustainable resource management in aquaculture (Waller *et al.*, 2015).

Essentially, the use of extractive species can improve water quality and reduce the environmental footprint of finfish culture while providing diversification of products for the farmer. In addition, the total biomass of product cultured at one site is increased, along with higher profitability and reduced economic risk (Barrington *et al.*, 2009).

1.2 Integrated production in Earthen ponds

The concept of integrated aquaculture was documented thousands of years ago, in China, and has been standard practice in South East Asia for hundreds of years (Ferreira et al., 2012). For example, here the concept has been applied in systems combining shrimp and seaweed, milkfish and seaweed, tilapia and rice (Largo et al., 2016) and other integrated crop/livestock/fish (mainly carp) systems developed in ponds (FAO, 1985).

Over the last three decades there has been a growing interest shown by several countries with a fishing tradition in researching into IMTA, with experimental works being conducted in Canada, Chile, South Africa, Israel and China (Barrington et al., 2009). More recently, the interest expanded to other countries such as Scotland, Ireland, Spain, Portugal, France, Norway, Denmark, Turkey, Japan, Korea, Thailand, U.S.A and Mexico (Guerrero & Cremades, 2012). However, most of the research is being applied to ocean cages, with exception to the examples presented before in South East Asia, where the focus is on pond aquaculture.

In Portugal, semi-intensive fish culture in saltwater ponds are the main production system used (Cunha *et al.*, 2011). This can be mainly explained by the difficulty to find appropriate locations to implement open water sea cages along the Portuguese coast, due to strong maritime swells and currents originated from the Atlantic Ocean. Moreover, there is a high availability of salt pans located in the coast that are relatively easy to convert into pond aquaculture systems. However, production costs of extensive and semi-intensive fish culture in earthen ponds are still too high to offer sustainable economic activity due to its low productivity (Cunha *et al.*, 2011). By adopting IMTA practices and diversifying the production – through the culture of extractive species such as oysters and macroalgae directly in the ponds – the total biomass of product cultured at one site can be increased, along with higher profitability and reduced economic risk.

On the environmental level, it is important to highlight the potential performance of extractive species used in earthen ponds when compared to open water systems. In fact, the uptake of dissolved inorganic nutrients (DIN) and particulate organic matter (POM) is thought to be more efficient in earthen ponds than in sea cages. Ammonia waste streams can be detected close to the fish cages but can quickly attenuate, while POM may not extend

much beyond a few hundred meters from the farm (Hughes & Black, 2016). Consequently, any direct ecological benefit with direct trophic transfer of nutrients needs to take place within a limited zone around the fish farm (Hughes & Black, 2016). While this may be difficult to achieve in sea-cages, earthen ponds provide a closed system where DIN and POM are more easily contained thus used up by the extractive species, increasing its use efficiency and performing a truly in-situ bio mitigation.

In the South, IPMA's Aquaculture Research Station in Olhão (EPPO) has been researching the implementation of IMTA directly in earthen ponds, rearing all the species together in the same system. More precisely, during the project SEAFARE (Sustainable and Environmentally Friendly Aquaculture in the Atlantic Region of Europe) it was attempted a co-cultivation of oyster (*Crassostrea angulata* and *C. gigas*), sea cucumber (*Holothuria tubulosa*), five species of seabreams (*Sparus aurata*, *Diplodus sargus*, *D. cervinus*, *D. vulgaris* and *D. puntazzo*) and meagre (*Argyrosomus regius*), with promising results.

Results of the project showed that the integration of gilthead seabream or meagre with the oysters may be the optimal combination for IMTA in earthen ponds. In comparison to normal polyculture tanks, fish in IMTA system were more robust according to the condition index. *Holothuria tubulosa* could be an interesting species to introduce in IMTA ponds, given its known contribute on reducing the load of organic matter into the system improving remineralization by feeding on phytobenthos and decomposed organic matter. However, high mortality was observed during the trial which may be linked with anoxic conditions on the bottom sediment and high water temperatures in the summer. Mesh bags hanged on floats close to the water surface are the most suitable grow-out structure for oysters in earthen ponds. In comparison with conventional fish culture ponds, IMTA ponds which included the presence of oysters, led to a more efficient use of primary production leading to higher benthic diversity with help in the remineralization processes and subsequent increases water quality (Cunha *et al.*, 2011). The overall picture reveals a promising future for IMTA in countries with strong focus on earth pond aquaculture in Europe (Cunha *et al.*, 2011).

1.3 Farmed species characterization

Next are characterized the farmed species in terms of biology and state of the art regarding their production.

1.3.1 Meagre – *Argyrosomus regius* (Asso, 1801)



*Figure 1. Representation of *Argyrosomus regius* (Adapted from FAO).*

Argyrosomus regius is a carnivore found in pelagic coastal areas. It is distributed in the Eastern Atlantic from Norway to Gibraltar and Congo, including the Mediterranean and the Black Sea (Chao & Trewavas, 1990). It can grow up to 2 meters and 50 kilograms. However, this species grows especially in the summer, while during winter months it almost stops feeding (Quemener, 2002). Overall, it presents good characteristic for aquaculture, including a fast growth of approximately 700 g after 12 months and 2–2.5 kg after 24 months (Ribeiro *et al.*, 2013) with a low feed conversion ratio of 0.9 – 1.2 (Monfort, 2010) and established induced spawning protocols to produce viable eggs and a relatively easy larval rearing (Estévez *et al.*, 2015). The meat exhibits low fat content with high lipid quality (Quemener, 2002), excellent taste and firm texture, while presenting an attractive fish shape (Monfort, 2010).

Its production began in the late 90s simultaneously in France and Italy, and is nowadays developing in other countries like Spain and Portugal. There was a meaningful increase in the last years, and is expected to grow fast in the medium term (Monfort, 2010). From a market point of view, there is a need for diversification of provided products beyond the whole fresh fish, to address socioeconomic factors identified as bottlenecks (Monfort, 2010). Nevertheless, it has the potential to change from niche market segments with limited production where small volumes are sold at relatively high prices, to become one of the best

candidates for aquaculture in Europe, given the favourable culture conditions and a predictable price drop under the pressure of increasing supply (Monfort, 2010).

1.3.2 White Sea Bream – *Diplodus sargus sargus* (Linnaeus, 1758)



Figure 2. Representation of Diplodus sargus sargus (Adapted from FAO).

Diplodus sargus is an omnivore inhabiting coastal rocky reef areas. It is distributed in the Eastern Atlantic: Mediterranean and Black Sea, and in the Eastern Central Atlantic: Madeira Island (Bauchot, 1987). It can grow up to 45 cm and 1.9 kilograms.

First attempts to culture white sea bream were carried out in early 1980s (Divanach *et al.*, 1982). Since 1995, only small quantities have been reported to be produced mainly by Greece, but also France and Spain. Furthermore, restocking experiments in artificial reefs were undertaken both in Gulf of Castellamare in Italy (D'anna *et al.*, 2004) and on the Algarve coast, in Portugal (Santos *et al.*, 2010) suggesting the species adequacy for restocking purposes.

Broodstock maturation and spawning in captivity does not represent major constraints, and eggs can be easily obtained by spontaneous spawning (Abellán & Garcia-Alcázar, 1995). It has better growth performance during larval stages (60-90 days) than other sparids such as the Sharpsnouth sea bream (*Diplodus puntazzo*) and Gilthead sea bream (*Sparus aurata*), however lower growth performances have been observed during the on growing phase (Divanach *et al.*, 1993; Abellán & Garcia-Alcázar, 1995), resulting in the white sea bream to be considered a poor candidate for aquaculture. However, this short performance could be explained by the fact that this and other species of the same genus have been fed with commercial diets optimized for Gilthead sea bream (Pavlidis & Mylonas, 2011).

Recent concerns from the aquaculture industry on the future availability of fishmeal and fish oil, and rising prices have renovated the interest toward omnivorous sparid species, such as the White Sea bream (Pavlidis & Mylonas, 2011). In fact, the experimental production of sparid species has been conducted for several years in the Portuguese Institute for the Sea and Atmosphere's (IPMA) Aquaculture Research Station (EPPO). Most relevant, the integration of such species in IMTA in earthen ponds semi-intensive systems was studied during 2010 and 2011, with the SEAFARE project.

During this experiment, tanks in a plain polyculture system (with several *Diplodus* species) were compared to IMTA tanks with the same species but also additional species, such as the Portuguese oyster (*Crassostrea angulata*) and Sea cucumber (*Holothuria tubulosa*). Results for *Diplodus sargus* showed a higher performance in the IMTA system, where fish presented higher growth rate, weight and condition factor (Cunha *et al*, 2011). In addition, the substantial quantity of seagrass and seaweeds found in *D. sargus* stomach suggests that they are mostly omnivores. They can take advantage of the plants that grow in the IMTA tanks, indicating a better degree of nourishment in these ponds (Cunha *et al*, 2011). The results are promising for optimizing the species aquaculture performance by their integration in IMTA systems.

1.3.3 Flathead Grey Mullet – *Mugil cephalus* (Linnaeus, 1758)



Figure 3. Representation of *Mugil cephalus* (Adapted from FAO).

M.cephalus normally forms large schools over sand or mud bottoms, occurring in coastal waters of tropical, subtropical and temperate areas of all seas. In the eastern Atlantic Ocean, it is distributed from the Bay of Biscay to South Africa, including Mediterranean Sea and the Black Sea (Thomson, 1966). Being a euryhaline species, it can be found in lower

sections of streams and estuaries as well in inland areas. Maximum length reached is 120 cm and maximum weights of 8 kg.

Adults feed during the day, on the bottom but also on surface water particle accumulations (Odum, 1970). The diet consists of microalgae (epiphytic and benthic), plant detritus, inorganic sediment particles and benthic animals to a lesser extent (Thomson, 1966; Odum, 1970). The ingestion of sediment particles is believed to function as a grinding substrate in the degradation of plant cell walls in the stomach (Thomson, 1966).

It is widely cultivated in freshwater and brackish ponds. A variety of grow out methods are practiced throughout the world, consisting in extensive, polyculture, and intensive or monoculture (Tamaru, 1993). This is an important aquaculture species, particularly in many Asian and Mediterranean countries (Fischer & Bianchi, 2012). Being a detritivore, it has been stocked in fish ponds to improve sediment quality and avoid oxygen depletion, thus being an excellent candidate for enhancement of aquaculture in earthen ponds, coastal lagoons and Salinas that exist throughout the EU Mediterranean countries. (Koven *et al.*, 2015)

Nevertheless, the main bottleneck in the culture of mullets is the difficulty to reproduce in captivity. The current industry is capture-based, relying exclusively on capture of wild fry grown out to market weight (Koven *et al.*, 2015). Spontaneous spawning in captivity is not possible due to inhibition at early stages of vitellogenesis and final oocyte maturation and ovulation (Koven *et al.*, 2015). DIVERSIFY-Fish project (2015) aimed to evaluate effectiveness of hormone-based treatments for induced spawning and optimize scaled-up breeding in captivity under natural and manipulated photo-thermal regimes. In fact, hormone-therapy based on GnRHa and dopamine D2 antagonist (females) or r-FSH and Methyl-testosterone (males) improved and synchronized gonadal development and induced captive mullets to reproduce. Results show good signs for the possibility of control over the reproductive cycle in captivity, although larval rearing protocols are still necessary to achieve supply of high quality juveniles (Koven *et al.*, 2015)

Overall, prospects for the species are very good. The market is expanding around the Mediterranean, since flathead grey mullet can provide not only whole fish and fillets but especially “bottarga”, a high value product with Italian origin which consists in dehydrated fish roe. In addition, feeding at the lower trophic levels makes it ideal for aquaculture,

representing an inexpensive source of animal protein (Tamaru, 1993). In this way, the species would be more acceptable to an increasingly aware consumer public that demands sustainability and lower environmental impact (Koven *et al.*, 2015).

1.3.4 Pacific Oyster – *Crassostrea gigas* (Thunberg, 1793)



Figure 4. Representation of *Crassostrea gigas* (Adapted from FAO).

Crassostrea gigas is an epifaunal, immobile organism found in rocky shores and coastal areas, growing individually or in dense mats on rocks and soft substrates in temperate tidal and sub-tidal zones (Harris, 2008). It is native to the Pacific Coast of Asia, but following the 1970s oyster crisis due to the fast disappearance of diseased *Crassostrea angulata* in European waters, the Pacific oyster was introduced saving the collapsing industry (Gouletquer & Héral, 1992). In several countries, uncontrolled natural reproduction has led to a significant species expansion and natural breeding stocks (Kater & Baars, 2004). The natural hybridization between genetically differentiated populations of *C. gigas* and *C. angulata* was demonstrated by Huvet *et al.* (2004), as a result, *C. angulata* is believed to be at threat from current culture development and extensive transfers of *C. gigas* (Huvet *et al.*, 2000)

Pacific oysters are hardy filter feeders that can reach a maximum of 30 cm in size, with a normal length of 8-15 cm (FAO, 2005). They tolerate broader ranges of temperature and salinity than native oysters, making them extremely desirable for commercial culture and extremely successful at invasion (Harris, 2008). The concerns about negative impacts on native oysters has lead towards development of methods of mitigation. Moreover, the development of triploid oysters prevents the production and spread of larvae, while presenting better quality meat (Harris, 2008).

The main constraint is systems carrying capacity, thus the existence of large food availability resulting from high primary productivity facilitates more intensive rearing densities. Given the tolerance to high ranges of seawater temperatures and salinities, it has the capacity to grow in variable environments from estuarine areas to brackish waters to offshore areas in oceanic waters (Cabi, 2014).

The grow-out is almost entirely sea-based, with a variety of techniques, including bottom culture, off-bottom culture, suspended and floating cultures. Those grown in suspended culture, are commonly subjected to the process of hardening for a period of up to three or four months prior to harvesting. The process allows daily periods of exposure to air, improving the meat content and quality once harvested (FAO, 2005). In Portugal, the most common grow-out technique is performed in the intertidal areas of Formosa, Alvor and Aveiro Lagoons, using mesh bags in an off-bottom culture. In the last years, IPMA's Aquaculture Research Station (EPPO) started to implement mesh bags floating at the surface of fish culture ponds, with promising growth results achieved so far. It was found that the bacterial load in fish mucus is highly reduced by the presence of oysters (Cunha *et al.*, 2011). Moreover, bivalves have often been used in integrated fish-farming to enhance economical value of by-products and improve water quality (Lefebvre, 2000), for example, researchers in France studied the suitability of oysters in IMTA systems and found that *Crassostrea gigas* could feed on the detritus/waste of fish farm effluents, allowing farmers to recapture the lost organic product of intensive fish farming, and grow economically valuable species. (Barrington *et al.*, 2009).

1.3.5 Sea Lettuce – *Ulva flexuosa* (Roth, 1800)



Figure 5. Representation of *Ulva* spp. (Adapted from “Sowerby’s Botany”, 1814).

Ulva is a genus of marine and brackish water green macroalgae (Nikolaisen *et al.*, 2011). It grows attached to stones or other substrates, but it easily detaches and grows well free floating, often forming dense mats, sometimes called “green tides”. The genus presents a cosmopolitan distribution in many climate and ecological conditions (Ben-Ari *et al.*, 2014). It has relatively high growth rate compared to other algae, both in nature and in cultivation facilities (Nikolaisen *et al.*, 2011). The specific *Ulva* species used in the study was found to be *Ulva flexuosa* by DNA barcoding analysis.

Seaweeds have been proposed as a biomass source to produce food, animal feed, bioactive ingredients, bioenergy, pharmaceuticals and cosmetics (Ben-Ari *et al.*, 2014). *Ulva* has been cultivated successfully for the food industry given it represents an important source of dietary fibres, mainly soluble (Ben-Ari *et al.*, 2014), and has also been widely used for bioremediation of waste water from land-based aquaculture (Nikolaisen *et al.*, 2011). In fact, *Ulva* has many of the basics conditions required to be a part of an IMTA system. It is a local species, forms lamellar, and has promising rate of growth in high nitrogen and ammonium concentrations (Guerrero & Cremades, 2012). However, there is a co-limitation of growth by light and nitrogen, indeed incoming irradiance must be high to utilize greater N concentrations and achieve maximal growth rates. When not limited by availability of nutrients, yields are directly correlated to incoming light (Nikolaisen *et al.*, 2011). The life cycle is also known and controllable (Guerrero & Cremades, 2012), however, in mass

cultures, sporulation may occur in response to seasonal environmental cues and reduce the biomass of vegetative thalli by more than half within a few days (Nikolaisen *et al.*, 2011).

Methods for the cultivation of *Ulva*, with the use of fish effluents, normally consist in independent tanks with bottom-aeration. In this case, the effluents flow in and out continually with a determined water exchange rate, while the aeration in the bottom stirs the algae vertically and evens the exposure of all thalli to light even in relatively deep tanks with high algae densities (Ben-Ari *et al.*, 2014). Nevertheless, this method is energy-intensive, constituting a big percentage of total algal culture cost (Ben-Ari *et al.*, 2014). The method attempted at EPPO and studied in this work, is totally different from the previous attempted, given it consists in floating structures that are placed directly inside the fish culture ponds, with no use of aeration. The thalli are manually fixed and let grown free-floating. The advantage of this method is the cost reduction, given no energy is spent for aeration, and no additional tanks are needed. The disadvantage seems to be the high labour necessary to fix the thalli. Preliminary results of the weekly trials showed promising results. Specific growth rates averaging from 15-35% were similar and sometimes even higher than previous studies conducted in tanks with aeration, such as 18-31% (Ben-Ari *et al.*, 2014), 19-21% (Neori *et al.*, 2000 and 2003), 11-37% (Msuya & Neori 2008) and 12-32% (Bruhn *et al.*, 2011). The potential for growth in this way was proven and its optimization may lead to a new promising method for *Ulva* production in earthen ponds. Overall, *Ulva* shows good growth rates important for biomass production and increased profit, good photosynthetic rates interesting for increasing availability of oxygen at aquaculture sites, while the inorganic nutrient removal rates are important for effluent mitigation (Barrington *et al.*, 2009)

1.4 Fatty Acids

1.4.1 Definition and nomenclature

Fatty Acids (FA), along with proteins, are the major contributors to aquatic organism's tissues, serving as the building blocks of lipids, such as TAG-triacylglycerols (lipids stored for energy) and phospholipids (structural components of membranes in cell walls) (Sobiechowska, 2008). Additionally, FAs are among the most important molecules transferred across the plant-animal interface in aquatic food webs (Brett *et al.*, 2009) being acquired directly from the diet and stored at relatively high levels.

FAs consist of a terminal methyl group (CH₃), a carbon chain (usually between 14 and 24 carbons) and a terminal carboxyl group (COOH) (Gurr *et al.*, 2002). In terms of classification, saturated FAs (SFA) are those with no double bonds while monounsaturated FAs (MUFA) have one double bond. Polyunsaturated (PUFA) have two or more double bonds. Throughout this work, the nomenclature used to name specific FAs will be as follows. For example, C18:1 ω 6, identifies a FA with 18 carbon atoms and two double bonds, while the notation " ω 6" (omega six) gives the position of the first double bond counting from the methyl end (Figure 6).

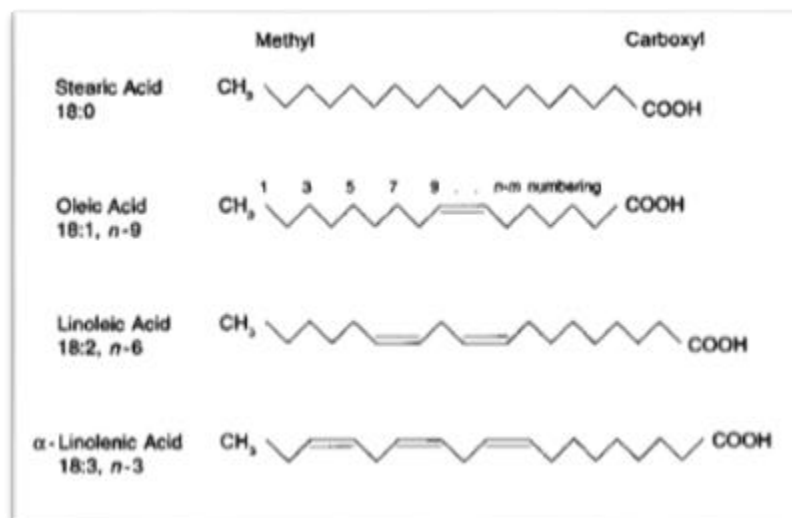


Figure 6. Molecular structure of stearic acid, oleic acid, linoleic acid and alpha-linolenic acid.

1.4.2 Essential fatty acids

Essential fatty acids (EFA) are among the dietary nutrients with greater direct impact on the composition of its consumer. Their importance is not only linked to their impact on growth and product quality, since they play a diverse role in the physiological and biochemical processes within aquatic animals (Watanabe, 1982). Lipids in general, and EFA particularly, play important roles in the processes of ingestion and digestion, cell synthesis, neural development, endocrine function and control, ionic regulation, immune function and reproduction. Their influence on growth is greatest in larval fish and crustaceans, due to their reduced ability to digest and absorb lipids, but also because of a higher demand for EFA in the development of neural tissues (Glencross *et al.*, 2009). Docosahexaenoic acid (DHA, 22:6 ω 3) has consistently been shown to provide the greatest EFA value to most species. In addition, Eicosapentaenoic (EPA, 20:5 ω 3) and Arachidonic (ARA, 20:4 ω 6) acids also have great nutritional value, while Linoleic (LA, C18:2 ω 6) and Alpha-Linolenic (ALA, C18:3 ω 3) acids have been shown to have slightly less important nutritional value, nevertheless all have been considered as essential to most species since they are precursors of EPA and DHA (Glencross *et al.*, 2009). The ideal quantities of these compounds to include in diets is directly related with whether the species origin is the marine or freshwater environment. While the latter can synthesize DHA, EPA and ARA from their precursors LA and ALA (Izquierdo, 2005), marine animals have a very limited ability to synthesize PUFA from the ω 3 family such as DHA and EPA, thus being considered essential fatty acids for their normal growth and development (Langdon, 1981), requiring them to be included in their diets. In humans, PUFAs from the ω 3 family such as ALA, C18:4 ω 3, DHA and EPA are considered essential, both due to the limitations in their biosynthesis, and because they play an important role in the prevention of cardiovascular diseases, osteoarthritis, diabetes, and possess important properties such as antimicrobial, antiviral, anti-inflammatory and antitumoral (Kendel *et al.*, 2015).

1.4.3 Fatty acid trophic marker concept

Identifying the diet of animals is important for understanding their basic ecology, characterizing trophic interactions, and predicting community-level consequences of biotic and abiotic change (Kelly & Scheibling, 2012).

Several methods can be used to characterize the feeding relationships within whole communities. Direct methods, more traditional, comprise the observation of trophic behaviours, food choice experiments and especially gut content analysis. However, gut contents analysis possesses several limitations (Deb, 1997), the most important being the fact that they tend to provide mere snapshots of diets at points in time and space (Hart & Reynolds, 2002), affecting extrapolation of results, considering that an organism feeding behaviour might vary temporally in function of prey abundance. Furthermore, traditional stomach analyses suffer from the fact that food items in the gut are frequently difficult to identify and are quantitatively biased due to differential digestion rates of soft and hard parts (Dalsgaard *et al.*, 2003). Gut contents therefore offer a poor basis for elucidating the detailed interactions which must exist in food webs (Hart & Reynolds, 2002).

Indirect methods consist on the use of trophic biomarkers. The perfect trophic marker would be a compound whose origin can be uniquely and easily identified, that is inert and nonharmful to the organisms, that is not selectively processed during food uptake and incorporation, and that is metabolically stable and hence transferred from one trophic level to the next in both qualitative and quantitative manner, providing essential insight into the dynamics of ecosystems (Dalsgaard *et al.*, 2003). However, such markers are unfortunately rare if non-existent, and instead researchers have become content with less ideal components, such as fatty acids.

Fatty acids analysis is an indirect method that can yield useful information about a consumer's diet (Hart & Reynolds, 2002). FAs are in many circumstances incorporated into consumers in a conservative manner, thereby providing information on predator-prey relations (Dalsgaard *et al.*, 2003). In fact, the fatty-acid trophic biomarker concept was developed based on the observation that marine primary producers biosynthesize and lay down certain fatty acid patterns that may be transferred through aquatic food webs, thus being recognized in their primary consumers, followed by their dynamics in fish, who directly

incorporate or transform dietary FA (Dalsgaard *et al.*, 2003; Bergé & Barnathan 2005). Nevertheless, the technique presents some constraints. Firstly, no single FA can be assigned uniquely to any one species (Dalsgaard *et al.*, 2003). Secondly, depending on the metabolic strategy, certain consumers might be able to transform dietary FA by the action of specific enzymatic activity (elongases and desaturases), or might even produce new FA by *de novo* biosynthesis. For example, benthic invertebrates have the capacity to significantly modify their dietary FAs and thereby obscure markers for food sources (Kelly & Scheibling, 2012).

By last, even if the diet is known to be the main factor to influence an organism FA composition (Chaguri, 2013), the temporal dynamics, or turnover rate of individual FA, is strongly linked to traits such as size, age, sex, state of maturity, migration and other factors like food abundance, water temperature and salinity. Consequently, FA have so far only been used as qualitative food web marker, while for a quantitative study (e.g. food-web, carbon mass-balance calculations) more information would be needed on how the various FA are selectively metabolized as they are conveyed through aquatic food webs (Brett *et al.*, 2009). For example, combining FA analysis with other lines of evidence, such as stable isotope analysis, offers a more reliable approach for examining trophic interactions (Kelly & Scheibling, 2012).

1.5 Expected fatty acids

1.5.1 Phytoplankton

Autotrophic organisms biosynthesize all their cellular constituents *de novo* including FA. Their FA signature is an expression of both genotypic and phenotypic characteristics. Additionally, large qualitative and particularly quantitative fluctuations, both within and between species, are observed in FA patterns being related to the combined effects of environmental conditions along with the physiological state of the algae (Dalsgaard *et al.*, 2003). Considering the present study, in fish culture ponds, there is a consistent influx of nutrients, thus primary producers in these areas are light limited rather than nutrient limited. Specifically, local environmental conditions such as temperature and light availability also play major influence.

Specific fatty acids or combinations of them cannot be used as indicators at the species-specific level, but can be associated with certain taxonomic classes of phytoplankton (Hart & Reynolds, 2002) such as *Bacillariophyceae* (diatoms), *Dinophyceae* (dinoflagellates), *Prymnesiophyceae* and to a lesser extent by *Chlorophyceae* (Dalsgaard *et al.*, 2003). Among well documented fatty acids, C18:3 ω 3 (ALA) is characteristic for chlorophytes. Diatoms are characterized by high concentration of EPA, C16:1 ω 7 and C18:1 ω 7 while having low levels of DHA and virtual absence of C₁₈ PUFAs. Dinoflagellates are rich in DHA and C18:4 ω 3. These differences provide the potential for identifying the transfer of different sources of phytoplankton through trophic levels (Pazos *et al.*, 1996; Hart & Reynolds, 2002; Pogoda *et al.*, 2013)

1.5.2 Green Macroalgae

Seaweeds chemical composition may vary according to the species, habitat, environmental conditions and other factors (Cruz-Suárez *et al.*, 2010). A study by Khotimchenko (2002) which compared FA compositions of algae from different regions of the world showed that, in general, and independently of the geographical source, algae had similar FA profiles typical of each phylum of seaweeds. Green algae contain large amounts of C₁₆ PUFAs, in particular 16:4 ω 3 is taxonomically characteristic of green macrophytic algae (Kendel *et al.*, 2015). Considering the order *Ulvales*, C₁₈ PUFAs such as C18:4 ω 3 and C18:3 ω 3 are also considered characteristic. Moreover, it was found in some studies that C18:4 ω 3 is predominant in *Ulva intestinalis*, while C18:3 ω 3 is predominant in *Ulva lactuca* (identical morphotype to *Ulva flexuosa* analysed on the present study) (Khotimchenko, 2002). In the order *Ulvales*, PUFAs can represent up to 66% of total FA (Cruz-Suárez *et al.*, 2010), therefore, and considering the huge and renewable biomass, seaweeds are a potential source of FAs for biotechnology and a potential dietary source of essential fatty acids (Khotimchenko *et al.*, 2002).

1.5.3 Zooplankton

The concept of Fatty Acid Trophic Marker (FATM) has been frequently applied to marine invertebrates, especially herbivorous zooplankton that represent a key link between primary producers and higher trophic levels (Dalsgaard *et al.*, 2003). The fatty acid composition of zooplankton is determined by taxonomic affiliation, changed by diet, and modified by starvation or temperature (e.g. higher proportions of DHA and EPA with decreasing temperatures). Additionally, zooplankton might have the ability to bioconvert some FA into other FA molecules (Brett *et al.*, 2009). In general, FA patterns of carnivorous and omnivorous copepods cannot be as easily linked to diet as is the case for herbivorous copepods from temperate regions to polar regions (Brett *et al.*, 2009). Even though, there is a considerable overlap in the FA composition of the major phytoplankton groups, being particularly difficult to attribute particular dietary sources based on individual FA (Brett *et al.*, 2009). Generally, in zooplanktonic FA signatures, it is assumed that abundant levels of C16:1 ω 7 and EPA represent diatom consumption, while C18:4 ω 3 and DHA represent dinoflagellate consumption. It has also been suggested that C14:0, C16:0 and C18:1 ω 9 are trophic markers for omnivorous feeding on ciliates and C18:1 ω 7 indicates bacterial consumption (Brett *et al.*, 2009). However, seasonal variability for zooplankton dietary sources increases uncertainty within diet-zooplankton patterns. In addition, their feeding strategies, turnover FA rates, and life history traits (including biosynthetic pathways) still needs to be better understood to allow a more accurately interpretation of patterns observed in field studies (Brett *et al.*, 2009).

1.5.4 Polychaeta

The composition of the diet is a decisive factor determining the FA composition of marine polychaetes such as *Hediste diversicolor* (Luis & Passos, 1995). However, polychaetes are omnivorous organisms, and depending on the environmental conditions, are able to change feeding mode from filter feeding to deposit feeding or active carnivorous feeding on small benthic organisms, while cannibalism is also quite common (Bischoff *et al.*, 2008). Even though some potential food items have characteristic FA compositions that could suggest their consumption, like primary producers, uneaten fish feed or sediment borne bacteria, the fact that polychaetes consume mixed diets poses problems for distinguishing

different items contribution to their diet due to overlap in FA patterns (Kelly & Scheibling, 2012).

In a study considering a recirculating aquaculture system, where solid waste originating from a fish culture system (including faecal material, uneaten fish feed and bacterial biofilms) was provided to *H. diversicolor*, it was found that FA profiles of the worms reflected those of the provided food sources. In addition, analysed samples revealed high levels of C18:1 ω 9 and DHA, proving that marine polychaetas are able to recycle valuable feed nutrients, which were otherwise discharged (Bischoff *et al.*, 2008). In another experiment, *H. diversicolor* was fed uniquely with a commercial fish diet, reflecting its high levels of C18:1 ω 9 and DHA, and to a lesser extent C18:2 ω 6 and EPA (Barragão, 2013). Overall, it has been evidenced that marine polychaetas are a suitable organism for recycling FA from fish culture systems waste. From another perspective, polychaetas could also be used as feed for a variety of cultured fish species, due to their valuable source of dietary fatty acids (Bischoff *et al.*, 2008). In fact, *H. diversicolor* has been utilized in the feeding of fish and crustaceans, providing an adequate nutrition to broodstock in captivity, having important role in gonadal maturation of different species such as *Solea vulgaris*, *S. senegalensis* and *Penaeus kerathurus* (Bagarrão, 2013).

1.5.5 Oyster

Bivalves assimilate FAs in relation to the FA composition in their diets (Gao, 2006). In *Crassostrea gigas* cultured in Galicia, the fluctuations in FA pattern through the year was found to be correlated both to phytoplankton concentration and sexual cycle. Moreover, PUFAs were negatively correlated with temperature, while presenting great fluctuations in ω 3's but in a minor way in ω 6's who showed no seasonal variation (Pazos, 1996). In another study, high concentration of EPA was found to be a reflect of a diet based on diatoms (Sobiechowska, 2008). In another study, comparing FA patterns in *C. gigas* sampled both in Japan and France, all contained high levels of PUFA in their tissue, and specifically high levels of EPA and DHA (Saito & Marty, 2010). *C. gigas* cultivated offshore in Germany also showed high levels of DHA and EPA (Pogoda *et al.*, 2013). In Italy, cultured *C. gigas* were characterized by predominance of PUFA's over SFA's and MUFA's, and high ω 3/ ω 6 ratios

(Orban *et al.*, 2004). Waldock (1984) showed that besides being acquired in the diet, adult *C. gigas* could elongate and desaturate ω 3 FA to produce DHA and EPA at low rates.

1.5.6 Fish

Marine fish have limited abilities to synthesize FA (elongation, desaturation), consequently they need to get FA from lower trophic levels. Indeed, dietary FA are incorporated into their tissue with little or no modification (Bischoff *et al.*, 2008). The global composition of FAs can vary by consequence of several factors, including species, age, seasonality and production system. Nevertheless, diet represents the main factor to influence their composition of FAs, following the rule for all cultured fish species (Tocher, 2003; Chaguri, 2013). Generally, C16:0 and C18:0 are the predominant SFA that occur naturally in animal fats, including cultured fish, although a range of chain lengths from C₁₂ to C₂₄ can be found (Tocher 2003). The main MUFA generally found is C18:1 ω 9, being its accumulation mainly a direct result of consumed diet and to a lesser extent a product of desaturase activity from C18:0 (Grigorakis, 2011). High levels of C18:2 ω 6 are usually observed in cultured fish because of the feed-incorporated plant oils, such as sunflower-seed, linseed, rapeseed, soybean, olive and palm oils (Miller *et al.*, 2008). This negatively influences fish's ω 3/ ω 6 ratio, thus requiring ω 3 LC-PUFA, in particular EPA and DHA, to be included in their diets to achieve proper FA profiles with higher ω 3/ ω 6 ratios to meet the requirements of fish and to maximize human health benefits (Miller *et al.*, 2008).

Studies on the quality of meagre reared in sea cages in Italy by Piccolo (2010) and in Greece by Grigorakis (2011) and Fountoulaki (2017), found that FA composition of fish muscle reflected dietary FA, confirming the general rule directly relating the fish tissue FA composition to the dietary FA's. In particular, C18:1 ω 9 and C18:2 ω 6 were the most abundant FA both in diet and fish muscle in all studies. DHA was slightly higher in fish than in feed, showing that meagre retains well ω 3 PUFA. EPA however had only half of the amount in fish than in the feed. While it is also well retained since it is a ω 3 PUFA, however it could be transformed to other ω 3 FA in fish flesh.

2 Material and Methods

2.1 Experimental design

The system was developed in the south coast of Portugal at the *Estação Piloto de Piscicultura de Olhão* – EPPO which is forms part of the *Instituto Português do Mar e Atmosfera* – IPMA (Figure 7). The experiment took place for 9 months - from April to December 2016 - being developed in 6 equally sized rectangular earthen ponds, with $435\text{m}^2 \times 1.5\text{m}$ deep (750 m^3) each and in a conventional open-air semi-intensive system. Water directed from the Ria Formosa lagoon system to the station's reservoir was pumped continually to the earthen ponds. Depending on the levels of water environmental parameters (mainly dissolved oxygen and parasites) the rate of water renewal was adjusted ranging from 20% to 100%. Water renewal rates were maintained equal for all ponds by controlling the flux at each one's entry.



Figure 7. Aerial view of *Estação Piloto de Piscicultura de Olhão* – EPPO. (arrows indicate the six ponds used for the experiment)

The same number of fish, consisting of meagre (*Argyrosomus regius*), white seabream (*Diplodus sargus*) and mullet (*Mugil cephalus*) were introduced in each of the 6 earthen ponds. In Table 1 is presented detailed information of the fish quantities introduced per pond.

Table 1: Initial quantity, average weight (g), biomass (kg) and density (kg.m^{-3}) of each fish species introduced per tank.

	Quantity	Average weight (g)	Biomass (kg)	Density (kg.m^{-3})
<i>Argyrosomus regius</i>	1458	200	292	0,389
<i>Diplodus sargus</i>	918	65	60	0,080
<i>Mugil cephalus</i>	581	100	58	0,077

Three treatments were tested in duplicated ponds. Differences between treatments reside in the addition of other harvestable species additionally to the fish species, namely, pacific oysters (*Crassostrea gigas*), and/or green macroalgae (*Ulva flexuosa*). In the first treatment – **F+A**, were cultured fish and algae (tanks 3 and 5), in the second treatment – **F+O**, were cultured fish and oysters (tanks 2 and 4), while in the third treatment – **F+O+A**, all species were cultured together: fish, oysters and algae (tanks 1 and 6). Treatments configuration is presented in Figure 8. Table 2 shows detailed information of the oysters and algae introduced.

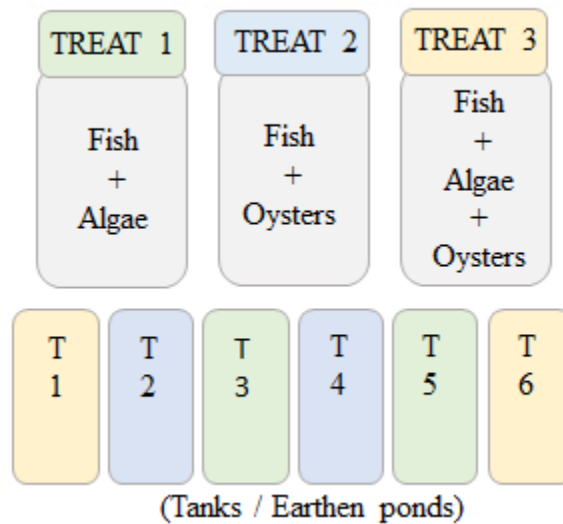


Figure 8. Diagram of tanks 1 to 6 with respective treatment. (F+A: Fish and algae, F+O: Fish and oysters, F+O+A: Fish, algae and oysters)

Table 2: Initial quantity, average weight (g), biomass (kg) and density (kg.m⁻³) of oysters introduced per tank in tanks 1, 2, 4 and 6, and algae introduced per tank in tanks 1, 3, 5 and 6.

	Quantity	Average weight (g)	Biomass (kg)	Density (kg.m-3)
<i>Crassostrea gigas</i>	17500	3	53	0.070
<i>Ulva flexuosa</i>			0.030	

Note: *Ulva* was harvested always one week after seeding (14 cycles in total). Three different initial biomasses were tested, consisting in 25, 30 or 50 grams of drained weight (d.w.).

2.2 Earthen ponds and grow-out management

Water parameters such as temperature, salinity, dissolved oxygen (DO), pH and turbidity were manually measured twice daily (9h/17h) with a portable logging multiparameter meter (*Hanna Instruments Multiparameter 9829*) always near the tank water outlet. Two automatic probes placed close to the area also contributed with continuous information on water temperature and dissolved oxygen. Each tank had an air injector programmed to work automatically at any given time in case the dissolved oxygen dropped below 4.5 to 3.5 ppm.

Fish

A commercial feed was provided during all the experiment by automatic feeders. Feed analytic constituents and raw materials are presented in Table 3.

Table 3: Feed description (analytic constituents and additives per kilogram of feed), along with raw materials of the feed. Information provided by the manufacturer (Alpis©)

Analytic constituents	(%)	Additives per Kg of feed	
Crude Protein	40.3	Vitamin D3	1200 UI
Crude Fat	15.3	Vitamin A	6000 UI
Moisture	8.3	Vitamin E	100 mg
Ashes	7.1	Vitamin C	50 mg
Crude Cellulose	5.4	CU	3 mg
Phosphorus	0.9		

Raw materials	(%)
Fish meal	32
Soya meal	27.5
Wheat	15
Soya oil	10
Feather meal	6
Rapeseed meal	4
Algae meal	4
Fish oil	1

Fish were fed daily at an average of 1.5% body weight. Every month, biometric measurements (weight and length) were performed in 50 individuals of meagre in each tank. Feeding quantities and feed pellet diameter (which varied from 3 to 4.5 cm) were adjusted accordingly to fish biometric measurements equally in all tanks. Parasite analysis was performed every 15 days in 3 individuals of meagre per tank, which involved the sacrifice of the fish and the removal of the first two left branchial arches for parasite identification and counting under microscope visualization.

Oysters

Triploid Pacific oysters (*Crassostrea gigas*) were proceeding from ‘Formoso & Alegre’ farm in Olhão. They were introduced in ponds number 1, 2, 4 and 6. Oysters were cultured in suspended mesh bags attached to a long-line at the surface of the earth pond (Figure 9).



Figure 9. Long-lines of oyster bags floating at the surface.



Figure 10. Oyster bags being submerged in water.

On a weekly basis, oyster bags were manually shaken in the water and turned upwards on the floating structure, being exposed to air for 24-hour period, after which were turned downwards to be again submerged in water (Figure 10). At the middle of the experiment period, oyster bags were all opened and inspected for dead individual's removal and afterwards placed again in the respective tanks. Final biometry (weight and length) was executed in 50 individuals of oysters in each tank.

Algae

A green macroalgae, *Ulva flexuosa*, was regularly collected from EPPO settling tank and cultured in ponds number 1, 3, 5 and 6 (additionally on these same ponds, two natural occurring algae, such as *Rhizoclonium riparium* and *Ulva Intestinalis*, were let to grow in the pond's margins, while in ponds 2 and 4 they were regularly removed).

Ulva flexuosa was collected from the settling tank (Figure 11.a), washed with freshwater, cut in pieces of approximately 15 cm by 15 cm, and squeezed drained. It was then manually fixed with known densities in a net (Figure 11.b), and placed at depths ranging 0-20 cm in a floating structure (Figure 11.c). Each ponds structure contained 6 fields of equal area (1 m by 1 m). After 7 days of grow-out, harvest and measurement of the final drained

weight was performed, along with specific growth rates and biomass generated per week in each structure, however those results are not reported in this work.



a)



b)



c)

Figure 11. *a) Collection of new *Ulva flexuosa* from the station's settling tank. b) Manual fixation on the net. c) Structure overview*

2.3 Sampling

Table 4: Number of samples collected. Triplicates were collected for each pond, thus six samples collected for each treatment (“F+O”, “F+A” and “F+O+A”)

	F+O	F+A	F+O+A	Totals
Commercial feed				3
Sediment	pooled across all tanks			3
Polychaeta	pooled across all tanks			12
Phytoplankton	6	6	6	18
Suspended matter	6	6	6	18
Zooplankton	6	6	6	18
<i>Rhizoclonium riparium</i>		6	6	12
<i>Ulva flexuosa</i>		6	6	12
<i>Ulva intestinalis</i>		6	6	12
<i>Crassostrea gigas</i>	6		6	12
<i>Argyrosomus regius</i>	6	6	6	18
<i>Diplodus sargus</i>	6	6	6	18
<i>Mugil cephalus</i>	6	6	6	18
				174

Sampling of all species was executed during the first two weeks of December. All fish species were sampled across each tank, consisting in *Argyrosomus regius* (weight: 530 ± 96 g; length: 37 ± 1.8 cm), *Diplodus sargus* (weight: 174 ± 14.5 g; length: 21 ± 0.6 cm) and *Mugil cephalus* (weight: 189 ± 26.9 g; length: 27 ± 1.3 cm). From each individual, a small rectangular piece of the dorsal white muscle was cut and the skin excluded. Macroalgae species were collected in tanks 3 and 5 (respective to treatment “F+A”) and in tanks 1 and 6 (respective to treatment “F+O+A”). *Ulva flexuosa* (cultured sea lettuce) was collected from the growing structures. *Ulva intestinalis* and *Rhizoclonium riparium* were collected from the rocks delimiting the tanks where these algae grow naturally. All algae samples were cleaned with distilled water to remove organic sediment and undesired organisms such as epiphytic crustaceans. Oysters of similar size (mean total weight: 62 ± 12.3 g) were collected from the culture bags in tanks 2 and 4 (respective to treatment “F+O”) and in tanks 1 and 6 (respective to treatment “F+O+A”). Stomach was excluded from all individuals. Phytoplankton, suspended matter and zooplankton were collected across all tanks. Identical volumes of water (2 L) collected from each tank were filtered with 0.7 µm fiberglass filters for phytoplankton

sampling, while 0.4 μm fiberglass filters were used for suspended matter sampling. A nylon net was transported horizontally in circles around each tank for zooplankton sampling. Polychaetes and sediment were collected once the tanks were emptied at the end of the experiment period. Several holes were excavated with a small shovel in random locations of the tanks and all the visible polychaetas were collected, which belonged to three different genres (*Diopatra spp.*, *Marphysa spp.* and *Nereis spp.*). Sediment top-layer samples were also randomly collected in different locations of the tanks. Since both sediment and polychaetas were only sampled in three random tanks and then pooled together, no differentiation between treatments is possible to be presented, thus each compartment profile represents an overall average of the system.

A total of 174 samples were collected for fatty acids extraction and analysis. All samples were frozen in liquid nitrogen immediately after collection, freeze-dried in a lyophilizer, and finally stored at -80°C . Filters for phytoplankton and suspended matter were stored without liquid nitrogen step.

2.4 Fatty acids extraction

Lipids extraction and preparation of fatty acid methyl esters (FAME's) was performed using the "one step acid transesterification", based on the previous works by Idarti *et al* (2005), Lewis *et al* (2000) and Christie (2003). For each sample, 4 mL of solution of methanol/sulfuric acid (95%)/dichloromethane-BHT (1.7:0.3:2, v/v) was added to the dry material (around 100-500 mg depending on the samples lipid richness, ANNEX 1). Samples were heated for 1h30 at 90°C causing lipids to be extracted and fatty acids to undergo methylation into FAME's. After addition of 1 mL of pure water (*milli-Q*) per tube, one round of centrifugation (1300 rpm, 5 minutes at 4°C) was performed. The inferior organic phase was then collected from the biphasic system and kept in new tubes chilled on ice. To fully collect the remaining FAME's from the biphasic system, 2 mL of heptane/dichloromethane (4:1, v/v) was added per tube, followed by new centrifugation (1300 rpm, 5 minutes at 4°C) after which the superior organic phase was collected (step repeated 3 times). After the collection of all the organic material, the residues on the initial tube were discarded. To clean

the collected organic phases, 4 mL of Potassium carbonate (2%) was added per tube and centrifugation was again performed. After centrifugation, 6 mL of the clean organic phase was collected in new glass tubes and fully evaporated using a nitrogen evaporator. Between 0.1-1 mL of solvent (heptane) was added (depending of FA richness, ANNEX 1) and transferred to proper vials for analysis in the gas chromatograph.

2.5 Analysis with GC-FID

Samples were injected into a Varian Star 3900 CP Gas Chromatograph (United States), equipped with an auto sampler and a flame ionization detector (FID). Initial column temperature was 100°C, increasing to 180°C at 8°C/min and finally to 220°C at 4°C/min. Peaks were identified by comparison of retention times with those of analytical standards of Sigma-Aldrich: Menhaden oil, Supelco C4, C16:4 ω 3 and BAME. Integration area of each peak was converted in percentage of Total Fatty Acids (%TFA). Between each three replicates was calculated the average, standard deviation and coefficient of variation. In case the coefficient of variation of the major peak was above 10, the replicates were re-extracted and/or re-analysed.

2.6 Data interpretation and statistical analysis

Environmental parameters

Environmental parameters (temperature, salinity, dissolved oxygen, pH and turbidity) of all ponds were measured twice daily, first in the morning (around 9 am) and secondly in the afternoon (around 5 pm). Data is presented for each tank as mean \pm standard deviation (s.d.) during all the experimental period. For each environmental parameter measured, statistical analysis was firstly executed to assess differences between paired tanks of the same treatment with two-way ANOVA with repetition (factors: tanks and periods) and additional statistical analysis was performed to assess differences between treatments with one way ANOVA (factor: treatments). Since there was a large number of observations, each

observation being randomly generated in a way that does not depend on the values of the other observations, and that the arithmetic average of the observed values is computed. If this procedure is performed many times, the central limit theorem says that the computed values of the average will be distributed according to a normal distribution.

Fatty acids

Considering the sampled species, a total of 174 samples were analysed thus for each was obtained a fatty acid profile. Samples of each species were grouped by tank and treatment. Multivariate statistical methods have advanced to become particularly useful for interpreting large data sets such as the ones presented in this work, where typically arrays of more than 30 fatty acids are determined simultaneously from one or more samples (Dalsgaard *et al.*, 2003). For this reason, PRIMER software (Plymouth Routines in Multivariate Ecological Research, version 7 with PERMANOVA+ add-on) was used to interpret the data sets. MDS (multidimensional scaling) plots were created to visualize each set of data. The purpose of MDS is to display the data points in a multi-dimensional space, by configuring data in a similarity/dissimilarity matrix. Samples are placed in a two-dimensional plot in such a way that the distance between samples reflects the rank order of the matching similarity/dissimilarity taken from a similarity matrix. Therefore, MDS provided a visual representation of the similarities of fatty acid profiles among samples of each species in each treatment. MDS was also used to visualize similarities of fatty acid profiles among selected groups of species. Pearson's correlation vectors, corresponding to individual fatty acids, are presented in MDS. Vectors are directionally oriented in the plot, indicating an association between the specific fatty acid and the sample near the vector. No overall transformation was applied to the data prior to analysis. Resemblance matrix was obtained with the Euclidean distance measure.

For each individual species, overall differences between treatments were analysed with ANOSIM one factor (ANOSIM is a multivariate analysis that uses a resemblance matrix, and carries out an approximate analogue of ANOVA, to test statistically whether there is a significant difference between two or more groups of sampling units). Pairwise

comparisons were also done with ANOSIM one factor (pairwise treatments). After identified which pair of treatments were different, SIMPER analysis was done to determine which fatty acids contributed more to the dissimilarities. In particular, the factor Sq. Dist/SD (Square Distance over standard deviation) was taken in consideration since it is a measure of how consistently each variable (fatty acids) contributed to the characterization of differences between groups, with higher values indicating more dissimilarity. This way, all fatty acids with values of Sq. Dist/SD over 0.90 were selected as being the ones who contributed more to dissimilarities. In addition, these fatty acids were tested in R software to look if their differences between paired treatments were significant. For this, Mann-Whitney test (non-parametric) or T-Student test (parametric) were used depending on each fatty acid homogeneity and homostacity, checked with Shapiro and Barlett tests.

3 Results

3.1 Environmental Parameters

Data from environmental parameters is presented for each tank in terms of mean values (\pm s.d.) throughout the 8 months of experiment in both periods of the day (morning and afternoons). Tanks 1 and 6 represent treatment with fish, oysters and algae (F+O+A), while tanks 2 and 4 represent treatment with fish and oysters (F+O) and tanks 3 and 5 represent treatment with fish and algae (F+A). Data is presented graphically along with tables with mean and s.d. values (a) and results from the statistical analysis (b).

3.1.1 Temperature

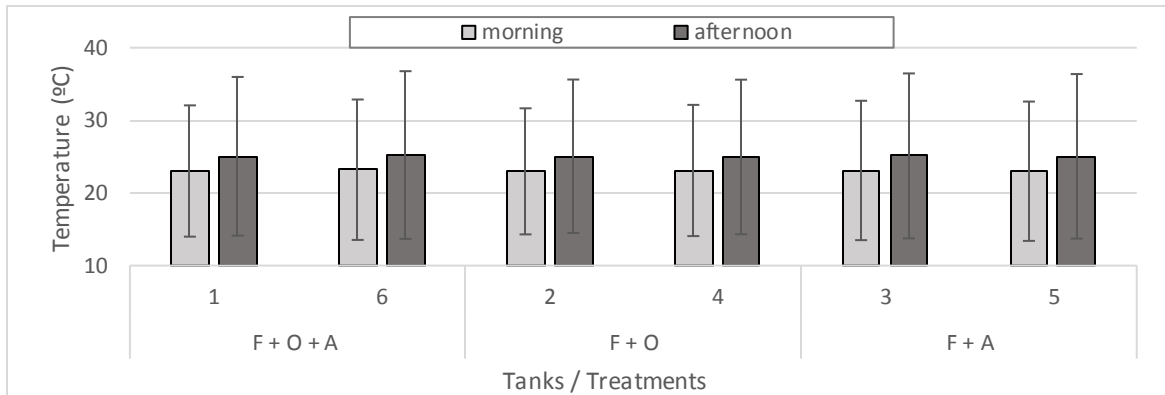


Figure 12. Temperature mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.

Table 5: a) Temperature mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). **b)** P -values obtained in statistical analysis (“bold”: $p < 0.05$).

a)								b)							
	(morning)				(afternoon)				(double-way ANOVA)						
	n	mean	\pm	s.d.	n	mean	\pm	s.d.	T1 / T6	T2 / T4	T3 / T5				
Tank 1	126	23.0	\pm	9.0	126	25.0	\pm	10.9	7.95E-12	1.54E-11	3.05E-11				
Tank 6	126	23.1	\pm	9.7	126	25.1	\pm	11.5				Periods	0.544	0.971	0.783
Tank 2	120	23.0	\pm	8.7	120	25.1	\pm	10.6				Tanks	0.984	0.702	0.943
Tank 4	120	23.1	\pm	9.0	120	25.0	\pm	10.6	(one-way ANOVA)						
Tank 3	118	23.1	\pm	9.6	118	25.1	\pm	11.4	Treatments						
Tank 5	118	23.0	\pm	9.6	118	25.0	\pm	11.3	0.883						

No statistically significant interaction was found between paired tanks and periods ($p > 0.05$, ANOVA test) (Table 5.b). Significant differences were found for temperature between periods of the day ($p < 0.05$, ANOVA test) (Table 5.b) with afternoon temperatures being always higher compared to the morning (Table 5.a). No significant differences were found for temperature between paired tanks, and between treatments ($p > 0.05$, ANOVA test).

3.1.2 Salinity

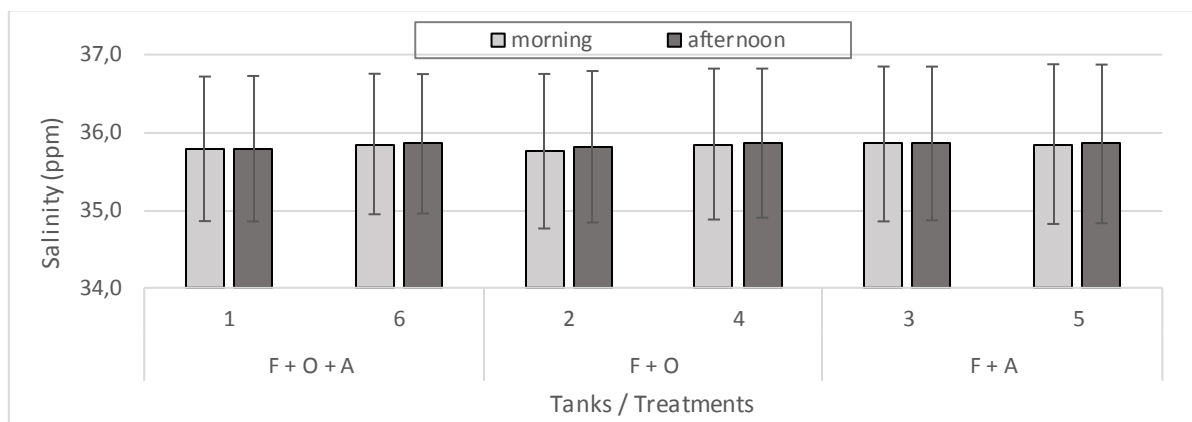


Figure 13. Salinity mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.

Table 6: a) Salinity mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). **b)** P-values obtained in statistical analysis (“bold”: $p < 0.05$).

	a)				b)					
	morning		afternoon		(double-way ANOVA)					
	count	mean	\pm s.d.	count	mean	\pm s.d.	T1 / T6	T2 / T4	T3 / T5	
Tank 1	126	35.8	\pm 1.0	126	35.8	\pm 1.0				
Tank 6	126	35.9	\pm 1.0	126	35.9	\pm 1.0	Periods	0.994	0.913	0.957
Tank 2	120	35.8	\pm 1.0	120	35.8	\pm 1.0	Tanks	0.480	0.622	0.964
Tank 4	120	35.8	\pm 1.0	120	35.9	\pm 0.9	Interaction	0.984	0.979	0.979
Tank 3	118	35.9	\pm 0.9	118	35.9	\pm 0.9	(one-way ANOVA)			
Tank 5	118	35.8	\pm 0.8	118	35.9	\pm 0.8	Treatments	0.968		

No statistically significant interaction was found between paired tanks and periods ($p > 0.05$, ANOVA test) (Table 6.b) No significant differences were found between periods, between paired tanks, or between treatments ($p > 0.05$, ANOVA test) (Table 6.b).

3.1.3 Dissolved oxygen

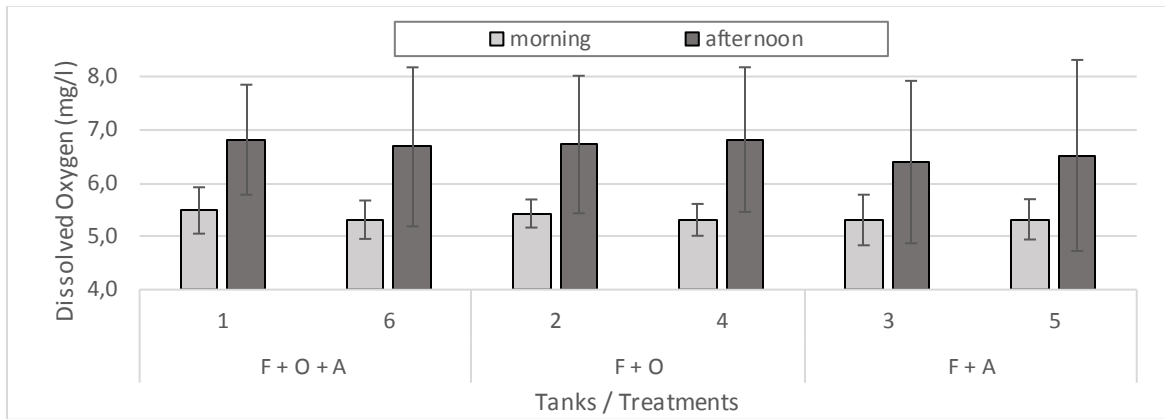


Figure 14. Dissolved oxygen mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.

Table 7: a) Dissolved oxygen mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). **b)** P -values obtained in statistical analysis (“bold”: $p < 0.05$)

	a)				b)			
	(morning)		(afternoon)		<i>(double-way ANOVA)</i>			
	n	mean \pm s.d.	n	mean \pm s.d.	T1 / T6	T2 / T4	T3 / T5	
Tank 1	126	5.5 \pm 0.4	126	6.8 \pm 1.0				
Tank 6	126	5.3 \pm 0.4	126	6.7 \pm 1.5	Periods	1.09E-48	2.65E-51	9.13E-30
Tank 2	120	5.4 \pm 0.3	120	6.7 \pm 1.3	Tanks	0.057	0.922	0.466
Tank 4	120	5.3 \pm 0.3	120	6.8 \pm 1.4	Interaction	0.796	0.176	0.551
Tank 3	118	5.3 \pm 0.5	118	6.4 \pm 1.5	<i>(one-way ANOVA)</i>			
Tank 5	118	5.3 \pm 0.4	118	6.5 \pm 1.8	Treatments	0.035		

No statistically significant interaction was found between paired tanks and periods of the day ($p > 0.05$, ANOVA test) (Table 7.b). Significant differences were found between periods for all paired tanks ($p < 0.05$, ANOVA test) (Table 7.b) with concentrations in the afternoon being always higher compared to the mornings. No significant differences were found between paired tanks ($p > 0.05$, ANOVA test), however significant differences were found between treatments ($p < 0.05$, ANOVA test). Average values in the morning were 5.3 ± 0.4 ppm, and were 6.4 ± 1.5 ppm in the afternoon.

3.1.4 pH

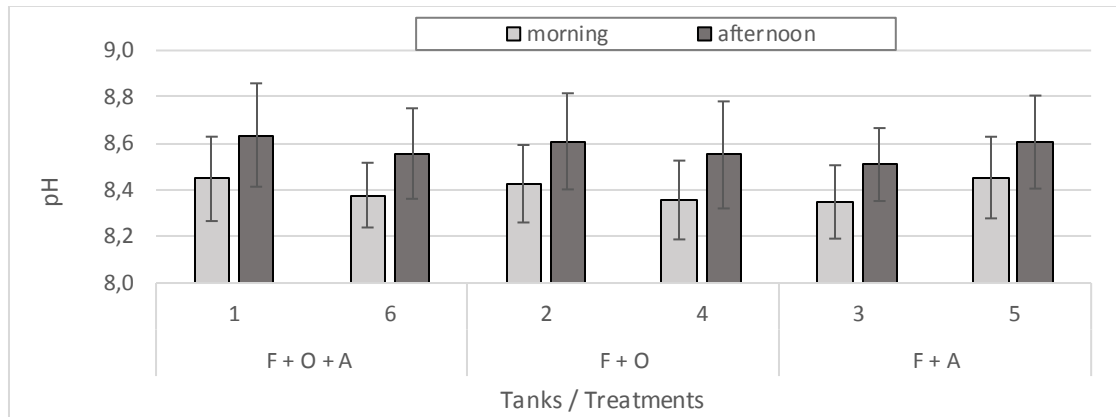


Figure 15. pH mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.

Table 8: a) pH mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). b) P-values obtained in statistical analysis (“bold”: $p < 0.05$)

	a)				b)					
	morning		afternoon		(double-way ANOVA)					
	count	mean	\pm s.d.	count	mean	\pm s.d.	T1 / T6	T2 / T4	T3 / T5	
Tank 1	126	8.5	\pm 0.02	126	8.6	\pm 0.03				
Tank 6	126	8.4	\pm 0.03	126	8.6	\pm 0.04	Periods	5.09E-28	4.20E-24	3.21E-18
Tank 2	120	8.4	\pm 0.03	120	8.6	\pm 0.04	Tanks	1.54E-06	2.10E-04	1.04E-08
Tank 4	120	8.4	\pm 0.03	120	8.6	\pm 0.05	Interaction	0.754	0.843	0.810
Tank 3	118	8.3	\pm 0.03	118	8.5	\pm 0.05	(one-way ANOVA)			
Tank 5	118	8.5	\pm 0.02	118	8.6	\pm 0.04	Treatments	0.103		

No statistically significant interaction was found between paired tanks and periods ($p > 0.05$, ANOVA test) (Table 8.b) Significant differences were found between periods of the day ($p < 0.05$, ANOVA test) (Table 8.b) indeed pH values were always higher in the afternoons compared to mornings (Table 8.a). Also, significant differences were found between paired tanks ($p < 0.05$, ANOVA test) (Table 8.b).

3.1.5 Turbidity

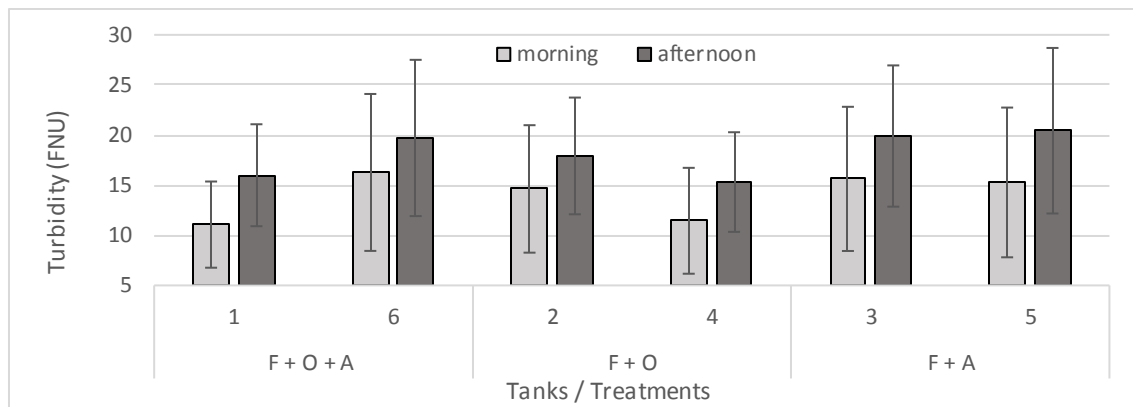


Figure 16. Turbidity mean values (\pm s.d.) are presented for each tank in both periods of the day (morning/afternoon) throughout the 8 months of experiment.

Table 9: Turbidity mean values (\pm s.d.) of each tank in both periods of the day and respective total counts (n). Table on the right – P-values obtained in statistical analysis (“bold”: $p < 0.05$)

	a)				b)					
	morning		afternoon		(double-way ANOVA)					
	count	mean	\pm s.d.	count	mean	\pm s.d.	T1 / T6	T2 / T4	T3 / T5	
Tank 1	126	11.1	\pm 20.1	126	15.9	\pm 26.1				
Tank 6	126	16.0	\pm 62.7	126	19.5	\pm 61.3	Periods	2.86E-12	1.10E-11	3.22E-11
Tank 2	120	14.6	\pm 41.0	120	17.9	\pm 34.2	Tanks	1.07E-12	3.20E-08	0.898
Tank 4	120	11.4	\pm 28.1	120	15.3	\pm 24.8	Interaction	0.302	0.529	0.524
Tank 3	118	15.6	\pm 52.2	118	19.9	\pm 50.1	(one-way ANOVA)			
Tank 5	118	15.3	\pm 56.3	118	20.4	\pm 68.8	Treatments	8,31E-08		

No statistically significant interaction was found between paired tanks and periods ($p > 0.05$, ANOVA test) (Table 9.b) Significant differences were found between periods of the day ($p < 0.05$, ANOVA test) (Table 9.b), indeed turbidity values were always higher in the afternoons compared to mornings (Table 9.a). Also, significant differences were found between paired tanks ($p < 0.05$, ANOVA test) and between treatments (Table 9.b)

3.2 Fatty acids profiles

3.2.1 Feed

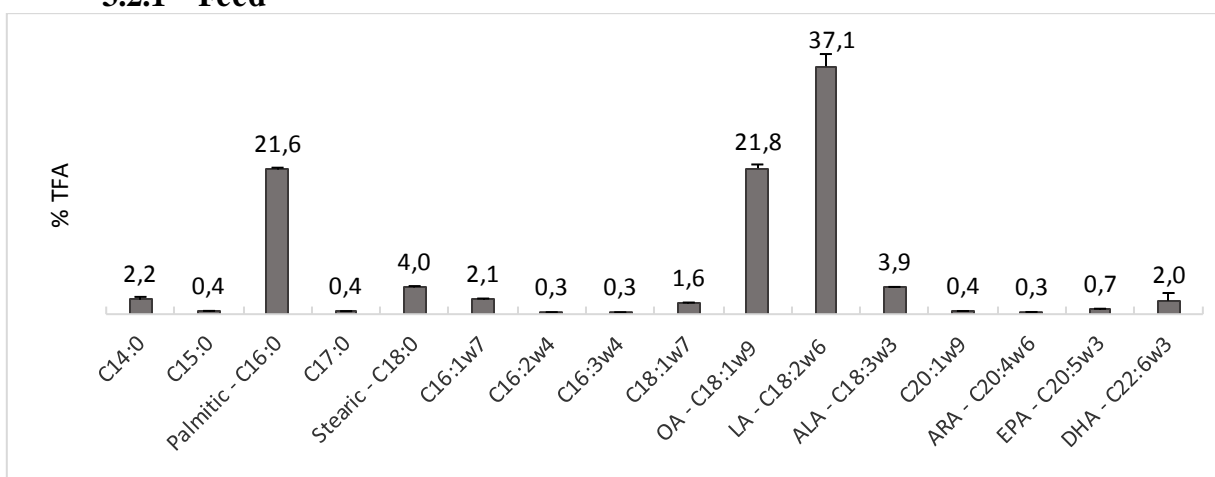


Figure 17. Fatty acid profile of the feed. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the analysis of three replicates. Fatty acids with percentages lower than 0.3% were considered as trace and are not presented. (Legend: OA = Oleic acid, LA = Linoleic acid, ALA = alpha-Linolenic acid, ARA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid).

Sixteen fatty acids were identified in the commercial feed analysed. The FA profile was dominated by one saturated fatty acid, C16:0, one monounsaturated fatty acid, C18:1 ω 9 (Oleic acid), and one polyunsaturated fatty acid, C18:2 ω 6 (Linoleic Acid). These three fatty acids accounted together for more than 80% of the total of fatty acids (ANNEX 4). Other less abundant fatty acids (with abundances varying from 1-5%) include C14:0, C18:0, C16:1 ω 7, C18:1 ω 7, C18:3 ω 3 (ALA) and C22:6 ω 3 (DHA). Overall, PUFA were predominant in the feed composition, with PUFA ω 6 having 37.4%, while PUFA ω 3 only account for 6.8%, thus showing a ω 3/ ω 6 ratio of 0.18. The SFA and the MUFA show similar abundances of 29.1% and 27% respectively (ANNEX 5)

3.2.2 Sediment

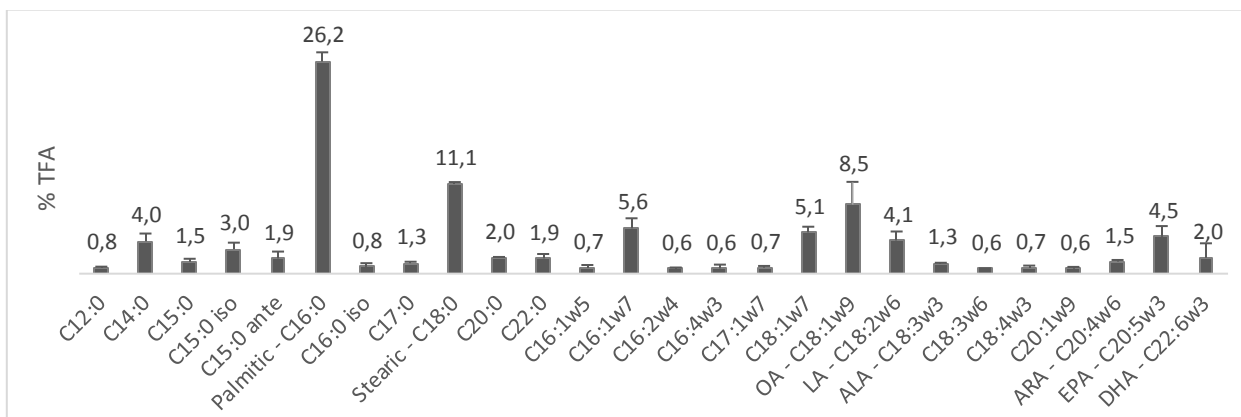


Figure 18. Fatty acid profile of the sediment. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the analysis of three replicates. Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. (Legend: OA = Oleic acid, LA = Linoleic acid, ALA = alpha-Linolenic acid, ARA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid).

Twenty-six fatty acids were identified in the sediment composition. The profile is dominated by three saturated fatty acids, C14:0, C16:0 and C18:0, three monounsaturated, C16:1 ω 7, C18:1 ω 7 and C18:1 ω 9 and two polyunsaturated fatty acids, C18:2 ω 6 and C20:5 ω 3 (EPA) (ANNEX 4). In addition, fatty acids of bacterial origin such as C15:0, C15:0iso, C15:0 ante, C16:0iso and C17:0 were also found. Together, all mentioned fatty acids accounted for around 80% of the total of fatty acids in the sediment.

Overall, SFA dominate the profile with 61.6%, mainly due to high levels of C16:0 and C18:0, along with and a great variety of other minor fatty acids described before. MUFA were the second more represented group, with 22.2%, while PUFA ω 6 and PUFA ω 3 represent 6.6% and 9.3% respectively, thus a ω 3/ ω 6 ratio of 1.41 (ANNEX 5).

3.2.3 Polychaeta

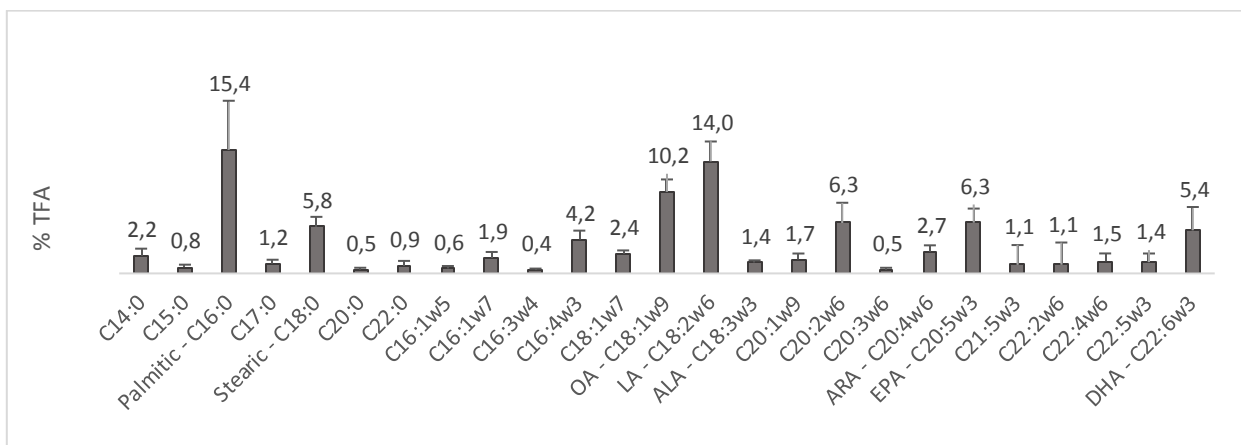


Figure 19. Fatty acid profile of polychaeta. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of twelve analysed samples. Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. (Legend: OA = Oleic acid, LA = Linoleic acid, ALA = alpha-Linolenic acid, ARA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid).

Twenty-five fatty acids were identified in polychaetas composition. The profile is dominated by two SFA, C16:0 and C18:0, one MUFA, C18:1 ω 9 (OA), and five PUFA, C16:4 ω 3, C18:2 ω 6 (LA), C20:2 ω 6, C20:5 ω 3 (EPA) and C22:6 ω 3 (DHA). Together these fatty acids account for 80% of the total fatty acids (ANNEX 4).

Overall, PUFAs are predominant with 45.9%, especially with high levels of PUFA ω 6 (28.5%), due to considerable abundances of the LA, and other minor contributors such as C20:2 ω 6 and C20:4 ω 6 (ARA). PUFA ω 3 account for 17.4%, but since levels of PUFA ω 6 are too high, the ω 3/ ω 6 ratio is low (0.61). The SFA represent 34.5% while MUFA only represent 19.7% (ANNEX 5).

3.2.4 Phytoplankton

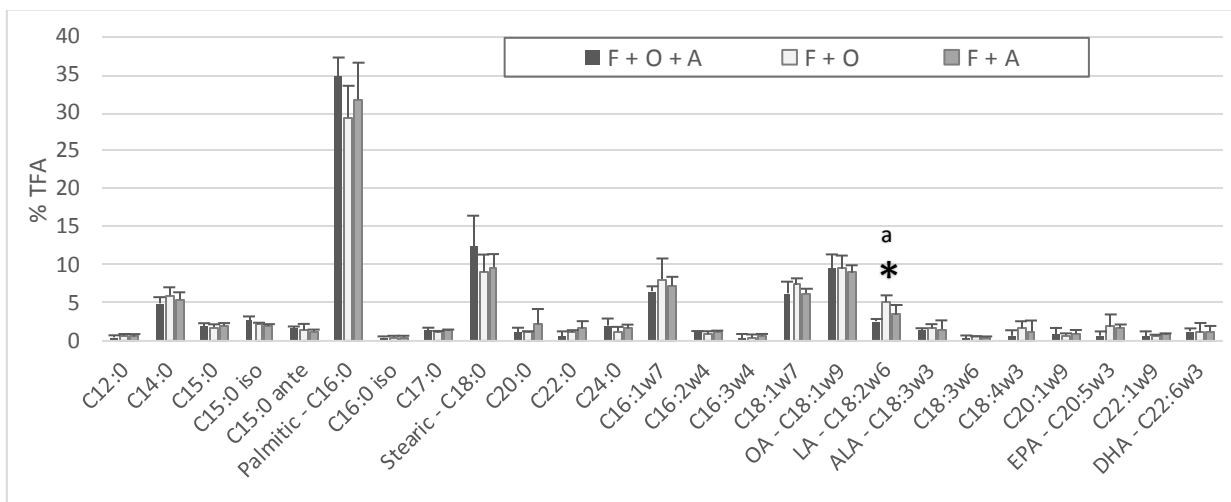


Figure 20. Fatty acid profile of the Phytoplankton in the three treatments. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of 6 samples from each treatment. Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. (Legend: OA = Oleic acid, LA = Linoleic acid, ALA = alpha-Linolenic acid, ARA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid). Significant differences between paired treatments are marked as follows: * $p < 0.05$, T-Student test; a: FOA \neq FO.

Twenty-five fatty acids were identified in phytoplankton samples. FA profiles were dominated by three saturated fatty acids, C14:0, C16:0 and C18:0, three monounsaturated fatty acids, C16:1 ω 7, C18:1 ω 7 and C18:1 ω 9, and one polyunsaturated fatty acid, C18:2 ω 6. These fatty acids accounted together for more than 80% of the total of fatty acids (ANNEX 4). Other noteworthy fatty acids were those of bacterial origin such as C15:0, C15:0iso, C15:0ante, C16:0iso and C17:0.

Overall differences between treatments profiles were not significant ($p = 0.068$, ANOSIM test), but showing a significance value close to 0.05. In fact, significant differences between paired treatments FOA - FO ($p < 0.05$, ANOSIM pairwise test) while no significant differences between FOA-FA or FO-FA ($p > 0.05$, ANOSIM pairwise test)

SIMPER analysis confirms the difference found, indeed the average squared distances between treatments FOA - FO were the highest when compared to other paired treatments (ANNEX 2).

Looking at the differences found between treatments **FOA-FO**, SIMPER analysis showed that the fatty acids which contributed more to the dissimilarity (all those with Sq.Dist/SD > 0.90), were by order of importance, C18:2w6, C16:1w7, C18:1w7, EPA and C16:0. (ANNEX 2). Significant differences in these specific fatty acids, between treatments FOA-FO, were only found for C18:2w6 ($p < 0.05$, Mann Whitney test) with a 2.5% variation.

3.2.5 Suspended particulate organic matter

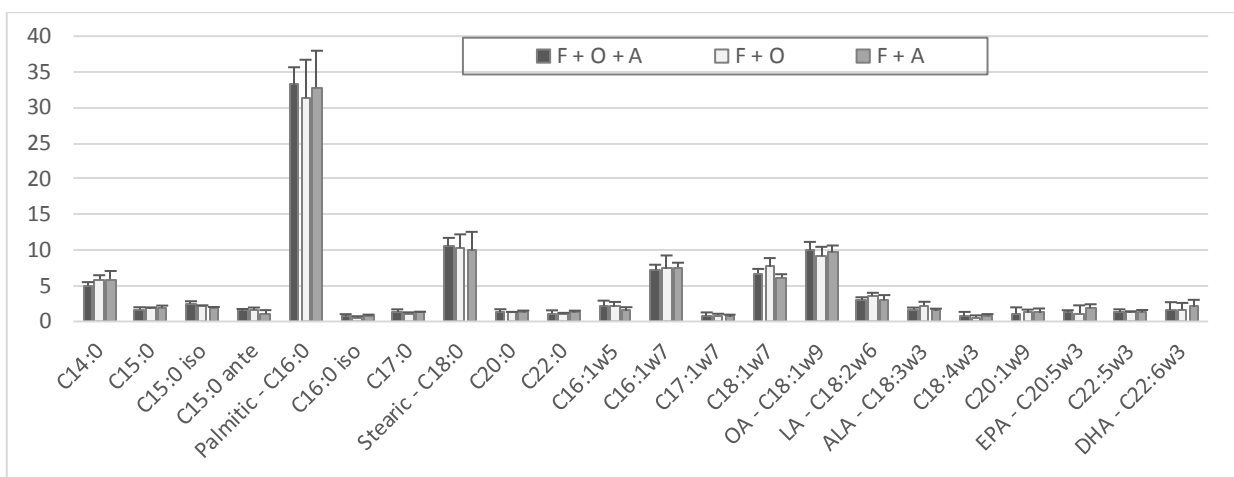


Figure 21. Fatty acid profile of suspended particulate organic matter in the three treatments. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of 6 samples from each treatment. Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. (Legend: OA = Oleic acid, LA = Linoleic acid, ALA = alpha-Linolenic acid, ARA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid).

Twenty-two fatty acids were identified in suspended matter samples. FA profiles were dominated by three saturated fatty acids, C14:0, C16:0 and C18:0, by three monounsaturated fatty acids, C16:1w7, C18:1w7 and C18:1w9 (OA), and by one polyunsaturated fatty acid, C18:2w6 (LA). These fatty acids accounted together for more than 80% of the total of fatty acids (ANNEX 4). Other noteworthy fatty acids were those of bacterial origin such as C15:0, C15:0 iso, C15:0 ante and C17:0. Overall differences between treatments profiles were not significant ($p > 0.05$, ANOSIM test). Additionally, no significant differences were found between paired treatments: FOA-FA, FOA-FO or FO-FA ($p > 0.05$, ANOSIM pairwise test) (ANNEX 2).

3.2.6 Zooplankton

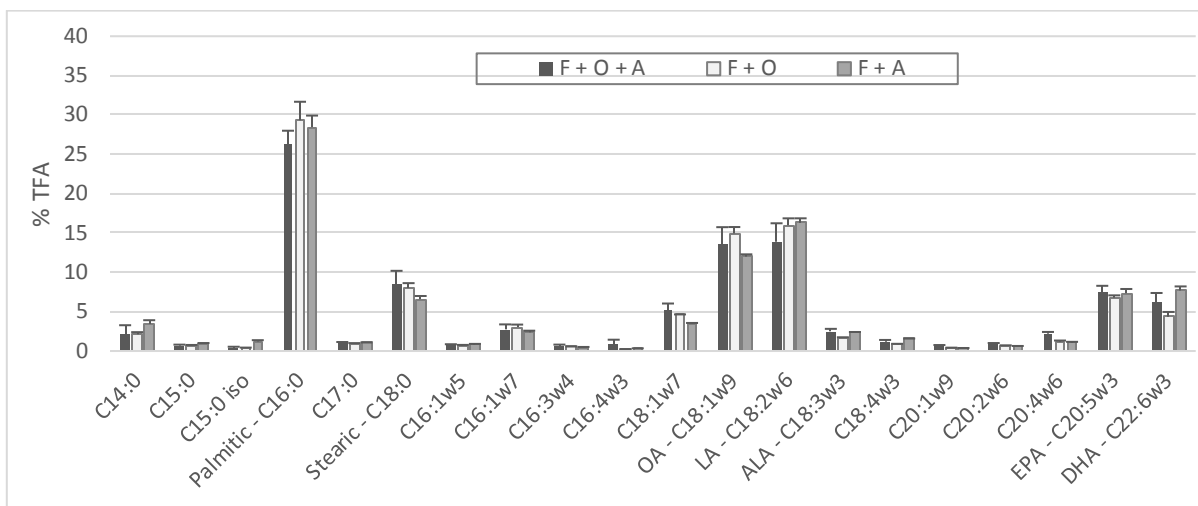


Figure 22. Fatty acid profile of the zooplankton in the three treatments. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of 6 samples from each treatment. Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. (Legend: OA = Oleic acid, LA = Linoleic acid, ALA = alpha-Linolenic acid, ARA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid).

Twenty fatty acids were identified in zooplankton samples. FA profiles were dominated by two saturated fatty acids, C16:0 and C18:0, by one monounsaturated, C18:1 ω 9 (OA), and by three polyunsaturated fatty acid, C18:2 ω 6 (LA), 20:5 ω 3 (EPA) and 22:6 ω 3 (DHA). These fatty acids accounted together for more than 80% of the total of fatty acids (ANNEX 4).

Overall differences between treatments profiles were not significant ($p > 0.05$, ANOSIM test). Additionally, no significant differences were found between paired treatments: FOA-FA, FOA-FO or FO-FA ($p > 0.05$, ANOSIM pairwise test) (ANNEX 2).

3.2.7 *Rhizoclonium riparium*

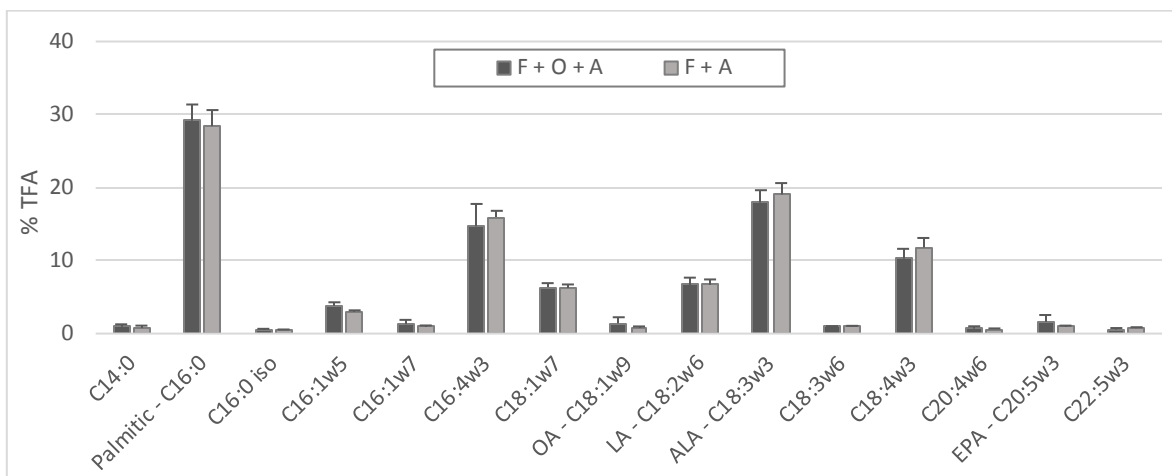


Figure 23. Fatty acid profile of the *Rhizoclonium riparium* in the two treatments. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of 6 samples from each treatment. Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. (Legend: OA = Oleic acid, LA = Linoleic acid, ALA = alpha-Linolenic acid, ARA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid).

Fifteen fatty acids were identified in *Rhizoclonium riparium*. Profiles were dominated by one SFA, C16:0, two MUFA, C16:1 ω 5 and C18:1 ω 7, and four PUFA, C16:4 ω 3, C18:2 ω 6 (LA), C18:3 ω 3 (ALA) and C18:4 ω 3. Together they accounted for more than 80% of the total fatty acids (ANNEX 4)

No significant differences were found between the two treatments FOA-FA ($p > 0.05$, ANOSIM test) (ANNEX 2).

3.2.8 *Ulva intestinalis*

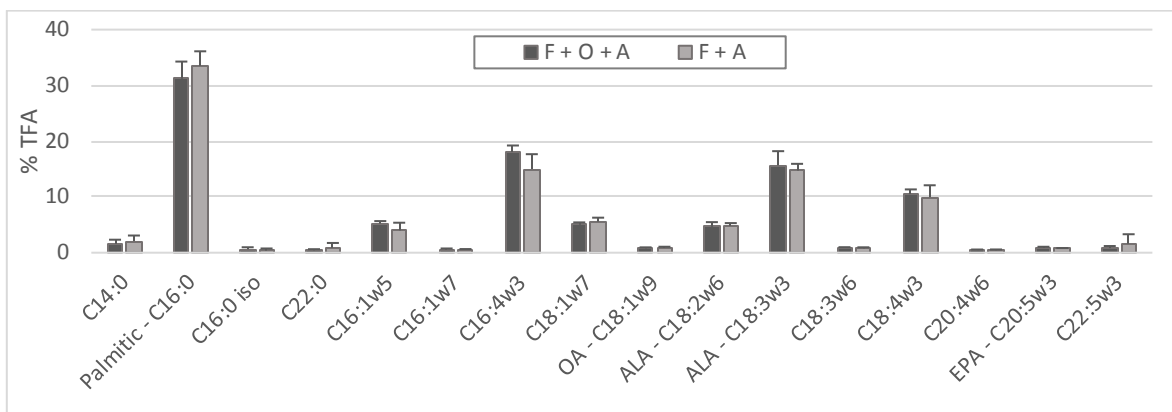


Figure 24. Fatty acid profile of the *Ulva intestinalis* in the two treatments. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of 6 samples from each treatment. Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. (Legend: OA = Oleic acid, LA = Linoleic acid, ALA = alpha-Linolenic acid, ARA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid).

Sixteen fatty acids were identified in *Ulva intestinalis*. Profiles were dominated by one SFA, C16:0, two MUFA, C16:1 ω 5 and C18:1 ω 7, and four PUFA, C16:4 ω 3, C18:2 ω 6 (LA), C18:3 ω 3 (ALA) and C18:4 ω 3. Together they accounted for more than 80% of the total fatty acids (ANNEX 4)

No significant differences were found between the two treatments FOA-FA ($p > 0.05$, ANOSIM test) (ANNEX 2)

3.2.9 *Ulva flexuosa* (cultured algae)

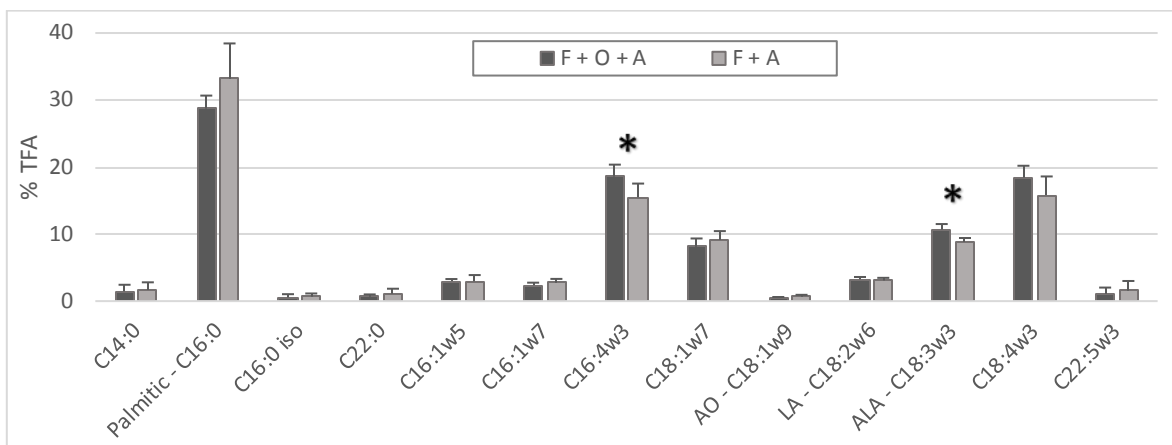


Figure 25. Fatty acid profile of the *Ulva flexuosa* in the two treatments. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of 6 samples from each treatment. Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. (Legend: OA = Oleic acid, LA = Linoleic acid, ALA = alpha-Linolenic acid, ARA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid). Significant differences between treatments are marked as follows: * $p < 0.05$, T-student or Mann Whitney test.

Thirteen fatty acids were identified in *Ulva flexuosa*. Profiles were dominated by one SFA, C16:0, one MUFA, C18:1 ω 7, and three PUFA, C16:4 ω 3, C18:3 ω 3 (ALA) and C18:4 ω 3. Together they accounted for more than 80% of the total fatty acids (ANNEX 4)

Significant differences were found between treatments **FOA-FA** ($p < 0.05$, ANOSIM test). Looking at the differences found between treatments FOA-FA, SIMPER analysis showed that the fatty acids which contributed more to the dissimilarity (all with Sq.Dist/SD > 0.90), were by order of importance, C18:3 ω 3, C16:4 ω 3 and C18:4 ω 3 (ANNEX 2). Significant differences in these specific fatty acids were only identified in C16:4 ω 3 and C18:3 ω 3 ($p < 0.05$, T-student test). Their levels were both higher in treatment FOA, with more 3.1% and 1.8%, respectively.

3.2.10 *Crassostrea gigas*

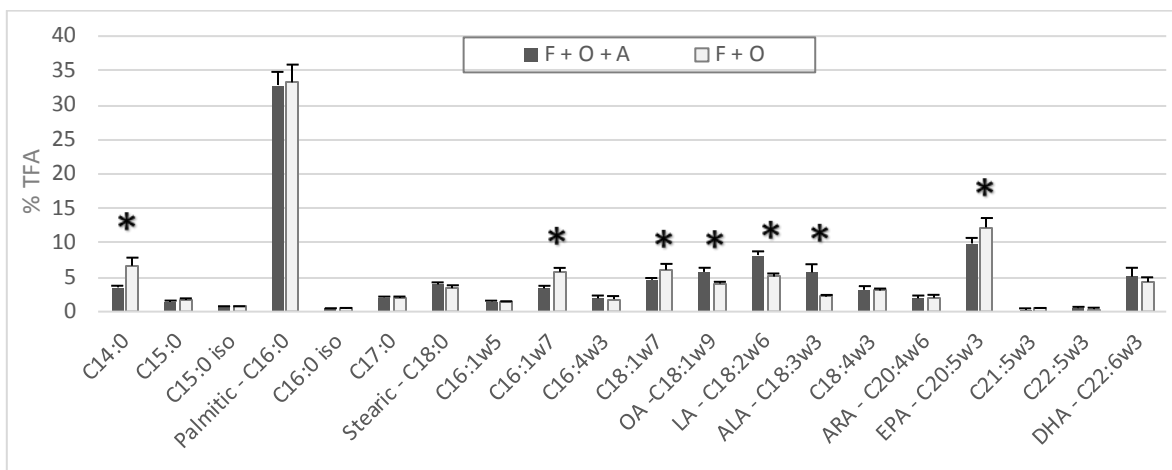


Figure 26. Fatty acid profile of *Crassostrea gigas* in the two treatments. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of 6 samples for each treatment. Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. Significant differences between treatments are marked as follows: * $p < 0.05$, T-student or Mann Whitney test

Twenty fatty acids were identified in *Crassostrea gigas*. Profiles were dominated by one saturated fatty acid, C16:0, three monounsaturated fatty acids, C16:1 ω 7, C18:1 ω 7 and C18:1 ω 9 (OA), and four polyunsaturated fatty acids, C18:2 ω 6 (LA), C18:3 ω 3 (ALA), C20:5 ω 3 (EPA) and C22:6 ω 3 (DHA). Together they account for more than 80% of the total of fatty acids (ANNEX 4).

Significant differences were found between treatments **FOA-FO** ($p < 0.05$, ANOSIM test). Looking at the differences found between treatments FOA-FO, SIMPER analysis showed that the fatty acids which contributed more to the dissimilarity (all with Sq.Dist/SD > 0.90), were by order of importance, C18:2 ω 6, C16:1 ω 7, C14:0, C18:3 ω 3, C18:1 ω 9, EPA and C18:1 ω 7. Indeed, significant differences were identified in all these specific fatty acids between treatments ($p < 0.05$, T-student or Mann Whitney test). In terms of percentage variation, in treatment FOA there were higher levels of C18:1 ω 9 (+1.7%), C18:2 ω 6 (+3.1%) and C18:3 ω 3 (+3.5%), while in treatment FO, there were slightly higher levels of C14:0 (+3.1%), C16:1 ω 7 (+2.4%), C18:1 ω 7 (+1.4%) and EPA (+2.3%).

3.2.11 Meagre

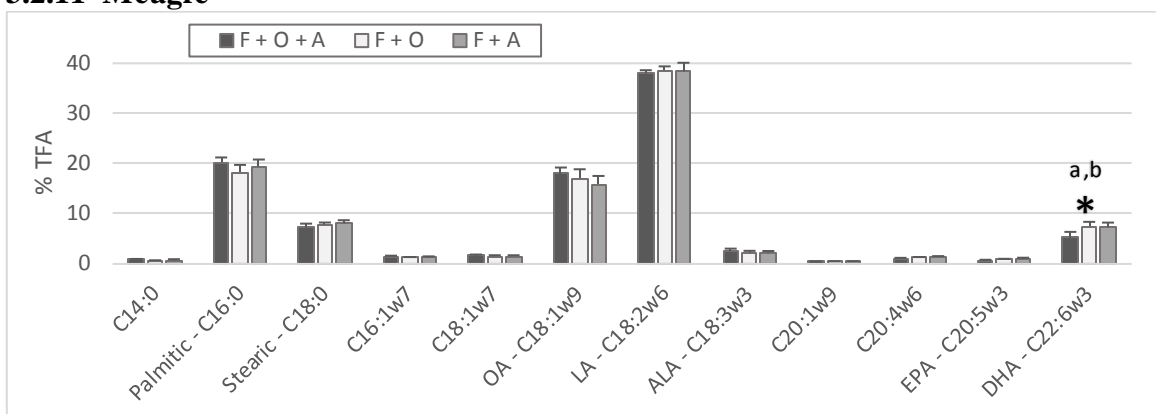


Figure 27. Fatty acid profile of the *Meagre* in the three treatments. Values are presented in %TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of each treatment samples ($n=6$). Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented. Significant differences between treatments are marked as follows: * $p<0.05$ (a: FOA \neq FO, b: FOA \neq FA, c: FO \neq FA, T-Student test)

Twelve fatty acids were identified in meagre. Profiles were dominated by two saturated fatty acids, C16:0 and C18:0, one monounsaturated, C18:1 ω 9 (OA), and one polyunsaturated, 18:2 ω 6 (LA). Together they account for more than 80% of the total of fatty acids. Other relevant fatty acid was the polyunsaturated C22:6 ω 3 (DHA) (ANNEX 4).

Overall differences between treatments profiles were significant ($p<0.05$, ANOSIM test). Additionally, significant differences were found between paired treatments **FOA-FO and FOA-FA** ($p<0.05$, ANOSIM pairwise test) while no significant differences between FO-FA ($p>0.05$, ANOSIM pairwise test). SIMPER analysis confirms the differences found, indeed the average squared distances between treatments FOA-FO and FOA-FA were similar and higher when compared to FO-FA (ANNEX 2)

Looking at the differences found between treatments FOA-FO and between treatments FOA-FA, SIMPER analysis showed that the fatty acids which contributed more to the dissimilarity (all those with Sq.Dist/SD > 0.90), were by order of importance, DHA and C18:2 ω 6. However, significant differences for these specific fatty acids were only found in DHA, between paired treatments FOA-FO and between FOA-FA ($p < 0.05$, T-student test), showing that DHA levels were significantly lower (-2.5%) in treatment FOA when compared to other treatments (ANNEX 2).

3.2.12 White seabream

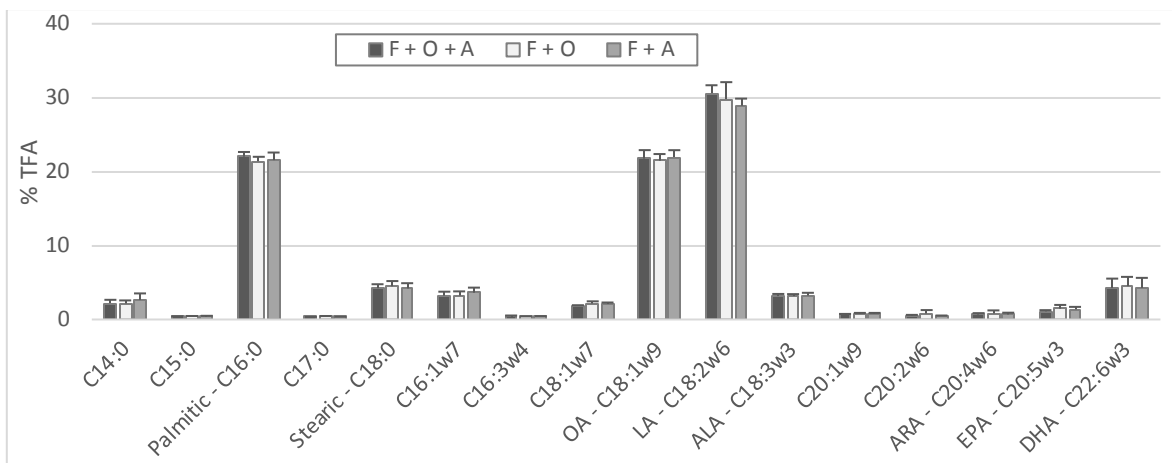


Figure 28. Fatty acid profile of white seabream in the three treatments. Values are presented in %TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of each treatment samples ($n=6$). Fatty acids with percentages lower than 0.4% were considered as trace, and are not presented.

Sixteen fatty acids were identified in white seabream. Profiles were dominated by two saturated fatty acid, C16:0 and C18:0, one monounsaturated, C18:1 ω 9 (OA), and one polyunsaturated, 18:2 ω 6 (LA). Together they account for more than 80% of the total of fatty acids. Other relevant fatty acid was the polyunsaturated C22:6 ω 3 (DHA) (ANNEX 4)

Overall differences between treatments profiles were not significant ($p>0.05$, ANOSIM test). Additionally, no significant differences were found between paired treatments: FOA-FA, FOA-FO or FO-FA ($p>0.05$, ANOSIM pairwise test) (ANNEX 2).

3.2.13 Mullet

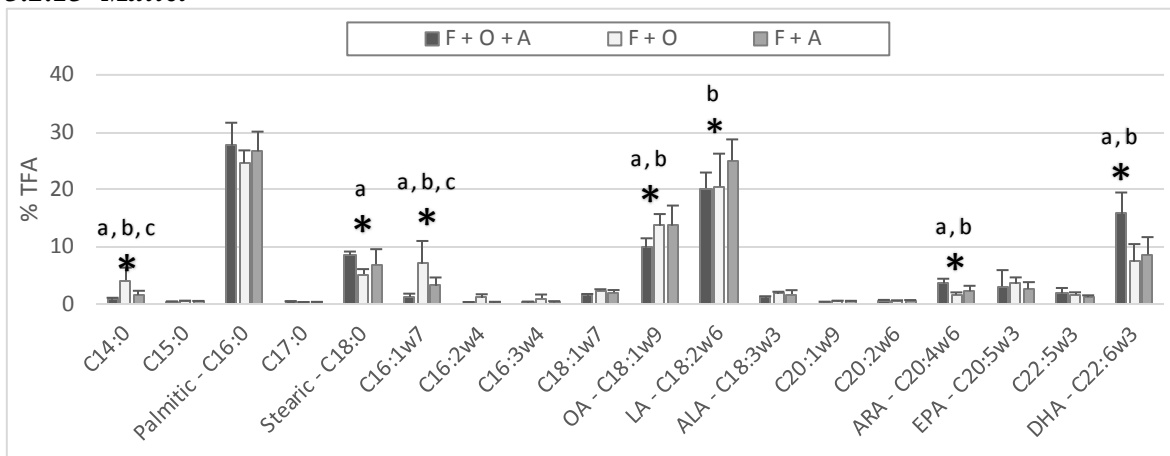


Figure 29. Fatty acid profile of Mullet in the three treatments. Values are presented in %TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of each treatment samples ($n=6$). Fatty acids with percentages lower than 0.4% were considered a trace, and are not presented. Significant differences between treatments are marked as follows: * $p < 0.05$ (a: FOA \neq FO, b: FOA \neq FA, c: FO \neq FA; T-student or Mann Whitney test)

Eighteen fatty acids were identified in mullet. Profiles were dominated by two saturated fatty acids, C16:0 and C18:0, two monounsaturated, C16:1 ω 7 and C18:1 ω 9 (OA), and two polyunsaturated, C18:2 ω 6 (LA) and C22:6 ω 3 (DHA). Together they account for more than 80% of the total of fatty acids (ANNEX 4).

Overall differences between treatments profiles were significant ($p < 0.05$, ANOSIM test). Significant differences were found between paired treatments **FOA-FO** and **FOA-FA** ($p < 0.05$, ANOSIM pairwise test) while no significant differences were found between FO-FA ($p > 0.05$, ANOSIM pairwise test). SIMPER analysis confirms the differences found, indeed the average squared distances between treatments FOA-FO and FOA-FA were higher when compared to FO-FA (ANNEX 2). Looking at the differences found between treatments (FOA-FO and FOA-FA), SIMPER analysis showed that the fatty acids which contributed more to the dissimilarity (all with Sq.Dist/SD > 0.90), were C14:0, C18:0, C16:1w7, C18:1w9, C18:2w6, ARA and DHA. Statistical analysis between paired treatments in these specific fatty acids, show that treatment FO had significantly higher C14:0 and C16:1w7 compared to other treatments ($p < 0.05$, Mann Whitney test). While treatment FOA had significantly higher ARA and DHA, and lower C18:1w9 than other treatments ($p < 0.05$, T-student test) (ANNEX 2).

3.2.14 Comparison between all species

In Figure 30 is presented an MDS plot with all the samples collected in all tanks.

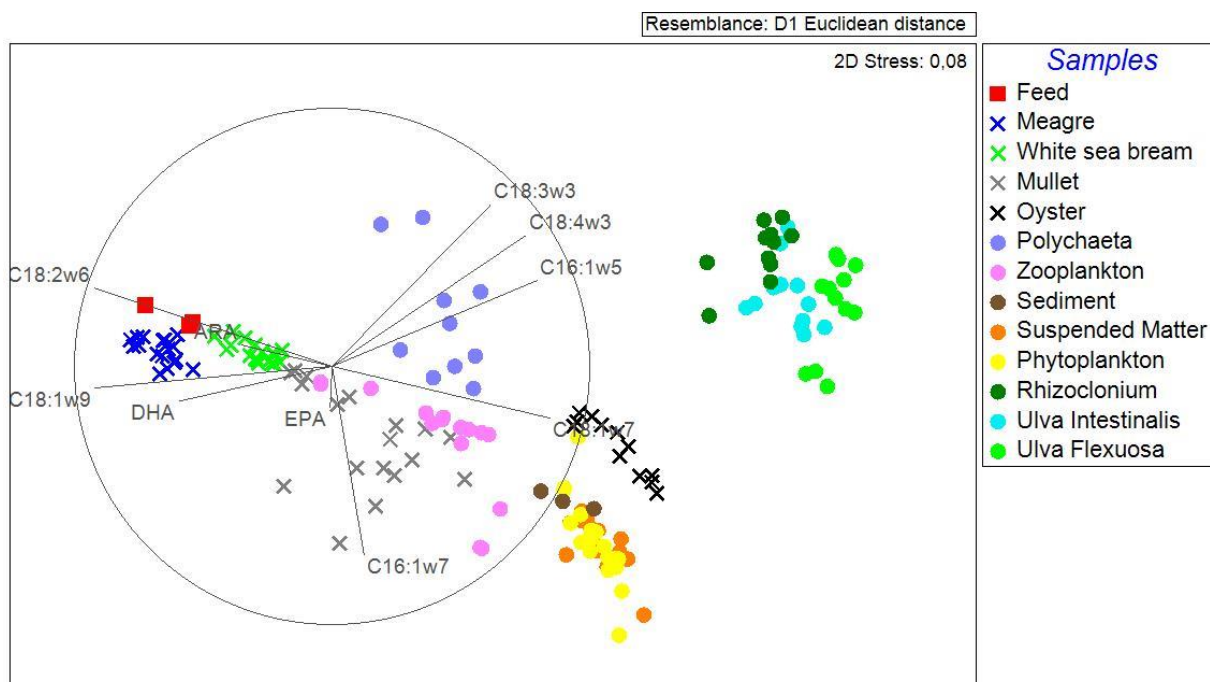


Figure 30. MDS plot of samples of all organisms sampled in the final period. $n=3$ for Feed and sediment, $n=18$ for Meagre, White sea bream, Mullet, Zooplankton and Phytoplankton, $n=12$ for oysters, Polychaeta, Rhizoclonium, Ulva intestinalis and Ulva Flexuosa

In figure 30 is possible to see the strength of the relation between samples and fatty acids. More precisely, C18:2 ω 6, C18:1 ω 9, ARA and DHA vectors position on the left indicate their strong relation especially with samples of feed, meagre and white sea bream. Pointing downwards, EPA and C16:1 ω 7 vectors indicate these fatty acids strong relation with samples of mullet, zooplankton, oysters, sediment, suspended matter and phytoplankton. Vector of fatty acid C18:1 ω 7 coincides perfectly with the samples of oyster, indicating their strong relation. By last, position of C18:3 ω 3, C18:4 ω 3 and C16:1 ω 5 vectors indicate their strong relation with all the samples of green macroalgae (*Rhizoclonium riparium*, *Ulva intestinalis* and *U. flexuosa*).

4. Discussion

4.1 Environmental Parameters

Temperature

Significant differences were found between periods of the day, with temperatures in the afternoon being always higher compared to those in the morning. The heating of approximately 1-2 °C during the day was mainly due the increase in the atmosphere temperature and to sunlight radiation.

Salinity

No significant differences were found between periods, between tanks and between treatments. Slight overall decreases in salinity in all tanks were found to be directly linked with periods of precipitation, by direct comparison with the data collected from the meteorological station (data not presented).

Dissolved oxygen

Significant differences were found between periods of the day, being always higher in the afternoons compared to mornings (fluctuations ranging from 1 to 2 mg/L). In fact, dissolved oxygen levels increase during the daylight hours when photosynthesis is occurring – resulting in higher concentrations in the afternoon – and decreases at night when respiration continues but photosynthesis does not – resulting in lower values in the morning (FAO, 1984).

Significant differences were found between treatments, with higher values detected in the treatments with oysters (FOA and FO) in comparison with the treatment without oysters (FA). The solubility of oxygen in fish pond's water is mostly influenced by water temperature (FAO, 1984), however water temperature was not different between treatments, thus this factor could not explain the variations observed. On the other hand, abiotic variables such as light, temperature and nutrients availability are the most important aspects regulating productivity and growth in phytoplankton (Geider *et al.*, 1998). Moreover, while light and temperature did not differ between treatments, the presence of filter feeding organisms such as oysters in treatments FOA and FO, might have boosted the remineralization process, thus

represent an additional output of dissolved inorganic nutrients. This way, the higher nutrient availability in treatments with oysters (FOA and FO) might have resulted in higher primary production in these treatments, explaining their higher dissolved oxygen values when compared with the treatment without oysters (FA).

pH

Significant differences were found between periods of the day, with values in the afternoon being always higher than those in the morning. In fact, with the increase of the photosynthetic rate during the day, more CO₂ is consumed thus less will remain in the water, meaning a higher pH value (FAO, 1984).

Turbidity

Significant differences found between periods of the day, with higher turbidity values detected in the afternoon. This could be explained by the increase of phytoplankton concentration during the day, knowing that turbidity represents both particulate matter and phytoplankton in the water.

In relation to differences found across treatments, the highest turbidity in treatment FA may be explained by the human interference when placing and harvesting the algae on the floating structures. In fact, walking inside the tanks resulted in particulate matter from the sediment to be resuspended in the water column. Additionally, the lack of oysters in these tanks means that no particulate matter is being filtered. On the contrary, treatment FO presented the lowest turbidity, assumed to be directly linked with the oysters' filtering action. By last, treatment FOA presented a mean value between the other two treatments, where indeed the human interference is balanced by the oysters' filtering action. This goes along with previous studies from researchers in France who studied the suitability of oysters in IMTA systems and found that *Crassostrea gigas* has the ability to feed on the detritus/waste of fish farm effluents thus improving water quality (Barrington *et al.*, 2009).

4.2 Fatty acids

Feed

Soya meal, soya oil and wheat, account together with half of the raw materials used in the feed composition (Table 3). This large quantity of vegetable sources used in the feed is directly related to the high abundances of LA (C18:2 ω 6) and OA (C18:1 ω 9) present in its profile (Figure 1), given that these fatty acids are known to be the major constituents of soybean and wheat (Johannes, 1985; Morrison, 1978; FAO, 1992). In addition, ALA (C18:3 ω 3) is also found in vegetable sources, however in lower amounts, thus being equally reflected on the feed profile. Other noteworthy FAs, which however were only found in lower amounts in the feed (between 1.6-2% of TFA), such as C16:1 ω 7, C18:1 ω 7 and DHA, have their origin in the fish meal and fish oil used as raw materials for the feed composition. Overall PUFA ω 6 are predominant, while PUFA ω 3 have low abundances, resulting in a poor ratio ω 3/ ω 6 of 0.18. Saturated fatty acids are very abundant and common across organisms of different trophic levels (Tocher, 2003), this way they cannot be considered as dietary tracers. On the contrary, all other fatty acids present in the feed are expected to be reflected in consumers with similar proportions found on the feed composition.

Autotrophic organisms

Phytoplankton and suspended particulate organic matter

Both phytoplankton and suspended matter were sampled by filtering water from the tanks (0.7 μ m fiberglass filter was used for phytoplankton and a 0.4 μ m fiberglass filters for suspended matter). The difference in the mesh size of filters might not have been the enough, thus the filter similarity resulted in practically the same range of particles sampled, which resulted in almost identical fatty acid profiles having the same fatty acids identified as most abundant (ANNEX 4), thus an overlap of samples in MDS plot (ANNEX 7). This way, every time phytoplankton is referenced in this discussion, the suspended particulate organic matter is also implied.

Profiles were dominated by SFA (62%) and MUFA (25%), in fact, samples were collected in December, after the smaller fall bloom, where reducing light intensity conditions are known to lead to the accumulation of SFA and MUFA in phytoplanktonic communities (Dalsgaard *et al.*, 2003). In terms of identification of taxonomic classes present in the phytoplanktonic community, the higher levels of C16:1 ω 7 and C18:1 ω 7 suggest the dominance of diatoms, given their ability to biosynthesize these FAs, along with low levels of C₁₈ PUFAs (Dalsgaard *et al.*, 2003). The hypothesis of diatom dominance could also be validated by Gonçalves *et al.* (2012) who observed a dominance of diatoms in a temperate estuary during autumn and winter. Other taxonomic classes possibly present in the studied phytoplankton community, include dinophytes and prymnesiophytes, especially due to the levels of C18:1 ω 9 and C18:4 ω 3 (Dalsgaard *et al.*, 2003). In addition, also chlorophytes could be reflected in lower amounts, being discriminated by 18:3 ω 3 and 18:2 ω 6 (Dalsgaard *et al.*, 2003). The presence of bacterial markers C15:0, C15:0iso, C15:0ante, C16:0iso and C17:0 is linked with the presence of bacterial activity (Mayzaud *et al.*, 1989)

In terms of overall **variations across treatments**, no significant differences were found. However, one difference was found in paired test, between treatments FOA-FO. Within the fatty acids that contributed more to the dissimilarity of treatments, only C18:2 ω 6 was significantly different, showing higher value in treatment with only oysters (FO). This could be a reflection of higher presence of chlorophytes in this treatment, since C18:2 ω 6 is characteristic of this taxonomic class, however this is just a hypothesis.

Overall, the FA that were considered characteristic of phytoplankton, and thus could be used as **trophic markers**, are C14:0, C16:1 ω 7 and C18:1 ω 7. However, they also represent considerable amounts in other possible food sources. Specifically, C14:0 and C16:1 ω 7 are also present in minor amounts in sediment, while C18:1 ω 7 is also present in sediment, macroalgae and zooplankton, thus further predator-prey relations discussed have to consider the feeding ecology of the heterotrophic species. Stable isotope analysis could have provided further insights to distinguish trophic levels of food sources.

Macroalgae

Macroalgae profiles were dominated by C16:0, with values near 30%. This was also observed in other studies (Khotimchenko *et al.*, 2002; Kendel *et al.*, 2015) where C16:0 was the most abundant FA with levels ranging from 24-42% in algae of the genus *Ulva*. Additionally, other dominant fatty acids across macroalgae profiles were C16:4 ω 3, C18:3 ω 3 and C18:4 ω 3 (ANNEX 8a). In fact, green algae are known to contain large amounts of C₁₆ PUFA, in particular C16:4 ω 3 is taxonomically characteristic of green macrophytic algae (Kendel *et al.*, 2015), while C₁₈ PUFA (such as C18:4 ω 3 and C18:3 ω 3) are commonly considered characteristic of the *Ulvales* order (Khotimchenko *et al.*, 2002). Moreover, the differences in these two C₁₈ PUFA (C18:4 ω 3 and C18:3 ω 3) can be used to distinguish species within the *Ulvales*. Khotimchenko *et al.* (2002) observed that while C18:4 ω 3 is predominant *Ulva intestinalis*, C18:3 ω 3 is predominant in *Ulva lactuca* (identical morphotype to sampled *Ulva flexuosa*), accordingly the same results were observed in the present study (ANNEX 8a and 8b). Other similarities of the analysed algae in this study to what Khotimchenko *et al.* (2002) and Kendel *et al.* (2015) observed, where the levels of C18:1 ω 7 and C18:2 ω 6 in the 5-10% range. Overall, these similarities confirm what Khotimchenko *et al.* (2002) had showed previously, affirming that in general, and independently of the geographical source, algae have similar FA profiles typical of each phylum of seaweeds.

In terms of **variations of each species across treatments**, no significant differences were found in *Rhizoclonium riparium* and *Ulva intestinalis*, however, significant differences were found in *Ulva flexuosa*. In fact, treatment FOA had significantly higher C16:4 ω 3 and C18:3 ω 3 than treatment FA.

Overall, the FA that were considered characteristic of the algae, and thus could be used as **trophic markers** for predator-prey relations, include C16:4 ω 3, C18:3 ω 3 and C18:4 ω 3, since these fatty acids are much less abundant in all other analysed food sources.

Heterotrophic organisms

Polychaeta

Polychaetas consume mixed diets, being an omnivorous organism that can change feeding mode from filter feeding to deposit feeding or active carnivorous on small benthic organisms (Bischoff *et al*, 2008). The mixed diet is reflected on its profile, being rich in many different fatty acids, posing difficulty for distinguishing different items contribution to its diet (Kelly & Scheibling, 2012). A feeding ecology study of a marine polychaeta (*Hediste diversicolor*) in estuarine and lagoon environments in the Southwest Coast of Portugal, showed that its main gut content was mucus (a food complex including organic matter, bacteria, fungi and phytoplankton), while also sand and vegetable detritus (parts of algae) were found in minor abundances (Fidalgo & Costa, 2006). Considering this, several hypotheses of food sources used by polychaetas in the present study can be suggested.

The high abundances of C18:1 ω 9 and C18:2 ω 6 can be directly linked to the consumption of uneaten commercial feed deposited in the bottom of the ponds. In addition, the abundance of 16:4 ω 3 could be related with the consumption of macroalgae detritus. Sediment is also believed to be consumed, due to the similar levels of ARA, DHA and EPA found in both samples. The considerable level of 20:2 ω 6 found in polychaetas, however, instead of being linked with food sources, might be a reflect of polychaetas ability to synthesize this fatty acid from its precursor 18:2 ω 6, by enzymatic activity (2-carbon chain elongation), however this hypothesis needs to be confirmed with controlled feeding studies.

Even if being a heterotrophic organism, marine polychaetas could also represent a food source for higher trophic levels organisms present in the ponds such as fish. Furthermore, the fatty acid 20:2 ω 6 can be used as **trophic marker** for predator-prey relations, since it is found in polychaetas at considerable levels ($6 \pm 2.5\%$), while in other possible food sources profiles it represents always less than 1% of total fatty acids.

In terms of pond aquaculture, it can be suggested that marine polychaetas are a suitable organism for recycling valuable feed nutrients from fish culture systems which were otherwise discharged (Bischoff *et al.*, 2008). Furthermore, polychaeta had 17.4% of PUFA

ω 3, more than the double compared to feed (6.8%), showing potential to be included in fish feeds, by representing a valuable source of dietary fatty acids such as PUFAs. In fact, *Hediste diversicolor* has been utilized in the feeding of fish and crustaceans, providing an adequate nutrition to broodstock in captivity, having important role in gonadal maturation of different species such as *Solea vulgaris*, *Solea senegalensis* and *Penaeus kerathurus* (Bagarrão, 2013).

Zooplankton

Zooplankton fatty acid composition is determined by taxonomic affiliation, changed by diet and modified by starvation and temperature (Brett *et al*, 2009).

Considering food sources, the levels of DHA ($6.1 \pm 1.5\%$) found in zooplankton profile could indicate the consumption of dinoflagellates, while the presence of EPA ($7.3 \pm 0.7\%$) could indicate the consumption of diatoms (Brett *et al*, 2009), even if both EPA and DHA had low amounts ($< 1.5\%$) in the profile of phytoplankton. In fact, these highly unsaturated fatty acids cannot be synthesized *de novo* (Gonçalves *et al.*, 2012), thus must have been acquired by the diet and accumulated, knowing that there is a higher tendency for accumulation of polyunsaturated fatty acids with decreasing temperatures in zooplankton (Brett *et al*, 2009).

The presence of fatty acids C16:1 ω 7, C18:1 ω 7 can be linked with the consumption of phytoplankton (and suspended particulate organic matter) since these two fatty acids are considered trophic markers of phytoplankton in this study. The higher amounts of C18:1 ω 9 ($13.5 \pm 1.9\%$) and C18:2 ω 6 ($14.9 \pm 2.1\%$) found in many heterotrophic organisms in this study is generally a reflect of direct consumption of the commercial feed, given that these two fatty acids are considered markers of the commercial feed. However, this hypothesis seems not likely to happen in zooplankton considering its feeding ecology. A more plausible explanation is by the consumption of phytoplankton (and suspended particulate organic matter), which also present these fatty acids in their profile but in lower amounts, but being probably accumulated over time in zooplankton explaining its higher amounts. By last, the presence of markers C15:0, C15:0iso and C17:0, and trace amounts of C15:0ante and C16:0iso indicate bacterial feeding (Dalsgaard *et al.*, 2003).

No **significant differences were found between treatment** profiles in zooplankton, thus their food sources are believed to be the same across treatments. Even if being a heterotrophic organism, zooplankton could also represent a food source for higher trophic levels organisms present in the ponds, such as oyster and mullet, however, the lack of any fatty acid considered as **trophic marker** poses difficulties to identify zooplankton exclusively in any given consumers diet.

Oyster

Sampled *Crassostrea gigas* presented similar fatty acid profiles to those found in other studies (Orban *et al.*, 2004; Saito & Marty, 2010; Pogoda *et al.*, 2013) being characterized by the same fatty acids accounting for at least 80% of the total of fatty acids, such as C16:0, C16:1 ω 7, C18:1 ω 7, C18:1 ω 9 and C18:2 ω 6, C18:3 ω 3, C18:4 ω 3, ARA, EPA and DHA. Moreover, the presence of these fatty acids can be linked with the consumption of phytoplankton, suspended particulate organic matter and zooplankton (ANNEX 7). In addition, the presence of C15:0, C15:0iso, C16:0iso and C17:0 reflects bacterial consumption. In fact, *C.gigas* is known to derive nutrition from all these sources (Le Gall *et al.*, 1997) where phytoplankton is normally predominant, but any other particle of appropriate size and palatability may be taken in and eaten.

The quantities of ARA, DHA, but especially EPA, were higher in *C. gigas* profile compared to the quantities found in their food sources, confirming that these fatty acids accumulate in *C. gigas* tissues (Saito & Marty, 2010). Furthermore, EPA could also be a result of biosynthesis from shorter ω 3 PUFA (Zhukova, 1991), such as elongase from C18:4 ω 3 to C20:4 ω 3 and further desaturase to C20:5 ω 3 (EPA). In similar way, ARA could be a result of biosynthesis from shorter ω 6 PUFA (ANNEX 10).

Significant differences were found between treatments profiles. In *C. gigas* from treatment FOA, there were significantly higher levels of C18:1 ω 9 (+1.7%) and C18:2 ω 6 (+3.1%). Considering its food sources, these two fatty acids are more abundant in zooplankton than phytoplankton, thus the higher levels reflected in *C. gigas* could indicate a

higher consumption of zooplankton in this treatment. On the contrary, significantly higher levels of C16:1 ω 7 (+2.4%) and C18:1 ω 7 (+1.4%) found in *C. gigas* from treatment FO could be a reflect of higher phytoplankton consumption.

Fish

The fatty acid composition of muscle tissue in all sampled fish species reflected the dietary fatty acids of the feed to great extent. Not only the feed's characteristic fatty acids, C18:1 ω 9 and C18:2 ω 6, but also other less abundant fatty acids such as C16:1 ω 7, C18:1 ω 7, C18:3 ω 3, EPA and DHA were reflected in similar percentages to those found in the feed. Saturated fatty acids such as C14:0, C16:0 and C18:0 showed the same pattern. Results observed confirm that dietary fatty acids of the feed are incorporated in marine fish tissue with little or no modification (Bischoff *et al.*, 2008). Additionally, all species reflected higher percentages of ARA and EPA, but especially DHA, than those found in the feed, indicating their ability to accumulate these fatty acids in their tissue. Lower samples dispersion within groups in meagre and bream suggest they had a more constant diet across ponds, while bigger dispersion in mullet samples suggest a more varied diet across ponds. (ANNEX 9a, and ANNEX 9b)

In **meagre**, differences found in treatments were attributed to less -2.5% of DHA in treatment FOA. However, this variation is probably due to natural variability in fatty acid composition, and is not believed to reflect any change in food sources of the meagre across treatments, which fed only on the commercial feed. This result was expected, in fact, the grow-out of meagre with only commercial feeds is common practice not only in sea cages Grigorakis *et al.* (2011) both also in earthen ponds (Ribeiro *et al.*, 2013).

In **white seabream**, no significant differences were found between treatments, thus no variation of food sources other than the commercial feed could be identified. Given its omnivorous feeding, it would be expected that white seabream could look for other food sources. However, it did not show any algal or polychaeta trophic marker. These items could

have been occasionally eaten by bream, however in insufficient amounts to be reflected on its fatty acid composition.

In **mullet**, the higher dispersion of samples suggests a more varied diet across ponds. While feed was the main dietary item consumed, the presence of higher abundances of ARA, EPA and DHA could be attributed to phytoplankton, suspended matter, zooplankton, sediment and polychaetas consumption. In fact, studies about the culture of this species in brackish semi-intensive fish ponds observed that it can utilize both supplemental feed and natural food, such as diatoms, algae, dinoflagellates, protozoa, plant detritus, copepods, nematodes, sand and mud (Mondal *et al.*, 2015).

The reasons for these higher abundances may instead be related with the species own ability to transform dietary fatty acids, for example the biosynthesis of ARA (20:4 ω 6) from C18:2 ω 6, or even the biosynthesis of DHA (20:5 ω 3) and EPA (22:6 ω 3) from its precursor C18:3 ω 3, however only controlled feeding studies could provide a conclusive answer to evaluate to what extent mullet possesses *desaturases* and *elongases* capable of such enzymatic activity (ANNEX 11).

The differences found between treatments in mullet were attributed to significantly higher C14:0 and C16:1 ω 7 in treatment FO, which may be linked to higher consumption of phytoplankton, suspended matter and sediment. Instead, a significantly higher ARA and DHA, in treatment FOA, could be either linked to higher consumption of polychaetas and sediment, or to a higher biosynthesis and accumulation of these fatty acids.

5. Conclusions

Regarding measured parameters, the dissolved oxygen was higher in treatments with oysters, possibly because they represent an additional output of dissolved inorganic nutrients resulting in higher primary productivity in these ponds. Treatments with oysters also had lower turbidity confirming their ability to feed on farm effluents thus improving water quality. In relation to the determination of dietary tracers, the commercial feed was mainly characterized by C18:1 ω 9 and C18:2 ω 6 (Oleic and Linoleic acids). Considering the autotrophic species, in phytoplankton, the community was possibly dominated by diatoms along with other taxonomic classes such as dinophytes, prymnesiophytes, chlorophytes and bacteria. Fatty acids that were considered characteristic of phytoplankton, and thus could be used as trophic markers, were C14:0, C16:1 ω 7 and C18:1 ω 7. However, they also represent considerable amounts in other possible food sources such as suspended particle organic matter, sediment and zooplankton. Trophic markers identified in macroalgae include C16:4 ω 3, C18:3 ω 3 and C18:4 ω 3. Considering the heterotrophic species food sources, polychaetas showed dietary tracers of the commercial feed, macroalgae, sediment and additionally is suggested the ability to biosynthesize 20:2 ω 6. In zooplankton, the dietary tracers found reflect a consumption of phytoplankton, suspended matter and bacteria, while the higher levels of DHA and EPA could be linked with a higher accumulation of PUFA with decreasing temperatures. Oysters reflected trophic markers of phytoplankton, suspended matter, zooplankton and bacteria, while higher levels of ARA, DHA and EPA than those found in their food sources confirm that these fatty acids accumulate in oyster tissues. In addition, ARA and EPA could be a product of biosynthesis from shorter PUFA ω 6 and PUFA ω 3, respectively. The differences found between treatments indicate a higher consumption of zooplankton in treatment FOA, and a higher consumption of phytoplankton in treatment FO. All fish species reflected the feed dietary tracers to a great extent. While meagre and white seabream consumed the commercial feed predominantly, the higher dispersion of mullet samples might suggest the consumption of additional dietary items (such as phytoplankton, suspended matter, zooplankton, sediment and polychaetas). Additionally, all species reflected higher percentages of ARA and EPA, but especially DHA, than those found in the feed, indicating their ability to accumulate these fatty acids in their tissue. In mullet, might also exist an ability to synthesize PUFA from shorter fatty acids.

References

Abellán E. & García-Alcázar A. (1995) Pre-growout and growout experience with seabream (*Diplodus sargus sargus*) and sharpnose seabream (*Diplodus puntazzo*). Marine Aquaculture Finfish Species Diversification Vol.16, pp. 57-63.

Bagarrão R. (2013) Reprodução e crescimento do poliqueta *Hediste diversicolor* sob diferentes condições ambientais. Tese de mestrado - Instituto politécnico de Leiria. pp 65.

Barrington K., Chopin T., Robinson S. (2009) Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. In Soto D. (eds.) Integrated mariculture: a global review. FAO Fisheries and Aquaculture Technical Paper, Rome. No. 529, pp. 7–46.

Barrington K., Ridler N., Chopin T., Robinson S., Robinson B. (2010) Social aspects of the sustainability of integrated multi-trophic aquaculture. Aquaculture International Vol. 18, pp. 201-211.

Bauchot M.L. (1987) Poissons osseux. In Fischer W., M.L. Bauchot M.L., Schneider M. (eds.) Fiches FAO d'identification pour les besoins de la pêche. (rev. 1). Méditerranée et mer Noire. Zone de pêche 37. Commission des Communautés Européennes and FAO, Rome. Vol. 2, pp. 891-1421.

Ben-Ari T., Neori A., Ben-Ezra D., Shauli L., Odintsov V., Shpigel M. (2014) Management of *Ulva lactuca* as a biofilter of mariculture effluents in IMTA system. Aquaculture No. 434, pp. 493-498.

Bergé J.P. & Barnathan G. (2005). Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects. Adva biochem Engin/Biotechnol No. 96, pp. 49-125.

Brett M., Muller-Navarra D.C., Persson J. (2009) Crustacean zooplankton fatty acid composition. Lipids in Aquatic ecosystems pp. 115-142.

Bischoff A.A., Fink P., Waller U. (2008) The fatty acid composition of *Nereis diversicolor* cultured in an integrated recirculated system: possible implications for aquaculture. Aquaculture No. 296, pp. 271-276.

Cabi (2014) Invasive species compendium: *Crassostrea gigas* datasheet (retrieved from: <http://www.cabi.org/isc/datasheet/87296>).

Chaguri M.P. (2013) Rastreabilidade em pescado: influência do grau de domesticação, origem e sazonalidade na diferenciação de peixes. Universidade Estadual Paulista UNESP.

Chao L.N. & Trewavas E. (1990) Check-list of the fishes the eastern tropical Atlantic (CLOFETA). JNICT, Lisbon; SEI, Paris; and UNESCO, Paris. Vol. 2

Chopin T. (2006) Commentary: Integrated Multi-Trophic Aquaculture, what it is, and why you should care...And don't confuse with Polyculture. Northern Aquaculture.

Cunha M.E., Quental-Ferreira H., Leão A.C., Ribeiro L., Soares F., Baptista F., Matias D., Joaquim S., Pousão-Ferreira P. (2011) SEAFARE, Sustainable and

Environmentally Friendly Aquaculture in the Atlantic Region of Europe. Land-based IMTA with Plankton/Oysters/Sea cucumber/Fish in earthen ponds. Project final report.

Cruz-Suárez L.E., León A., Peña-Rodríguez A., Rodríguez-Peña G., Moll B., Ricque-Marie D. (2010) Shrimp/*Ulva* co-culture: A sustainable alternative to diminish the need for artificial feed and improve shrimp quality. *Aquaculture* No. 301, pp. 64-68.

D'Anna G., Giacalone V.M., Badalamenti F., Pipitone C. (2004) Releasing of hatchery-reared juveniles of the white seabream *Diplodus sargus* in the Gulf of Catellammare artificial reef area (NW Sicily). *Aquaculture* No. 233, pp. 251-268

Divanach P., Kentouri M., Paris J. (1982) Etapes du developpement embryonnaire et larvaire du sar, *Diplodus sargus* en elevage. *Aquaculture* No. 274, pp. 339-353.

Divanach P., Kentouri M., Charalambakis G. (1993) Comparison of growth performance of six Mediterranean fish species reared under intensive farming conditions in Crete (Greece), in raceways with the use of self-feeders. *European Aquaculture Society* No. 18.

DebD. (1997) Trophic uncertainty vs parsimony in food web research. *Oikos* No. 78, pp. 191-4.

Estévez A., Duncan N., Campoverde C., Andree K., Roque A., Gisbert E., Afonso J.M., Robaina L., Izquierdo M., Tsigenopoulos C., Mylonas C., Papandroulakis N., Katharios P., Chatzifotis S., Papadakis Y., Tsertou M.I. (2015) DIVERSIFY Fish Project: New advances in Meagre (*Argyrosomus Regius*) culture research. *Aquaculture Europe 2015 Conference*.

FAO (1984) Inland Aquaculture engineering: Chapter 21 Aeration and oxygenation in aquaculture. FAO (Food and Agriculture Organization of the United Nations) Fisheries and Aquaculture Department, Rome.

FAO (1992) Technology of production of edible flours and protein products from soybeans: chapter 1, The soybeans. FAO (Food and Agriculture Organization of the United Nations) Agriculture and Consumer protection department, Israel.

FAO (2016) The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. FAO (Food and Agriculture Organization of the United Nations) Fisheries and Aquaculture Department, Rome.

FAO (2005) Cultured Aquatic Species Information Programme: *Crassostrea gigas*. FAO (Food and Agriculture Organization of the United Nations) Fisheries and Aquaculture Department, Rome.

Fischer W., Bianchi G. (2012) *Mugil cephalus* (Linnaeus, 1758). FAO (Food and Agriculture Organization of the United Nations) Fisheries department, Rome.

Fountoulaki E., Grigorakis K., Kounna C., Rigos G., Papandroulakis N., Diakogeorgakis J., Kokou F. (2017) Growth performance and product quality of meagre (*Argyrosomus regius*) fed diets of different protein/lipid levels at industrial scale. *Italian Journal of Animal Science*.

Gao Q.F., Shin P.K.S., Lin G.H., Chen S.P., Cheung S.G. (2006) Stable isotope and fatty acid evidence for uptake of organic waste by green-lipped mussels *Perna viridis* in a polyculture fish farm system. *Marine Ecol Prog Ser* Vol. 317, pp. 273-283.

Geider Q.F, Macintyre H.L., Kana T.M. (1998) A dynamic regulatory model of phytoplankton acclimation to light, nutrients and temperature. *Limnol Oceanogr* Vol 43, pp. 679-669

Glencross B. D. (2009) Exploring the nutritional demand for essential fatty acids by aquaculture species. *Reviews in Aquaculture* Vol. 1, pp. 71-124.

Gonçalves A.M.M., Azeiteiro U.M., Pardal M.A., De Troch M. (2012) Fatty acid profiling reveals seasonal and spatial shifts in zooplankton diet in temperate estuary. *Estuarine, Coastal and Shelf Science* Vol. 109, pp. 70-80.

Gouletquer P. & Héral M. (1992) The ecology of *Crassostrea gigas* in Australia, New Zealand, France and Washington State. *Oyster Ecology Workshop*

Grigorakis K., Fountoulaki E., Vasilaki A., Mittakos I., Nathanailides C. (2011) Lipid quality and filleting yield of reared meagre (*Argyrosomus regius*). *International Journal of Food Science and Technology* No.46, pp. 711-716.

Guerrero S. & Cremades J. (2012) Integrated multi-trophic aquaculture: A sustainable, pioneering alternative for marine cultures in Galicia.

Gurr MI., Harwood J.L., Frayn K.N. (2002) Lipid biochemistry. Blackwell Science, Oxford

Harris J. (2008) Pacific oyster aquatic invasive species profile. *Aquatic invasion Ecology*.

Hart P.J.B. & Reynolds J.D (2002) Handbook fish biology and fisheries, Vol 1.

Hughes A.D. & Black K.D. (2016) Going beyond the search for solutions: understanding trade-offs in European integrated multi-trophic aquaculture development. *Aquaculture Environment Interactions* Vol. 8, pp. 191-199.

Huvet A., Fabioux C., McCombie H., Lapègue S., Boudry P. (2004) Natural hybridization between genetically differentiated populations of *C.gigas* and *C.angulata* highlighted by sequence variation in flanking regions of a microsatellite locus. *Marine Ecol. Prog. Ser.* 272, pp. 141-152.

Huvet A, Lapegue S, Magoulas A, Boudry P. (2000) Mitochondrial and nuclearDNA phylogeography of *Crassostrea angulata*, the portuguese oyster endangered in Europe. *Conservation Genetics* Vol.1, pp. 251-262.

Izquierdo M. (2005) Essential fatty acid requirements in Mediterranean fish species. *In: Mediterranean fish nutrition* (eds. Basurco, B., Nengas, I., Alexis, M., Izquierdo, M.). Zaragoza: CIHEAM (Cahiers Options Méditerranéennes) Vol. 63, pp. 91-102.

Kater B.J., Baars J.M.D.D. (2004) The potential of aerial photography for estimating surface areas of intertidal Pacific oyster beds (*C. gigas*). J. Shellfish Res 23, pp. 773-779.

Koven B., Rosenfeld H., Fauvel C., Nixon O., Tandler A., Meiri-Ashkenazi I., Bitan A., Gisbert E., Estévez A., Kotzamanis Y., Robles R., Shafran G., Papaioannou N. (2015) DIVERSIFY Fish project: Achievements in grey mullet culture obtained during 2014 and 2015. Aquaculture Europe 2015 Conference.

Lefebvre S., Barillé L., Clerc M. (2000) Pacific oyster (*Crassostrea gigas*) feeding responses to a fish-farm effluent.

Le Gall S., Bel Hassen M., Le Gall P. (1997) Ingestion of a bacterivorous ciliate by the oyster *Crassostrea gigas*: protozoa as a trophic link between picoplankton and benthic suspension-feeders. Mar Ecol Prog Ser 152, pp.301-306

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

Mayzaud, P., Chanut, J. P. and Ackman, R. G. (1989). Seasonal changes of the biochemical composition of marine particulate matter with special reference to fatty acids and sterols. Mar. Ecol. Prog. Ser.56, pp 189–204

Monfort M.C. (2010) Present market situation and prospects of meagre (*Argyrosomus regius*) as an emerging species in Mediterranean aquaculture. Studies and Reviews. General Fisheries Commission for the Mediterranean. FAO. Rome.

Morrison W.R. (1978) Wheat lipid composition. Cereal Chem Vol. 55, pp. 548-558.

Miller M.R., Nichols D.P., Carter C.G. (2008) Oil sources for use in aquaculture – alternatives to the sustainable harvest of old fish. Nutrition Research Reviews Vol 21., pp 85-96

Nikolaisen L., Jensen P.D., Bech K.S., Dahl J., Busk J., Brødsgaard T., Rasmussen M.B., Bruhn A., Bjerre A.B., Nielsen H.B., Albert K.R., Ambus P., Kadar Z., Risø S.H., Sander B., Schmidt E.R. (2011) Energy Production from Marine Biomass (*Ulva lactuca*). National Environmental Research Institute and Danish Technological Institute.

Neori A., Chopin T., Troell M., Buschmann A.H., Kraemer G.P., Halling C., Shpigiel M., Yarish C. (2004) Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture No. 231, pp. 361-391.

Odum W.E. (1970) Utilization of the direct grazing and plant detritus food chains by the striped mullet *Mugil cephalus*. In Steele J.J. (eds.) Marine food chains. Edinburgh, Scotland. pp 222–240.

Orban E., Di Lena G., Masci M., Navigato T., Casini I., Caproni R., Gambelli L., Pellizzato M. (2004) Growth, nutritional quality and safety of oysters *Crassostrea gigas* cultured in the lagoon of Venice (Italy). *J Sci Food Agric* Vol. 84, pp. 1929-1938.

Pavlidis M. & Mylonas C. (2011) *Sparidae*: Biology and aquaculture of gilthead seabream and other species.

Pazos A.J., Ruíz C., García-Martin O., Abad M., Sánchez J.L. (1996) Seasonal variations in lipid content and fatty acid composition of *Crassostrea gigas* cultured in El Grove, Galicia, N.W.Spain. *Comp Biochem Physiol* Vol 114, pp. 171-179.

Pogoda B., Buck B.H., Saborowski R., Hagen W. (2013) Biochemical and elemental composition of the offshore-cultivated oyster *Ostrea edulis* and *Crassostrea gigas*. *Aquaculture* No.400, pp. 53-60.

Piccolo G., Bovera F., De Riu N., Marono S., Salati F., Cappuccinelli R., Moniello G. (2010) Effect of two different protein/fat ratios of the diet on meagre (*Argyrosomus regius*) traits. *Italian Journal of Animal Science* Vol 7, pp. 363-371.

Quèmenèr L. (2002) Le maigre commun (*Argyrosomus regius*). Biologie, pêche, marche et potential aquacole. Editions Ifremer, Plouzané, France.

Ribeiro L., Quental Ferreira H., Soares F., Pousão P. (2013) Portuguese Research Studies Meagre Production in Earthen Ponds. *Global Aquaculture Advocate* Vol 16. pp. 38-41

Santos M., Leitão F., Moura A., Cerqueira M., Monteiro C.C. (2010) *Diplodus spp* on artificial reefs of different ages: influence of the associated microbenthic community. *ICES journal of Marine Science* No. 68, pp. 87-97.

Saito H., Marty Y. (2010) High levels of Eucosapentaenoic acid in the lipids of oyster *Crassostrea gigas* ranging over both Japan and France. *J. Oleo Sci* No.59, pp. 281-292.

Sobiechowska M. (2008) Algal pigments as biomarkers in the aquatic food web: phytoplankton divisions and their utilization by benthic invertebrates and fish in Lake Erie 2003-2005. PhD thesis. Univ. at Albany, NY.

Stibor H. & Sommer U. (2004) Ocean food webs and trophic dynamics. UNESCO – EOLSS sample chapters.

Soto D. (2009) Integrated mariculture, a global review. FAO (Food and Agriculture Organization of the United Nations) Fisheries and Aquaculture Technical Paper, Rome. No.529

Troell M., Halling C., Neori A., Chopin T., Buschmann A.H., Kautsky N., Yarish C. (2003) Integrated mariculture: asking the right questions.

Thomson J.M. (1966) The Grey Mullet. *Oceanogr. Mar. Biol. Ann. Rev.* 4, pp. 301–335.

Tamaru C.S., FitzGerald W.J., Sato V. (1993) Hatchery manual for the artificial propagation of striped mullet. Guam aquaculture development and training center, and the oceanic institute.

Waller U, Buhmann K.A., Ernst A., Hanke V., Kulakowski A., Wecker B., Orellana J., Papenbrock J. (2015) Integrated multi-trophic aquaculture in a zero-exchange recirculation aquaculture system for marine fish and hydroponic halophyte production.

Zhukova N.V. (1991) The pathway of the biosynthesis of non-methylene-interrupted dienoic fatty acids in molluscs. *Comp Biochem Physiol* Vol.100, pp. 801-804.

ANNEXES

ANNEX 1: Weight of dry material used to begin extraction and volume of solvent added for each type of sample.

sample	dry material (mg)	solvent (mL)
Feed	135	0.8
Sediment	500	0.1
Phytoplankton	filter	0.1
suspended matter	filter	0.1
zooplankton	200	0.8
<i>polychaeta</i>	200	0.8
<i>Ulva flexuosa</i>	200	0.8
<i>Ulva intestinalis</i>	100	0.8
<i>Rhizoclonium riparium</i>	150	0.8
<i>Crassostrea gigas</i>	125	0.8
<i>Argyrosomus regius</i>	200	0.8
<i>Diplodus sargus</i>	130	1
<i>Mugil cephalus</i>	150	0.8

ANNEX 2: Average squared distances between paired treatments and fatty acids with Sq.Dist>0.90 (SIMPER analysis), along with indication of significant differences (see Annex 12 and Annex 13 for detailed information on statistics analysis)

	(average squared distances)			(fatty acids with: Sq.Dist/SD > 0.90)								
	FOA - FO	FOA - FA	FO - FA									
phytoplankton	120.1*	86.9	84.9	(FOA - FO)	C18:2ω6*	C16:1ω7	C18:1ω7	EPA	C16:0			
suspended matter	54.6	51.3	77.4									
zooplankton	46.7	44.5	34.2									
<i>ulva flexuosa</i> *		93.5*			C18:3ω3*	C16:4ω3*	C18:4ω3					
<i>ulva intestinalis</i>		60.1										
<i>rhizoclonium riparium</i>		35.2										
oyster*	67.2*				C18:2ω6*	C16:1ω7*	C14:0*	C18:3ω3*	C18:1ω9*	EPA*	C18:1ω7*	
meagre*	21.4*	22.9*	16.5	(FOA - FO)	DHA*	C18:0	C18:2ω6	C16:0				
				(FOA - FA)	DHA*	18:2ω6	C18:0					
bream	14.5	14.2	15.8									
mullet*	262.8*	193.4*	165.9	(FOA - FO)	C18:0*	DHA*	C18:2ω6	C16:1ω7*	C14:0*	C18:1ω9*	ARA*	
				(FOA - FA)	C18:0	DHA*	C18:2ω6*	C16:0	C18:1ω9*	C14:0*	ARA*	

* indicates significant differences overall between treatments (ANOSIM one factor)

* indicates significant differences between paired treatments (ANOSIM pairwise)

*indicates significant differences in specific fatty acids between paired treatments (T-student or Mann Whitney)

	Feed	Sediment	Phytoplankton	Rhiparium	U.Flexuosa	U.Intestinalis	Zooplankton	Polychaeta	Oyster	Meagre	Bream	Mullet
C14:0	2.2 ± 0.4	4.0 ± 1.0	5.5 ± 0.5	0.9 ± 0.3	1.6 ± 1.0	1.8 ± 0.9	2.4 ± 1.1	2.2 ± 0.9	5.1 ± 1.8	0.7 ± 0.2	2.3 ± 0.6	2.2 ± 2.0
C15:0	0.4 ± 0.1	1.5 ± 0.4	1.9 ± 0.3				0.8 ± 0.1	0.9 ± 0.3	1.6 ± 0.2		0.4 ± 0.1	0.4 ± 0.1
C15:0 iso		3.0 ± 0.8	2.3 ± 0.4				0.6 ± 0.4		0.7 ± 0.1			
C15:0 ante		1.9 ± 0.8	1.4 ± 0.5									
C16:0	21.6 ± 0.3	26.2 ± 1.1	32.5 ± 0.1	28.8 ± 2.1	30.9 ± 4.5	32.3 ± 3.0	27.4 ± 2.2	15.4 ± 6.1	33.1 ± 2.2	18.2 ± 1.5	21.7 ± 0.8	26.3 ± 3.4
C16:0 iso		0.8 ± 0.5	0.5 ± 0.2	0.6 ± 0.1	0.6 ± 0.5	0.5 ± 0.4						
C17:0	0.4 ± 0.1	1.3 ± 0.2	1.3 ± 0.3		0.6 ± 0.1		1.1 ± 0.1	1.3 ± 0.5	2.1 ± 0.1		0.4 ± 0.1	0.4 ± 0.1
C18:0	4.0 ± 0.2	11.1 ± 0.2	10.4 ± 0.7	0.4 ± 0.2			7.9 ± 1.5	5.8 ± 1.3	3.7 ± 0.4	7.3 ± 0.7	4.3 ± 0.6	6.9 ± 2.1
C20:0		2.0 ± 0.1	1.5 ± 1.2					0.5 ± 0.2				
C22:0		1.9 ± 0.5	1.1 ± 0.7		0.9 ± 0.6			1.1 ± 0.6				
C16:1w5		0.7 ± 0.4		3.5 ± 0.5	3.0 ± 0.7	4.6 ± 1.2	0.8 ± 0.1	0.6 ± 0.3	1.2 ± 0.1			
C16:1w7	2.1 ± 0.2	5.6 ± 1.2	7.4 ± 0.6	1.1 ± 0.5	2.5 ± 0.5	0.6 ± 0.1	2.7 ± 0.5	1.9 ± 0.7	4.3 ± 1.3	1.3 ± 0.2	3.4 ± 0.6	3.8 ± 3.4
C16:4w3		0.7 ± 0.2		15.3 ± 2.3	17.0 ± 2.4	16.5 ± 2.5	0.6 ± 0.5	4.2 ± 1.1	1.9 ± 0.4			
C18:1w7	1.6 ± 0.1	5.1 ± 0.7	6.7 ± 0.7	6.3 ± 0.5	8.6 ± 1.3	5.3 ± 0.6	4.6 ± 1.0	2.4 ± 0.4	5.4 ± 1.0	1.5 ± 0.2	2.1 ± 0.2	2.0 ± 0.5
C18:1w9	21.8 ± 0.5	8.5 ± 2.8	9.4 ± 0.3	1.1 ± 0.7	0.6 ± 0.2	0.9 ± 0.2	13.5 ± 1.9	10.2 ± 1.5	4.9 ± 1.0	16.0 ± 1.7	21.7 ± 1.0	12.4 ± 3.0
C18:2w6	37.1 ± 1.8	4.1 ± 1.1	3.7 ± 1.3	6.7 ± 0.8	3.2 ± 0.4	4.7 ± 0.7	14.9 ± 2.1	14.0 ± 2.5	6.7 ± 1.7	36.2 ± 1.1	29.8 ± 1.6	22.0 ± 4.6
C18:3w3	3.9 ± 0.2	1.3 ± 0.1	1.5 ± 0.1	18.5 ± 1.7	9.7 ± 1.2	15.2 ± 1.9	2.3 ± 0.4	1.4 ± 0.3	3.9 ± 2.0	2.2 ± 0.4	3.2 ± 0.3	1.5 ± 0.5
C18:4w3		0.7 ± 0.3	1.2 ± 0.5	11.0 ± 1.5	17.1 ± 2.6	10.2 ± 1.6	1.2 ± 0.3		3.1 ± 0.4		0.4 ± 0.1	0.3 ± 0.2
C20:1w9	0.4 ± 0.1	0.6 ± 0.2	0.8 ± 0.5				0.5 ± 0.2	1.9 ± 0.7	0.3 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
C20:2w6							0.7 ± 0.2	6.3 ± 2.5			0.6 ± 0.3	0.6 ± 0.1
ARA C20:4w6	0.3 ± 0.1	1.5 ± 0.2		0.7 ± 0.2		0.5 ± 0.1	1.7 ± 0.6	2.7 ± 0.8	2.0 ± 0.4	1.1 ± 0.2	0.7 ± 0.3	2.5 ± 1.1
EPA C20:5w3	0.7 ± 0.1	4.5 ± 1.3	1.4 ± 0.6	1.3 ± 0.8		0.8 ± 0.2	7.3 ± 0.7	6.3 ± 1.7	11.1 ± 1.6	0.9 ± 0.2	1.3 ± 0.4	3.1 ± 1.8
C21:5w3								1.1 ± 2.4	0.4 ± 0.1			
C22:2w6												
C22:5w3				0.6 ± 0.2	1.3 ± 1.1	1.2 ± 1.2	0.3 ± 0.4	1.5 ± 1.1	4.7 ± 1.1	6.4 ± 1.4	0.4 ± 0.2	1.6 ± 0.6
DHA C22:6w3	2.0 ± 1.1	2.0 ± 1.8	1.2 ± 0.1	0.6 ± 0.2			6.1 ± 1.5	5.4 ± 2.9			4.3 ± 1.2	10.6 ± 4.9

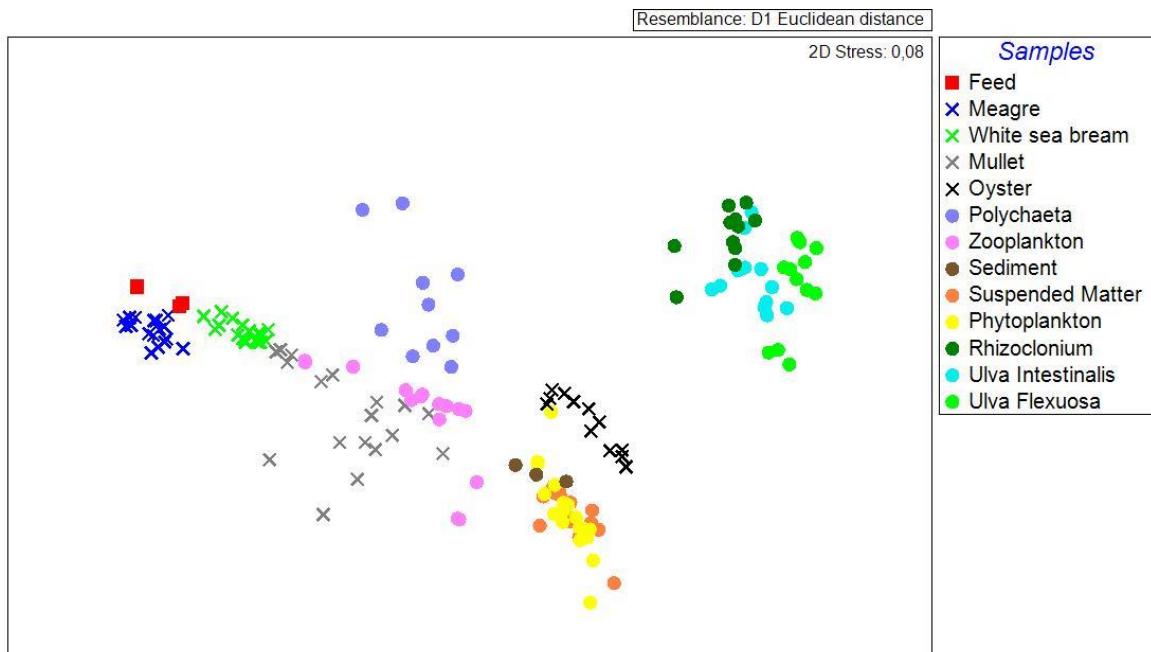
ANNEX 4: Fatty acids accounting for at least 80% of the total fatty acids

	SFA	MUFA	PUFA
feed	C16:0	C18:1w9	C18:2w6
sediment	C14:0 C16:0 C18:0	C16:1w7 C18:1w7 C18:1w9	C18:2w6 EPA
(autotrophs)			
phytoplankton	C14:0 C16:0 C18:0	C16:1w7 C18:1w7 C18:1w9	C18:2w6
suspended matter	C14:0 C16:0 C18:0	C16:1w7 C18:1w7 C18:1w9	C18:2w6
R. riparium	C16:0	C16:1w5 C18:1w7	C16:4w3 C18:2w6 C18:3w3 C18:4w3
U.Intestinalis	C16:0	C16:1w5 C18:1w7	C16:4w3 C18:2w6 C18:3w3 C18:4w3
U. flexuosa	C16:0	C18:1w7	C16:4w3 C18:2w6 C18:3w3 C18:4w3
(heterotrophs)			
zoo	C16:0 C18:0	C18:1w9	C18:2w6 EPA DHA
polychaeta	C16:0 C18:0	C18:1w9	C16:4w3 C18:2w6 EPA DHA 20:2w6
oyster	C16:0	C16:1w7 C18:1w7 C18:1w9	C18:2w6 C18:3w3 EPA DHA
meagre	C16:0 C18:0	C18:1w9	C18:2w6 DHA
bream	C16:0 C18:0	C18:1w9	C18:2w6 DHA
mullet	C16:0 C18:0	C16:1w7 C18:1w9	C18:2w6 DHA

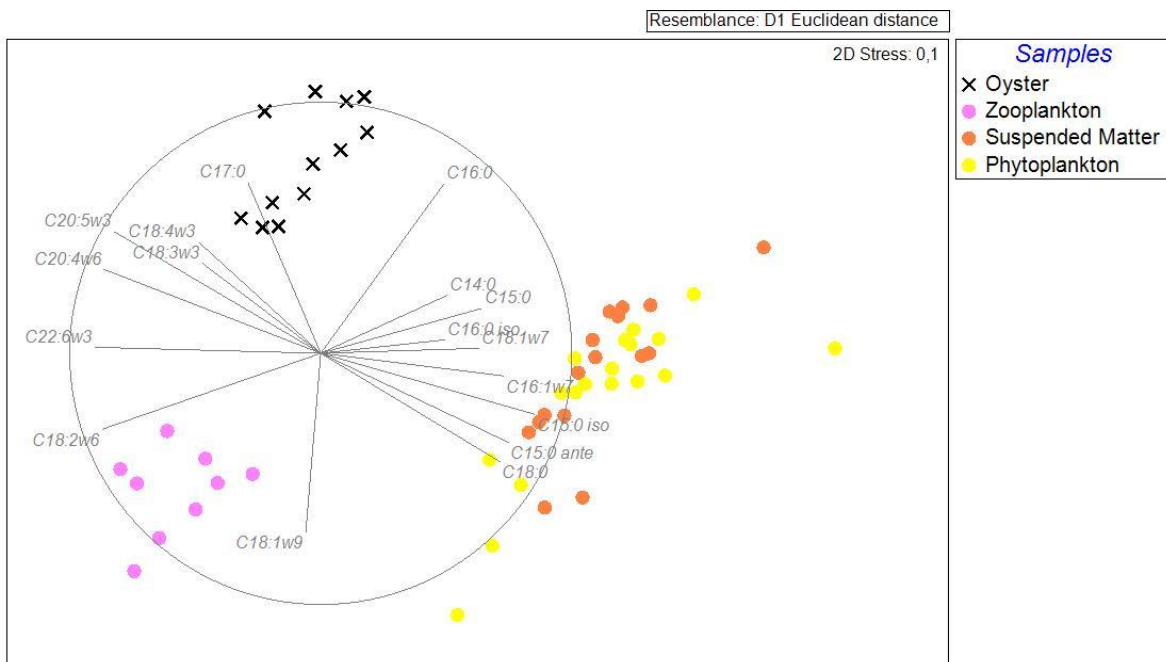
ANNEX 5: Total percentages of SFA, MUFA, PUFA, PUFA w3 and PUFA w6.

	SFA (%)	MUFA (%)	PUFA (%)	ω3 (%)	ω6 (%)	ω3/ω6
<i>FEED</i>	29.1	26.0	44.9	6.8	37.4	0.18
<i>Sediment</i>	61.6	21.2	17.2	9.3	6.6	1.41
<i>Polychaeta</i>	34.5	18.7	31.4	17.4	28.5	0.61
<i>Phytoplankton</i>	62.1	25.5	12.1	5.9	4.2	1.39
<i>Zooplankton</i>	40.6	21.6	37.6	18.9	17.8	1.06
<i>Rhizoclonium</i>	31.9	12.3	55.8	46.9	8.5	5.51
<i>U.Intestinalis</i>	36.9	11.9	51.2	44.5	6.4	6.92
<i>U.Flexuosa</i>	35.5	15.1	49.4	45.6	3.5	12.95
<i>Oyster</i>	46.9	16.3	36.3	25.9	9.3	2.78
<i>Meagre</i>	28.4	20.5	51.1	10.5	39.9	0.26
<i>Bream</i>	29.3	28.1	42.6	10.0	31.7	0.32
<i>Mullet</i>	36.6	19.0	44.3	17.4	25.5	0.68

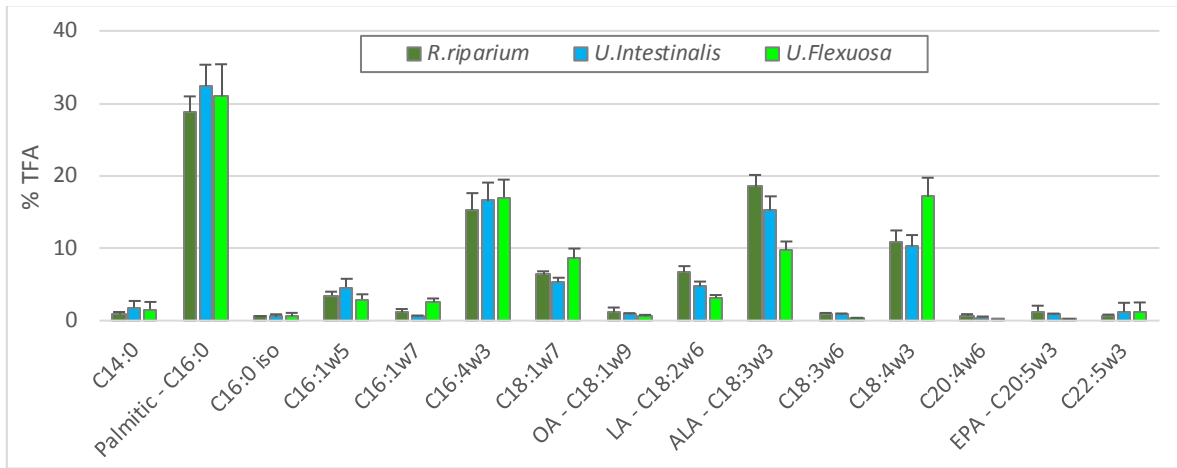
ANNEX 6 - MDS plot of samples of all organisms sampled in the final period. n=3 for Feed and sediment, n=18 for Meagre, White sea bream, Mullet, Zooplankton and Phytoplankton, n=12 for oysters, Polychaeta, Rhizoclonium, *Ulva intestinalis* and *Ulva Flexuosa*.



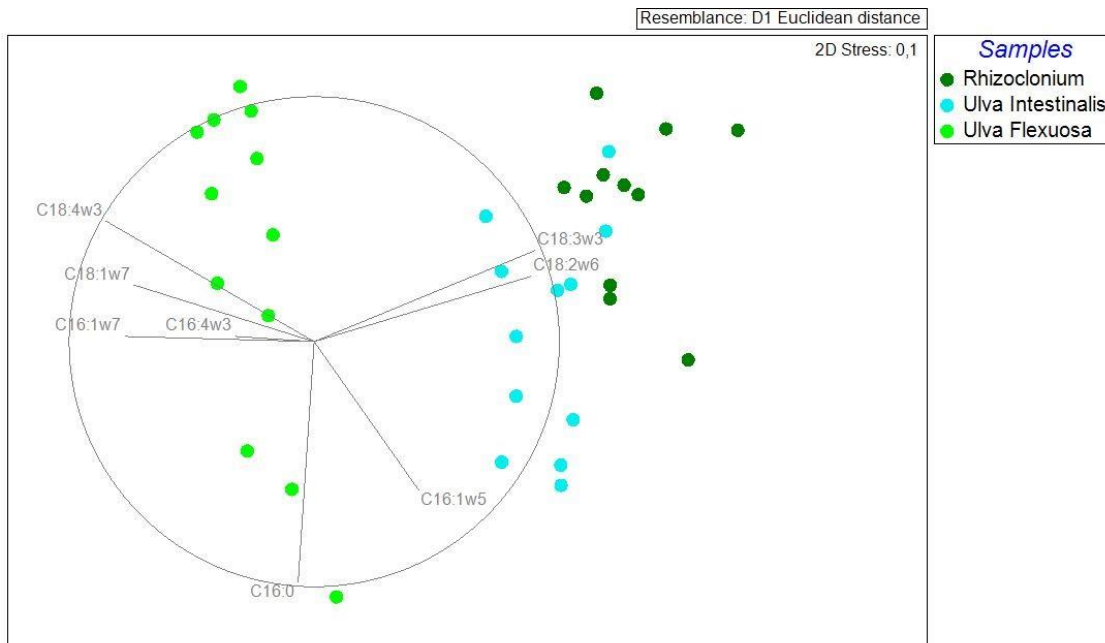
ANNEX 7: MDS (Multidimensional scaling) plot with comparison between samples of oyster (n=12), zooplankton (n=18), suspended matter (n=18), and phytoplankton (n=18). Each point represents one sample. Vectors represent Pearson's correlation.



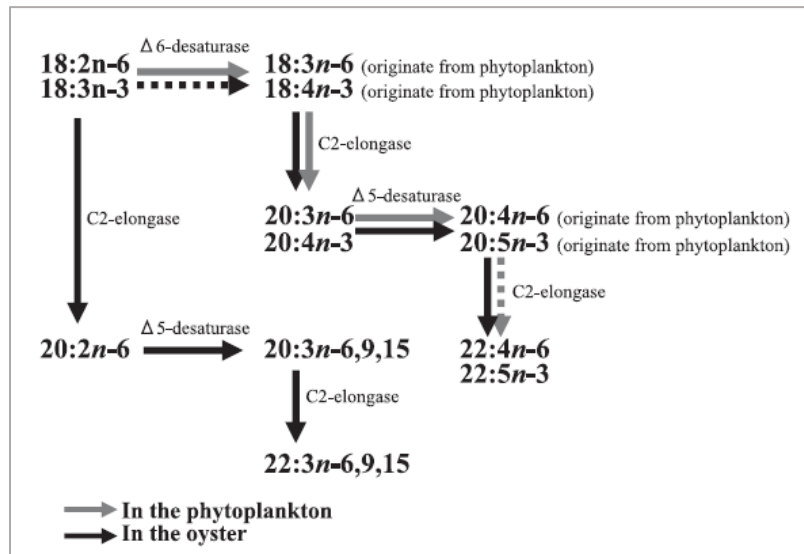
ANNEX 8a: Graph with comparison between the single profile of three algal species. Values are presented in % of TFA (percentage of total fatty acids) \pm s.d., and were obtained from the average of 12 samples for each species



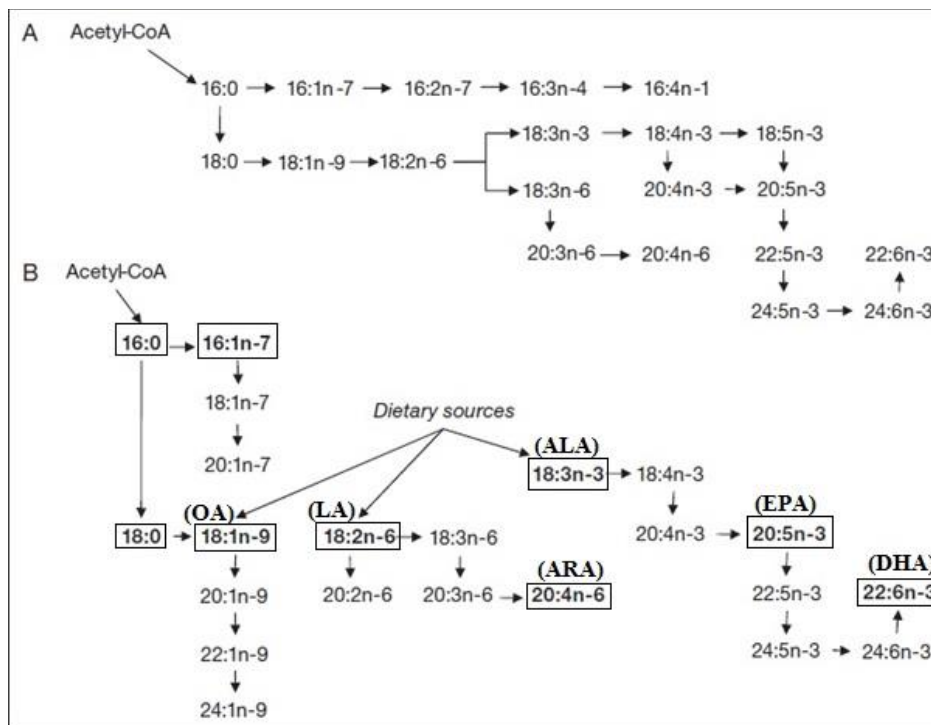
ANNEX 8 b: MDS (Multidimensional scaling) plot with comparison between all the samples of *Rhizoclonium riparium* (n=12), *Ulva intestinalis* (n=12) and *Ulva flexuosa* (n=12). Each point represents one sample. Vectors represent Pearson's correlation.



ANNEX 10: Biosynthesis of PUFA in oyster (Adapted from Saito & Marty, 2010)



ANNEX 11. Major fatty acid biosynthetic pathways in (A) plants and algae and (B) animals. FAs highlighted in (B) tend to accumulate at relatively high concentrations in animal tissues. Horizontal arrows represent desaturation, downward vertical arrows represent 2-carbon chain elongation, and upward vertical arrows represent β -oxidation. (Adapted from Kelly and Scheibling, 2012)



ANNEX 12: Statistics resume of ANOSIM with pairwise tests.

Global Test **PHYTOPLANKTON**

Sample statistic (Global R): 0,122

Significance level of sample statistic: **6,8%**

Number of permutations: 999 (Random sample from 2858856)

Number of permuted statistics greater than or equal to Global R: 67

Pairwise Tests

Groups	Statistic	R	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
FOA, FO	0,276		4,3	462	462	20
FOA, FA	-0,044		69,9	462	462	323
FO, FA	0,12		11,7	462	462	54

Global Test **SUSPENDED MATTER**

Sample statistic (Global R): 0,02

Significance level of sample statistic: 30,8%

Number of permutations: 999 (Random sample from 1009008)

Number of permuted statistics greater than or equal to Global R: 307

Pairwise Tests

Groups	Statistic	R	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
FOA, FO	0,032		34,9	126	126	44
FOA, FA	0		40,3	462	462	186
FO, FA	0,005		34,2	462	462	158

Global Test **ULVA FLEXUOSA**

Sample statistic (Global R): 0,291

Significance level of sample statistic: **3,2%**

Number of permutations: 462 (All possible permutations)

Number of permuted statistics greater than or equal to Global R: 15

Global Test **ULVA INTESTINALIS**

Sample statistic (Global R): 0,22

Significance level of sample statistic: 6,5%

Number of permutations: 462 (All possible permutations)

Number of permuted statistics greater than or equal to Global R: 30

Global Test **RHIZOCLONIUM RIPARIUM**

Sample statistic (Global R): -0,064

Significance level of sample statistic: 68%

Number of permutations: 462 (All possible permutations)

Number of permuted statistics greater than or equal to Global R: 314

Global Test **OYSTER**

Sample statistic (Global R): 0,967

Significance level of sample statistic: **0,2%**

Number of permutations: 462 (All possible permutations)

Number of permuted statistics greater than or equal to Global R: 1

Global Test **ZOOPLANKTON**

Sample statistic (Global R): 0,049

Significance level of sample statistic: 34,7%

Number of permutations: 378 (All possible permutations)

Number of permuted statistics greater than or equal to Global R: 131

Pairwise Tests

Groups	Statistic	R	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
FOA, FA	-0,073		52,4	21	21	11
FOA, FO	0,018		38,1	21	21	8
FA, FO	1		33,3	3	3	1

Global Test **MEAGRE**

Sample statistic (Global R): 0,224

Significance level of sample statistic: **3,3%**

Number of permutations: 999 (Random sample from 2858856)

Number of permuted statistics greater than or equal to Global R: 32

Pairwise Tests

Groups	Statistic	R	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
FOA, FO	0,493		0,9	462	462	4
FOA, FA	0,309		4,8	462	462	22
FO, FA	-0,059		61,5	462	462	284

Global Test **WHITE SEABREAM**

Sample statistic (Global R): -0,025

Significance level of sample statistic: 54,9%

Number of permutations: 999 (Random sample from 2858856)

Number of permuted statistics greater than or equal to Global R: 548

Pairwise Tests

Groups	Statistic	R	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
FOA, FO	-0,046		59,7	462	462	276
FOA, FA	0,067		19	462	462	88
FO, FA	-0,061		61,3	462	462	283

Global Test **MULLET**

Sample statistic (Global R): 0,388

Significance level of sample statistic: **0,3%**

Number of permutations: 999 (Random sample from 2858856)

Number of permuted statistics greater than or equal to Global R: 2

Pairwise Tests

Groups	Statistic	R	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
FOA, FO	0,624		0,2	462	462	1
FOA, FA	0,406		1,3	462	462	6
FO, FA	0,167		11,7	462	462	54

ANNEX 13: P-values obtained for fatty acids that contributed more for dissimilarities between paired treatments.

phytoplankton	FOA-FO	C18:2 ω 6* 0.002165	C16:1 ω 7 0.6991	C18:1 ω 7 0.1494	EPA 0.1447	C16:0 0.0649		
<i>ulva flexuosa</i>	FOA-FA	C18:3 ω 3* 0.001757	C16:4 ω 3* 0.02034	C18:4 ω 3 0.08751				
oyster	FOA-FO	C18:2 ω 6* 0.002165	C16:1 ω 7* 0.00217	C14:0* 0.005	C18:3 ω 3* 0.00217	C18:1 ω 9* 0.00217	EPA* 0.00656	C18:1 ω 7* 0.0082
meagre	FOA-FO	DHA* 0.007292	C18:0 0.113	C18:2 ω 6 0.1823	C16:0 0.0755			
	FOA-FA	DHA* 0.0125	18:2 ω 6 0.6164	C18:0 0.153				
mullet	FOA-FO	C18:0* 3.24E-05	DHA* 0.00193	C18:2 ω 6 0.9073	C16:1 ω 7* 0.00213	C14:0* 0.00217	C18:1 ω 9* 0.00515	ARA* 0.00091
	FOA-FA	C18:0 0.1943	DHA* 0.00515	C18:2 ω 6* 0.0274	C16:0 0.6523	C18:1 ω 9* 0.04511	C14:0* 0.04113	ARA* 0.02311