Universidade de Aveiro
Ano 2022

Luisa da
Costa Marques

Avaliação do potencial das ascídias em sistemas de aquacultura multi-trófica integrada e sua distribuição na Ria de Aveiro

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

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Aos meus filhos e marido - obrigada

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palavras-chave

Bioincrustação, IMTA, Tunicados, Ácidos gordos essenciais, Sustentabilidade, Taxa de filtração, Espécies extrativas

resumo

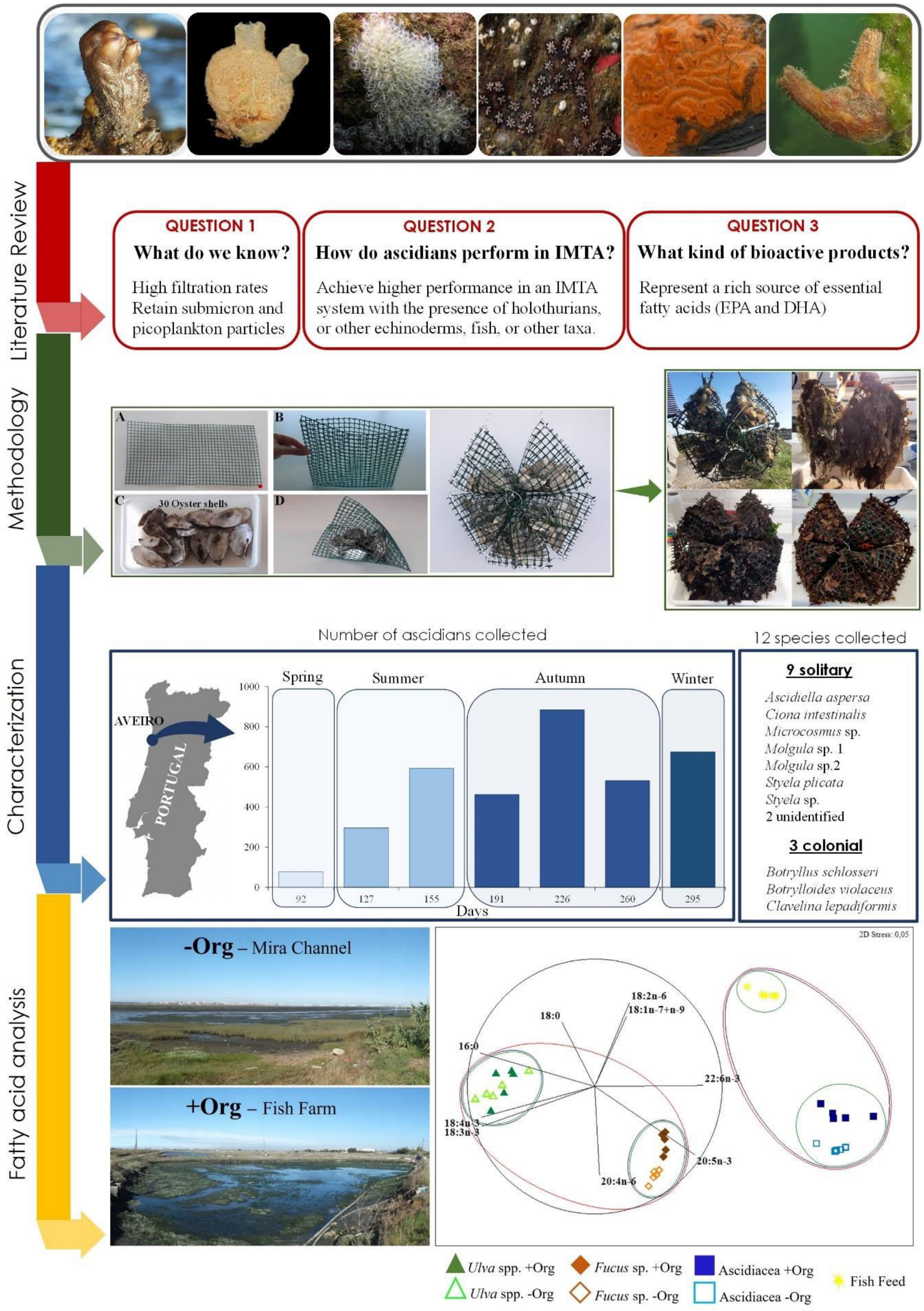
A intensificação da produção da aquacultura requer o uso de mais ração por área, aumentando assim a produção de efluentes. Inevitavelmente, a aquacultura irá gerar sempre estas descargas, as quais muitas vezes são um obstáculo para o meio ambiente e os quais representam um encargo económico para o produtor. A preocupação para que a aquacultura seja mais sustentável tem se tornado de interesse público. Num sistema operacional de Aquacultura Multi-trófica Integrada (IMTA), a recuperação e reciclagem de nutrientes não consumidos, excretados e não assimilados são realizados por espécies extrativas que pertencem a diferentes níveis tróficos. Este sistema possibilita a combinação de diferentes espécies que poderão representar um manancial de biomassa adicional com elevado valor de mercado. O principal objetivo deste estudo foi investigar o potencial de ascídias como espécies extrativas em sistemas de IMTA. Para atingir este objetivo, o presente estudo foi dividido em três tarefas principais. A primeira foi focada numa revisão bibliográfica sistemática de modo a compreender a importância ecológica das ascídias como filtradoras eficientes, investigar a sua contribuição como espécies extrativas, estabelecer um padrão do seu valor nutricional e determinar o seu potencial para a indústria da aquacultura. Posteriormente, foi realizado um estudo para monitorizar a comunidade bioincrustrante de ascídias existente na Ria de Aveiro através do uso de uma unidade personalizada 3D de substrato artificial (ASU), recorrendo a conchas de ostras como substrato natural, um co-produto resultante da atividade ostrícola no local de estudo. A última etapa foi a realização de uma avaliação do perfil lipídico das ascídias (Ascidiacea) e de algas marinhas (Ulva spp. e Fucus sp.) presentes na Ria de Aveiro, sob a influência de efluentes ricos em matéria orgânica provenientes da atividade de produção piscícola, com o objetivo de identificar e quantificar os ácidos gordos presentes. O presente estudo revelou que nos últimos anos, as ascídias ganharam popularidade no que diz respeito ao seu papel na indústria da aquacultura. De um modo geral, as ascídias apresentam uma alta capacidade de filtração, o que as torna excelentes espécies extrativas em sistemas de IMTA. Além disso, as ascídias têm demonstrado ser candidatas promissoras como ingredientes para a formulação de rações, visto serem ricas em ácidos gordos essenciais, melhorando assim a qualidade do peixe produzido em aquacultura. Os 295 dias da experiência de campo, que decorreram na Ria de Aveiro, permitiram a recolha de 12 espécies de ascídias. Duas destas espécies, Clavelina lepadiformis e Ascidiella aspersa foram recolhidas e registadas pela primeira vez para Portugal continental. O estabelecimento das ascídias em um determinado substrato é influenciado pela hidrodinâmica, sazonalidade e pela presença de locais ricos em nutrientes. Adicionalmente, as ascídias mostraram preferência por locais com algum grau de atividade aquícola. A ASU utilizada pode ser explorada como uma estrutura de apoio eficiente para a monitorização a curto ou longo prazo da comunidade de ascídias, bem como de comunidades incrustantes. Relativamente ao perfil de ácidos gordos, este revelou que as ascídias e algas marinhas que se encontram sob a influência de efluentes ricos em matéria orgânica apresentam diferenças significativas no seu perfil lipídico. Verificou-se ainda que estas quando geradas em ambientes de atividade piscícola são fontes valiosas de ácidos gordos, promotores da saúde humana. O conhecimento científico obtido contribuiu para um primeiro passo na validação do uso de ascídias em sistemas IMTA, evidenciando a valorização da sua biomassa e o contributo para o crescimento económico.

keywords Biofouling, IMTA, Tunicates, Essential fatty acids, Sustainability, Filtration rate, Extractive species

abstract

Intensification of aquaculture production requires the use of more feed per area, increasing waste generation. Unavoidably, aquaculture productions will always generate waste inputs and they are often a nuisance to the environment with no economic value. The concern for aquaculture to be sustainable has made it a public interest. In an operational Integrated Multi-Trophic Aquaculture (IMTA) system, the recovery and recycling of uneaten feed and wastes are performed by extractive species belonging to different trophic levels. These systems enable the combination of different species, and they represent valuable market potential. The main objective of this thesis was to investigate the potential of ascidians as an extractive species in an IMTA framework. To achieve these goals, the present study was divided into three main tasks. The first step was focused on a systematic review to understand the ecological importance of ascidians as efficient filter-feeders, investigate their potential contribution as extractive species, set the benchmark for their nutritional value and potential added value to the aquaculture industry. Afterward, a field survey to monitor and explore the existing ascidian biofouling community present in the Ria de Aveiro was performed through a customized 3-D artificial substrate unit (ASU) using oyster shell waste as a nature-based substrate, an available co-product of oyster farming from the study site. The final and third step examined the lipid profile of ascidians (Ascidiacea) and seaweed (Ulva spp. and Fucus sp.) present in the Ria de Aveiro under the influence of organic-rich effluent originated from fish farming activities, to identify and quantify the fatty acids present. The present study revealed that in recent years, ascidians have gained popularity concerning their role in the aquaculture industry. Overall, ascidians present a high ability to filtrate large quantities of water, enabling them to be excellent potential extractive species in an IMTA framework. Furthermore, ascidians have shown to be promising candidates as ingredients for aquafeed formulations, as they are rich in essential fatty acids, improving the quality of fish produced in aquaculture. The 295-day field research, conducted in the Ria de Aveiro, allowed the retrieval of 12 ascidian species. Two of which, Clavelina lepadiformis and Ascidiella aspersa were for the first time, collected and registered for mainland Portugal. Ascidian establishment showed to be influenced by hydrodynamics, seasonality, and by the availability of nutrient-rich locations. Additionally, ascidians showed a preference for locations with some degree of aquaculture activity. The ASU employed can be explored as an efficient support structure for short- or long-term monitoring of the ascidian community, as well as fouling communities. Investigation regarding the fatty acid profile revealed that ascidians and seaweeds under organic-rich effluents display significant differences in their lipid profile. Furthermore, when generated in such environment, they are valuable sources of fatty acids, promoters of human health. The scientific knowledge achieved contributes to an initial step toward incorporating ascidians in IMTA systems, providing marine biomass valorization and economic growth, using its by-products and simultaneously reducing the environmental impact caused by waste from aquaculture farms.

Graphical Abstract



QUESTION 1

What do we know?

High filtration rates
Retain submicron and
picoplankton particles

QUESTION 2

How do ascidians perform in IMTA?

Achieve higher performance in an IMTA system with the presence of holothurians, or other echinoderms, fish, or other taxa.

QUESTION 3

What kind of bioactive products?

Represent a rich source of essential fatty acids (EPA and DHA)

12 species collected

9 solitary

Ascidiella aspersa
Ciona intestinalis
Microcosmus sp.
Molgula sp. 1
Molgula sp.2
Styela plicata
Styela sp.
2 unidentified

3 colonial

Botryllus schlosseri
Botrylloides violaceus
Clavelina lepadiformis

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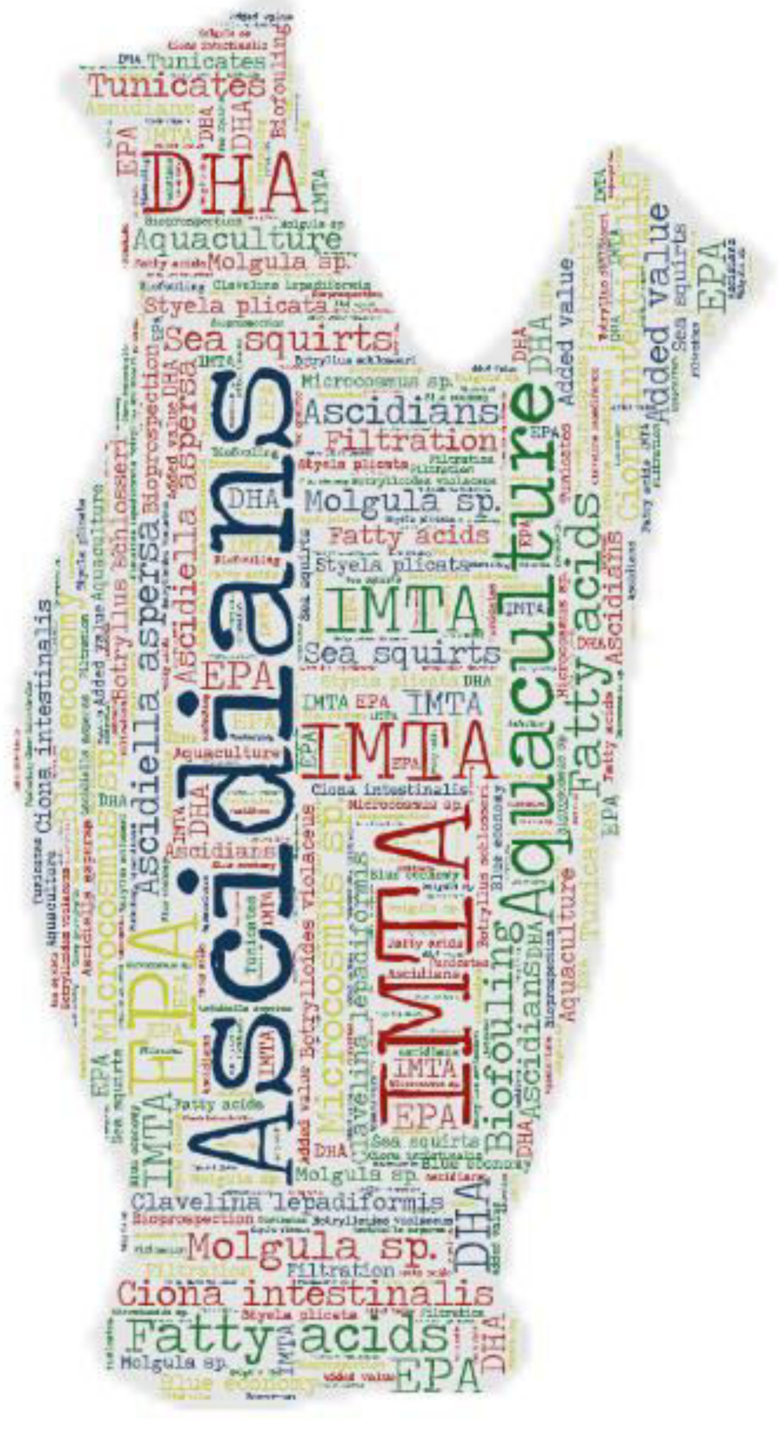
List of Fatty Acid Nomenclature

Class	Shorthand formula	Common name
Branched fatty acid	*iso* 14:0	12-methyl-tridecanoate
	iso 15:0	13-methyl-tetradecanoate
	anteiso 15:0	12-methyl-tetradecanoate
	iso 16:0	14-methyl-pentadecanoate
	anteiso 16:0	13- methyl-pentadecanoate
	iso 17:0	15-methyl-hexadecanoate
	anteiso 17:0	14-methyl-hexadecanoate
Saturated fatty acid	14:0	Tetradecanoic acid
	15:0	Pentadecanoic acid
	16:0	Palmitic acid
	17:0	Margaric acid
	18:0	Stearic acid
	20:0	Arachidic acid
	21:0	Heneicosanoic acid
	22:0	Behenic acid
	24:0	Lignoceric acid
Monounsaturated fatty acid	15:1	Pentadecenoic acid
	16:1	Palmitoleic acid
	16:1*n*-9	7-hexadecenoic acid
	16:1*n*-7	Palmitoleic acid
	17:1	Margaric acid
	17:1*n*-9	8-heptadecenoic acid
	methyl-heptadecanoate	Heptadecanoic acid
	18:1*n*-9	Oleic acid
	18:1*n*-7	Vaccenic acid
	20:1	Eicosenoic acid
	20:1*n*-9	Gondoic acid
	20:1*n*-7	13-eicosenoic acid
	22:1*n*-11	11-docosenoic acid
	22:1*n*-9	Erucic acid
	24:1*n*-9	Nervonic acid
Polyunsaturated fatty acid	16:2	Hexadecadienoic acid
	16:2*n*-6	7,10-hexadecadienoic acid
	16:3*n*-3	7,10,13-hexadecatrienoic acid
	18:2	Octadecadienoic acid
	18:2*n*-6 (LA)	Linoleic acid
	18:3*n*-6	γ-linolenic acid
	18:3*n*-3 (ALA)	α-linolenic acid
	20:2*n*-6	Eicosadienoic acid
	20:3*n*-6 (DGLA)	Dihoma-γ-linoleic acid
	20:3*n*-3 (ETE)	Eicosatrienoic acid
Highly unsaturated fatty acid	16:4*n*-3	4,7,10,13-hexadecatetraenoic acid
	18:4*n*-3	Stearidonic acid
	20:4*n*-6 (ARA)	Arachidonic acid
	20:4*n*-3 (ETA)	Eicosatetraenoic acid
	20:5*n*-3 (EPA)	Eicosapentaenoic acid
	22:4	Adrenic acid
	22:5*n*-6	Docosapentaenoic acid
	22:5*n*-3 (DPA)	Docosapentaenoic acid
	22:6*n*-3 (DHA)	Docosahexaenoic acid

Acronym List	
3D	Three-Dimensional
AFDW	Ash Free Dry Weight
ASU	Artificial Substrate Unit
BCFA	Branched Fatty Acids
BIC	Bayesian Information Criterion
CO_2	Carbon dioxide
DHA	Docosahexaenoic acid
DistLM	Distance-Based Linear Model
DNA	Deoxyribonucleic acid
EPA	Eicosapentaenoic Acid
EU	European Union
FA	Fatty Acid
FAME	Fatty Acids Methyl Esters
FH	Fishing Harbour
GC-MS	Gas Chromatography-Mass Spectrometry
GFFC	Glass Fiber Filters
HCT116	Human colon cancer cells
IMTA	Integrated Multi-trophic Aquaculture Farm
IMTA	Integrated Multi-trophic Aquaculture
M	Marina
MDS	Multidimensional Scaling
MUFA	Monounsaturated Fatty Acids
n-*3*	Omega-3
n-*6*	Omega-6
NIS	Non-Indigenous Species
$N\text{-}NH_4$	Ammonium
$N\text{-}NO_x$	Nitrogen oxides
OF	Oyster Farm
OSPAR	Convention for the Protection of the Marine Environment of the North-East Atlantic
P(MC)	Monte Carlo p
POM	Particulate Organic Matter
$P\text{-}PO_4$	Orthophosphate
PUFA	Polyunsaturated Fatty Acids
PVC	Polyvinyl Chloride
RAS	Recirculatory Aquaculture System
SD	Standard Deviation
SDG	Sustainable Development Goals
SFA	Saturated Fatty Acids
SIMPER	Similarity Percentage Species Contributions
SPM	Suspended Particulate Matter
TG	Tide Gauge
UN	United Nation

Chapter 1

Introduction


Ascidians
IMTA
Aquaculture
DHA
EPA
Fatty acids
Tunicates
Sea squirts
Molgula sp.
Styela plicata
Ciona intestinalis
Clavelina lepadiformis
Ascidiella aspersa
Botryllus schlosseri
Botrylloides violaceus
Microcosmus sp.
Biofouling
Bioprospection
Filtration
Added value
Blue economy

1. Introduction

1.1 Aquaculture worldwide

In a world with an ever-growing human population, the aquaculture industry has undergone one of the main food production systems of the future, thus its recognition as one of the fastest-growing animal-food-producing sectors. Despite its impressive growth rate over the past decades (FAO, 2022), the increasing need for protein delivery is a major challenge that concerns researchers and leaders worldwide. To meet these demands, aquaculture production will depend on new research, improved management practices, proper technology, and financial resources, along with balancing the environmental impacts. According to Chopin and Tacon (2021), aquaculture represents 54.1% of total world fisheries and aquaculture production, and marine and coastal aquaculture represent 55.2% of total world aquaculture production. Considering only marine and coastal aquaculture, seaweed represented 51.3%, followed by mollusks (27.4%), finfish (11.6%), crustaceans (9.1%), and other aquatic animals (0.6%) in 2018 (FAO, 2022).

In Europe, marine aquaculture accounts for about 20% of fish and shellfish, with seven countries contributing to total production (Norway, Spain, Turkey, United Kingdom, France, Italy, and Greece, in descending order) (European Environment Agency, 2021, accessed 06 June 2022, https://www.eea.europa.eu/). The most cultivated species are Atlantic salmon, blue mussels, rainbow trout, European seabass, Gilthead Sea bream, oysters, and carp. In 2019, the consumption of fish and seafood by EU citizens was about 24 kg and circa one-quarter of this amount originated from aquaculture. However, consumption varies between member states in the EU, with Portugal being by far the leader in terms of consumption per capita (more than twice the average EU level) (EUMOFA, 2019). Despite these commercial prospects, the EU's contribution to world aquaculture production represented less than 2% of the global output in 2018 (FAO, 2022).

The intensification of global aquaculture production has raised many issues regarding the three pillars of sustainability: economic, social, and environmental (Neori et al., 2007). Fortunately for the aquaculture industry and the planet’s well-being, much progress has been made in the name of sustainability. Following the Millennium Development Goals, in 2015 the United Nations (UN) released the Sustainable Development Goals (SDG) to improve global sustainability by the year 2030 (United Nations A/RES/70/1, 2015). This common vision will guide governmental authorities in promoting sustainable economic strategies, safety and security, well-being, and education. Consequently, aquaculture has a key role to play in contributing to achieving several of the SDG’s goals. Out of the 17 goals, seven are the most related to the marine aquaculture industry. SDG1 “no poverty”: aquaculture can provide a significant socio-economic contribution in coastal and rural communities; SDG2 “zero hunger”: seafood production may increase by one-third by 2030; SDG3 “promoting health and well-being”: fish and shellfish are a rich source of protein, omega-3 fatty acids, vitamins and minerals; SDG12 “responsible consumption and production”: the search for alternative aquafeeds is a growing market; SDG13 “climate change”: onshore facilities enable seafood to be produced close to large marketplaces and urban areas, reducing the distance of transport thus reducing CO_2 emissions; SDG14 “life below water”: land-based production of seafood can lessen the pressure on oceans as well; SDG17 “partnership for the goals”: forming partnerships and cooperations with national and international organizations and governments will allow interconnectivity among countries, thus promoting international trades and financial support. Conversely, new research has alerted that the socio-economic fallout caused by the global pandemic - COVID-19 could have major impacts, delay, or even aggravate the SDG's initial goals (Munasinghe, 2020; Sumner et al., 2020; Oxfam International, 2021). Furthermore, a UN study indicates that, if done correctly, aquaculture can increase food production, promote economic growth, and improve nutrition for the population (Stead, 2019).

The aftermath of the COVID-19 pandemic revealed many systemic issues, in particular, that our food system cannot be resilient to such a crisis. The pandemic highlighted the importance of redesigning and placing our food system on a sustainable path. Therefore, the European Green Deal established a goal to make Europe the first climate-neutral continent by 2050 (The European Green Deal, 2019). To accomplish this goal, a set of principles to boost the economy, improve quality of life and health, and care for nature were laid out. The Farm to Fork Strategy is at the core of the European Green Deal aiming to make food systems fair, healthy, and environmentally friendly, and to strengthen their resilience (Farm to Fork Strategy, 2020). The main purpose of this strategy is to aid the transition to a sustainable food system by promoting sustainable food production, processing and distribution, consumption, and reducing food waste (Farm to Fork Strategy, 2020).

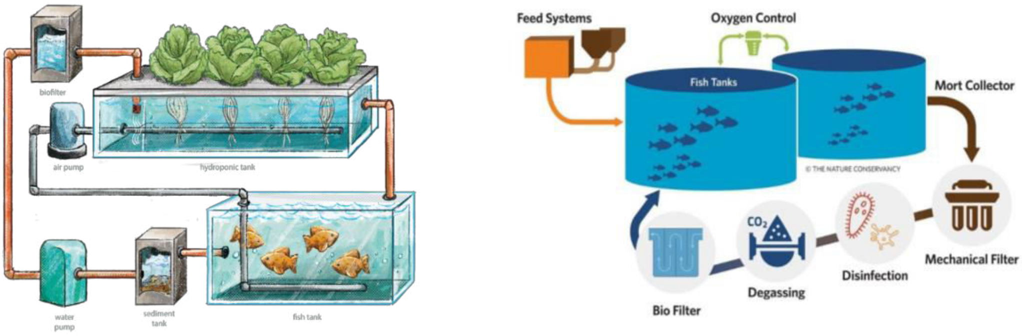
1.2 Aquaculture farming

Aquaculture is a simple word and yet an overly complex concept. Aquaculture production is commonly practiced using extensive, semi-intensive, and intensive systems (Welcomme and Bartley, 1998). Extensive aquaculture (Fig. 1.1A), also known as open systems, involves fish and shellfish production at density levels identical to those found in nature. These systems require limited maintenance: farm ponds are not fertilized or aerated, nor are aquafeeds provided (Naylor et al., 2000). Semi-intensive aquaculture systems (Fig. 1.1B) produce aquatic organisms at higher density levels naturally seen in nature. They are considered mid-level technology systems as they require a greater degree of maintenance. Hands-on management and energy investment such as aeration and aquafeeds are supplemented. Therefore, these systems usually have a higher return rate of production (Lin et al., 1990). Intensive aquaculture systems (Fig. 1.1C), are high-tech systems considering that farmers must provide for all the biological needs of the cultured organism and all maintenance requirements: continuous aeration, filtrations, and waste management. Yields from these systems can greatly exceed those of extensive and semi-intensive systems, as they have very high production costs associated but, on the other hand, allow total control over the production (Lin et al., 1990). Examples include recirculating aquaculture systems and aquaponics. The practice of intensive aquaculture farming is gaining distinction as technological advances allow better risk management (Tucker, 2008).

A) Extensive **B) Semi-Intensive** **C) Intensive**

Figure 1.1: Aquaculture types of systems. A) Extensive (source: aquacultures.wordpress.com); B) Semi-intensive (source: bentoli.com); C) Intensive (source: en.wikipedia.org).

Aquaponics is an ecologically applied combination of hydroponics (soilless plant production) with aquaculture (fish farming) in a closed-loop system (Figure 1.2A). In aquaponics, the nutrients are provided primarily from fish waste byproducts, rather than the inorganic soluble nutrient salts used in traditional hydroponics (Lennard, 2015). Furthermore, aquaponic systems are extremely water-conscious, conserving up to 90% of the water (Diver, 2006). Because the plants remove the nutrients from the water, aquaponic systems do not require water exchange. In addition, advantages such as reduced land and fertilizer usage, dependence on the weather conditions, no soil and therefore no weeding is necessary, plant growth is accelerated, extra income for the aquaponic farmers, food can be grown all year, and reduced carbon footprint are all equally important features in these systems (Lennard, 2015; Palm et al., 2018).



A) Aquaponic System **B)** Recirculating Aquaculture System (RAS)

Figure 1.2: Conceptual representation of two closed-looped systems: (A) aquaponic system (source: earth.org) and (B) recirculating aquaculture system (source: asagriaqua.com).

Recirculatory Aquaculture System (RAS) is a technology where water is recycled and reused after mechanical and biological filtration (Figure 1.2B). This method can be used for the culture of various species of fish, typically reared in indoor/outdoor tanks in a controlled environment (Badiola et al., 2012). The removal of suspended matter and metabolites are either removed or converted into non-toxic products by the system components and the purified water is subsequently saturated with oxygen and returned to the fish tanks (Martins et al., 2010a). The management of these systems relies mainly on the quantity and quality of feed and the type of filtration, with the overall goal of providing good water quality for cultured aquatic organisms. This technology has become a solution for large-scale and sustainable fish production and offers several significant advantages: reduction of disease contamination due to the controlled environment in which fish are produced; healthier fish, as no antibiotics or disease treatments are applied; reduction of transportation costs due to facilities built closer to markets; ability to harvest year-round, and faster growth with lower production costs (Badiola et al., 2012). However, the main disadvantage is the high investment necessary for infrastructures, operating costs, system maintenance, and the high-power supply dependency (Badiola et al., 2012; Turcios and Papenbrock, 2014).

1.3 Aquaculture and sustainable development

Large-scale aquaculture has evolved substantially in the past 20 years (Naylor et al., 2021). At the beginning of the 21st century, as the search for innovative and sustainable practices kept growing, Chopin (2004), introduced the concept of the practice of Integrated Multi-trophic Aquaculture (IMTA).

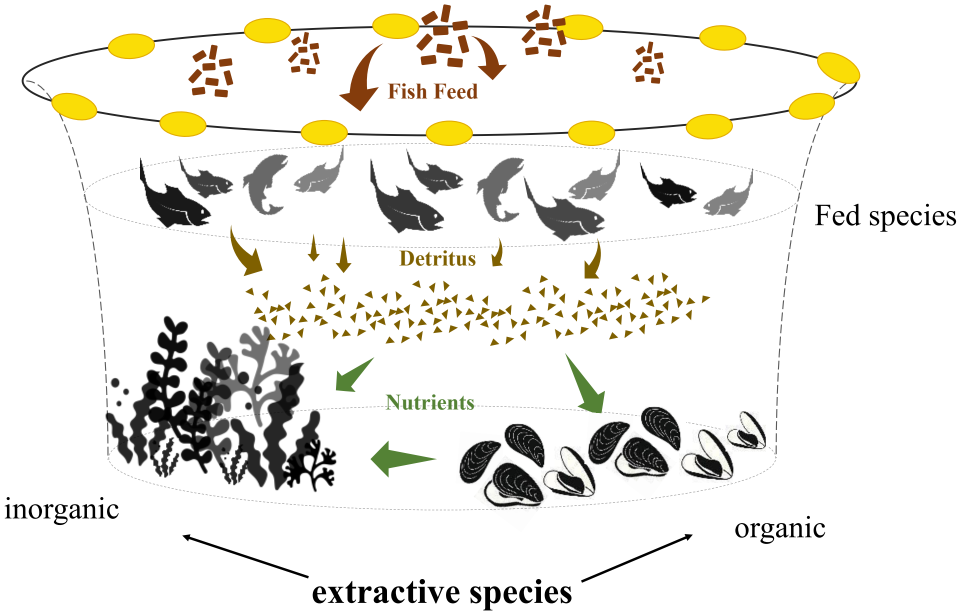


Figure 1.3: Conceptual representation of an Integrated Multi-Trophic Aquaculture (IMTA) system.

This concept is extremely broad, flexible, and evolving as it can be applied worldwide to open-water or land-based systems, marine or freshwater environments, and temperate or tropical climates (Chopin, 2021). An IMTA framework (Figure 1.3) is a nature-based solution in which the by-products, wastes, uneaten feed, and nutrients from one species are reused and converted to become fertilizer, feed, and energy for the growth of another species, commonly at a different trophic level (Naylor and Burke, 2005). These systems may include several different combinations of co-cultured species. IMTA aims at mimicking a natural ecosystem by combining and incorporating complementary species from different trophic levels in the same productive environment. Extractive species uptake excess organic and inorganic matter contributing to reduce costs to comply with environmental regulations. In addition, their potential market value (*e.g.*, food, feed, pharma) might provide extra economic benefits to farmers. Selected species should be cultured at densities that optimize nutrient uptake, promote a stable balance between biological and chemical processes improve ecosystem's health, and should be economically important as aquaculture products (Alexander et al., 2016). However, making the concept of carrying capacity operational presents multiple challenges to farmers.

Nonetheless, an IMTA framework also presents numerous benefits, including the decrease in waste outputs from overall farming activities (Resende et al., 2022), the additional production of a marketable product for little or no additional input cost, and more importantly, environmentally sustainable farming operations (Barrington et al., 2009; Troell et al., 2009; Khanjani et al., 2022). The open key question concerns the optimization of the uptake of particulate and dissolved organic matter from uneaten/undigested feed and feces (Nederlof et al., 2022). This excess of nutrients may ultimately create a bio-deposit and lead to eutrophicated waters (Chopin et al., 2001; Buschmann and Hernandez-Gonzalez, 2021) or represent an economic burden to fish farmers (Fry et al., 2016).

Over the last 15 years, scientific knowledge addressing IMTA systems associated with different extractive species has increased (Figure 1.4), with a particular focus on extraction capacity, growth performance, and overall production improvement (Alexander et al., 2016; Ju et al., 2016; Fossberg et al., 2018; Grosso et al., 2021; Jerónimo et al., 2021; Mazón-Suástegui et al., 2022).

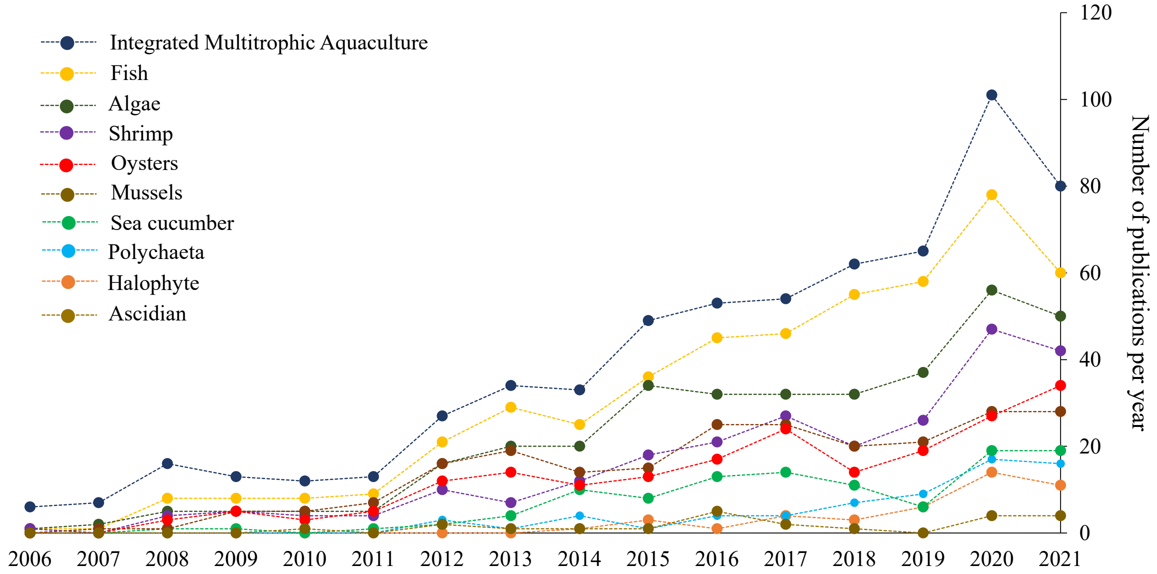


Figure 1.4: Number of publications per year retrieved using "integrated multitrophic aquaculture" OR "integrated multi-trophic aquaculture” OR "IMTA" AND fish, *algae, shrimp*, oyster*, mussel*, “sea cucumber”, polychaet*, halophyte*, and ascidian* OR tunicate* OR “sea squirt*” as search topic in Scopus between 2006 and 2021.

Some of the most studied organisms used in these systems are various fish species (Wang et al., 2013; Buck et al., 2018; Ellis and Tiller, 2019), macro and microalgae (Holdt and Edwards, 2014; Andreotti et al., 2017; Milhazes-Cunha and Otero, 2017; Li et al., 2019; Shpigel et al., 2019; Laramore et al., 2022), shrimp (Omont et al., 2020; Pinheiro et al., 2020), oysters (Ferreira et al., 2012; Biswas et al., 2020; Omont et al., 2020), mussels (Cranford et al., 2013; Sterling et al., 2016; Sanz-Lazaro and Sanchez-Jerez, 2017), sea cucumbers (Yu et al., 2012; Zhen et al., 2014; Zamora et al., 2018; Israel et al., 2019), polychaetes (Carvalho, 2007; Marques et al., 2018; Jerónimo et al., 2020, 2021), halophytes (Webb et al., 2012; Buhmann and Papenbrock, 2013; Marinho et al., 2015; Marques et al., 2017; Pinheiro et al., 2020), and ascidians (Zhen et al., 2014; Ju et al., 2015; Petersen, 2016).

1.4 Ascidians – an overview

Ascidians, most commonly known as sea squirts, are marine filter-feeder organisms with fast growth rates (Millar, 1952) that can be found from shallow water to the deep sea. There are no freshwater species, as they do not tolerate salinities below 25 (Lambert, 2005; Watts et al., 2015). There are approximately 3000 described species worldwide. The class Ascidiacea consists of three suborders based on the structure of the adult branchial sac: Aplousobranchia, Phlebobranchia, and Stolidobranchia, Lahille 1886. Some ascidians can be solitary while others may form colonies with many small individuals called zooids, and these are classified as colonial ascidians (Petersen, 2007).

They are round or cylindrical organisms ranging from about 5 to 200 mm in length (Gasparini and Ballarin, 2018). These organisms generate a one-way current through their bodies, filtering particulate organic matter from the water column via an oral siphon and expelling filtered water through the atrial siphon (Jørgensen and Goldberg, 1953; Jørgensen, 1954). Ascidians are hermaphrodites and reproduce by external or internal fertilization (Honegger, 1986). Despite living the great majority as sessile individuals, however, they have a short lifespan and mobile larval state (Barnes, 1990). The microscopic lecithotrophic larvae are incapable of long-distance dispersal (Petersen and Svane, 1995); however, they may travel long distances due to human intervention, such as through ballast waters and fouling on the hulls of ships or due to translocations of cultivated shellfish for aquaculture purposes (Lambert, 2001; Locke et al., 2007). For this reason, ascidians are considered important bioindicators of anthropogenic transport (Marins et al., 2010).

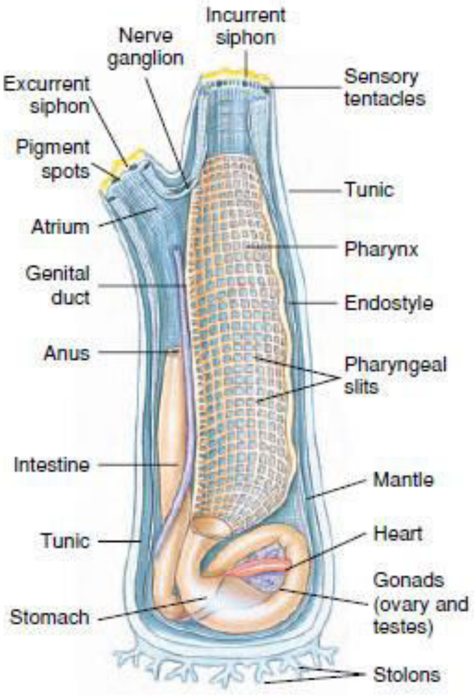


Figure 1.5: Structure of a common ascidian, *Ciona* sp. (source: biocyclopedia.com).

Furthermore, several ascidians are wild-harvested or cultured with the purpose to be eaten raw, cooked, dried, or pickled (Hirose et al., 2009; Lambert et al., 2016). In many countries, ascidians are considered delicious delicacies. Ascidian *Halocynthia roretzi* Drasche, 1884, commonly known as "sea pineapple" is produced in Japan and Korea and it is most commonly eaten raw as sashimi being served with vinegared soy sauce. It can also sometimes be salted, smoked, grilled, deep-fried, or dried (Lambert et al., 2016). *Styela clava*, Herdman, 1881 is used in Korean cuisine by being added to various seafood dishes (Sool and Seok, 1998). From the Mediterranean Sea, *Microcosmus*, Heller, 1877, species are eaten in France, Italy, and Greece in many diverse ways, for example, raw with lemon, or in salads with olive oil, lemon, and parsley (Voultsiadou et al., 2007). *Pyura chilensis*, Molina, 1782, is used in Chilean cuisine, eaten both raw and in stews (Davis, 1995). In Australia, the former food source *Pyura praeputialis,* Heller, 1878, is now mainly used as fishing bait (Lambert et al., 2016). Ascidians can colonize a variety of natural and artificial substrates (Millar, 1971; Lambert, 2007). Many ascidians are highly invasive because they possess strong competitive abilities, have wide environmental tolerances, can rapidly expand their geographic range, and are readily transported by human activities (Bullard et al., 2007).

Figure 1.6: Examples of some ascidian dishes (source: sercblog.si.edu; misschinesefood.com; onagawa-senrei.co.jp; neurosciencenews.com).

1.5 Biofouling ascidians and the aquaculture industry

Biofouling has been a worldwide problem for as long as humans have been sailing throughout the oceans. Marine biofouling is the gradual colonization of unwanted aquatic organisms in particular barnacles, polychaetes, bivalves, bryozoans, seaweeds, and ascidians on natural and/or artificial surfaces immersed in, or exposed to, the aquatic environment (Callow and Callow, 2002; Fitridge et al., 2012; Sievers et al., 2017). Generally, biofouling organisms can cause negative impacts, such as degradation and corrosion of structures, also being able to decrease the efficiency of moving parts. Furthermore, biofouling on other organisms can cause negative impacts on growth, cause deformities, and increase mortality (Fitridge et al., 2012; Sievers et al., 2017). In consequence, biofouling in marine aquaculture is one of the main barriers to achieve efficient and sustainable production, due to the serious economic impacts and spread of non-indigenous species (NIS) (Fitridge et al., 2012).

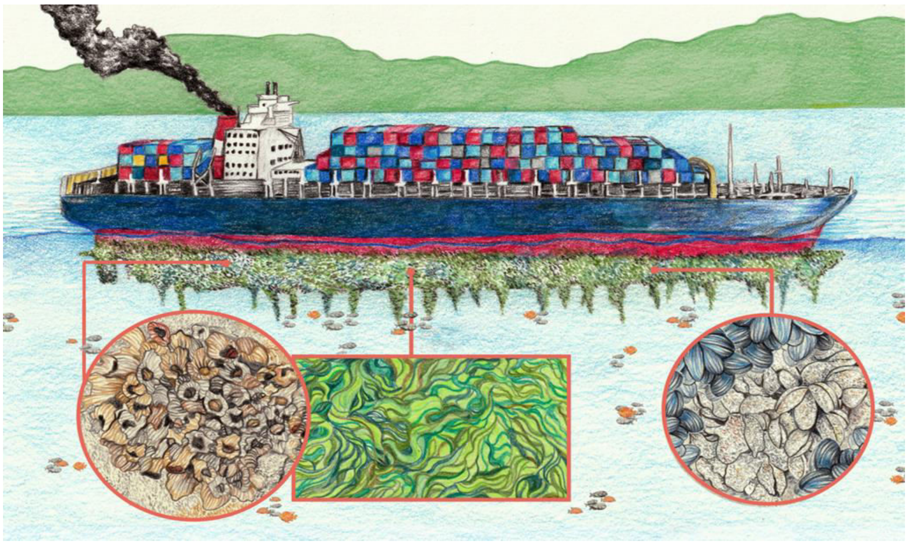


Figure 1.7: Example of biofouling marine organisms, how they are transported by ship, and the area that is the most susceptible to biofouling on a typical ship (source: chinadialogueocean.net/).

The discharge of ballast water and carriage of biofouling by vessels are the two most important pathways, through which potentially harmful marine organisms enter their non-native range (Hewitt et al., 2004; Bell et al., 2011). Traits like mobility, larvae dispersal, rapid growth, and tolerance to a broad range of environmental conditions such as salinity, temperature, and strength of ocean currents are characteristics of a biofouling capability. Once settlement occurs, biofouling organisms can expand rapidly outcompeting for space, food, or other factors with the native species or when natural enemies are absent (Bannister et al., 2019). However, these organisms do not always establish, spread, and cause impacts.

Globally, ascidians are recognized as significant contributors to benthic marine fouling communities. Nevertheless, it was only in the early 21st century that reports emerged addressing an unfamiliar slime-like fouling organism infesting offshore structures in locations where this phenomenon had not been previously noted (Kott, 2002). Biofouling ascidians are a serious problem as they are ubiquitous in coastal ecosystems (Lambert and Lambert, 1998) and are among the main colonizers of aquaculture gear, such as pipes, rope lines, buoys, cages, and nets (Rosa et al., 2013). Also, ascidians are amongst the most devastating biofoulers to shellfish and finfish aquaculture operations (Lambert, 2007; Adams et al., 2011). Some ascidian species often comprise over 80% of the organisms in the fouling community (Arsenault et al., 2009). Currently, there are many species responsible for mass fouling events; among these, the most common genera include *Didemnum*„ Savigny, 1816, *Ciona*, Fleming, 1822, *Styela*, Fleming, 1822, *Botrylloides*, Milne Edwards, 1841, *Botryllus*, Gaertner, 1774, and *Eudistoma*, Caullery, 1909, with the first genus being the most problematic of all (Aldred and Clare, 2014).

Figure 1.8: Example of marine organisms fouling polychaete production, mussels, ropes, aquaculture gear, ship hulls, buoys, and oyster nets (sources: www.marinebiosecurity.org.nz/; eurofish.dk/; www.ices.dk/; www.marinespecies.org/; mlcalliance.org/; web.whoi.edu/).

Regulating and mitigating biofouling organisms is a specific and complex problem, as it varies with cultures species, location, and the native species already established (Adams et al., 2011). Nonetheless, the implementation of biofouling mitigation measures and monitoring programs allows for better fouling management. Additionally, this process is a heavy financial burden to the aquaculture industry. On average, 15% of total operating costs are spent annually on the prevention or removal of fouling organisms (Lacoste and Gaertner-Mazouni, 2015). Currently, there is a high demand to investigate innovative, eco-friendly, inexpensive, and rapid-reaction technological solutions that target fouling organisms, whether for prevention, treatment, removal, and/or avoidance.

In Portugal, most of the biofouling research has been addressed concerning diverse methods of anti-fouling treatments. Several investigations have dedicated resources to assess the level of efficacy and toxicity of anti-fouling nanomaterials in various marine species: microalgae, diatoms, mussels, oysters, polychaetes, rotifers, crustaceans, and echinoderms (Silva et al., 2016; Avelelas et al., 2017; Figueiredo et al., 2019; de Campos et al., 2021, 2022).

Figure 1.9: Image of several different organisms fouling the steel pillars of the rack-and-bag production of oyster *Magallana gigas*, occurring in the Ria de Aveiro, Portugal. Photograph was taken in November 2018, Aveiro (Photograph by Luisa Marques).

Despite these novel investigations, preliminary studies concerning the monitorization of community dynamics, ecology, reproductive cycles, and identification of the major environmental drivers are paramount to better understand how a specific fouling organism may behave in a certain location.

In recent years, few studies have approached monitorization surveys in the Azores and Madeira Archipelago (Canning-Clode et al., 2013; Ramalhosa et al., 2021) and mainland Portugal (Saldanha et al., 2003; Fragoso and Icely, 2009; Peck et al., 2015; Azevedo et al., 2020). These studies were able to assist aquaculture managers in implementing anti-fouling strategies and adjustments to their productions, helped with recurring biofouling monitoring programs in ports and harbours, and revealed new records of NIS from many locations. However, in Portugal, research on ascidian biofouling communities is scarce and strongly required. Knowledge of the ascidian community's composition, abundance, preferences, and distribution is fundamental to understand and implement enhanced mitigation programs and actions.

1.6 Ascidians and their role in an IMTA framework

Currently, there is a large market for cultured ascidians, especially amongst Asian populations. Ascidian cultivation is scarce worldwide and limited only to Japan and Korea (Lambert et al., 2016). Many hurdles can be found throughout the way, even for countries that have dominated ascidian production: climate disasters such as tsunamis and hurricanes can greatly affect Asian countries (Fujii et al., 2019). More recently Sweden and Norway started pilot projects that aimed to turn ascidian production into a major industry (Towers, 2013; Marin Biogas, 2018). In Sweden, the project Marin Biogas aims to continuously produce *Ciona intestinalis* with the collection of fertilized eggs, investigating this ascidian preference of substrate, and maintaining stock culture are just some of the many tasks required (Hackl et al., 2015). Also, project *Integrerad akvakultur – Innovativt marint produktions* system has received financial support and has established a consortium of stakeholders in order to build the integrated system. (IVL, 2016). In the neighboring country, Uni Research and UiB have teamed up with the Research Council of Norway to help commercialize ascidians (Towers, 2013). At an early stage, they aimed to cultivate the most biomass possible to produce feed for ruminants and salmon. However, various methods of harvesting, washing and pressing were primarily tested out to maximize production and profit. (Towers, 2013).

Notwithstanding, the development of an effective IMTA framework where ascidians are included is yet to be accomplished. Few studies have already demonstrated that the newly developed IMTA system can allow for mutually beneficial ascidian (*S. clava*) and sea cucumbers (*Apostichopus japonicus*) growth, and therefore can be more economically viable (Ju et al., 2015, 2016; Lin et al., 2016). To summarize the importance of such a valuable resource it is worth pointing out the benefits and drawbacks of using ascidians as an extractive species. The strengths, weaknesses, opportunities, and threats (SWOT) analysis of this practice are outlined in Figure 1.9. It is worth pointing out that ultimately ascidians tip the scale toward more positive applications. Ascidians work as water filtration systems, renewing the water circulation, reducing organic matter content, and thus providing benefits to the co-extractive species, whether they are fish, sea cucumbers, or macroalgae. Additionally, ascidians can provide added value in the forms of biofuel, biogas, protein source, omega-3 essential fatty acids for fish feed, and many other bio compounds.

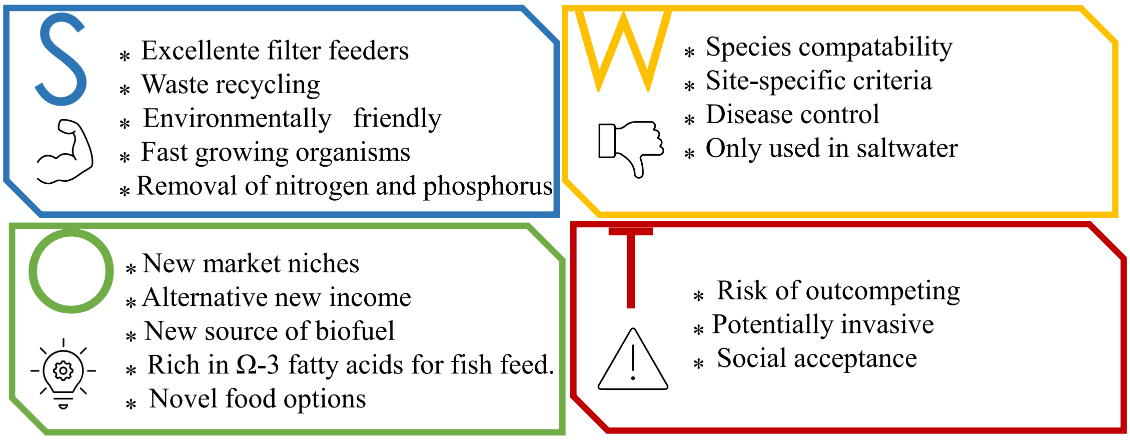


Figure 1.10: SWOT analysis of the incorporation of ascidians within an IMTA framework (own elaboration).

1.7 Scope, objectives, and thesis outline

The world's population is growing at a fast pace and, according to FAO (2022) could grow to around 9.7 billion in 2050. As aquaculture contributes more and more to the world's population feeding maintenance with highly nutritious products, this fast-growing trend generates several issues such as increasing demand for proteins, the search for alternative sustainable feeds and marine ingredients, and space management to farm marine species. However, the focus on the aquaculture industry to be increasingly more efficient, eco-friendly, and practice sustainable activities also has a major interest.

The concept of IMTA systems was introduced by Thierry Chopin (2004) aiming to use natural processes to simultaneously break down waste, produce diversified crops, and provide a host of benefits for both people and the planet. It is a win-win situation where cultured species and extractive species are both benefited and IMTA has the potential to support the tendency for aquaculture's growth.

Ascidians are sessile filter-feeders that display fast growth rates, feed on living and non-living organic material, inhabit a variety of substrates, and can be recognized as a potential extractive species in an IMTA system with potential added value as bioresources. In this context, the main objective of the present thesis is to study the ascidians occurring in the Ria de Aveiro coastal lagoon and to evaluate their potential added value concerning their use as an ingredient for fish feed, as well as an important source for both human nutrition and for aquaculture. These organisms were chosen due to the growing awareness they currently deserve worldwide, their variety of applications in many different industries, and due to their abundance in Ria de Aveiro coastal lagoon, Portugal.

To successfully address the objectives of the present thesis, the research was divided into four complementary Chapters. Chapters 2 to 5 feature the most relevant findings from each task and Chapter 6 summarizes concluding remarks and future perspectives

Chapter 2: provides a systematic review that aims to answer three main questions: 1) What do we know about ascidians' biological and ecological importance as filter feeders? 2) If ascidians were implemented in an IMTA framework, which are the best combinations of extractive species that may better amplify their performance and understand how will ascidians function under this framework? 3) What kind of bioactive products can ascidians provide regarding their nutritional value and potential added value to the aquaculture industry?

Chapter 3: aims to monitor and establish a reference for the ascidian biofouling community present in the Ria de Aveiro (Portugal) coastal lagoon. To achieve this end, an innovative customized 3-D star-shaped structure composed of five triangular-based pyramids made with oyster shells was used. This unit was a nature-based structure, simulating a natural habitat and thus allowing ascidians to settle, establish, and grow. The main objective of the work was to monitor ecosystem shifts in ascidian diversity.

Chapter 4: presents an individual technical report of the main features of each ascidian species observed in the previous study (Chapter 3). This report addresses a total of six species (*Clavelina lepadiformis*, *Ciona intestinalis*, *Ascidiella aspersa*, *Botrylloides violaceus*, *Botryllus schlosseri*, *Styela plicata*, and two genera (*Molgula* sp., and *Microcosmus* sp.). Features such as biology and ecology, habitat preferences and environmental tolerances, native origin and global distribution, reproduction and life stages, and some applications and curiosities. In addition, two ascidians, *C. lepadiformis* and *A. aspersa* were for the first time, collected and registered for mainland Portugal.

Chapter 5: explores the potential of ascidians and macroalgae as valuable sources of essential fatty acids. The primary goal of this chapter was to explore the differences in the fatty acid profiles of ascidians (Ascidiacea) and macroalgae (sea lettuce, *Ulva* spp. and bladderwrack, *Fucus* sp.) cultured under the influence of organic-rich effluents provided by a semi-intensive fish farm activities *versus* without the influence of organic-rich effluents.

Chapter 6: summaries a discussion of the main research questions of the thesis, provides a general conclusion from previous chapters, and offers a point of view on an upcoming future for ascidians in the aquaculture industry, with perspectives for future research, recommendations, and opportunities.

Chapter 2

Chapter 2

Potential of ascidians as extractive species and their added value in marine integrated multi-trophic aquaculture systems – from pests to valuable blue bioresources

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2. Potential of ascidians as extractive species and their added value in marine integrated multi-trophic aquaculture systems – from pests to valuable blue bioresources

Abstract

Ascidians are considered filter-feeder biofouling pests that negatively affect aquaculture facilities. However, they can also be recognized as a potential co-cultured/extractive species for integrated multi-trophic aquaculture (IMTA) with potential added value as bioresources. A systematic review aiming to understand the ecological importance of ascidians as efficient filter-feeders [What?]; their potential contribution as extractive species [How?]; and to set the benchmark for their nutritional value and potential added value to the aquaculture industry [For what?] is a timely contribution to advance the state of the art on these largely overlooked bioresources. In the last two decades, there has been an overall increase in publications addressing ascidians in aquaculture, namely their negative impacts through biofouling, as well as their role in IMTA, environmental status, and microbiology. While *Ciona intestinalis*, a solitary ascidian, has been the most studied species, overall, most ascidians present high filtration and fast-growth rates. As ascidians perform well under IMTA, competition for resources and space with other filter-feeders might occur, which may require additional management actions to optimize production. Studies addressing their bioactive products show that ascidians hold great potential as premium ingredients for aquafeed formulations, as well as dietary supplements (e.g., amino acids, fatty acids). Further research on the potential use of ascidians in IMTA frameworks should focus on systems carrying capacity.

Keywords

Tunicates, Bioresource, IMTA, Filtration rate, Fatty acids, Retention efficiency.

2.1 Introduction

Aquaculture is an important source of food, nutrition, income, and livelihood for hundreds of millions of people worldwide (FAO, 2020). With the continuous increase of the world's population, aquaculture production needs to increase by 21–44 million tonnes by the year 2050 (Costello et al., 2020). An idealistic scenario, and a major challenge for the aquaculture industry, is to be profitable, product-diversified, socially beneficial, and yet ecologically efficient and environmentally friendly, *i.e.*, to cope with the principles of sustainable development. Integrated multi-trophic aquaculture (IMTA) has the potential to achieve such a goal.

An IMTA framework is a nature-based solution in which the by-products, wastes, uneaten feed, and nutrients from one species are recycled and converted to become fertilizer, feed, and energy for the growth of another (Naylor and Burke, 2005). These systems can be land-based or open-water, use marine, brackish or freshwater, and may include several different combinations of co-cultured species (Neori et al., 2004). IMTA aims at mimicking a natural ecosystem by combining and incorporating complementary species from different trophic or nutritional levels in the same productive environment. In an operational IMTA system, extractive species uptake organic and inorganic matter contributing to reducing costs and complying with environmental regulations (Reid et al., 2020). In addition, their potential market value (*e.g.*, food, feed, pharma) might provide extra economic benefits to farmers (Barrington et al., 2009; Béné et al., 2015). Selected species should be cultured at densities that optimize nutrient uptake, promote a stable balance between biological and chemical processes improving the ecosystem's health, and should be economically important as aquaculture products (Alexander et al., 2016).

However, implementing a healthful and balanced concept can present multiple challenges to farmers. Nonetheless, an IMTA framework also presents numerous benefits, including the decrease in waste outputs from overall farming activities, the additional production of a marketable product for little or no additional input cost, and more importantly, environmentally sustainable farming operations (Barrington et al., 2009; Troell et al., 2009).

The open key question concerns the optimization of the uptake of particulate and dissolved organic matter from uneaten/undigested feed and feces. Organic nutrients can nitrify the benthic-pelagic community (Albert et al., 2021) and the excess of inorganic nutrients (ammonia, nitrate, nitrite) may ultimately create a bio-deposit and lead to eutrophicated waters (Chopin et al., 2001) and/or represent an economic burden to fish farmers (Fry et al., 2016).

Ascidians, commonly known as sea squirts or tunicates, are found in all marine habitats from shallow water to the deep sea (Shenkar and Swalla, 2011) and there are approximately 3000 described species (Shenkar and Swalla, 2011). Currently classed under Phylum Chordata, ascidians hold a unique evolutionary position as the sister group of vertebrates. These organisms are benthic suspension feeders that filter particulate organic matter from the water column via an oral siphon and expelled filtered water through the atrial siphon (Jørgensen and Goldberg, 1953; Jørgensen, 1954). They present a wide variety of forms, (from small colonies to big solitary forms), colors, shapes (from cone-shaped, elongated, globular, or oval), and sizes (generally from 5 to 200 mm) (Petersen, 2007; Shenkar and Swalla, 2011). The body is always covered with a tunic, a protective layer that may be translucid, brightly colored or dull, covered by various kinds of spines, and contain calcareous spicules (Lambert and Lambert, 1987), or even be covered by a dense layer of sand grains (Young, 1989). Most solitary ascidians are hermaphrodites and reproduce by external fertilization (Honegger, 1986). They develop a free-swimming tadpole-like larva that swims for a short period, settles on a wide variety of habitats, and finally matures into a sessile adult (Shenkar and Swalla, 2011). Colonial specimens can reproduce both sexually and asexually (Gasparini et al., 2015). Ascidians often present an invasive behavior, representing the most dominant fouling species worldwide, colonizing natural and artificial substrates (Ordóñez et al., 2013).

While several ascidian species present a preference to settle on natural substrates (Hirose and Sensui, 2021), others settle on artificial structures, such as ship hulls, floating docks (Zvyagintsev et al., 2007), and aquaculture infrastructures, (Hodson et al., 2000; Khalaman, 2001; Bullard et al., 2013; Rosa et al., 2013) process known as biofouling. At times some species even grow on other organisms being farmed, such as on the shells of mollusks (Dijkstra and Nolan, 2017; Casso et al., 2018). Hereupon, these organisms hold great potential as co-cultured/extractive species in IMTA frameworks, with the potential to contribute to more efficient, profitable, and sustainable aquaculture systems. Benthic fish contribute to sediment resuspension while searching for food or shelter (Yahel et al., 2008; Carvajalino-Fernández et al., 2020). Although these resuspension events can be brief and localized (Yahel et al., 2002), in an IMTA scenario ascidians, as excellent filter-feeders, can rapidly uptake nutrient recycling and contribute to a positive outcome.

The main objective of this systematic review is to understand how ascidians may no longer be regarded as pest organisms, whose biofouling negatively impacts aquaculture ventures, but rather as important extractive species in IMTA frameworks that yield premium biomass for high-end uses. For this purpose, we surveyed the scientific literature to answer the following three questions: [What do we know?] To better understand the biological and ecological importance of ascidians as filter-feeders in an IMTA framework; [How do ascidians perform in IMTA?] to evaluate which combination of species will contribute the most to enhance the performance of ascidians in IMTA frameworks; and [For what kind of bioactive products?] to recognize ascidians as potential bioresources in different high-end fields, namely blue biotechnology and human nutrition.

Here, special attention will be given to fatty acids (FA), as both omega-3 (*n*-3) and omega-6 (*n*-6) FAs are essential components for food, feed, and pharma industries. The analysis of these three questions will enable us to discuss and conclude the potential of ascidians as extractive species and their added value in marine IMTA frameworks.

2.2 Literature review

In January 2020, a systematic literature review, with no year restriction, was performed using the databases Thomson Reuters Web of Science (Core Collection) (Topic) and Scopus (Article title, Abstract, Keywords). The strategy used was to search within a combination of specific terms: Filtration AND (tunicate OR ascidian OR 'sea squirt*'); Aquaculture AND (tunicate OR ascidian OR 'sea squirt*'); Fatty acid* AND (tunicate OR ascidian OR 'sea squirt*') to achieve the review's goal.

A schematic representation of the selection process is summarized in Figure 2.1. Overall, a total of 566 publications were retrieved (after excluding duplicates from the two databases), and a spreadsheet with the bibliographic information of each reference was created for further analysis to ascertain their relevance for this study. The review selection consists of two sections.

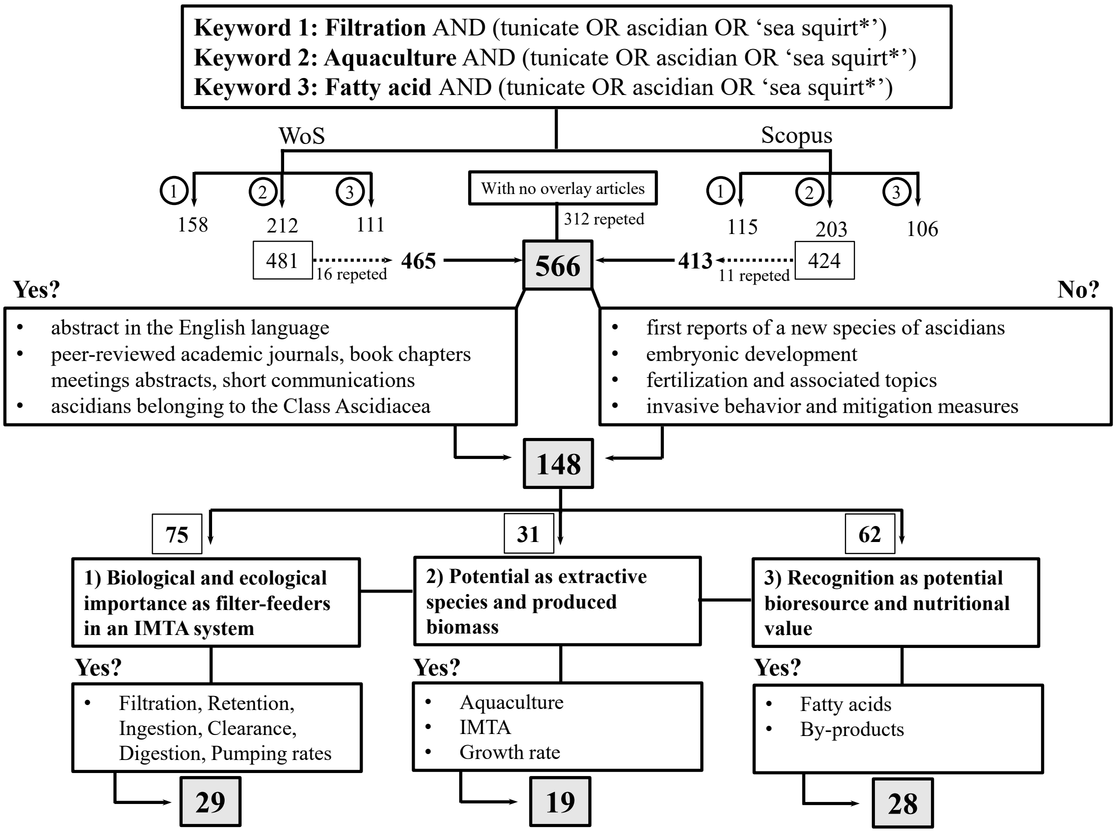


Figure 2.1: Schematic representation of the process employed for the selection of relevant publications retrieved from each database (Web of Science, WoS; Scopus).

The first article selection aimed to retain publications with, at least, the abstract in the English language, peer-reviewed academic journals, book chapters, meeting abstracts, short communications, and reports on ascidians within Class Ascidiacea. When studies addressed ascidians in a general way, these were registered as “Ascidiacea”. Furthermore, studies addressing the following topics were excluded from the present review: i) first reports on the occurrence of a new species of ascidian in a given location and their geographic distributions, ii) embryonic development, iii) fertilization, reproduction and associated topics, iv) invasive behavior of ascidians and mitigation measures.

A total of 148 publications (Table SI 2.1) were considered relevant and selected for further analysis. Ten research categories (aquaculture, biochemistry, biofouling, biology, biotechnology/methods, diseases, environmental, IMTA, microbiology, and review) were created and assigned to each of the 148 publications, with a maximum of four categories being attributed per publication.

The rationale for this procedure is detailed in Table 2.1. Additionally, each publication was also assigned to one of the three questions (occasionally two) initially established: (question 1 [What?]: 75 publications, question 2 [How?]: 31 publications, and question 3 [For what?]: 62 publications). Subsequently, each of the publications assigned to each of the three questions was further screened as detailed in Figure 2.1.

Briefly, concerning question 1, only publications addressing filtration, retention, ingestion, clearance, digestion, and water pumping rates were selected, for a total of 29 publications. Regarding question 2, only publications addressing topics such as aquaculture, IMTA, and growth rates were included, for a total of 19 publications. Finally, for question 3, publications referring to FAs and other potential co-products were considered, for a total of 28 publications. Blue biotechnology may focus on a plethora of potentially bioactive compounds (Vieira et al., 2020).

As this review targets marine species and they can be seen as sources of the essential *n*-3 and *n*-6 FAs, special attention will be given to these bioactive compounds as they can represent an added value for food, feed, and pharma industries.

Table 2.1: Research categories considered and their respective criteria.

Research Category	
Aquaculture	Refers to farming of ascidians, impacts that other species may have and economic value
Biochemistry	Refers to proximate composition, lipid composition, fatty acid identification and nutrition information
Biofouling	Refers to biofouling ascidians in aquaculture sites and its impacts on produced species
Biology	Refers to biological and ecological traits such as growth, filtration, clearance, retention rates, natural diets, population interactions, and habitat preferences
Biotechnology /Methods	Refers to models created and tested, development of technology towards the study of ascidians
Diseases	Refers to diseases associated with ascidians
Environmental	Refers to environmental parameters and their impact on ascidians, pollution, toxicity and bioremediation
IMTA	Refers to farming ascidians with one or more different trophic groups, along with their interactions and impacts
Microbiology	Refers to the identification, characterization, and isolation of bacteria from ascidians
Review	Refers to any published review on ascidians

2.3 Results

Out of the 148 publications, 80 fell into the research category "Biology", hence demonstrating the importance of understanding the morphology, biology, and anatomy of ascidians in a general manner (Figure 2.2).

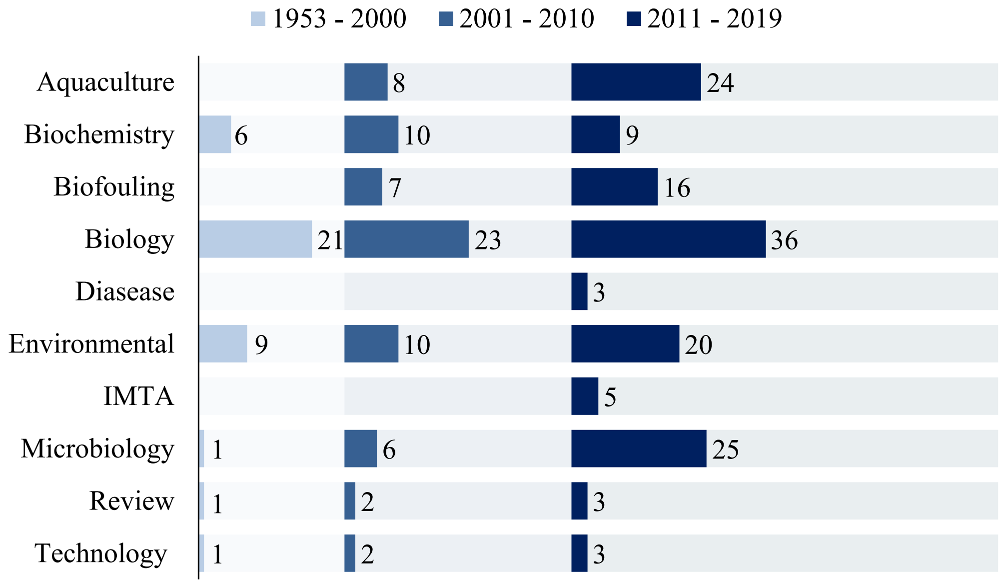


Figure 2.2: The number of publications (n=148), from 1953 to 2019, was assigned to each research category.

Since the 21st century, an overall increase in all research categories is noted, but it is worth mentioning a gradual and joint increase of publications in "Aquaculture", "Biofouling" and "IMTA" categories (44%, 25%, and 7%, respectively) as these are correlated with each other. In addition, bacteria and associated diseases with ascidians are a growing concern, as seen with the increase in the number of publications within the category "Microbiology". A total of 45 species, belonging to 3 orders and 12 families (Table SI 2.2) were present in this review, in which solitary ascidians represented 72% (Figure 2.3A) and merely 28% were colonial ascidians (Figure 2.3B).

Despite the high number of ascidians from the marine realm, solely three species dominated the focus of scientists throughout the years. *Ciona intestinalis*, a translucent column-like tunicate, was by far the most studied species, followed by *Halocynthia roretzi* and *Styela clava* (Figure 2.3A).

A detailed analysis was performed regarding the three questions (Table 2.2). Ascidians *C. intestinalis* and *S. clava* were the two most studied species for their biological and ecological importance as filter-feeders [What?] and as the most effective combination of species for IMTA [How?]; while *H. roretzi* and *Halocynthia aurantium*, were mostly studied for their potential as bioresource [For what?] (26% and 11%, respectively).

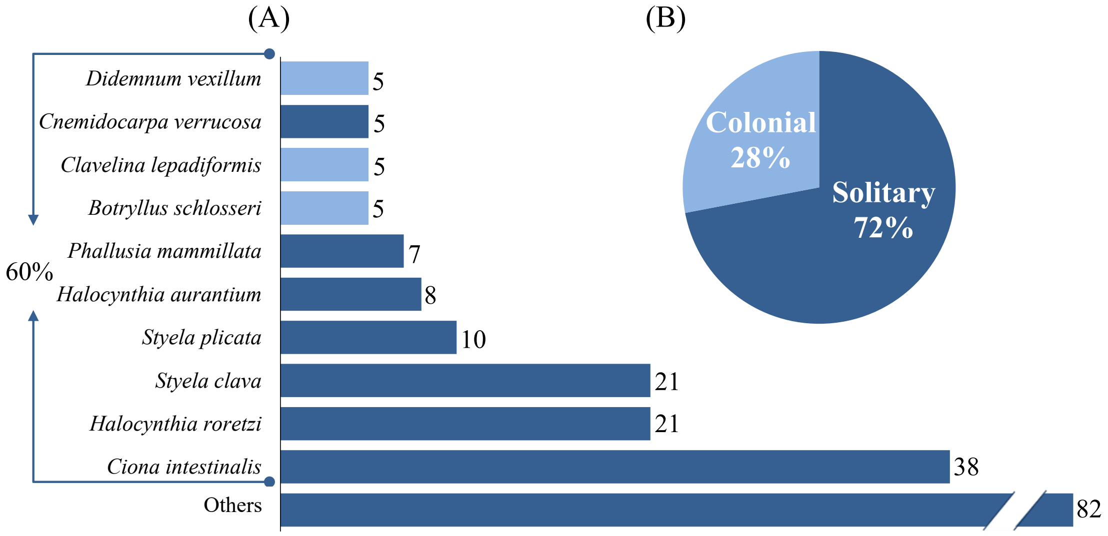


Figure 2.3: (A) Top ten studied ascidian species and the total number of publications addressing them. Light blue represents colonial species, dark blue represents solitary species; (B) Percentage of solitary and colonial ascidians addressed in the 148 publications surveyed. See supporting information for a complete list of studied species (Table SI 2.2)

Table 2.2: Percentages of the top two research categories, countries, and ascidian species that most contributed to each of the questions [What?], [How?], [For what?], addressed in the 148 publications surveyed.

Questions	Category	Country	Species
1) [What?]	Biology (52%)	France (18%)	*Ciona intestinalis* (21%)
	Environmental (21%)	Canada (12%)	*Styela clava* (8%)
2) [How?]	Aquaculture (30%)	Canada (36%)	*Ciona intestinalis* (33%)
	Biology (27%)	China (21%)	*Styela clava* (28%)
3) [For what?]	Biochemistry (44%)	South Korea (27%)	*Halocynthia roretzi* (26%)
	Microbiology (43%)	Japan (21%)	*Halocyhthia aurantium* (11%)

[What do we know?] (Question 1)

To comprehend the role of ascidians as filter-feeders and their importance, a better understanding of basic biology is needed. Essentially, water filtrations rates were present in most of the 29 publications analyzed (Figure 2.1), with these referring to 31 different species (allocated to 17 different genera). Solitary ascidians, such as *C. intestinalis*, *Phallusia mammillata*, and *Styela plicata*, were the most investigated species accounting for 17.5%, 9.5%, and 7.9% of the publications, respectively. France is the leader both in the number of ascidian species being studied, as well as in the number of studies performed (Table 2.3).

Filtration rates presented a great variability between the different species of ascidians addressed, with intraspecific variability also being recorded, for example for *C. intestinalis* with values ranging from 3.5 L h^{-1} to 11.9 L h^{-1} (Fiala-Médioni, 1974; Petersen and Riisgärd, 1992) and for *P. mammillata* with values ranging from 4.4 L h^{-1} to 11.9 L h^{-1} (Fiala-Médioni, 1973; Hily, 1991) (Table 2.3). Nakai et al. (2018) demonstrated that water filtration rate increases with size, while Ribes et al. (1998) showed that filtration rates may vary seasonally, displaying an increase with rising water temperatures. Just 30% of publications registered retention rate values. A total of 17 species were investigated, with only two species being colonial ascidians. Particle retention varied from 1.7 to 4.71 µm (mean value).

Table 2.3: Summary of the main features of filtration, pumping, and retention rate of the studied ascidians addressed in the 29 publications selected regarding question 1 [What?]. LPS: Low particulate suspension; HPS: High particulate suspension; RE: Retention efficiency; CR: Clearance rate; FE: Filtration efficiency. *Pumping rate; **adapted from (Petersen, 2007).

Studied species	**Country**	**Filtration rate/Pumping rate**	**Retention efficiency**	**Reference**
Ascidiacea	NA	similar in different species suspension feeding is highly efficient		Petersen (2007)
Ascidia challengeri	Antarctic	304 ml.h AFDW*	1.2 - 2 µm	Kowalke (1999)
Ascidia virginea	Sweden	5.2 L.h^{-1}.g^{-1}**		Petersen & Svane (2002)
Ascidiella aspersa	Denmark	5.4 L.h^{-1}.g^{-1}**	2 - 3 µm completely retained; RE decreased 70% for 1 µm	Randløv & Riisgärd (1979)
Ascidiella aspersa	France	6.28 h^{-1}		Hily (1991)
Ascidiella aspersa	United Kingdom	5.26 L.h^{-1}.g^{-1} at about 10 000 cells ml^{-1}	decrease > 4.5 µm	Pascoe et al. (2007)
Ascidiella scabra	United Kingdom	at LPS: 0.71 h^{-1}; decreased with increasing suspension load		Robbins (1983)
Ascidiella scabra	United Kingdom	FE: at HPS unchanged		Robbins (1984)
Boltenia echinata	Sweden	3.8 L.h^{-1}.g^{-1}**		Petersen & Svane (2002)
Ciona intestinalis	USA		1 - 2 µm	Jørgensen & Goldberg (1953)
Ciona intestinalis	France	3.5 L.h^{-1}.g^{-1}		Fiala-Médioni (1974)
Ciona intestinalis	France	4.3 L.h^{-1}.g^{-1}; FE(mean)= 74% 5.9 L.h^{-1}.g^{-1}*		Fiala-Médioni (1978)a
Ciona intestinalis	Sweden	7.7 L.h^{-1}.g^{-1} **	2 - 3 µm completely retained; RE decreased 70% for 1 µm	Randløv & Riisgärd (1979)
Ciona intestinalis	United Kingdom	at LPS: 0.21 h^{-1}(mud); 0.11 h^{-1} (*Fucus*); decreased with increasing suspension load		Robbins (1983)
Ciona intestinalis	United Kingdom	FE: at HPS unchanged		Robbins (1984)
Ciona intestinalis	Denmark	11.9 L.h^{-1}.g^{-1} ** 4 - 21ºC increased, > 21ºC decrease		Petersen & Riisgärd (1992)
Ciona intestinalis	Sweden	8.4 L.h^{-1}.g^{-1}**		Petersen & Svane (2002)
Ciona intestinalis	United Kingdom	4.61 L.h^{-1}.g^{-1} at about 5000 cells ml^{-1}	similar to 2 - 5.5 µm	Pascoe et al. (2007)
Ciona intestinalis	USA	0.07–0.97 L.h^{-1}*		Du Clos et al. (2017)
Ciona intestinalis	France	positively related to food concentration		Hoxha et al. (2018)
Ciona robusta	France	positively related to food concentration CR higher than *C. intestinalis*		Hoxha et al. (2018)
Ciona savignyi	Japan	0.125 L.h^{-1} ind^{-1} (ind 3.5 cm) 0.359 L.h^{-1} ind^{-1} (ind 5.3 cm) 1.05 L.h^{-1} ind^{-1} (ind 6.4 cm) optimal at 24-25ºC		Nakai et al. (2018)
Clavelina lepadiformis	France	2.5 L.h^{-1}.g^{-1}		Fiala-Médioni (1974)

Clavelina lepadiformis	Denmark		2 - 3 µm completely retained; RE decreased 70% for 1 µm	Randløv & Riisgärd (1979)
Clavelina lepadiformis	Sweden	8.9 L $^{-1}$.g^{-1}**		Petersen & Svane (2002)
Cnemidocarpa verrucosa	Antarctic	348 ml.h AFDW*	1.4 - 4 µm	Kowalke (1999)
Cnemidocarpa verrucosa	Antarctic		0.2 - 2 µm	Lesser & Slattery (2015)
Corella eumyota	Antarctic	251 ml.h AFDW*	1.2 - 5 µm	Kowalke (1999)
Corella parallelogramma	Sweden	7.0 L.h^{-1}.g^{-1}**		Petersen & Svane (2002)
Didemnum sp.	Australia	reduced heterotrophic bacteria		Pile (2005)
Halocynthia papillosa	France	6.3 L.h^{-1}.g^{-1}		Fiala-Médioni (1974)
Halocynthia papillosa	Spain	3.0 - 3.6 L.h^{-1}.g^{-1}**	0.6 - 7 µm	Ribes et al. (1998)
Halocynthia pyriformis	Canada	136 ml.min^{-1} DW (1g)	2 - 5 µm: increased 5 - 15 µm: decreased	Armsworthy et al. (2001)
Halocynthia sp.	Australia	only reduced <3 µm		Pile (2005)
Halocynthia spinosa	Israel		1 µm at 95% efficiency; 0.3 µm at 50% efficiency	Jacobi et al. (2018)
Herdmania momus	Israel		1 µm at 95% efficiency; 0.3 µm at 50% efficiency	Jacobi et al. (2018)
Microcosmus sabatieri	France	6.9 L.h^{-1}.g^{-1}		Fiala-Médioni (1974)
Microcosmus exasperatus	Israel		1 µm at 95% efficiency; 0.3 µm at 50% efficiency	Jacobi et al. (2018)
Molgula manhattensis	Denmark	Higher than *C.intestinalis* and *A. aspersa*	2 - 3 µm completely retained; RE decreased 70% for 1 µm	Randløv & Riisgärd (1979)
	Sweden	2.1 L.h^{-1}.g^{-1}**		Petersen & Svane (2002)
Molgula pedunculata	Antarctic	349 ml.h AFDW*	1.2 - 6.5 µm	Kowalke (1999)
Phallusia julinea	Australia	reduced heterotrophic bacteria		Pile (2005)
Phallusia mammillata	France	4.4 L.h^{-1}.g^{-1} (ind 10 - 12 cm)		Fiala-Médioni (1973)
Phallusia mammillata	France	4.8 L.h^{-1}.g^{-1} ; FE(mean)= 76% 6.3 L.h^{-1}.g^{-1} *		Fiala-Médioni (1978)a
Phallusia mammillata	France	15 ºC: 4.3 L.h^{-1}.g^{-1}** 20 ºC: 1.6 L.h^{-1}.g^{-1}** (mean): 10 ºC: 3.56, 15 ºC: 5.79, 20 ºC: 2.63 ml.h^{-1}.g^{-1} DW*		Fiala-Médioni (1978)b
Phallusia mammillata	France	pO_2 > 119 mg Hg: decrease pO_2 > 98 mg Hg: decrease faster FE: 77-79%		Fiala-Médioni (1979)
Phallusia mammillata	France	11.9 L.h^{-1} .g^{-1}		Hily (1991)
Phallusia mammillata	NA	825 - 5100 ml.h (ind 8 - 128 g WW)		Carlisle (1996)
Phallusia nigra	Israel		1 µm at 95% efficiency; 0.3 µm at 50% efficiency	Jacobi et al. (2018)
Polyandrocarpa zorritensis	Italy	max: 1.745 L.h^{-1}.g^{-1} DW	RE: 41%, removed bacterial biomass of 16.34 + 1.71 µg.C.L^{-1}.g^{-1} DW)	Stabili et al. (2016)
Polycarpa mytiligera	Israel		1 µm at 95% efficiency; 0.3 µm at 50% efficiency	Jacobi et al. (2018)
Polycarpa pedunculata	Australia	only reduced <3 µm		Pile (2005)
Polycarpa sp.	Australia	reduced heterotrophic bacteria		Pile (2005)
Pyura microcosmus	France	1.94 h^{-1}		Hily (1991)

Pyura sp.	Australia	only reduced <3 µm		Pile (2005)
Pyura tessellata	Sweden	3.0 L.h^{-1}.g^{-1}**		Petersen & Svane (2002)
Styela clava	New Zealand	declined after 3wks (sedimentation)		Lohrer et al. (2006)
Styela clava	South Korea	0.477J d^{-1} mean DW (310 mg) at 5–15 °C 0.687 J d^{-1} mean DW (310 mg) at 15–25 °C		Kang et al. (2015)
Styela plicata	France	8.8 L.h^{-1}.g^{-1} ; FE(mean)= 80% (mean): 10.7 L.h^{-1}.g^{-1} *		Fiala-Médioni (1978)a
Styela plicata	USA	*Nannochloropsis* sp.: 10^5+10^6 cells: 3158 ml.h^{-1}; *Escherichia coli*: 10^5+10^6 cells: 3475ml.h^{-1};		Draughon et al. (2010)
Styela plicata	USA	<10 µm: decreased (fast and slow flow speeds); >10 µm: decreased (flow speed from 3 to 22 cm.s^{-1}) maximal at intermediate flow speeds 12 cm.s^{-1}		Sumerel & Finelli (2014)
Styela plicata	Italy	max: 1.4 L.h^{-1}.g^{-1} DW	RE: 81% removed bacterial biomass of 32.28 + 2.15 µg C.L^{-1}.g^{-1} DW)	Stabili et al. (2016)
Styela plicata	Israel		1 µm at 95% efficiency; 0.3 µm at 50% efficiency	Jacobi et al. (2018)

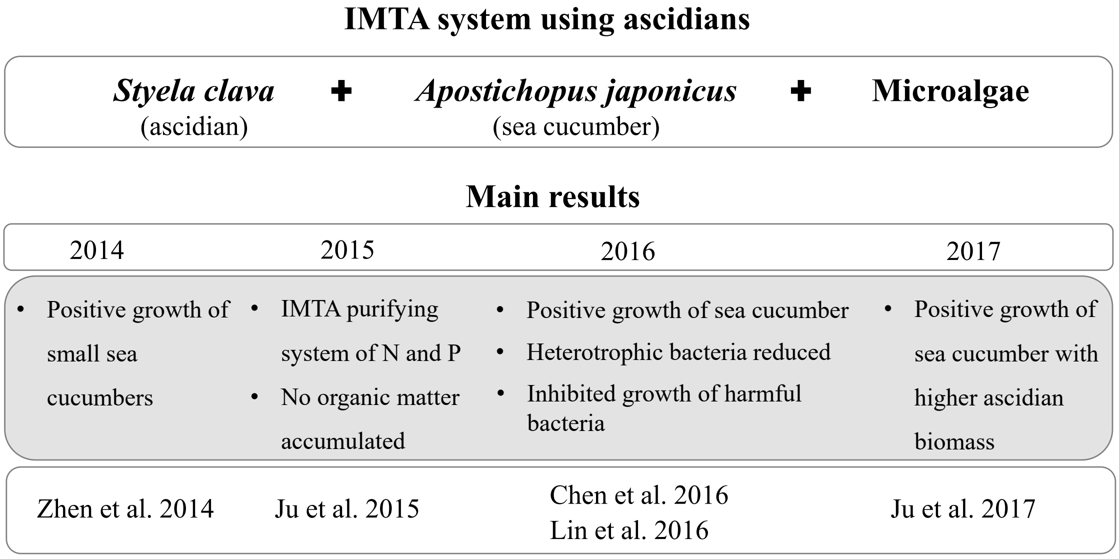
Table 2.4: Summary of the main impacts produced by ascidians in aquaculture scenarios addressed in the 19 publications selected regarding question 2 [How?].

Studied species	Country	Aquaculture species	Main results	Reference
Ciona intestinalis	Australia	*Mytilus galloprovincialis*	Small mussels: 4% shorter in shell length; 21% reduced flesh weight; Large mussels: 3.9% shorter in shell length, flesh weights not reduced	Sievers et al. (2013)
Styela clava			Large mussels: 4.4% shorter in shell length, flesh weights not reduced	
Botrylloides violaceus *Botryllus schlosseri* *Ciona intestinalis*	Canada	Mussel	*C. intestinalis*: 80% more coverage on unfouled plates *C. intestinalis*: <10% coverage on pre-settled plates Higher individual growth on pre-settled plates than on unfouled plates	Paetzold et al. (2012)
Ciona intestinalis *Styela clava*	Canada	*Mytilus edulis*	*C. intestinalis* has a negative impact, replacing *S. clava*	Ramsay et al. (2008)a
Ciona intestinalis	Canada	*Mytilus edulis*	*C. intestinalis* was marginally higher in August; Mussel loss 50-60% for all treatments	Ramsay et al. (2008)b
Ciona intestinalis	Canada	*Mytilus edulis*	*C. intestinalis:* abundance: 98.4-828.6 ind/0.3m mussel sock; Negative effect on epifaunal species, primarily on sessile organisms	Lutz-Collins et al. (2009)
Molgula sp.			*Molgula* sp.: colonized the mussel socks in lower numbers and an opposite spatial pattern of *C. intestinalis*	
Ciona intestinalis	Canada	*Mytilus edulis*	Size and condition decreased with increasing ascidian densities; 50% mussel mortality observed under heavy ascidian fouling	Daigle & Herbinger (2009)
Ciona intestinalis	Canada	*Mytilus edulis*	*C. intestinalis* can dominate mussel biomass and contribute to organic sedimentation	Guyondet et al (2016)
Didemnum sp. *Herdmania momus*	France	*Pinctada margaritifera*	Competition between oysters and ascidians was not a limiting factor, in spite of a diet overlap for nanophytoplankton	Lacoste et al. (2016)
Ascidiella aspersa	Japan	Scallop	*A. aspersa* settle as larvae in early summer and grows well until winter, resulting in overgrowth on scallops in the harvest season	Kanamori et al. (2017)
Ciona savignyi	Japan	*Mizuhopecten yessoensis*	Filtration increased with size increase; *C. savignyi* has the potential to negatively impact the growth of the Japanese scallop through competition for food.	Nakai et al. (2018)
Didemnum vexillum	New Zealand	*Perna canaliculus*	Mussels may only be vulnerable to direct *D. vexillum* fouling impacts at early stages of production	Fletcher et al. (2013)
Ciona intestinalis	Norway	*Mytilus edulis*	In forced upwelling conditions: positive effect on both species; ascidians would be more efficient at extracting resources due to their lower metabolic cost and higher filtration capacity.	Filgueira et al. (2019)
Ciona intestinalis	South Africa	*Mytilus galloprovincialis*	Competitive exclusion of the mussel in dark, sheltered areas and physiological exclusion of the ascidian elsewhere	Rius et al. (2011)
Ascidiacea	Spain	Oyster	15 spp. were identified	Casso et al. (2018)

[How do ascidians perform in IMTA?] (Question 2)

Question 2 focused on understanding which combination of species with ascidians contributes the most to enhance the performance of IMTA frameworks, their extractive ability, and their impacts on other cultured species. Only five out of the 19 relevant publications (Figure 2.1) specifically addressed IMTA. These five publications presented similar aspects, as they were all performed in China and addressed *S. clava*.

The main goal of these publications was to optimize the commercial production and growth of the sea cucumber *Apostichopus japonicus* (Figure 2.4). The remaining 14 publications addressed issues associated with the impact of biofouling promoted by ascidians on cultured species, namely mussels, oysters, and scallops were addressed (Table 2.4). *Ciona intestinalis* was the most discussed ascidian regarding this topic.



IMTA system using ascidians

Styela clava (ascidian) **+** ***Apostichopus japonicus*** (sea cucumber) **+** **Microalgae**

Main results

2014	2015	2016	2017
• Positive growth of small sea cucumbers	• IMTA purifying system of N and P • No organic matter accumulated	• Positive growth of sea cucumber • Heterotrophic bacteria reduced • Inhibited growth of harmful bacteria	• Positive growth of sea cucumber with higher ascidian biomass
Zhen et al. 2014	Ju et al. 2015	Chen et al. 2016 Lin et al. 2016	Ju et al. 2017

Figure 2.4: Schematic representation of the main features of integrated multitrophic aquaculture (IMTA) frameworks using ascidians addressed in 5 of the 19 publications selected regarding question 2 [How?].

[For what kind of bioactive products?] (Question 3)

The recognition of the nutritional value *sensu lato* of ascidians and their potential as bioresources was considered in 28 publications (Figure 2.1), addressing 25 species belonging to 15 genera. Ascidians being addressed under this scope mostly originated from Asian countries (China, Japan, South Korea, and North Korea), with a major focus on *C. intestinalis*, *Halocynthia* sp., and *Styela* sp. Amongst the various studies, 16 of them addressed specifically the FA composition of ascidians, including 20 species belonging to 13 genera. From these, few analyzed the tunic and inner body separately (Zhao et al., 2015; Zhao and Li, 2016), with the remaining analyzing the whole body of ascidians.

A wide range of FAs was identified with percentages varying from 0.06 to 44% of total FA (Jeong et al., 1996; Zlatanos et al., 2009), nonetheless, palmitic acid (16:0), stearic acid (18:0), arachidonic acid (AA - 20:4 (*n*-6)), eicosapentaenoic acid (EPA – 20:5*n*-3) and docosahexaenoic acid (DHA – 22:6*n*-3) were consistently recorded (Figure 2.5), see Table SI 2.3 for further detail. Fatty acids 16:0 and 18:0 were constantly higher in all studied ascidians, however, in several species, EPA and DHA presented high values as well (Carballeira et al., 1995; Jeong et al., 1996; Zhao and Li, 2016). Out of the studies analyzed, biocompounds such as didemnilactones A and B and neodidemnilactone (Niwa et al., 1994), 2,3-dihydroxy FA glycosphingolipids (Aiello et al., 2003), anticancer ecteinascidin 743 (Mendola, 2003), pentylphenols, cyclopropane FA, and cyclopentenones (Rob et al., 2011) were proven to originate from ascidians (Table 2.5). Cytotoxicity against human solid tumor cell lines (Bao et al., 2009), against HCT116 cells (human colon cancer cells), and inhibition of the division of fertilized sea urchin eggs (Rob et al., 2011) are just some examples of these compound functionalities.

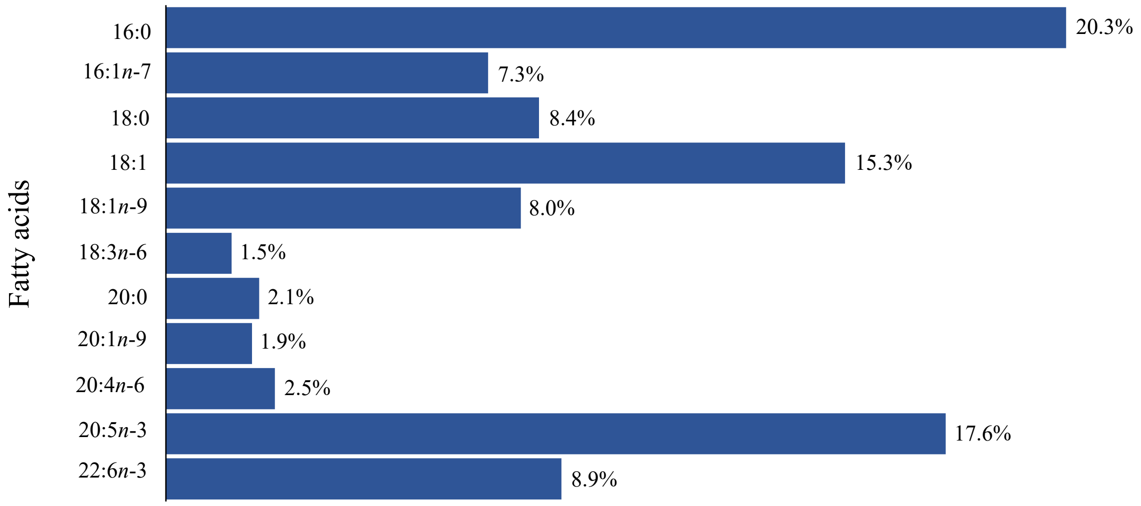


Figure 2.5: Mean relative percentage values of total fatty acids of the ascidians are addressed in this review. See Table SI 2.3 for further details.

Table 2.5: Summary of the main attributes of the bioactive compounds of ascidians and other features addressed in the 28 publications selected regarding question 3 [For what?].

Country	Studied species	Bioactive compounds and others	Reference
Greece	*Microcosmus sulcatus*	protein 0.8%, moisture: 81.1%, fat: 1.0%, ash: 7.5%; glutamic acid: 1.05 g.100g freeze-dried	Zlatanos et al. (2009)
Italy	*Microcosmus sulcatus*	2,3-dihydroxy fatty acid glycosphingolipids	Aiello et al. (2003)
India	*Didemnum psammathodes*	protein: 3.78 μg.ml^{-1}; total carbohydrate: 2.15 μg.ml^{-1}; crude fiber: 9.2 μg.ml^{-1}; total free amino acid: 3.2 μg.ml^{-1}; leucine: 540.9 mg.g, arginine: 401.2 mg.g, lysine: 385.4 mg.g	Sri Kumaran & Bragadeeswaran (2014)
	Eudistoma viride	protein: 3.62 μg.ml^{-1}; total carbohydrate: 12.2 μg.ml^{-1};	

		crude fiber: 7.9 μg.ml^{-1}; total free amino acid: 3.9 μg.ml^{-1}; leucine: 582.3 mg.g, arginine: 365.4 mg.g, lysine: 344.5 mg.g	
Japan	*Didemnum moseleyi*	Didemnilactone and Neodidemnilactone	Niwa et al. (1991)
Japan	*Didemnum moseleyi*	Didemnilactones A and B and Neodidemnilactone	Niwa et al. (1994)
Japan	*Diplosoma* sp.	Pentylphenols 1 (inhibited the division of fertilized sea urchin eggs) and 2, cyclopropane fatty acid 3, and cyclopentenones 4 (cytotoxicity against HCT116 cells) and 5	Rob et al. (2011)
Morocco	*Cynthia savignyi*	Cholesterol was the main sterol: 40.8%	Maoufoud et al. (2009)
	Cynthia squamulata	Cholesterol was the main sterol: 59.5%	
NA	Ascidiacea	Man-made glue	Pennati & Rothbacher (2015)
NA	Ascidiacea	edible ascidians: raw, cooked, dried, or pickled	Lambert et al. (2016)
Norway	*Ciona intestinalis*	Cellulose: 96%; (g.100g DW): glutamic acid: 5.27; leucine: 2.54; glycine: 2.31	Hassanzadeh (2014)
Norway	*Ciona intestinalis*	Cholestanol: (32.54% tunic,15.81% inner body); Cholesterol (29.63% tunic, 33.11% inner body)	Zhao et al. (2015)
South Korea	*Halocynthia roretzi*	Up to 80% of fishmeal could be replaced with tunic meal of sea squirt without retardation in growth. Optimal growth was fishmeal 20 diet	Choi et al. (2018)
South Korea	Polyclinidae	1- Aplidic acid A; 2- Aplidic acid B; 3- 4Z-Aplidic acid B; 4- Aplidic acid C; 5- 4Z-Aplidic acid C; 6- Aplidamide A	Bao et al. (2009)
South Korea	*Halocynthia roretzi*	Abalone fed the sea tangle (ST) 400 diet achieved the best growth	Jang et al. (2018)
Turkey	*Phallusia* sp.	Cholesterol: 32%; Volatiles: Hydrocarbons: 48.4%	Slantchev et al. (2002)
	Styela sp.	Cholesterol: 42.3%; Volatiles: Phenols: 46.2%	
USA	*Styela clava*	US retail price (frozen): ($3.63/kg)	Karney et al. (2009)
USA	*Ecteinascidia turbinate*	Anticancer ecteinascidin 743; Commercial-scale in-sea proved cost effective	Mendola (2003)

2.4. Discussion

Ascidians as organic matter extractive species

Over the years, ascidian's biology and functionality have been of growing interest and several studies have addressed water filtration, clearance, retention, pumping, ingestion, and digestion rates. According to Fiala-Médioni (1978a), the definition of filtration rate is the volume of water that has been cleared of particles in a given time frame. Authors have gradually replaced the term "filtration rate" with clearance rate and although this topic has been widely addressed, previous reports have shown considerable variation in the results being reported.

Petersen (2007) complied information on the suspension-feeding of ascidians and concluded that "filtration rates in different species at identical conditions will not vary more than within the same species of different sizes" and also suggests that ascidians are more efficient in non-turbid conditions. Moreover, this present review revealed that since Petersen's (2007) work, there is a generalized lack of studies on this topic. In the last decade, only six new publications have addressed filtration rates, mainly in the genus *Styela* and *Ciona* (Draughon et al., 2010; Sumerel and Finelli, 2014; Kang et al., 2015; Stabili et al., 2016; Hoxha et al., 2018; Nakai et al., 2018), and therefore further research is urgently needed.

Testing filtration rates can be very complex in several ways and several variables must be taken into consideration. Robbins (1983) suggested that with an increase in food concentration, the filtration rate would decrease. Randløv & Riisgärd (1979) observed that the presence of a folded pharynx in *Molgula manhattensis* increased the area of the water-transporting structure, thus allowing for higher filtration rates. The lag-phase phenomenon was not perceived by Randløv & Riisgärd (1979) leading to lower rates being reported and ultimately to an overall misinterpretation of their findings and not allowing comparison with other studies. Therefore, the need for a lag phase with an appropriate time (20 to 140 min) is highly recommended (Petersen and Riisgärd, 1992). Moreover, Petersen & Svane (2002) measured the filtration rate of seven ascidians and concluded that the area of the branchial basket and the length of the ciliary band lining the stigmata openings also contributes to higher filtration rates.

Ascidians are very sensitive organisms to any chemical or mechanical disturbance, which can cause them to close their siphons and thereby stop filtration, thus generating unrealistic filtration rates. Several studies in the 1970s (Fiala-Médioni 1973, 1974, 1978b, 1978a, Randløv & Riisgärd 1979) concluded that undisturbed ascidians filter water very efficiently and at constant rates, a feature that will unquestionably optimize their performance if these are employed in the IMTA framework.

Most often, it is not easy to evaluate if filtration rates are at their optimal by merely recording the appearance of ascidians (unlike what occurs for some bivalves, such as mussels) (Petersen and Riisgärd, 1992). As environmental variables play an important role in the filtration process, several investigations aimed to elucidate the relationship between filtration rate, body size, temperature, and particle concentration (Fiala-Médioni, 1978b; Petersen and Riisgärd, 1992; Kang et al., 2015). In sum, the standardization of the methodology used to investigate filtration rate is in high demand to better evaluate and compare data from different research.

Consistent results were observed allowing us to affirm that as ascidians increase in size, their filtration rate will also increase, and filtration rate decline with temperatures above 20-21 ºC, this being true for ascidians from temperate waters. Moreover, the optimal temperature for ascidians' filtration rate may vary with the species being addressed and with the local conditions. Nakai et al. (2018) registered an optimal filtration at temperatures of 24-25 ºC for *Ciona savignyi*. Several reports focus on the deleterious effects of biofouling by ascidians on mussel farming and their potential competition as filter feeders for trophic resources. One study compared ascidian and mussel filtration rates and highlighted that at 16 °C and 19 °C these are similar (Daigle and Herbinger, 2009).

Conversely, allied with the filtration process is particle retention efficiency. Various approaches have shown that the diet of ascidians mainly comes from smaller particles (particulate organic matter (POM) <20 µm) (Ju et al., 2015, 2016), picophytoplankton (<2 µm), and phytoplankton biomass (Riisgärd and Larsen, 2016). Moreover, Lacoste et al. (2016) verified an overall lack of food selectivity. The retention efficiency increased for particle sizes 2-5 µm (Armsworthy et al., 2001), in which particles from 2-3 µm were completely retained (Randløv and Riisgärd, 1979) and retention efficiency decreased for particles above 4.5 µm (Pascoe et al., 2007).

In general, a threshold of 2-4 µm is observed. In a more recent study, with an *in situ* experiment using 6 different ascidian species, a 95% retention efficiency was registered for 1 µm particles and 50% efficiency for submicron particles (0.3 µm), thus widening ascidians' scope (Jacobi, 2018). The ability of *S. plicata* and *Polyandrocarpa zorritensis* to remove *Vibrio alginolyticus* from seawater has also been tested, with *S. plicata* showing a higher efficiency for bioremediation and restoring seawater quality (Stabili et al., 2016). The same authors also demonstrated that retention efficiency was higher in solitary ascidians (81%) than in colonial ones (41%). Lefebvre et al. (2000) used oysters in a land-based fish-farm effluent and confirmed that suspended feeders can improve water quality and add economic value. However, the retention efficiency of filter-feeders in an IMTA scenario must be dealt with caution as many parameters may influence the settling velocity of the suspended particles of organic matter (Reid et al., 2009).

Nonetheless, further research is much needed considering that our systematic review revealed the existence of few publications addressing this topic in colonial ascidians.

Ascidians incorporated in IMTA frameworks

Despite the increase in interest in IMTA frameworks over the last years, ascidians have rarely been addressed under this scope. Most publications on aquaculture mostly focus on ascidians as pests due to biofouling features and negative impacts on aquaculture facilities, mainly on shellfish productions (Carver et al., 2003). Cultured shellfish can be negatively affected by ascidian fouling in many ways, with these causing a reduction in mussel growth, flesh weight, and reduced overall size and condition (Daigle and Herbinger, 2009; Sievers et al., 2013; Guyondet et al., 2016; Nakai et al., 2018).

In extreme conditions, this may even lead to mussel mortality (Daigle and Herbinger, 2009). However, this scenario cannot be generalized, as Cordell et al. (2013) did not record any negative effects on mussel growth at four different locations and Sievers et al. (2013) observed no reduction of flesh weight was seen in larger mussels. Moreover, Lacoste et al. (2016) found that food competition between oysters and ascidians was not a limiting factor, which advises caution on making generalized assumptions about the negative impacts of ascidians on the farming of bivalves.

Indeed, several factors such as location, species involved, environmental parameters, sampling, and experiment conditions, among others must also be considered (Fletcher et al., 2013).

Furthermore, some ascidians present invasive traits, growing quickly, and therefore must be supervised to not overwhelm and overgrow the other culture species.

The solitary ascidian, *C. intestinalis*, was investigated in 60% of publications in this field, given that this is one of the most studied ascidian species. As an example, they present high tolerance to a wide range of salinities and temperatures (Lutzen, 1999; Shenkar and Swalla, 2011), allowing them a worldwide spatial distribution. This biofouling ascidian, with a fast-growing rate (Ramsay et al., 2008b; Lutz-Collins et al., 2009), contributes to organic sedimentation (Guyondet et al., 2016), and prefers unfouled sites, dark and sheltered areas (Paetzold et al., 2012) does not necessarily have negative impacts on all bivalves or other organisms, further research is needed.

Recently, some studies investigated the impacts of the presence of ascidian *S. clava* in an IMTA framework to optimize the growth of the sea cucumber *A. japonicus* (Zhen et al., 2014; Ju et al., 2015, 2016). These studies have shown that an IMTA framework consisting of ascidian-sea cucumbers-microalgae, not only has the potential to reduce organic matter in the surrounding sediment (Ju et al., 2015), but it can also reduce harmful bacteria (Lin et al., 2016) and purify the water body from dissolved nutrients such as nitrogen and phosphorus (Ju et al., 2015). Moreover, this framework can also have a positive impact on the growth performance of these sea cucumber species (Zhen et al., 2014; Chen et al., 2015; Ju et al., 2016). Available literature shows that only one ascidian species (*S. clava*) was addressed in these studies, and yet with very positive results.

How to incorporate and manage ascidians in an IMTA framework is an important issue with many critical factors that must be considered. Growth rate, spawning season, number of generations, settlement locations, and life span are some of these factors. As an example of how contrasting can these factors be for different ascidians, *C. intestinalis* can produce from 12000 to 100000 eggs over different spawning periods, whereas the colonial ascidian *Botryllus schlosseri* can only produce up to 50 eggs in 3 months (Paetzold et al., 2012). Solitary ascidians *Ascidiella aspersa*, *C. intestinalis* (Millar, 1952), and *Corella willmeriana* (Lambert, 1968) can develop into mature adults in just 3 months and reach up to 50 mm, 120 mm, and 12 mm respectively, with 1 or 2 generations and a life span of 12 to 18 months (*A. aspersa* and *C. intestinalis*) and 3 months (*C. willmeriana*).

The difficulty arises in the management of these biological and ecological characteristics due to the range of intra and interspecific variability and the potential environmental impacts that using ascidians may bring (*e.g.*, biofouling). The existence of a specific area that may promote the settlement of ascidians, such as longlines or PVC (polyvinyl chloride) plates, can be a simple solution to foster the production of biomass of these organisms and allow them to easily remove their biomass for multiple applications.

Exploring the possibility of using multiple combinations of different ascidian species with other taxa, such as fish, shellfish, or echinoderms (namely sea cucumbers) is paramount to test innovative IMTA frameworks with enhanced socio-economic and environmental performance.

Ascidians as bioresources for high-end uses

Considering the increase of wild-harvested or cultured ascidians for human consumption, mainly in Japan, South Korea, and Chile, knowledge of the proximate composition, biocompounds, and food safety issues are of greater relevance.

Over the last decade, an increasing concern on food safety issues associated with ascidians has led promptly several studies on the identification of bacteria associated with edible ascidians such as *H. aurantium* (Chen et al., 2018) and *H. roretzi* (Kumagai et al., 2011). Bacteria associated with ascidians can also be a source of bioactive secondary metabolites and biosurfactants with diverse biotechnology applications in the food-processing industry, among other high-end markets (Achieng et al., 2017).

Several natural products have been isolated from ascidians, for example, the cellulose that is present almost exclusively in the ascidian’s tunic and it is rich in carbohydrate contents (Zhao and Li, 2016), whereas the inner body is protein-rich (Berrill, 1950; Hassanzadeh, 2014). Many other compounds, for example, alkaloids, cyclic peptides, and polyketides, collagens, sulfated polysaccharides, glycosaminoglycans, sterols, among others, can be exploited as by-products in the pharmaceutical and chemical industry (Hassanzadeh, 2014; Monmai et al., 2018) due to their antibacterial, antifungal, antitumor and anti-inflammatory activities (Chen et al., 2018). Numerous biocompounds have successfully been retrieved from ascidians, a recent review on this matter describes “about 160 molecules endowed with antimicrobial activity produced by ascidians and/or by their associated microorganisms” (Casertano et al., 2020).

In recent years, the search for new chemical constituents derived from marine invertebrates has increased intensity (Datta et al., 2015). For instance, Pennati and Rothbächer (2015) investigated the ascidian’s larval bioadhesion properties to develop man-made glues and fouling-resistant surfaces from solitary and colonial ascidians. Nowadays, ascidians are used in multiple applications such as fishing bait, health supplement tablets, (Lambert et al., 2016), and as ornamental species for marine aquaria, fetching at high prices online (https://www.reefcleaners.org/; https://www.mysaltwaterfishstore.com/).

Looking at FAs in more detail, our review revealed that approximately 70% of publications regarding FAs focused on solitary ascidians, and once again ascidian *C. intestinalis* was the main focus. Many studies have drawn their attention to establishing ascidians as a new bioresource for *n*-3 FAs-rich marine lipids (Hassanzadeh, 2014; Zhao et al., 2015; Zhao and Li, 2016). Nonetheless, the profiling of FAs in ascidians, in general, is still poorly explored. Our study retrieved information from 20 species, with 13 ascidian species being addressed only once.

The overall results suggest that ascidians can be a good source of *n*-3 polyunsaturated FAs, namely essential FAs such as EPA and DHA, which were detected in most ascidians surveyed (Dagorn et al., 2010; Zhao et al., 2015). Therefore, ascidians present a high nutritional value, they are a healthy seafood choice due to their high protein levels and low calories (Lee et al., 1995; Kang et al., 2011). Hassanzadeh (2014) concluded that the composition profile of ascidian FAs seems to be similar to fish oil. Therefore, ascidian biomass may eventually be a good alternative to fish oil and fish meal in formulated aquafeeds.

Moreover, ascidians present an amino acid composition similar to egg albumin, suggesting a great potential and capability to be weighed as marine organisms' feed (Hassanzadeh, 2014). Indeed, the replacement of fish meal with ascidian biomass in aquafeeds has already started being addressed with Jang et al. (2017) and Choi et al. (2018) having partially or fully replaced the fish meal with the tunic of the ascidian *H. roretzi* in aquafeeds for the abalone *Haliotis discus* with compromising its growth performance.

2.5. Conclusions

In the past two decades, considerable insights have been achieved into ascidians' ecology and biology, including filtration and retention efficiencies. Their nutritional value and potential role in IMTA frameworks are also starting to be thoroughly investigated.

Despite the intra and interspecific variability recorded for ascidians' filtration rates, there is a consensus that these organisms do display high filtration rates, that they can retain submicron and picoplankton particles, and that they also present a fast-growing rate. As available scientific evidence suggests that these organisms are capable to perform well under an IMTA framework however, it is important to investigate if competition with other filter-feeders for trophic resources and space can occur, namely with mussels, scallops, and oysters. Furthermore, available studies to date suggest that ascidians achieve higher growth performances in IMTA frameworks when in the presence of sea cucumbers. The development of innovative IMTA frameworks is important to maximize the systems carrying capacity.

Finally, among other potentially bioactive compounds, ascidians represent a rich source of EPA and DHA, both being essential FAs paramount for human consumption, marine fish, and shrimp nutrition. Despite some cultural barriers in western countries, ascidians are increasingly regarded as a healthy seafood for human consumption, being an interesting source of essential amino and FAs. The use of ascidians as an alternative ingredient for the formulation of aquafeeds also looks promising and will certainly deserve further attention in the coming year.

Chapter 3

Using oyster shells for customized 3-D structures for monitoring ecosystem shifts on ascidians diversity

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3. Using oyster shells for customized 3-D structures for monitoring ecosystem shifts on ascidians diversity

Abstract

Biofouling communities are broadly distributed and there is a growing need to understand, monitor, and prevent their dispersal and colonization. Ascidians are a major group of fouling organisms but have remained poorly studied in this context. Furthermore, the search for improved sustainable practices regarding shipping networks, biofouling management, treatments, and monitorization has made headway rapidly.

The present study surveyed and established a baseline for the ascidian biofouling community in a coastal lagoon, by operationalizing the concept of artificial substrate units (ASU) through a customized 3-D unit with the shape of a triangular-based pyramid, a nature-based structure that simulates natural habitats, made with oyster shells sourced from local aquaculture farms. The ASU were grouped into a five-replicate star-shaped, to be collected at each sampling moment. Throughout the 295 days (from May to December 2020) of the present study covering five different locations of the Ria de Aveiro (Portugal) coastal lagoon, a total of 12 species of ascidians were collected.

While *Ascidiella aspersa*, *Microcosmus* sp., and *Molgula* sp. 1 were registered in all the locations surveyed, the remaining nine ascidian species were dominant only in specific locations of the coastal lagoon. Values of total abundance presented an overall increasing trend in all locations surveyed, with maximum values corresponding to summer periods. Two locations (Oyster Farm and Integrated Multi-Trophic Aquaculture Farm) recorded the highest abundance values. The present findings demonstrated that the ASU employed using oyster shells, a widely available co-product of oyster farming, can be considered an efficient support structure for short- or long-term monitoring of the ascidian community, as well as fouling communities in general.

Hydrodynamics, seasonality, and nutrient-enriched waters were the main contributors to the establishment of ascidians. For the first time, *Clavelina lepadiformis* and *A. aspersa* were collected and reported in the coastal waters of mainland Portugal. While preventing the settlement of fouling communities can be extremely difficult, an improved understanding of existing communities of these organisms can undoubtedly contribute to the development of improved management practices to control them. An updated list of all ascidian species recorded to date from coastal waters of mainland Portugal is also presented.

Keywords

Settlement, Coastal lagoon, Artificial substrate, Monitorization, Fouling community, Tunicates

3.1 Introduction

Ascidians are amongst the most significant groups of fouling organisms worldwide, a feature that has increased the interest of the research community in these organisms (Taylor et al., 1997; Fitridge et al., 2012). They are sessile filter-feeders that display fast growth rates (Millar, 1952), and inhabit a variety of substrates (Millar, 1971; Lambert, 2007). They can also be recognized as a potential co-cultured/extractive species for Integrated Multi-Trophic Aquaculture (IMTA) with potential added value as bioresources (Marques et al., 2022).

Ascidians present a short lifespan and their lecithotrophic larval development makes them an important bioindicator of anthropogenic transport (Marins et al., 2010). Their larvae can tolerate long-distance dispersal as a result of human intervention (Marshall et al., 2003) through ballast waters or translocations of cultivated shellfish for aquaculture purposes (Lambert, 2001; Locke et al., 2007). Ascidians can quickly spread and colonize extensive areas and new ecosystems, occupying artificial and natural substrates (Millar, 1971; Lambert, 2007). Fouling ascidians settle on aquaculture gear, piers, floating docks, marinas, and wharf piles from commercial ports causing deleterious impacts on those locations (Dumont et al., 2011; Koplovitz et al., 2016). Furthermore, ascidian fouling on mussel farms can negatively impact the growth of these bivalves (Guenther et al., 2006; Bullard et al., 2013), increase their mortality (Forrest et al., 2007; Bannister et al., 2019), induce shell deformities (Taylor et al., 1997), and by increasing the weight on aquaculture infrastructures (Ramsay et al., 2008a; Rodriguez and Ibarra-Obando, 2008) can cause negative ecological and economic impacts (Carver et al., 2003; Lutz-Collins et al., 2009; Lacoste et al., 2016).

Controlling and mitigating biofouling is a large financial burden to the aquaculture industry. Estimates indicate that 20- 30% of additional costs are spent annually on biofouling control, with these figures varying with cultured species and location (Lacoste and Gaertner-Mazouni, 2015). Thus, aquaculture activities demand the development of innovative technological solutions that target fouling organisms, to reduce their impacts and achieve more sustainable farming approaches. However, biofouling control in aquaculture must be assessed with caution, as the removal process can also be stressful and damaging for the species being cultured (Lacoste and Gaertner-Mazouni, 2015). Expectations are that these methods will evolve as the aquaculture industry moves forward (Bannister et al., 2019).

Therefore, one of the measures to assist this matter includes the establishment of monitorization programs and carrying out local surveys, as these will allow early detection and enable a rapid response. Implementation of monitoring programs is normally managed by international organizations, including the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) (Lehtiniemi et al., 2015).

Throughout the years, different monitoring programs for fouling communities have been addressed, such as metabarcoding analysis (Azevedo et al., 2020), the maintenance and surveillance of marine vessels (Gewing and Shenkar, 2017), proteomic profiling (Kuplik et al., 2019), and the use of oyster shells as a habitat collector (Outinen et al., 2019), all of which can significantly serve as an effective support structure for the early-detection of biofouling species. As such, it is essential to understand the biofouling communities, with emphasis on the ascidians community's composition, abundance, and distribution, and identify the major environmental drivers that shape the settlement dynamics of these communities for further implementing enhanced mitigation actions.

The present study aims to investigate three research questions: i) can customized 3-D ASU structures made of oyster shells support the monitoring of ecosystem shifts on ascidians diversity? ii) using Ria de Aveiro, a coastal lagoon (Portugal) as a case study, can this 3-D ASU be employed to survey and establish a baseline for the ascidian biofouling community? iii) can these 3-D ASU also be used in an aquaculture facility?

The main objective was to monitor and establish a benchmark for the ascidian biofouling community by using an innovative customized 3-D star-shaped structure composed of five replicated ASU using oyster shells as a nature-based substrate.

3.2 Material and Methods

3.2.1 Study Area

The study area is located in the shallow coastal lagoon Ria de Aveiro, Portugal (Figure 3.1), which forms four major channels (Ovar Channel, 29 km to the North; Espinheiro Channel, a highly modified water body, which corresponds to the Vouga estuary; the Ílhavo Channel, 15 km to the southeast; and the Mira Channel, 20 km to the south characterized by a typical estuarine gradient); and connects to the Atlantic Ocean through a narrow artificial navigation channel (Lopes et al., 2017).

Our experimental design consists of five distinct research locations distributed within Mira Channel and the premises of a facility that receives water from the Ílhavo Channel. The first sampling location, “Tide Gauge” (TG), is located at the Ocean boundary of the Ria de Aveiro, which receives the greatest influence from marine waters and features 1.3 km long, 350 m wide, and 20 m deep. The tidal currents' velocities can reach 1 m s^{-1} and progressively gets weaker in the many innermost canals (Martins et al., 2010b). Three sampling locations are allocated in the Mira Channel, characterized by the existence of intertidal zones, namely mudflats and salt marshes, where recreational and aquaculture activities occur, such as extensive natural banks of shellfish; "Fishing Harbour" (FH) (40° 37' 57.3" N. 8° 43' 57.8" W), where commercial vessels resort for shelter, landing catches of coastal fisheries and has a maximum capacity of 136 medium size fishery boats; "Marina" (M) (40° 37' 13.4" N. 8° 44' 54.2" W), where small recreational boats can dock on floating structures, with the capacity of 130 berths, and "Oyster Farm" (OF) (40° 37'06.7" N. 8° 44' 28.2" W), is established within a rack-and-bag culture oyster farm of *Magallana gigas*. This method is highly dependent on the tidal range.

Ílhavo Channel, with a 15 km length, is characterized by partially mixed waters depending on the volume of freshwater inflow from the Boco river. The fifth sampling location is in this channel "Integrated Multi-Trophic Aquaculture Farm" (IMTA), (40°36'44"N 8°40'3"W). A land-based semi-intensive fish farm that operates under an IMTA framework. At this facility, seabass and seabream are produced in earth ponds, with their nutrient-rich effluents being supplied to farm macroalgae in concrete tanks.

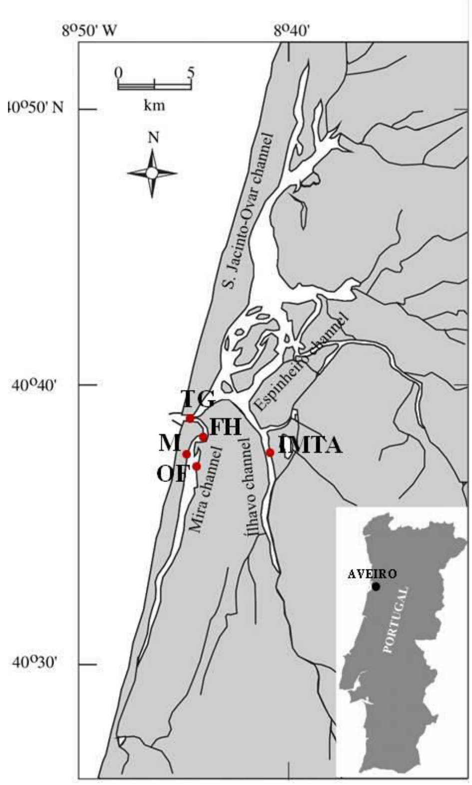

Figure 3.1: Ria de Aveiro coastal lagoon and the four sampling sites located in the Mira channel and the fifth sampling site located in Ílhavo channel, surveyed in the present study (red dots). TG, Tide Gauge; FH, Fishing Harbour; M, Marina; OF, Oyster Farm; IMTA, Integrated Multi-Trophic Aquaculture

3.2.2 Customized 3-D Artificial Substrate Units

Oyster shell-based units were developed to serve as a nature-based structure for ascidian larvae to settle (Figure 3.2). Initially, from a net roll with a 0.01 m mesh (Figure 3.2A), a rectangle with 0.2 m in length and 0.4 m in height was cropped (Figure 3.2B). Afterward, the rectangle was folded in half and the lateral parts were sewn together with a nylon fishing line (Figure 3.2C). Next, the superior edge was opened, 30 oyster shells (10 large + 20 small) were placed within each triangular-based pyramid (Figures 3.2D, E), and the opposite sides were joined and sewn together forming the final triangular-based pyramid (Figure 3.2F). Oyster shells (from *Magallana gigas*) were sourced from an oyster farm at Ria de Aveiro, where this is an abundant coproduct of oyster farming due to mortality during grow-out. To achieve the final star-shaped composed of five replicated ASU, five triangular-based pyramids were connected using a polypropylene nautical cord and a zip tie fastened through the center edges (Figure 3.2H).

Each pyramid was weighed to ensure similar weights amongst replicates. The final weight varied between 270 – 310 g with an average weight of 285 g. Each pyramid measured 0.2 m in length and 0.2 m in height and holds 1 L volume-wise. A total of 175 triangular-based pyramids were created, which were used to assemble 35 star-shaped composed of five replicated ASU.

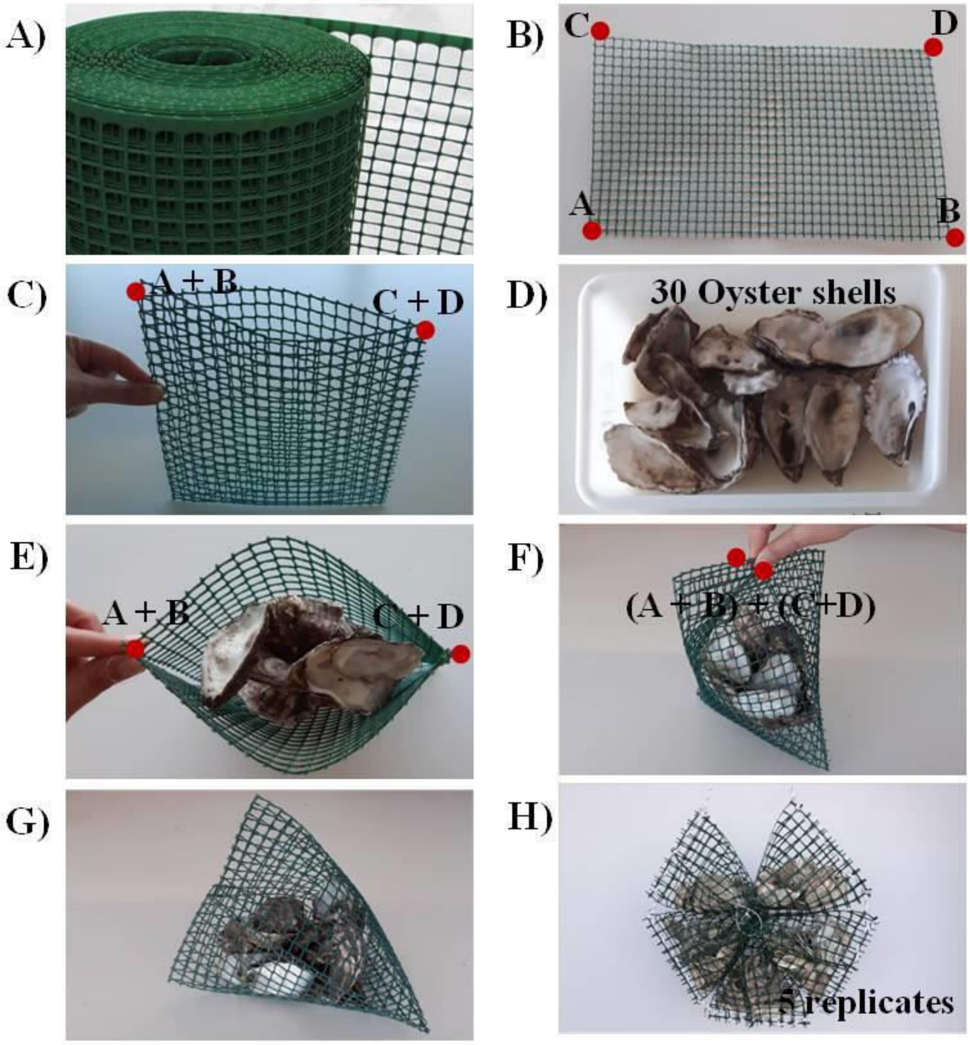

Figure 3.2: Representation of the steps to set up the triangular-based pyramid (A–G) and the final star-shaped artificial substrate unit (ASU) consisting of five replicates (H). Photographs were taken in February 2020, Aveiro. (Photographs by Luisa Marques).

3.2.3 Sampling and Laboratory Procedures

During February 2020 (late winter), seven star-shaped units, each composed of five replicated ASU, were deployed at each of the five research sites. These were randomly widespread and attached to existing structures, remaining fully or partially submerged during tides. During the experimental period, no intervention whatsoever (including cleaning) was performed on the ASU. The first sampling procedure occurred in May 2020, after 92 days, and it was repeated every five weeks until December 2020 (late autumn), with the final unit being sampled 295 days after being deployed. At each sampling moment, five ASU (corresponding to one star-shaped unit) was retrieved from each location, transported in a cooler with local water, and immediately processed upon arrival at the laboratory. From each star-shaped unit collected, every one of the five triangular-based pyramids was processed individually, thus allowing to retrieve five independent replicates from each location.

3.2.4 Nutrient Analysis

Salinity, water temperature (ºC), pH, and dissolved oxygen (mg L^{-1}) were measured *in situ* (Table SI 3.1) during each sampling event. Water samples, collected in triplicates, were transported under refrigerated conditions and immediately filtered with pre-weighed filters (Ahlstrom Munksjö GF/C, Ø47 mm dehydrated at 105°C). Afterward, filters were dried at 60°C for 48 h (suspended particulate matter: SPM) and combusted at 450°C for 5h (ash-free dry weight: AFDW), particulate organic matter (POM) was calculated by subtracting SPM and AFDW, following the EPA Method 160.2. Water aliquots were stored frozen at -20°C until analysis. A Skalar San++ Continuous Flow Analyzer (Skalar Analytical, Breda, The Netherlands) was used to determine dissolved ammonium ($N\text{-}NH_4$, mg L^{-1}), nitrogen oxides (N-NOx, mg L^{-1}) and orthophosphate ($P\text{-}PO_4$, mg L^{-1}) concentrations, using Skalar's standard automated methods for $NH_4\text{-}N$ (Modified Berthelot reaction for ammonia determination), N-NOx (Total UV digestible nitrogen/nitrate + nitrite/nitrite) and $P\text{-}PO_4$ (Total UV digestible phosphate/orthophosphate).

3.2.5 Ascidian Sampling

Biological samples were transported in a cooler with local water and immediately processed upon arrival at the laboratory. Ascidians from each replicate were retrieved from the net and the oyster shells were subsequently counted. Ascidians were carefully removed manually and placed in a Petri dish with seawater. Most ascidians exhibit a tactile response during measuring and may contract; therefore, for a more accurate measurement, ascidians were spread out on a tray and left for 15 minutes before measuring was performed using graph paper. Individual mass (wet weight) was registered for each replicate. Colonial ascidians for which removal from the net and oyster shells was not possible were photographed and coverage area was measured using the software ImageJ. Subsequently, ascidians were stored at -80°C and subsequently freeze-dried to determine their total biomass. Ascidians were identified to the lowest possible taxa, using identification guides and dichotomous keys (Alder and Hancock, 1905; Hayward and Ryland, 1990).

3.2.6 Diversity Indicators

A set of five indices was determined (Table SI 3.2): 1) to evaluate the number of species present in each location (species richness); 2) and 3) species richness and evenness were determined using Pielou's Evenness and Margalef's Richness Index (Margalef, 1968; Pielou, 1969), as these allow to evaluate the level of homogeneity within the ascidian community at each location; 4) and 5) to characterize species diversity we employed Shannon's and Simpson's index, as these account for both abundance and evenness of the species present at each sampling location ((Simpson, 1949; Shannon and Weaver, 1963).

3.2.7 Statistical Analyses

Data from the total abundance were log(x+1) transformed to reduce the skewness of our original data and a Bray-Curtis similarity matrix (Bray and Curtis, 1957) was assembled. A two-way PERMANOVA design was created with "location" and "sampling period" being used as fixed factors. Whenever significant differences were observed, two pairwise test analyses were performed: the first was to infer which sampling locations presented significant differences between the total abundance of ascidians collected, and the second was to determine which sampling periods within each location presented significant differences. The statistical significance of variance components was tested using 999 permutations of unrestricted permutations of data, with an a priori chosen significance level of $\alpha=0.05$. The Monte Carlo P value (P(MC)) was used whenever permutations were less than 100.

A multidimensional scaling (MDS) was performed to visualize the overall patterns and relationships between the biological matrices surveyed. Before the statistical analysis of environmental parameters (PO_4^{3-}, NH_4^+, NO_x, and POM), a resemblance matrix based on Euclidean distance was calculated and data were log(x+1) transformed, again, to reduce the skewness of our original data. Afterward, all parameters followed normalization. The relationship between all environmental variables (PO_4^{3-}, NH_4^+, NO_x, and POM) and the distribution of the ascidian community was explored by carrying out a Distance-Based Linear Model analysis (DistLM) with "Best" as the selection procedure and "BIC" (Bayesian information criterion) as the selection criterion. All multivariate analyses were performed using PRIMER 6 + PERMANOVA© software (software package from Plymouth Marine Laboratory, UK) (Anderson et al., 2008)

3.3 Results

A total of eight genera and 12 species of ascidians were collected from the ASU during the present study from all five locations (Figure 3.3). Three colonial ascidians *Botryllus schlosseri* (Pallas, 1766), *Botrylloides violaceus* (Oka, 1927), *Clavelina lepadiformis* (Müller, 1776), and nine solitary ascidian species, *Ascidiella aspersa* (Müller, 1776), *Ciona intestinalis* (Linnaeus,1767), *Styela plicata* (Lesueur, 1823), *Styela* sp. (Fleming, 1822), *Microcosmus* sp. (Heller, 1877), *Molgula* sp. 1 (Forbes, 1848), *Molgula* sp. 2 (Forbes, 1848) were identified (with two solitary ascidians having remained unidentified, due to their small size or slightly damaged body).

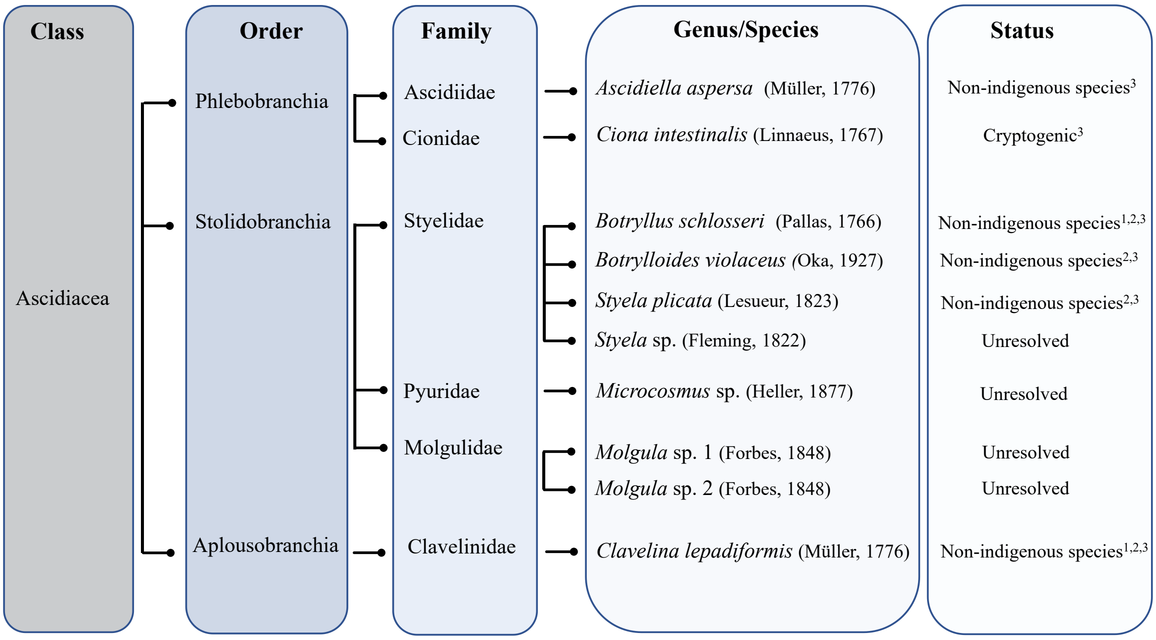

Figure 3.3: Taxonomic tree of the ten identified species retrieved from the Ria de Aveiro. Superscript numbers indicate reference: 1- Canning-Clode et al. (2013); 2 – Chainho et al. (2015); 3 – Ramalhosa et al. (2021).

The total number of ascidian species retrieved in each sampled location ranged from five to eight species at the end of the trial (Table 3.1). The ascidian species *A.aspersa*, *Microcosmus* sp., and *Molgula* sp. 1 were recorded in all sampling locations (Table 3.1). Nonetheless, these species displayed some variations in their abundance and biomass values (Figure 3.4). *Microcosmus* sp. and *Molgula* sp. 1 recorded the highest values of both abundance and biomass throughout the study period, in all sampling locations, with a particular highlight in Mira Channel locations (FH, M, and OF) (Table 3.2). Despite *Ciona intestinalis* being collected from four out of the five studied locations, this species registered abundance and biomass values at the inner Ílhavo Channel IMTA farm (Figure 3.4E), of 50.4% and 84.5% total respectively (Table 3.2). At the ocean boundary TG location, *C. lepadiformis* was dominant with 43% of the total abundance while *Microscosmus* sp. and *Molgula* sp. 1 dominated the biomass values with a combining percentage of 85% (Table 3.2). *Styela plicata* was solely retrieved from both farm locations at Mira and Ílhavo Channels (OF and IMTA), while *Styela* sp. was retrieved from the port locations at Mira Channel (FH and M) (Table 3.1).

Table 3.1: Presence/absence table of the collected ascidians throughout the duration of the experiment and their respective location within Ria de Aveiro. TG: Tide Gauge; FH: Fishing Harbour; M: Marina; OF: Oyster Farm; IMTA: Integrated Multi-Trophic Aquaculture.

	TG	**FH**	**M**	**OF**	**IMTA**
Ascidiella aspersa	●	●	●	●	●
Botryllus schlosseri					●
Botrylloides violaceus		●		●	
Ciona intestinalis		●	●	●	●
Clavelina lepadiformis	●	●			
Microcosmus sp.	●	●	●	●	●
Molgula sp. 1	●	●	●	●	●
Molgula sp.2	●	●		●	
Styela plicata				●	●
Styela sp.		●	●		
unknown 1					●
unknown 2	●				
Total	6	8	5	7	7

A particular trend towards an increase in total abundance over time, followed by a subsequent decrease was observed, with maximum values being recorded during sampling days 155 to 226 (from July to October) (Figure 3.4). However, this trend is not so evident concerning biomass values. In Mira Channel locations (FH, M, and OF), the species that presented the highest abundance also presented higher biomass values (Table 3.2).

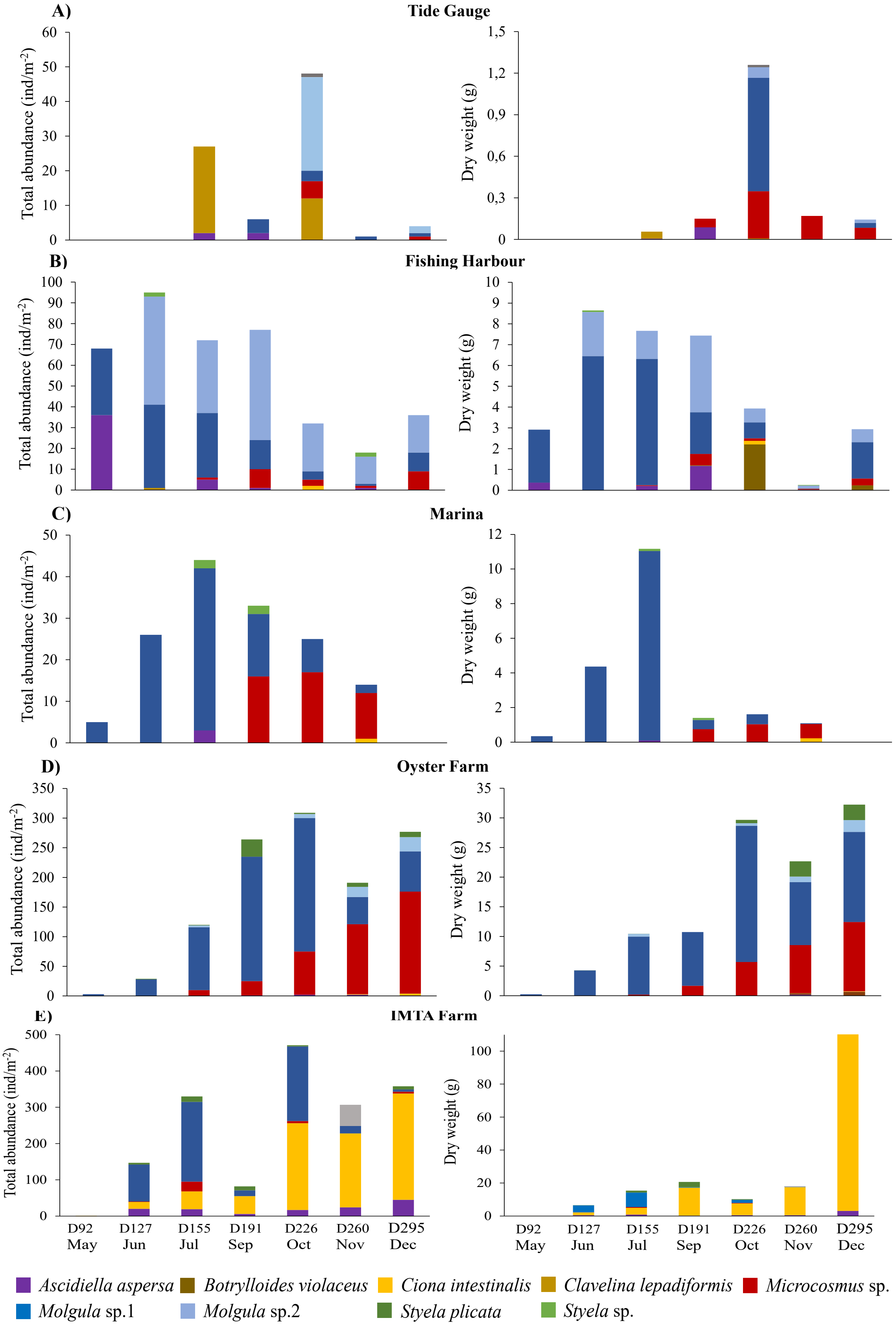

Figure 3.4: Total abundance (ind.m^{-2}) and dry weight (g) registered for each collected ascidian throughout the duration of the experiment, from each sampling location. D, day.

Interestingly, in all studied locations, the same combination of two species consistently dominated total abundance and total biomass, except for TG location: *C. lepadiformis* and *Molgula* sp. 2 represented 76.7% of the total abundance while *Microcosmus* sp. and *Molgula* sp. 1 represented 85% of the total biomass. In the remaining locations: at FH *Molgula* sp. 1 and *Molgula* sp. 2 represented 81.7% of the total abundance and 83.4% of the total biomass; at M and OF locations, *Microcosmus* sp. and *Molgula* sp. 1 dominated 94.6% and 90.8%, respectively of the total abundance and 97.2% and 90.2%, respectively of the total biomass; at IMTA site, *C. intestinalis* and *Molgula* sp. 1 represented 84% of the total abundance and 92.9% of the total biomass.

Table 3.2: Percentage of the total abundance and total biomass registered for each collected ascidian from each location, throughout the duration of the experiment. In bold is represented the two highest values for each location. TG: Tide Gauge; FH: Fishing Harbour; M: Marina; OF: Oyster Farm; IMTA: Integrated Multi-Trophic Aquaculture.

(%)	**TG**		**FH**		**M**		**OF**		**IMTA**	
	Abu	Bio	Abu	Bio	Abu	Bio	Abu	Bio	Abu	Bio
Ascidiella aspersa	4.7	5.2	10.8	5.3	2.0	0.5	0.5	0.2	7.7	3.1
Botrylloides violaceus	-	-	-	7.3	-	-	-	0.8	-	-
Clavelina lepadiformis	**43.0**	3.3	0.3	0.0	-	-	-	-	-	-
Ciona intestinalis	-	-	0.5	0.5	0.7	1.1	0.3	0.1	**50.4**	**84.5**
Microcosmus sp.	7.0	**36.9**	5.8	3.2	**29.9**	**13.0**	**33.3**	**24.8**	2.3	0.6
Molgula sp. 1	10.5	**48.1**	**32.9**	**58.0**	**64.6**	**84.1**	**57.5**	**65.4**	**33.7**	**8.4**
Molgula sp. 2	**33.7**	5.7	**48.7**	**25.4**	-	-	4.3	3.5	-	-
Styela plicata	-	-			-	-	4.1	5.2	2.5	3.1
Styela sp.	-	-	1.0	0.3	2.7	1.2	-	-	-	
unknown 1	-	-	-	-	-	-	-	-	3.4	0.2
unknown 2	1.2	0.8	-	-	-	-	-	-	-	-

Ascidian *B. schlosseri* was only collected at IMTA, registering a maximum area of 0.014 m^2 at sampling day 127. Similar values of the coverage area of *B. violaceus* were recorded at locations FH and OF, however, these were recorded at different sampling periods (0.0032 m^2 at sampling day 295 (December) and 0.0033 m^2 at sampling day 226 (October), respectively). Overall, significant differences were detected in the total abundance of the ascidian community between each sampling site and sampling period (Table 3.3). Furthermore, pairwise test analysis (Table SI 3.3) revealed that abundance values of *C. intestinalis* were significantly different between IMTA and the remaining locations, (IMTA/TG p=0.001; IMTA/FH p=0.001; IMTA/M p=0.001; IMTA/OF p=0.001), as was *C. lepadiformis* between the ocean boundary TG and other locations (TG/FH p=0.04; TG/M p=0.012; TG/OF p=0.015; TG/IMTA p=0.013). Likewise, the two species within the genus *Molgula* presented abundance values with significant differences amongst all locations.

The MDS ordination analysis showed a clear separation of the total abundance of the ascidian community between all five locations (Figure 3.5). Furthermore, a separation between the farm sites (OF and IMTA) with the remaining sampling locations is quite evident. The size and direction of species vectors indicate that *C. intestinalis* and *A. aspersa* are the main contributors to the IMTA site, whereas *C. lepadiformis* is responsible for the separation of the ocean boundary TG, but with lesser influence. *Molgula* sp. 2 is the main contributor to the FH location, *Microcosmus* sp. and *Molgula* sp. 1 are equally dominant at M and OF and are represented in the MDS ordination plot as such. The analysis of nutrient concentrations provided from the water samples retrieved from each sampling period demonstrated that PO_4^{3-} and NH_4^+ displayed a similar pattern. The OF and IMTA sites presented the highest mean values, both with statistically significant differences (1.68µmol/L and 1.69µmol/L for PO_4^{3}; 17.35 µmol/L and 21.86 µmol/L for NH_4^+ respectively) (Figure 3.6).

Furthermore, DistLM analysis unveiled that NH_4^+ best explained the variations in ascidians' total abundance amongst the farm locations (OF and IMTA) while NO_x best explained the remaining locations.

Table 3.3: Results of the two-factor permutational multivariate analysis of variance (PERMANOVA) of the Log(x+1) transformed data for the ascidian species collected throughout the present study in the Ria de Aveiro.

	Ascidiella aspersa		*Ciona intestinalis*		*Clavelina lepadiformis*		*Microcosmus* sp.		*Molgula* sp. 1	
	Pseudo *F*	$P_{(perm)}$	Pseudo *F*	$P_{(perm)}$	Pseudo *F*	$P_{(perm)}$	Pseudo *F*	$P_{(perm)}$	Pseudo *F*	$P_{(perm)}$
Site	50.70	**0.001**	399.91	**0.001**	5.32	**0.001**	42.60	**0.001**	66.78	**0.001**
Day	1.45	0.188	16.95	**0.001**	2.89	**0.011**	23.97	**0.001**	20.24	**0.001**

	Molgula sp. 2		*Styela plicata*		*Styela* sp.		unknown 1		unknown 2	
	Pseudo *F*	$P_{(perm)}$	Pseudo *F*	$P_{(perm)}$	Pseudo *F*	$P_{(perm)}$	Pseudo *F*	$P_{(perm)}$	Pseudo *F*	$P_{(perm)}$
Site	173.76	**0.001**	23.82	**0.001**	2.67	**0.028**	5.40	**0.001**	1	0.491
Day	23.64	**0.001**	3.276	**0.004**	0.912	0.506	5.40	**0.001**	1	0.504

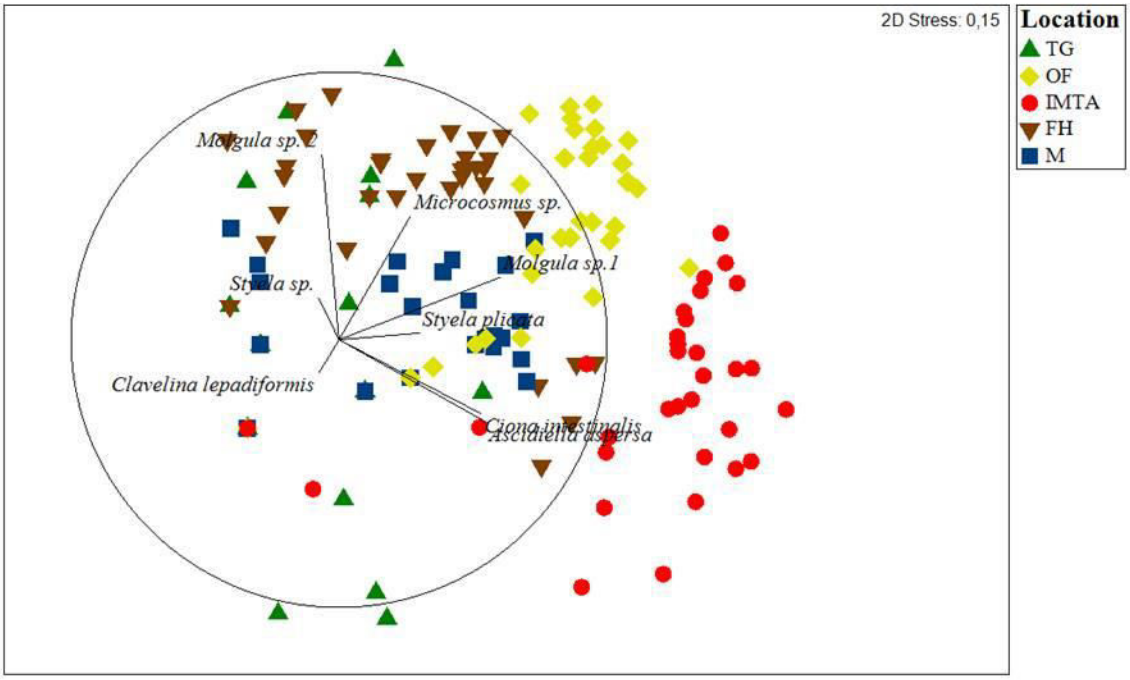

Figure 3.5: Multidimensional scaling plot (MDS) with each dot representing the abundance values for each ascidian retrieved from each of the sampled locations and their spatial distribution. TG, Tide Gauge; FH, Fishing Harbour; M, Marina; OF, Oyster Farm; IMTA, Integrated Multi-Trophic Aquaculture.

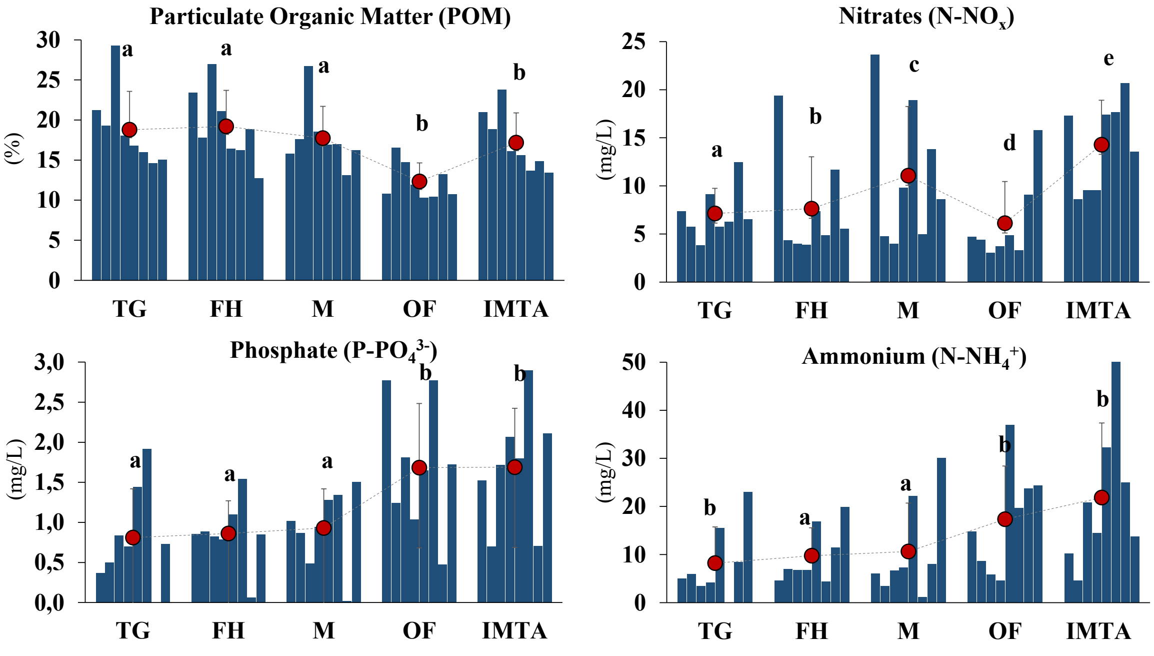

Figure 3.6: Environmental parameters [POM (%), NO$_x$ (μmol/L), PO$_4^{3-}$ (μmol/L), NH$_4^+$ (μmol/L)] registered at each sampled location throughout the duration of the experiment, in the Ria de Aveiro. Red dots represent the mean average with the respective standard deviation. Letters represent statistically significant differences. TG, Tide Gauge; FH, Fishing Harbour; M, Marina; OF, Oyster Farm; IMTA, Integrated Multi-Trophic Aquaculture.

Ecological diversity indices Shannon and Simpson revealed an equal pattern throughout the five locations, in which TG presented the highest values of biodiversity, followed by FH, IMTA, OF, and finally M with the lowest index values. Margalef's index indicated that the FH is the location with the highest species richness. Pielou's index demonstrated that TG and IMTA represent the two locations in which the ascidian communities are the most homogeneous (Table SI 3.4). Lastly, species richness indicated that the highest number of ascidian species recorded was eight within the FH location, while the lowest was five, within the M location.

A detailed list of ascidians reported from coastal waters from mainland Portugal with an indication of their specific location and where were they collected is provided (Table 3.4). A more detailed list of all ascidian species reported to date in Portuguese waters (mainland Portugal along with Madeira and Azores archipelagos) is also presented (Table SI 3.5). A total of 75 ascidian species were recorded in mainland Portuguese waters to date, being distributed over 40 different genera, 55% of which belong to order Stolidobranchia, 22.5% to order Aplousobranchia, and the remaining 22.5% to order Phlebobranchia. Moreover, 68% of all recorded species are solitary ascidians, with the other 32% being colonial organisms.

Table 3.4: List of recorded ascidian species from the coastal waters of mainland Portugal.

Species	**Type**	**Location**	**Reference**
Abyssascidia millari	Solitary	Portugal	Ramos-Esplá 1988
Aplidium albicans	Colonial	Portugal	Ramos-Esplá 1988
Aplidium densum	Colonial	Portugal	Ramos-Esplá 1988
Aplidium elegans[1]	Colonial	Portugal	Ramos-Esplá 1988
Aplidium enigmaticum	Colonial	Portugal	Ramos-Esplá 1988
Aplidium nordmanni[2]	Colonial	Portugal	Ramos-Esplá 1988
Aplidium pallidum	Colonial	Portugal	Ramos-Esplá 1988
Aplidium proliferum	Colonial	Portugal	Ramos-Esplá 1988
Aplidium punctum	Colonial	Portugal	Ramos-Esplá 1988
Aplidium sagresensis	Colonial	Sagres	Ramos-Esplá et al. 1993
Aplidium sp.	Colonial	Ria Formosa	Peck et al. 2015
Araneum sigma	Solitary	Portugal	Ramos-Esplá 1988
Asajirus indicus[1]	Solitary	Portugal	Ramos-Esplá 1988
Bathypyura celata[2]	Solitary	Portugal	Ramos-Esplá 1988
Bathystyeloides dubius	Solitary	Portugal	Ramos-Esplá 1988
Bathystyeloides enderbyanus	Solitary	Portugal	Ramos-Esplá 1988
Botrylloides violaceus	Colonial	Nazaré	Nagar et al. 2010

Botryllus schlosseri	Colonial	Lisboa	Saldanha et al. 1974
		Porto	Azevedo et al. 2020
		Faro Sesimbra Setubal	Ben-Shlomo et al. 2006
		Portugal	Ramos-Esplá 1988
Ciona intestinalis	Solitary	Porto	Azevedo et al. 2020
Cnemidocarpa bythia[2]	Solitary	Portugal	Ramos-Esplá 1988
Cnemidocarpa devia[2]	Solitary	Portugal	Ramos-Esplá 1988
Cnemidocarpa digonas[2]	Solitary	Portugal	Ramos-Esplá 1988
Cnemidocarpa platybranchia[2]	Solitary	Portugal	Ramos-Esplá 1988
Corella eumyota	Solitary	Algarve	Sofia Ruiz 2015
		Vila Praia de Âncora Póvoa de Varzim Nazaré Peniche Oeiras	Nagar et al. 2010
Corynascidia translucida	Solitary	Portugal	Ramos-Esplá 1988
Culeolus suhmi	Solitary	Portugal	Ramos-Esplá 1988
Dendrodoa grossularia	Solitary	Porto	Azevedo et al. 2020
		Portugal	Ramos-Esplá 1988
Diazona violacea	Colonial	Portugal	Ramos-Esplá 1988
Dicarpa pacifica	Solitary	Portugal	Ramos-Esplá 1988
Dicarpa simplex	Solitary	Portugal	Ramos-Esplá 1988
Didemnum coriaceum	Colonial	Portugal	Ramos-Esplá 1988
Didemnum maculosum	Colonial	Portugal	Ramos-Esplá 1988
Didemnum vexillum	Colonial	Porto	Azevedo et al. 2020
Diplosoma listerianum	Colonial	Portugal	Ramos-Esplá 1988
Distaplia rosea	Colonial	Portugal	Ramos-Esplá 1988
Distomus variolosus[2]	Colonial	Portugal	Ramos-Esplá 1988
Halocynthia papillosa[2]	Solitary	Portugal	Ramos-Esplá 1988
Hemistyela pilosa[1]	Solitary	Portugal	Ramos-Esplá 1988
Heterostigma separ	Solitary	Portugal	Ramos-Esplá 1988
Lissoclinum perforatum	Colonial	Portugal	Ramos-Esplá 1988
Microcosmus nudistigma	Solitary	Portugal	Ramos-Esplá 1988
Microcosmus polymorphus	Solitary	Portugal	Ramos-Esplá 1988
Microcosmus sabatieri	Solitary	Portugal	Ramos-Esplá 1988
Microcosmus squamiger	Solitary	Algarve	Sofia Ruiz 2015

		Cascais	Turon et al. 2007
Minipera pedunculata	Solitary	Portugal	Ramos-Esplá 1988
Molgula manhattensis	Solitary	Portugal	Ramos-Esplá 1988
Molgula occidentalis	Solitary	Algarve	Sofia Ruiz 2015
Molgula sp.	Solitary	Ria Formosa	Peck et al. 2015
Molguloides crenatum	Solitary	Portugal	Ramos-Esplá 1988
Octacnemus ingolfi	Solitary	Portugal	Ramos-Esplá 1988
Oligotrema lyra[1]	Solitary	Portugal	Ramos-Esplá 1988
Oligotrema unigonas[1]	Solitary	Portugal	Ramos-Esplá 1988
Phallusia mammillata	Solitary	Algarve	Oliveira et al. 2009
Polycarpa comata	Solitary	Portugal	Ramos-Esplá 1988
Polycarpa errans	Solitary	Portugal	Ramos-Esplá 1988
Polycarpa fibrosa	Solitary	Portugal	Ramos-Esplá 1988
Polycarpa gracilis	Solitary	Portugal	Ramos-Esplá 1988
Polycarpa pseudoalbatrossi	Solitary	Portugal	Ramos-Esplá 1988
Polycarpa violacea	Solitary	Portugal	Ramos-Esplá 1988
Polysyncraton lacazei	Colonial	Portugal	Ramos-Esplá 1988
Proagnesia depressa	Solitary	Portugal	Ramos-Esplá 1988
Protomolgula bythia	Solitary	Portugal	Ramos-Esplá 1988
Pseudodiazona abyssa	Colonial	Portugal	Ramos-Esplá 1988
Pyura tessellata	Solitary	Portugal	Ramos-Esplá 1988
Situla lanosa	Solitary	Portugal	Ramos-Esplá 1988
Styela canopus	Solitary	Algarve	Sofia Ruiz 2015
		Portugal	Ramos-Esplá 1988
Styela charcoti	Solitary	Portugal	Ramos-Esplá 1988
Styela clava	Solitary	Cascais Lisboa Porto	Davis & Davis 2005
		Sines	Nagar et al. 2010
		Portugal	Ramos-Esplá 1988
Styela crinita	Solitary	Portugal	Ramos-Esplá 1988
Styela loculosa	Solitary	Portugal	Ramos-Esplá 1988
Styela plicata	Solitary	Algarve	Sofia Ruiz 2015
		Albufeira Peniche Nazaré	Nagar et al. 2010
Styela rustica[1]	Solitary	Portugal	Ramos-Esplá 1988
Styela similis	Solitary	Portugal	Ramos-Esplá 1988

Synoicum duboscqui	Colonial	Portugal	Ramos-Esplá 1988
Synoicum pulmonaria	Colonial	Portugal	Ramos-Esplá 1988

previous ascidian species name is no longer accepted: *Aplidium elegans*; *Asajirus indicus*; *Hemistyela Pilosa*; *Oligotrema lyra*; *Oligotrema unigonas*; *Styela rustica*

previous ascidian species name was misspelled*: Aplidium nordmanni; Bathypyura celata; Cnemidocarpa bythia; Cnemidocarpa devia; Cnemidocarpa digonas; Cnemidocarpa platybranchia; Distomus variolosus; Halocynthia papillosa*

3.4 Discussion

The presence of *A. aspersa*, *Microcosmus* sp., and *Molgula* sp. 1 in all five locations demonstrates that, despite some variations in the abundance and biomass values throughout samplings, these ascidians are highly tolerant to the environmental fluctuations present in coastal ecosystems, such as Ria de Aveiro, a mesotidal coastal lagoon. Furthermore, *A. aspersa* and species belonging to the genus *Microcosmus* (*M. squamiger*, *M. plana*) are considered to be non-indigenous species (NIS) in Portugal (Figure 3.3), with invasive behavior (Chainho et al., 2015; Ramalhosa et al., 2021) and exhibit a wide distribution (Lambert et al., 2010). The sampling locations at Mira Channel (FH, M, and OF), registered the highest abundance of these three ascidians species, indicating the presence of favorable conditions for their settlement and growth.

However, species-specific traits must be taken into consideration. Abdul Jaffar et al. (2016) described that ascidians' distribution may not be influenced by hydrodynamic factors but rather by the type and availability of substrates. However, considering the distribution of the species recorded in our study, the hydrodynamic factors present in Ria de Aveiro (Lopes et al., 2017) may well be an explanation for such distribution. The highest total number of ascidians were collected at the OF and IMTA location (intertidal areas with poor or low water flow, (Dias et al., 2000), while the lowest number of ascidians were recorded at the TG, FH, and M locations (subtidal areas that exhibit a higher water flow). Therefore, these findings point out a strong relationship between water circulation and larval establishment in the existing infrastructures.

In coastal ecosystems, namely hosting boating and shipping activities, boats and ship's hulls and ship ballast water are commonly recognized for their potential as vectors of the introduction of biofouling organisms consequently making places such as marinas, ports, and wharves extremely susceptible to the fouling activities (Davidson et al., 2010; Hoxha et al., 2018). Furthermore, inshore aquaculture infrastructures can be targeted as well due to the loading of nutrients and the availability of artificial subtracts favorable for biofoulers organisms (Lambert, 2007; Atalah et al., 2014, 2020; Loureiro et al., 2021). In the Ria de Aveiro, colonial ascidian *B. violaceus* was registered only at the FH and OF sites, in line with what previous authors have reported regarding the pathways of introduction (Carver et al., 2006; Bock et al., 2011; Palanisamy et al., 2018b). Interestingly, *B.*

schlosseri was only registered at the IMTA site. These two species have been recognized as NIS in Portugal (Figure 3.3) (Canning-Clode et al., 2013; Chainho et al., 2015; Ramalhosa et al., 2021).

Ciona intestinalis total abundance dominated at the IMTA location, representing 50% of the total ascidian community collected throughout the present study, whereas at the remaining locations (FH, M, and OF) it presented extremely low abundance values (0.5%, 0.7%, 0.3%, respectively). Additionally, this location recorded the highest concentrations of dissolved inorganic nutrients. This evidence suggests that the nutrient-enriched waters produced by the fish cultivation system are favoring the growth and biomass accumulation of this species (Chatzoglou et al., 2020). On the other hand, the OF location overall registered high abundances, but on what concerns POM values, this location recorded the lowest values. A possible explanation for such a finding is the existing oyster production at this location that may contribute to a reduction of the POM from the water and consequently increase the ammonia signal (Dame et al., 1984).

The general trend of the increase in abundance values was observed in all the studied locations during sampling days 155 to 226 (July to October), which in turn corresponds to the period of ascidians settlement (Coma et al., 2000; Lambert, 2007). Several studies have verified that seasonality is a key element in the development of marine fouling communities (Lindeyer and Gittenberger, 2011; Sievers et al., 2013; Lezzi and Giangrande, 2018; Fortič et al., 2021) and that larval availability also varies with the season (Shenkar et al., 2008).

Our results are compatible with those found by other authors, who have demonstrated that ascidians can present different interspecific recruitment periods year-round. More specifically, Valentine et al. (2016) verified that recruits of colonial *B.violaceus* can be observed from September to October. Solitary *C. intestinalis* reaches a recruitment peak in August and can continue until late November (Ramsay et al., 2008b, 2009; Valentine et al., 2016). Lindeyer and Gittenberger (2011) documented the succession of native *versus* non-native fouling communities and verified that *Molgula socialis*, *Styela clava*, and *B. violaceus* settled mostly from June to December, while *C. intestinalis*, *A. aspersa*, and *B. schlosseri* settled from March to December. Therefore, in locations where predictable seasonal fouling patterns are present, the moment of retrieval of the substrate used to collect marine fouling communities is paramount.

Some biofouling populations can proliferate very rapidly and then gradually retreat. This is especially true for *C. intestinalis* and *S. clava* (Watts et al., 2015). However, environmental factors such as temperature and salinity (Vercaemer et al., 2011; Valentine et al., 2016), light (Gulliksen, 1972; Al-Sofyani and Satheesh, 2019), rainfall (Gama et al., 2006), substrate availability (Osman and Whitlatch, 1995) among others, may also play a significant role in the recruitment process. For example, according to Valentine et al. (2016), ascidians *C. intestinalis* and *A. aspersa* have a negative response to temperatures above 21 °C, but other ascidians such as *S. clava* are less sensitive to temperature fluctuations. Ascidian colonization has been positively correlated with warmer water temperatures (Rodriguez and Ibarra-Obando, 2008), and at the five locations selected for this experiment, temperatures varied on average from 15.4 °C-18.1 °C, indicating that ascidian colonization is benefiting from these temperatures.

Ecological indicators are mainly used as supporting information regarding a targeted ecosystem and to evaluate possible impacts on those ecosystems. These indexes provide data about an ecosystem, namely the biodiversity status (Karydis and Tsirtsis, 1996). The Simpson index considered a dominance indicator, revealed that three locations (TG, FH, and IMTA) presented dominant species, *C. lepadiformis*, *Molgula* sp. 2, and *C. intestinalis*, respectively. Despite the Shannon-Weiner index accounting for both species richness and its evenness, analogous results to the Simpson index were obtained. Moreover, the Pielou index displayed the locations TG and IMTA with the most uniformed ascidian community, possibly because of the abundancies registered at these locations of *C. lepadiformis* and *C. intestinalis*, respectively. Although the differences in species richness between each location were minor and Gamito (2010) revealed that this index is strongly affected by sampling effort and caution must be taken into consideration, the FH location was indicated as the location with the highest number of species. This evidence shows that it cannot be ruled out that this port, located in a loading dock and most probably other ports belonging to the Ria de Aveiro, are more susceptible to biofouling activities and constitute pathways for fouling introductions.

Notwithstanding, ascidian biofouling in marinas and ports should be greater when compared to natural locations as described by (Marins et al., 2010; McNaught and Norden, 2011). In our study, such observations were not entirely met. A possible explanation for such findings is that, in some way, the presence of our star-shaped ASU mimics the artificial infrastructures that are normally present in marinas and ports, and therefore artificializing natural locations. Furthermore, at OF and IMTA locations the presence of aquaculture activities may have also contributed to higher abundance values. Also, possibly a longer experimental trial would be needed to detect a higher biofouling presence under these artificial conditions.

Furthermore, the availability of non-colonized substrates and/or new artificial substrates can influence the recruitment and settlement of ascidian larvae (Osman and Whitlatch, 1995). In the present study, the substrate used (non-colonized oyster shells within a star-shaped unit) was the same in all locations. Therefore the element of preference for one type of substrate over another was eliminated, as other authors have previously described (Stoner, 1994; Chase et al., 2016). Future research involving the investigation of ecological ascidian succession from each location and the effectiveness of this ASU would imply a different experimental approach, such as longer experimental sampling periods, identification of the surrounding fauna and respective specie status, more frequent records of environmental variations, and ultimately standardization and method validation.

Previous review studies on antifouling techniques (Fitridge et al., 2012; Sievers et al., 2017; Bannister et al., 2019) revealed that the great majority of the methods that are employed are based on reactive treatments rather than proactive prevention of the fouling organisms. The most popular methods are water pressure, air exposure, coating technology, physical removal, biological control using grazers, heat, and acetic acid. Consequently, many farmers are having reservations concerning these methods due to the negative outcomes, such as efficiency, stock fitness, costs and profits, and environmental impact. However, few antifouling preventive methods are being discussed. Strategies such as the use of metals (copper, nickel, and tin) may promote negative impacts (Fitridge et al., 2012); despite investigations indicating that little environmental impact is caused by the use of natural compounds that inhibit larval metamorphosis, no commercial-scale trials to test the effectiveness have been achieved (Bannister et al., 2019); encapsulation technique is mostly applied to boat hulls, pontoons, and piles, and as the selective breeding of fouling resistant stock may be a prosperous option (Sievers et al., 2017), nevertheless, these methods can be time-consuming.

Our research supports the repurposing of oyster shells that otherwise would end up in a land-fill or inadequate disposal (Ramakrishna et al., 2018; Chilakala et al., 2019) and is an environmentally friendly practice and reduces the costs for farmers. For instance, in the United Kingdom, the disposal of oyster shell waste can cost £80 per ton (Morris et al., 2019). Repurposing oyster shell waste has been an increasingly studied topic with innumerous applications, such as biological filtration in marine aquariums (Cohen et al., 2021), desulfurization/denitrification sorbents (Jung et al., 2007), a substrate to collect non-indigenous ascidians species (Outinen et al., 2019), used as an artificial stone (Silva et al., 2019), restoration of oyster reefs (Burrows et al., 2005), eutrophication control (Kwon et al., 2017), reduction of environmental toxicity and as natural resources (e.g. limestone) (Chilakala et al., 2019).

The star-shaped ASU is easy-to-use, it can be employed not only by farmers but also by local authorities and can be deployed and retrieved at any time. Moreover, the ASU can be reused, just by letting it dry in the sun where all fouling organisms will shed off. This feature is a valuable asset if there is no intention of using ascidian biomass for other purposes. However, there is the possibility of extra benefits for farmers through the added value that ascidians' biomass may provide as they are rich in omega-3 fatty acids (Dagorn et al., 2010; Marques et al., 2021) and can be used as an aquafeed supplement (White et al., 2019).

To date, knowledge of the ascidian diversity, distribution, and status in mainland Portugal is scarce however, a recent study on fouling ascidians was carried out in Madeira Islands (Ramalhosa et al., 2021). Although 75 different species have already been reported for mainland Portuguese coastal waters, 68% of them have been recorded by Ramos Esplá (1988) more than 30 years ago. It is therefore likely that due to ongoing climate change and anthropogenic activity new species may have been established in the meantime and others may no longer be present. It is unquestionable that, at least for Portuguese waters, an updated survey of ascidian species occurring in this region is needed.

3.5 Conclusions

The present study revealed that the customized 3-D star-shaped ASU applied is effective for the monitorization of ecosystem shifts in the ascidian diversity and it can be used to survey and establish a baseline for the ascidian biofouling community. In addition, this structure can be applied in aquaculture facilities as well, allowing for the monitorization of ascidians fouling behavior. This study represents the first attempt to survey the coastal lagoon of Ria de Aveiro addressing the ascidian biofouling community, its distribution, and composition, resorting to oyster shells, an abundant co-product of oyster farming.

Evidence suggests that the geographic distribution of the ascidian community is conditioned by hydrodynamic variations, seasonality, and by nutrient availability. Furthermore, ascidians settled most in aquaculture environment locations, such as oyster production and at the IMTA farm facility.

Therefore, two species (*C. lepadiformis* and *A. aspersa*) were, for the first time, collected and reported for mainland Portugal.

Chapter 4

Biological and ecological traits of the ascidians present in the Ria de Aveiro

In preparation for publication in the Global Biodiversity Information Facility (GBIF) platform

4. Biological and ecological traits of the ascidians present in the Ria de Aveiro

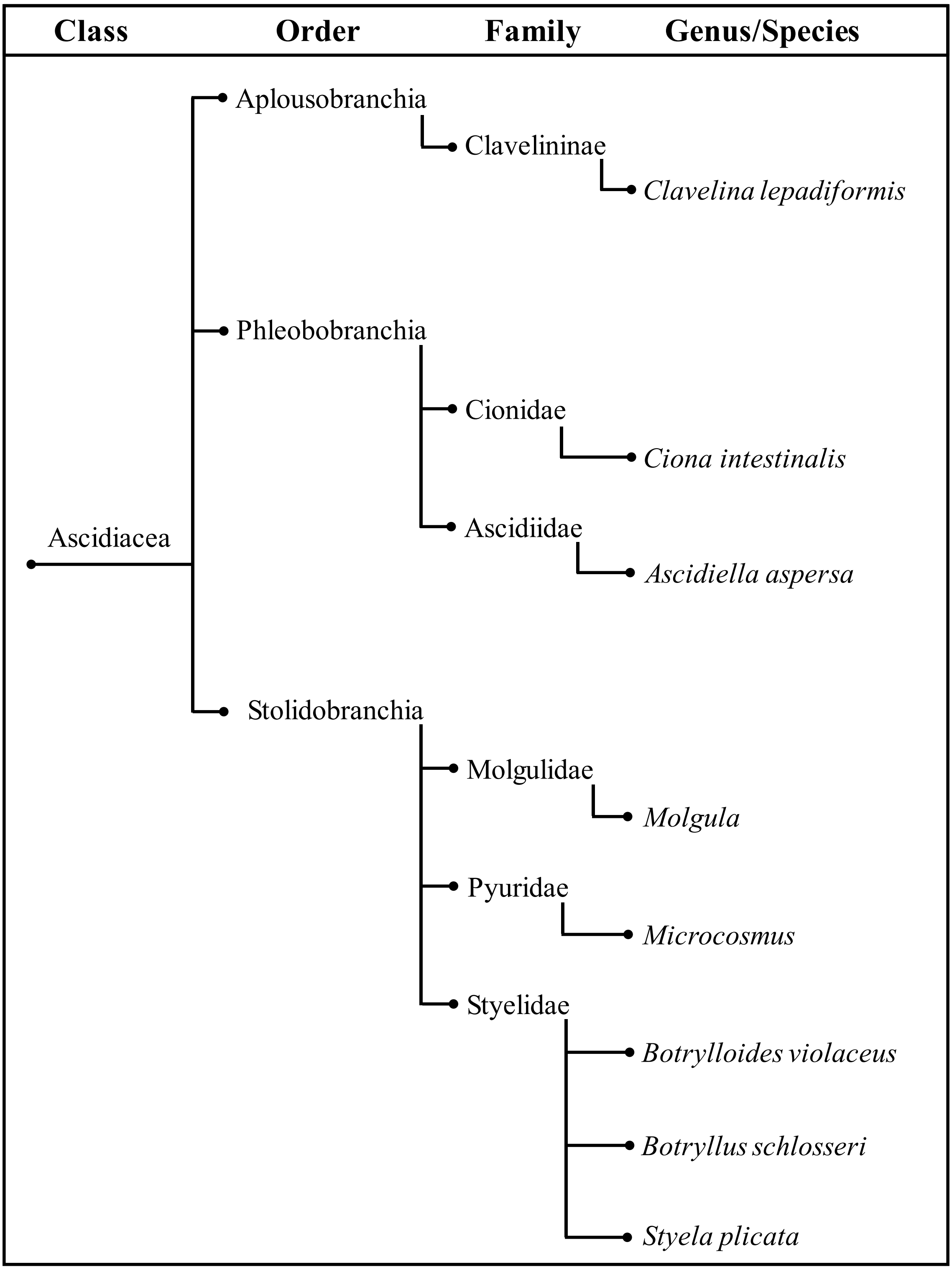

Figure 4.1: Taxonomic tree of the 8 ascidian species collected in the Ria de Aveiro. The tree is based on the World Register of Marine Species: WoRMS.

4.1. *Clavelina lepadiformis* (Müller, 1776)

Common Name

Light bulb sea squirt

Figure 4.2: Photograph of *Clavelina lepadiformis* was taken in July 2020, Aveiro, using Digital Microscope System Leica DMS300 **(**Photograph by Luisa Marques).

Description

Clavelina lepadiformis is a colonial ascidian that forms large clusters. Colonies of several independent transparent zooids are joined at the base by short stolons, which allow the observation of their internal organs (Berrill, 1950). Eggs and larvae vary in color from yellowish white and sometimes pink or red (Fish and Fish, 1996) and are visible in the atrial cavity. These zooids possess a white ring around the pharynx and have pale yellow or white longitudinal lines delineating the endostyle and dorsal lamina, giving them a light-bulb appearance (Barnes, 1963). Zooids can reach up to 25mm long x 5mm in diameter.

Habitat

Colonies are found in shallow rocky sublittoral habitats, occupying natural or artificial hard substrates attached to rocks, wooden docks, stones, and seaweed, to a depth of about 50 m, and are known to dominate disturbed habitats (Naranjo et al., 1996). Tolerant to a wide range of salinities from 14-40 (Millar, 1971; Fish and Fish, 1996; Mackenzie, 2011), and tolerance to cold and warm temperate areas.

Distribution

Clavelina lepadiformis is native to the northeast Atlantic coast, ranging from southern Norway to the Mediterranean Sea (Berrill, 1950; Seebens et al., 2017). It has been introduced to the Azores (Cardigos et al., 2006), South Africa (Monniot et al., 2001), South Korea (Pyo and Lee, 2011), and the United States East Coast (Reinhardt et al., 2010).

Reproduction and Life stages

Clavelina lepadiformis shows a yearly life cycle of growth that begins in October when new zooids start to grow, reaching their peak in February/April with larval production in late summer, followed by the disintegration of the zooids. Colonies grow rapidly and are full size after about two months (Riley, 2008).

Reproduction in *C. lepadiformis* is both sexual and asexual. The gonad is located in the intestinal loop. The fertilized eggs develop in the atrial cavity, and the tadpole larvae escape for a short free-swimming period, up to 3 h (Fish and Fish, 1996). In some areas, colonies regress in winter and can re-grow in the spring. Populations inside harbours were found to remain all year, while populations outside harbours presented a life span of 7 months (De Caralt et al., 2002).

Applications and others

Diazona violacea presents a similar size and white markings in a transparent zooid, most often mistaken for *C. lepadiformis*. Predators include bottom-feeding fish, carnivorous gastropods, starfish (Millar, 1971), and flatworm *Prostheceraeus moseleyi* (Turon, 2016). *Clavelina lepadiformis* has been noted to be markedly toxic to invertebrate larvae and bacteria (Teo and Ryland, 1995).

4.2. *Ciona intestinalis* (Linnaeus, 1767)

Common Name

Vase tunicate

Figure 4.3: Photograph of *Ciona intestinalis* was taken in December 2020, Aveiro (Photograph by Luisa Marques).

Description

Ciona intestinalis is a large solitary ascidian with a soft gelatinous cylindrical body, a thin and semi-transparent test, and large siphons. Scalloped yellow characteristic markings are present in the margins of the siphons (Van Name, 1921). This species may grow up to 150 mm in length and 30 mm in diameter and normally it is fixed by a broad foot-like stalk or base (Sato et al., 2012).

The brachial siphon has eight lobes, and the atrial siphon has six. The branchial siphon is higher up than the atrial siphon. The mantle has ten muscle bands that run the length of the body and pale orange internal organs are seen through the translucent body. When disturbed, siphons are rapidly retracted using strong longitudinal muscles located beneath the protective outer tunic (Fiala-Médioni, 1974). Populations of *C. intestinalis* can form dense aggregations with >5000 individuals/m^2 (Millar, 1971).

Habitat

Ciona intestinalis can be found in great abundance on rocks, wood, metal, concrete docks wrecks, buoys, ropes ships, sheltered areas with some current, and aquaculture gear (Millar, 1971; Ramsay et al., 2008a). This species can be found at depths up to 100 m (Dybern, 1965). It is considered to be primarily a cold-water species, but temporary or transient populations have been observed in tropical waters. Populations of *C. intestinalis* show great fluctuations over a period of years and can colonize rapidly new and cleared structures.

This ascidian can survive a broad range of temperatures, from -1 °C-30 °C (Dybern, 1965; Carver et al., 2003), and salinities from 8-40 (Dybern, 1967). However, mortality increases at temperatures of <10 °C and it cannot withstand extended periods with salinity below 11 (Dybern, 1967).

Distribution

Ciona intestinalis is believed to be native to the northeast Atlantic however, it is considered non-indigenous in northern Atlantic Canada (Locke et al., 2009). Major natural populations are found widely distributed in many European countries (Dybern, 1965; Gulliksen, 1972). However, *C. intestinalis* has been spread throughout all temperate regions mostly by shipping activities (Monniot and Monniot, 1994).

Reproduction and Life stages

Sexual mature adults are normally 40 mm in height (Dybern, 1965). The adults are hermaphrodites but do not self-fertilize. Eggs are externally fertilized. Eggs and sperm are released into the surrounding water column where fertilization takes place. The free-swimming tadpole larvae spawned into the water column and can persist for 1-2 days. Tadpole larva undergoes retrogressive metamorphosis to form an adult (Yamaguchi, 1975).

Dispersal distances are limited, to less than 6 km per generation (Kanary et al., 2011; Collin et al., 2013). Temperature variations are the main contributor for *Ciona intestinalis* to exhibit considerable differences in generation time and spawning phenology (Dybern, 1965). In colder waters, *C. intestinalis* can live up to two years and will spawn continuously throughout the year. In waters with seasonal temperature differences, this species lives roughly 1 year and spawns at temperatures higher than 8°C. Furthermore, in continuously warm waters generation time is short than 1 year and spawning is continuous throughout the year (Yamaguchi, 1975).

Applications and others

Many organisms prey upon this ascidian, for example, fish, crabs, and starfish. *Ciona intestinalis* is a model organism for developmental biologists and its genome was sequenced and published in 2002 (Dehal et al., 2002). It holds 14 pairs of chromosomes and 14.000 genes (Dehal et al., 2002; Shoguchi et al., 2006). The soft texture and yellow marks around the siphons are peculiar features of this species.

4.3. *Ascidiella aspersa* (Müller, 1776)

Common Name

European sea squirt

Fluted sea squirt

Figure 4.4: Photograph of *Ascidiella aspersa* was taken in September 2020, Aveiro (Photograph by Luisa Marques).

Description

Ascidiella aspersa is a solitary semi-transparent with a thick, rough, and finely papillated greyish-black to a brown tunic that covers the whole adult body (Kott, 1985). They can be found in clusters with basal attachment. *Ascidiella aspersa* presents two siphons: the oral siphon is 6-8 lobed and located at the terminal end while the atrial siphon is six lobed (Kanamori et al., 2017) and is located at one-third of the length of the body.

Several lighter markings are present around the edge of each siphon. The large oral tentacles can be seen inside the oral siphon when the siphon is expanded. The intestinal gut is located to the left of the brachial sac with numerous rows of straight stigmata (Kanamori et al., 2017). Adults can reach up to 50-100 mm (MarLIN, 2006).

Habitat

Ascidiella aspersa establishes in subtidal waters with a maximum of 90 m (Kanamori et al., 2017), however, they can also inhabit shallow waters like estuaries, harbors, and semi-enclosed embayments, as these protect from strong currents and tidal forces (NIMPIS, 2010). This species can tolerate salinities ranging from 18 to 40 (Petersen and Riisgärd, 1992). They are mostly found in soft bottoms and can be associated with hard substrates, such as pier pylons, rocks, and docks (Carman et al., 2010). It appears that the presence of native fauna does not inhibit their establishment as their population is increasing as global ocean water temperatures increase as well (Stachowicz et al., 2002).

Distribution

Ascidiella aspersa is native to the northeastern Atlantic, from the Mediterranean Sea to Norway (Lynch et al., 2016) however, its presence is scarce in the North Sea. In recent years, *A. aspersa* has expanded its range to several areas including the northwestern Atlantic coast of North America, New Zealand, Southern Australia, Tasmania, and India (NIMPIS, 2010).

Reproduction and Life stages

This species is hermaphroditic, with the male sex organs developing first (Millar, 1952), and has a lifespan of 18 months. Male and female organs are fully mature when they reach the size of 40 mm. Generally, one year after larval settlement one spawning season occurs (Millar, 1952).

During the spring and early summer male and female gametes are released into the water column where fertilization occurs (Lynch et al., 2016). Chemotaxis interactions occur between sperm and egg (Bolton and Havenhand, 1996). Larvae go through a short free-swimming stage and within just 24 h, they quickly metamorphose into juveniles. Afterward settlement takes place.

Applications and others

Ascidiella aspersa lacks predators (Currie et al., 1998), allowing for this species to form large populations and subsequent high amounts of biomass. They also compete directly with other native filter-feeding fauna (scallops, mussels, and oysters) with negative impacts on the economy (Currie et al., 1998).

4.4. ***Molgula* sp.** (Forbes, 1848)

Common Name

Sea grapes

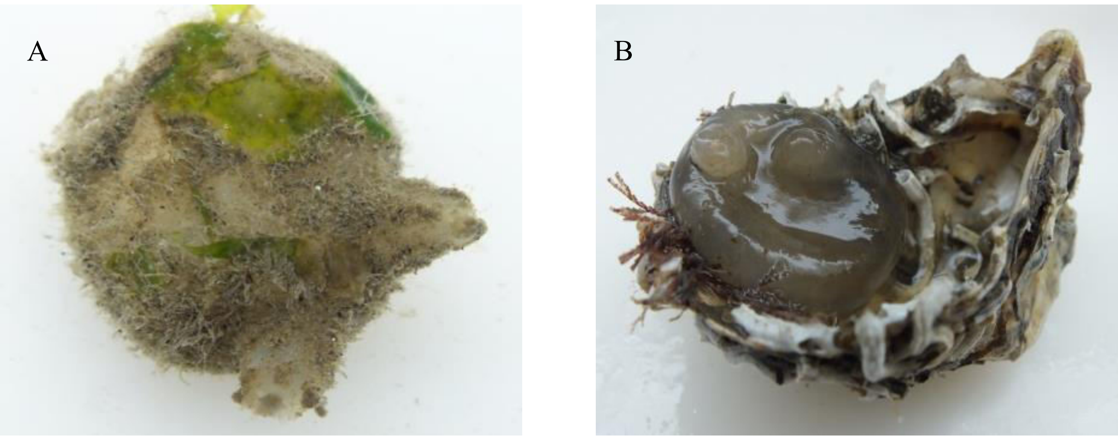

Figure 4.5: Photographs of **A)** *Molgula* sp. 1 and **B)** *Molgula* sp. 2 were taken in May and June 2020, respectively, Aveiro (Photograph by Luisa Marques).

Description

Specimens belonging to the genus *Molgula* are solitary ascidian species with a globular-like body with some lateral compression. Large specimens can measure 20-50 mm in length and 30 mm in height and often occurs in dense clusters (Van Name, 1921). The tunic is firm, translucent, tough, and moderately thick. The tunic presents a grey or greenish-blue color and some areas on the tunic are covered in small hair-like projections called papillae that may be attached with sand grains or shell fragments (Lambert, 2003).

The siphons are fairly close together at the anterior end. The oral siphon is shorter with six lobes while the atrial siphon has four lobes. These are prominent when expanded (Van Name, 1921).

Habitat

*Molgu*la can be found abundantly in shallow subtidal waters attached to natural substrates, such as bedrock, boulders, stones, and shells, or slow-moving artificial substrates such as buoys, ropes, and ship hulls. They can live in temperature ranges of about 10-26 ºC and salinity ranges of 10-35 (Wass, 1972). They are also known to tolerate high pollution levels or hypoxia-stressed situations (Weis and Weis, 1992). *Molgula* is found especially in ports and harbours up to 90 m in depth.

Distribution

Molgula is native to the East and Gulf Coasts of the United States of America (Van Name, 1921). Currently, this genus has a wide distribution and is very abundant. It has been introduced in Europe, Gulf of Mexico, Argentina, West Coast of North America, Japan, and Australia (Berrill, 1950; Hewitt, 1999; Haydar et al., 2011).

Reproduction and Life stages

Molgula is thought to have a lifespan of one year, during which time sexual maturity is reached rapidly and reproduction may occur several times (Zvyagintsev et al., 2003). Being a hermaphroditic genus, both eggs and sperm are released to the atrial chamber. Fertilized eggs hatch into a tadpole larva with a muscular tail, notochord, eyespots, and a set of adhesive papillae.

The lecithotrophic larvae can only live for a few days until it attaches to a hard surface (Grave, 1933). Most species of the genus *Mogula* present a poor larval dispersal capacity (Stamp, 2016). Once settled, the tail is absorbed, the gill basket expands, and the tunicate begins to feed by filtering (Barnes, 1963).

Applications and others

Some species of this genus are regarded as serious foulers, living on oyster stock and empty oyster shells (Andrews, 1973) and they can overgrow most other fouling community organisms (Calder and Brehmer, 1967; Otsuka and Dauer, 1982). *Molgula* can be a host for many copepods (*Doropygus laticornis*, *Enteropsis sphinx*, *Lichomolgus canui*, *Notodelphys canui*) and bacteria (*Streptomyces hyaluromycini).*

4.5. *Microcosmus* sp. (Heller, 1877)

Common Name

Scale-bearing sea squirt
Grooved sea squirt

Figure 4.6: Photograph of *Microcosmus* sp. was taken in July 2020, Aveiro (Photograph by Luisa Marques).

Description

Microcosmus species are relatively small solitary ascidians, up to 40-50 mm in diameter with a globular but irregular shape. The tunic is normally tough and leathery with wrinkles, often totally or partially covered by epibionts, and it is brown to reddish in color (Kott, 1985). The epibiotic community of can be very complex and consists mostly of algae, hydrozoans, bryozoans, and even other ascidians. The mantle is strongly muscular, and the two siphons are short and often concealed in contracted individuals. Internally, the number of folds in the branchial sac is variable. They can form dense aggregations of 2300 individuals/m^2 (Rius et al., 2009a).

Habitat

The majority of species of this genus prefer eutrophicated habitats of strong turbidity (Naranjo et al., 1996). They can thrive in many disturbed habitats tolerating pollution, stagnation, and low salinities (Naranjo et al., 1996; Lowe, 2002), causing this genus to hold fouling traits. The optimal temperature range is 10-30 ºC (Nagar and Shenkar, 2016) and the salinity range is 15-36 (Lowe, 2002).

Microcosmus are found in shallow littoral communities, with the highest abundance at 10 m, however, it has been registered at 35 m (Kott, 1985). *Microcosmus* can be found on rocky substrata and also on artificial substrata. They are mostly established inside marinas, harbours, and aquaculture facilities forming dense aggregates, and in both sheltered and exposed habitats (Kott, 1985). It can colonize adjacent natural communities outcompeting native species. This genus can withstand reduced salinity conditions (15-36) (Lowe, 2002).

Distribution

The genus *Microcosmus* has spread worldwide in temperate waters, however, they are most abundant in the Mediterranean climate (Turon et al., 2007). Some species are considered invasive in certain areas, while others are considered fouling organisms and represent potential threats to Mediterranean littoral communities (Rodriguez and Ibarra-Obando, 2008).

Reproduction and Life stages

All *Microcosmus* are hermaphrodites, and many are broadcast spawners resulting in a high reproduction rate. Eggs and sperm are released into the water column where external fertilization occurs. Afterward, the embryos hatch as tadpole larvae, which do not feed and have to settle in a matter of hours before exhausting their reserves. Larvae measure up to 1.3 mm, with a short trunk and well-developed tail (Rius et al., 2009a). Seasonal reproduction peak takes place in the summer. Most species present a two-year cycle (Rius et al., 2009b).

Applications and others

Gastropods *Stramonita haemastoma* and *Hexaplex trunculus* have adopted *M. squamiger* as one of their favorite sources of prey (Rius et al., 2009b). The idea of using these gastropods as pest control where *Microcosmus* is considered invasive is being discussed. On the other hand, ascidians *Molgula manhattensis* and *Styela canopus* are affected directly by *M. squamiger* through the monopolization of resources and shading (Lowe, 2002; Lambert and Lambert, 2003).

4.6. ***Botrylloides violaceus*** (Oka, 1927)

Common Name

Purple colonial tunicate
Violet tunicate
Orange sheath tunicate

Figure 4.7: Photograph of *Botrylloides violaceus* was taken in October 2020, Aveiro (Photograph by Luisa Marques).

Description

Botrylloides violaceus is a colonial ascidian, that creates flat sheets with an irregular form. Colonies are uniform in color and can be from pale yellow, pink, red, and orange, to bluish-purple, and it is these zooids that give the species that color (Millar, 1982). They present small bean-shaped and elongated individuals called zooids that are organized into meandering systems, each separated by a prominent ridge, embedded in a firm matrix.

Zooids are characterized by 16 branchial tentacles located on the inside of the oral siphon, and a pharynx with 10-11 rows of stigmata. Each zooid grows approximately 3 mm in length (Lambert and Lambert, 2003). The colony grows to form thick, encrusting patches that typically reach 3-4 mm and 20-300 mm in diameter (Okuyama and Saito, 2002).

Habitat

Botrylloides violaceus occupies a wide variety of habitats, including bays, docks, boat hulls, ropes, and harbours on both vertical and horizontal surfaces, more frequently reported in anthropogenic structures than in natural surfaces (Simkanin et al., 2012).

This species can be found 7 m deep. Due to its rapid growth, short larval dispersal, and acidic tunic, it reduces the availability of space for settlement for other fauna, strengthening their populations. However, predation can limit the spread of *B. violaceus* to natural habitats (Simkanin et al., 2012). This species is very resilient as it can tolerate a wide range of temperatures (-1-27 ºC) and salinity (15-34) (Carman et al., 2007; Dijkstra et al., 2008; Epelbaum et al., 2009).

Distribution

Botrylloides violaceus is native to the Northwest Pacific from northern Japan to southern Korea and northern China (Nishikawa, 1991; Rho et al., 2000), being first described in Japan in 1927. This species is now widely introduced and can be found in the Northeast Pacific, Italy, the Netherlands, the Mediterranean Sea, and British waters (Zaniolo et al., 1998; MarLIN, 2006; Nagar et al., 2010; Palanisamy et al., 2018a; Maguire, 2022).

Reproduction and Life stages

Botrylloides violaceus has two types of reproduction: asexual and sexual reproduction. Sexual reproduction happens between 10-12 ºC and begins when the gonads of both sexes develop on either side of the zooid (Zaniolo et al., 1998). The egg is fertilized in a brood pouch and develops until the larva escapes. Whole-body regeneration can occur in this species.

Larvae are quite large (0.01-0.02 mm), spherical in shape, brightly colored, and with 24-34 finger-like buds, called ampullae (Epelbaum et al., 2009). They are lecithotrophic, spending less than 24 h in the water column before attaching head-down on an appropriate substrate (Zaniolo et al., 1998). All *Botrylliodes* of the same generation appear, grow, and die simultaneously.

Applications and others

This species has provided a new source of prey for some organisms such as snails, gastropods, crabs, fishes, and starfishes (Osman and Whitlatch, 1995; Dijkstra et al., 2007).
Botrylloides violaceus can grow faster on artificial substrates (rubber and metal), rather than on natural substrates (shell, marble, slate) (Tyrrell and Byers, 2007).

4.7. ***Botryllus schlosseri*** (Pallas, 1766)

Common Name

Star ascidian
Golden star tunicate

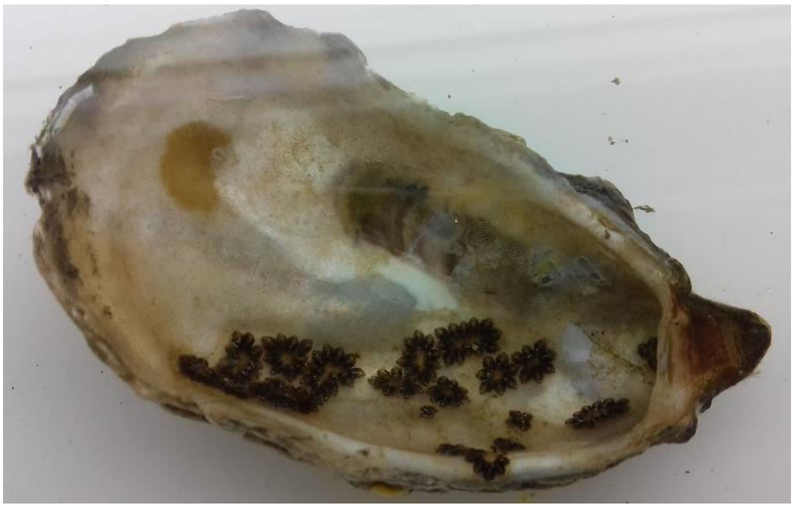

Figure 4.8: Photograph of *Botryllus schlosseri* was taken in May 2020, Aveiro (Photograph by Luisa Marques).

Description

Botryllus schlosseri is a colonial ascidian with fleshy colonies, forming compact sheets. Colonies vary greatly in color including orange, violet, brown, blue, and grey (Berrill, 1950). The zooids are elliptical, arranged in star systems, each with a central cloacal opening, with typically 6-12 zooids forming star-shaped systems (Carver et al., 2006).

Individual zooids may grow to 3 mm in size, with colonies reaching 50 mm long and up to 2 mm thick. A colony can be easily separated from the main body to form an independent colony usually referred to as a subclone (Nishikawa, 1991).

Habitat

Colonies grow on slow-moving, submerged objects in shallow waters and are generally found fixed to seaweeds, wharves, piers, ships, and artificial substrata (Andrews, 1973). *Botryllus schlosseri* are most commonly in locations with a considerable wave or current exposure.

This species can tolerate a wide range of temperatures (-1-30 ºC) and salinity (14-44) (Brunetti et al., 1980), and can be found in depths up to 200 m.

Distribution

The native origin of *B. schlosseri* is uncertain. It is globally widespread and can be found on the temperate coasts of Europe, Asia, North America, Chile, Argentina, South Africa, Australia, and on oceanic islands (Rinkevich et al., 2001; Ben-Shlomo et al., 2006; Lejeusne et al., 2011). This species presents a wide distribution in the Atlantic and the Mediterranean. Its range has spread over the last 100 years to a nearly worldwide extent. It is also considered the most common colonial tunicate in North America (Lejeusne et al., 2011; Maguire, 2022).

Reproduction and Life stages

Botryllus schlosseri matures in 50 days and presents a life span of 12 months. There is a pair of hermaphrodite gonads with reproduction being both sexual and asexual (Millar, 1952). Asexual reproduction occurs approximately every two weeks, during which a new bud will grow and begin to actively feed, while the adult is eventually re-absorbed.

When sexually productive, *B. schlosseri* are known to produce yellowish-white or pale-orange tadpole larvae. Self-fertilization is avoided while cross-fertilization is favored. The larval phase is under 24 h and has the potential dispersal of 1-10 km. (Barnes, 1963; Gasparini et al., 2015).

Applications and others

Botryllus schlosseri is used as a model organism. The genome has been sequenced. It is 580 megabases in length, organized into 16 chromosomes. Ascidians *B. schlosseri* and *B. violaceus* have a native impact on the eelgrass *Zostera marina* by fouling the leaves of the grass and reducing the availability of light (Wong and Vercaemer, 2012).

4.8. *Styela plicata* (Lesueur, 1823)

Common Name

Pleated Tunicate

Figure 4.9: Photograph of *Styela plicata* was taken in November 2018, Aveiro (Photograph by Luisa Marques).

Description

Styela plicata is an ovular solitary ascidian, with a greyish to tannish-white tunic (Kott, 1985; Nishikawa, 1991). Its tunic is firm and thick, with deep irregular ridges and a lumpy surface. It is largely composed of cellulose. *Styela plicata* is fixed to the substrate by the posterior end of its body, usually without roots or stalks.

The two short siphons present four lobes with red or purple stripes. Adults can reach sizes between 40-90 mm (Yamaguchi, 1975). *Styela plicata* is a protandric hermaphrodite, meaning that it is male earlier in life and later turns into a female (Lambert et al., 2005).

Habitat

Styela plicata is eurythermal. This species can live in a wide range of conditions: water temperatures from 10-30 °C and salinities between 22-34 (Sims, 1984; Thiyagarajan and Qian, 2003). They can also be found from the low intertidal zone to depths of 30 m and tolerate brackish waters and some levels of pollution (Naranjo et al., 1996).

Styela plicata is largely found on man-made structures such as marinas, docks, and vessel hulls, but is also known to settle on oyster reefs, mangroves, and rocky substrates. This ascidian can outcompete native encrusters and exclude them from hard substrates. In contrast, *S. plicata* is extensively cultured on long lines in Korea and Japan (Lambert et al., 2016).

Distribution

The native region is currently unknown however, evidence points to the Northwest Pacific (Carlton, 2006; de Barros et al., 2009). *Styela plicata* is distributed globally due to its ability to foul on ships' hulls and other hard substrates, traveling the oceans in this manner. Its known range distribution spans the coasts of North America, the Caribbean, Mexico, Brazil, Uruguay, Argentina, Mediterranean Sea, Senegal, Somalia, Japan, Australia, New Zealand, Indian Ocean (Lambert and Lambert, 1998; Carlton, 2006).

Reproduction and Life stages

This species presents external fertilization. *Styela plicata* reaches sexual maturity within 2-5 months, during the summer or winter, respectively (Yamaguchi, 1975). Adults are sexually mature at 40 mm (de Barros et al., 2009). *Styela plicata* must experience 8 h of darkness before the release of gametes. Spawning can occur between 11-28° C (West and Lambert, 1976). The eggs and sperm are released into the water column and the larvae (1.3 mm) attempts to find a suitable substrate (Yamaguchi, 1975).

The larvae have an extended swimming period of over 48 h, before settlement without a cost to metamorphosis (Thiyagarajan and Qian, 2003). Larval settlement is most successful in the spring and fall. The life span of less than one year, usually 5-9 months (Lambert and Lambert, 1998; de Barros et al., 2009). Breeding season occurs all year except during winter however, populations may be abundant one year and absent the next (Lambert and Lambert, 1998).

Applications and others

Styela plicata shelters the amphipod *Leucothoe spinicarpa* (Thiel, 1999). Snails, crustaceans, brittle stars, and fish have been known to prey on *S. plicata* however, the presence of secondary metabolites on the body wall of *S. plicata* causes it to be unpalatable to some predators, particularly fish (de Barros et al., 2009). *Styela plicata* also has the potential to be used for bioremediation (Cestone et al., 2008; Draughon et al., 2010).

Chapter 5

Screening for health-promoting fatty acids in ascidians and seaweeds grown under the influence of fish farming activities

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5. Screening for health-promoting fatty acids in ascidians and seaweeds grown under the influence of fish farming activities

Abstract

The present study aimed to contrast the fatty acid (FA) profile of ascidians (Ascidiacea) and seaweeds (sea lettuce, *Ulva* spp. and bladderwrack, *Fucus* sp.) occurring in a coastal lagoon with *versus* without the influence of organic-rich effluents from fish farming activities. Our results revealed that ascidians and seaweeds from these contrasting environments displayed significant differences in their FA profiles. The *n*-3/*n*-6 ratio of Ascidiacea was lower under the influence of fish farming conditions, likely a consequence of the growing level of terrestrial-based ingredients rich in *n*-6 FA used in the formulation of aquafeeds. Unsurprisingly, these specimens also displayed significantly higher levels of 18:1*n*-7+*n*-9 and 18:2*n*-6, as these combined accounted for more than 50% of the total pool of FAs present in formulated aquafeeds. The dissimilarities recorded in the FAs of seaweeds from these different environments were less marked (≈5%), with these being more pronounced in the FA classes of the brown seaweed *Fucus* sp. (namely polyunsaturated fatty acids). Overall, even under the influence of organic-rich effluents from fish farming activities, ascidians and seaweeds are a valuable source of health-promoting FAs, which confirms their potential for sustainable farming practices, such as integrated multi-trophic aquaculture.

Keywords

Aquafeeds, EPA, DHA, *n*-3/*n*-6 ratio, *n*-3 PUFA, IMTA

5.1 Introduction

Marine organisms are commonly perceived as a rich source of *n*-3 fatty acids (FA) (Strobel et al., 2012; Zhao et al., 2015; Marques et al., 2018; Lopes et al., 2020) whose consumption ensures health-promoting benefits against cardiovascular and neurological diseases. Additionally, consumers also acknowledge the anti-inflammatory, anti-coagulation, and anti-oxidative properties (among others) of *n*-3 FA originating from seafood, making them paramount for human nutrition (Simopoulos, 2002; Candela et al., 2011; Swanson et al., 2012; Béné et al., 2015).

As a result of the fast-growing trend of the world population (Béné et al., 2015; FAO, 2020) and the high request for nutritious and healthy marine food (Olsen, 2011; Strobel et al., 2012; Abreu et al., 2014) aquaculture activities are facing a major challenge in recent years to keep up with an ever-growing demand. Proportionally, there is also a growing focus on the improvement of aquaculture efficiency, as well as the promotion of environmentally and financially sustainable practices (Hasan and Halwart, 2009; Alexander et al., 2016; Engle et al., 2017; Custódio et al., 2020). As an example of this ongoing effort, one can refer to the reduction of the levels of marine-based ingredients, such as fishmeal and fish oil, in the formulation of aquafeeds for marine species aquaculture (namely finfish and shrimp) (Olsen, 2011; Hodar et al., 2020).

Indeed, a growing proportion of marine-based ingredients have been partially replaced by land-based ingredients (e.g., wheat, soy, corn) (Apper-bossard et al., 2013; Metwalli, 2013; Iqbal et al., 2021) and oils (e.g., palm oil, soybean oil, sunflower oil) (Ayisi et al., 2018; Soller et al., 2018). Nonetheless, aquafeeds for marine species production still include marine-based ingredients to achieve desirable FA profiles (Makkar et al., 2016). These marine-based ingredients, particularly fish oil, are a source of essential FAs, such as *n*-3 long-chain polyunsaturated FAs (PUFA) 20:5*n*-3 eicosapentaenoic acid (EPA), and 22:6*n*-3 docosahexaenoic acid (DHA), which are paramount to ensure the healthy development of species being farmed and, as such, safeguard that these remain a valid source of these important nutrients in human diets (Broadhurst et al., 2002; Glencross, 2009).

Consequently, the aquaculture industry has evolved to develop productive frameworks that target the co-production of extractive species that impair the loss of valuable nutrients (such as *n*-3 long-chain PUFA); this approach has been termed integrated multi-trophic aquaculture (IMTA) and has gained a growing interest in the scientific community. These productive systems benefit from the simultaneous farming of species occupying different trophic levels to sequester, recycle and remove excess nutrients originating from uneaten and undigested feed, as well as excretion products present in aquaculture effluents that shape the biochemical content of co-farmed species (Abreu et al., 2014). Extractive species produced under organic-rich effluents (Org) are responsive to their surrounding environment and experience more or less pronounced shifts in their biochemical composition (Aguado-Giménez et al., 2014; Sprague et al., 2016; Marques et al., 2018)

Consequently, FA analysis has become an excellent tool to trace the biochemical fingerprint of aquaculture effluents in aquatic environments and their species (Fernandez-Jover et al., 2011; White et al., 2019).

Ascidians are marine filter-feeders commonly investigated for marine natural products development, such as anti-cancer and anti-malarial drugs (Palanisamy et al., 2017). Knowledge on ascidians' FA profiling is still poorly explored. However, some studies have already confirmed that ascidians present a high *n*-3/*n*-6 ratio (Zhao et al., 2015; Monmai et al., 2018) and high values of EPA and DHA (Dagorn et al., 2010), establishing ascidians as a potential new bioresource for *n*-3 fatty acids-rich marine lipids (Hassanzadeh, 2014; Zhao et al., 2015; Zhao and Li, 2016) Hassanzadeh (2014) concluded that the FA profile of ascidians presented similar values to that of fish oil and, therefore, considered ascidians as a good alternative for fish oil in the formulation of aquafeeds. Additionally, ascidian biomass may even successfully replace fishmeal in the formulation of aquafeeds (Jang et al., 2017; Choi et al., 2018).

The use of seaweeds has been thoroughly explored in IMTA systems (Chatzoglou et al., 2020; Giangrande et al., 2020; Vega et al., 2020). Seaweeds production under this productive framework is receiving growing attention for mass production given their nutritional value and profile in natural bioactive metabolites (particularly with antioxidant properties) (Ashkenazi et al., 2019; Giangrande et al., 2020). Similar to ascidians, seaweeds are considered an important source of *n*-3 long-chain PUFA, especially α-linolenic acid (ALA; 18:3*n*-3) and EPA (Da Costa et al., 2019; Lopes et al., 2020), with their potential as ingredients for aquafeed formulations, also being increasingly acknowledged (Wan et al., 2019). Although the lipid content in seaweed is relatively low (1.27% to 9.13%) (Pirian et al., 2020), these organisms feature high *n*-3/*n*-6 ratios, making them an appealing source of a valuable source of essential FA in health-promoting diets (Moreira et al., 2020).

The present study aimed to compare the FA profile of ascidians (Ascidiacea) and seaweeds (sea lettuce, *Ulva* spp. and bladderwrack, *Fucus* sp.) sampled in a coastal lagoon with *versus* without the influence of organic-rich effluents from fish farming activities. Additionally, the FA profile of ascidians is also contrasted with that of the most commercially used fish aquafeed employed in the studied location to investigate if these filter-feeding marine organisms somehow mimicked the FA profile of those aquafeeds when grown under the influence of organic-rich effluents originating from fish farms.

5.2 Materials and Methods

5.2.1 Study Areas

Ria de Aveiro is a shallow coastal lagoon in the west margin of mainland Portugal that holds the Vouga river estuary and presents a complex and irregular geometry. This coastal lagoon has four main channels emerging from the sea entrance: S. Jacinto-Ovar, Espinheiro, Ílhavo, and Mira channels. The first sampling location surveyed was located at Mira channel (40°36'51"N, 8°44'25"W) without the influence of organic-rich effluents from fish farming activities and is herein referred to as -Org. The second sampling location surveyed was located at a land-based semi-intensive fish farm (40º36'43"N, 8º40'43"W) supplied by Ílhavo channel's waters (Figure 5.1). An IMTA framework is employed in this location, in which European seabass and Gilthead seabream are produced in earthen ponds, and seaweeds are produced in tanks supplied with organic-rich waters from these earthen ponds. This location will be referred to as +Org. Both channels of this coastal lagoon pre-sent strong salinity gradients with very low values at their upper reaches. Salinity, temperature, dissolved oxygen, and pH were registered *in situ* at the time of sampling. Environmental parameters are summarized as supplementary information (Table SI 5.1).

5.2.2 Sample collection

5.2.2.1 Ascidiacea

Ascidians were collected manually from both locations described above (Figure 5.1). The taxonomic identification of ascidians is complex, and producers are unable to readily sort them by species, namely if they target the production of small-sized specimens (when key diagnosing morphological features are incipient). While *Styela plicata* and *Ciona intestinalis* were certainly present among the ascidians collected, it is not impossible to rule out the presence of other species without using molecular tools (e.g., DNA barcodes) or taxonomic identification by experts. As such, ascidians were pooled into composite samples and will be simply termed Ascidiacea.

All specimens were left to depurate for 48 h after being sampled, in order to safeguard that their guts were emptied and, as such, avoid any bias on their FA profile from dietary prey. All specimens were depurated using filtered seawater (GFFC, glass microfiber filter 1.2 µm, Ø47 mm) from their sampling locations. After depuration, all specimens were washed thoroughly using tap water to eliminate any impurities and all five composite samples of 3 individuals each (of similar sizes) were selected per sampling location. All samples were freeze-dried and stored at -20 ºC. Before FA analysis, samples were grounded into a fine powder using a mortar and pestle.

5.2.2.2 Seaweeds

Specimens from the genus *Ulva* (Chlorophyceae) and *Fucus* (Phaeophyceae) were collected from the same locations as ascidians (-Org and +Org) (Figure 5.1). As already detailed above for ascidians, more than one species of *Ulva* can be present in one or both of the sampling locations surveyed in the present work. As such, all sea lettuce samples collected were termed *Ulva* spp. Concerning the samples of bladderwrack collected in the present work, all specimens of this brown seaweed could be easily identified to the species level (*Fucus vesiculosus*) but to keep consistency with the identification level of the green seaweed, it will be addressed as one species of the genus *Fucus*.

All seaweeds were washed using tap water to eliminate impurities and excess water was dried from samples. Five composite samples of five seaweeds each were separated by species and location, freeze-dried, and stored at -20 ºC. As for ascidians, seaweed biomass was also grounded into a fine powder using a mortar and pestle.

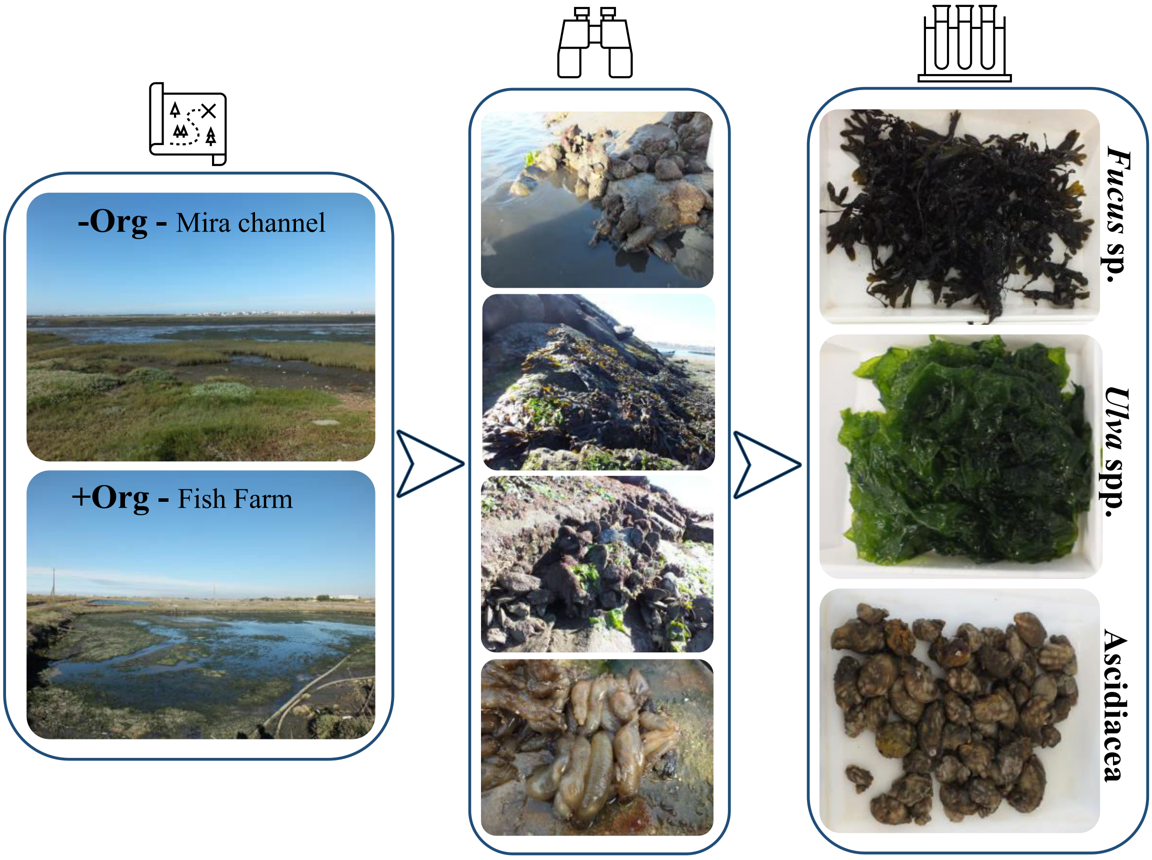

Figure 5.1: Sampling locations chosen for this study: Mira Channel (40°36'51"N, 8°44'25"W) without the influence of organic-rich effluents (-Org) and a land-based semi-intensive fish farm (40º36'43"N, 8º40'43"W) supplied by Ílhavo channel's waters, with the influence of organic-rich effluent (+Org). Images of the organisms collected at both sampling locations. Photographs were taken in November 2017, Aveiro. (Photographs by Luisa Marques).

5.2.2.3 Fish feed

The FA profile of the formulated fish feed (Standard Orange 4; SORGAL, Sociedade de óleos e rações, SA) supplied at the fish farm operating under an IMTA framework was determined using 250 mg of feed per each of the five replicates analyzed ((Table SI 5.2) for detailed composition). All storage and processing of these samples before FA analysis were identical to those described above for ascidians and seaweeds.

5.2.3 Total lipid extraction

Lipid extraction was performed by adding 3.75 mL of a mixture of methanol/chloroform (2:1, v/v) to 150 mg of ascidians and 250 mg of seaweeds (five biological replicates per biological matrix tested) in a glass test tube with a Teflon-lined screw cap. Samples were then homogenized and incubated in ice on a rocking platform shaker (Stuart Scientific STR6, Bibby, UK) for 2 h and 30 min. The mixture was centrifuged at 2000 rpm for 10 min., and after the organic phase was collected. The biomass residue was re-extracted two times with 2 mL of methanol and 1 mL of chloroform. Afterward, water was added (2.3 mL) to the total collected organic phase, centrifuged at 2000 rpm for 10 min and the organic (lower) phase was recovered.

Solvents were dried under a stream of nitrogen gas. A total lipid extract was estimated by gravimetry. Lipid extracts were stored in dark vials and stored at -20 °C before analysis by gas chromatography-mass spectrometry (GC–MS). Re-agents were purchased from Fisher Scientific Ltd. (Loughborough, UK). All other reagents were purchased from major commercial suppliers. Milli-Q water (Synergy, Millipore Corporation, Billerica, MA, USA) was used.

5.2.4 Fatty acid profiling

Fatty acid methyl esters (FAMEs) were prepared using a methanolic solution of potassium hydroxide (2.0 M) as described by Melo et al. (2015). Subsequently, 2.0 μL of a hexane solution containing FAMEs were analyzed by GC–MS on an Agilent Technologies 6890 N Network (Santa Clara, CA) equipped with a DB–FFAP column. The column was 30 m long, had 0.32 mm of internal diameter, and a film thickness of 0.25 μm (123-3232, J&W Scientific, Folsom, CA). The GC equipment was connected to a Mass Selective Detector (Agilent 5973 Network) operating with an electron impact mode at 70 eV and scanning the range m/z 50–550 in a 1 s cycle in a full scan mode acquisition. The carrier gas Helium was used at a flow rate of 1.4 ml min^{-1}. The elution relied on an increasing temperature gradient: 80 °C for 3 min, a linear increase to 160 °C at 25 °C min^{-1}, followed by a linear increase at 2 °C min^{-1} to 210 °C, then at 30 °C min^{-1} to 250 °C, standing at 250 °C for 10 min.

Identification of FAs was performed considering retention times and mass spectrometry spectra of FA standards (Supelco 37 Component Fame Mix, Sigma-Aldrich), as well as through mass spectrum comparison with those in Wiley 275 library and AOCS Lipid Library. The relative amounts of FAs were calculated by the percent area method with proper normalization, considering the sum of all areas of the identified FAs. Results were expressed as means ±SD.

5.2.5 Statistical analysis

Data from FA profiles was square-rooted transformed, and a Bray-Curtis matrix was assembled. A one-way PERMANOVA was used to test for differences between the FA profiles (for both all individual FAs, as well as FA classes) of Ascidiacea and seaweeds originating from +Org and -Org, with "sampling location" being used as a fixed factor. The statistical significance of variance components was tested using 999 permutations of unrestricted permutations of data, with an a priori chosen significance level of $\alpha=0.05$.

Individual differences in the relative abundance of FA (whose values recorded >5% of the total pool of FA in at least one of the biological matrices surveyed), FA classes, $\sum n$-3, $\sum n$-6, and the $\sum n$-3/$\sum n$-6 ratio from +Org and -Org were compared by either a t-test or the non-parametric Mann-Whitney U rank comparisons if samples were not normally distributed. A multidimensional scaling (MDS) was used to graphically visualize overall patterns and relationships between the different biological matrices survey. A SIMPER analysis was used to determine which FAs contributed the most to similarities and dissimilarities within Ascidiacea and seaweeds, at a cut-off of 50%. All analyses were performed using the PRIMER 6 + PERMANOVA© software (software package from Plymouth Marine Laboratory, UK).

5.3 Results

5.3.1 Ascidiacea

The total of lipid content of 8.1% for -Org and 2.8% for +Org was calculated. The FA profile of Ascidiacea revealed a total of 42 different FA (Table SI 5.3). Nonetheless, 4 FAs alone represented more than 50% of the total pool of FAs (Table 5.1). PERMANOVA test revealed the existence of significant differences in the FA profiles (p=0.006) and FA classes (p=0.011) of Ascidiacea from the two locations surveyed (Table 5.2). Furthermore, statistical differences were also recorded between all FA classes (Table 5.1).

Concerning the *n*-3/*n*-6 ratio, significant differences were detected between both sampling locations (p=0.002) (Table 5.1), with higher values being recorded for Ascidiacea sampled at -Org (5.77) (Figure 5.2). In general, all FAs presented a higher relative abundance at –Org, except for FA octadecenoic acid 18:1*n*-7+*n*-9, 18:2, 18:2*n*-6, and 20:1*n*-9, which displayed higher abundances at

+Org. The FAs EPA and DHA were the two most well-represented FAs (17.8% for +Org and 21.0% for -Org; 8.7% for +Org and 11.8% for -Org, respectively) (Table 5.1). Furthermore, the relative abundance of FAs 18:1*n*-7+*n*-9, 18:2*n*-6, and DHA were statistically significant between the two locations (Table 5.1).

Table 5.1: Fatty acid profile of ascidians (Ascidiacea) and seaweeds (sea lettuce, *Ulva* spp. and bladderwrack, *Fucus* sp.) sampled in locations with *versus* without the influence of organic-rich effluents from fish farming activities (+Org or -Org, respectively), as well as the formulated fish feed (FF) most supplied in fish farming activities in the study location. Values are expressed as a percentage of the total pool of fatty acids and are averages of five replicates (n=5) ± SD. Only fatty acids accounting for at least 5% of the total pool of fatty acids in one of the biological matrices surveyed are presented. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

	Ascidiacea			***Ulva* spp.**			***Fucus* sp.**			**Fish Feed**
	+Org	-Org		+Org	-Org		+Org	-Org		
14:0	0.94 ± 0.17	1.45 ± 0.12		0.68 ± 0.22	0.64 ± 0.17		8.04 ± 0.64	8.47 ± 0.27		1.53 ± 0.35
16:0	11.50 ± 1.31	12.56 ± 0.67		37.74 ± 1.14	38.05 ± 1.86		16.17 ± 1.29	15.03 ± 0.62		17.25 ± 0.68
16:1*n*-9	5.78 ± 0.62	5.37 ± 0.29		3.33 ± 0.27	2.67 ± 0.27		0.25 ± 0.04	0.29 ± 0.03		3.62 ± 0.18
16:4*n*-3	n.d	n.d		5.18 ± 0.33	4.27 ± 0.67		0.59 ± 0.06	0.59 ± 0.05		n.d
18:0	4.87 ± 1.23	5.89 ± 0.53		6.58 ± 3.99	8.70 ± 2.29		4.34 ± 1.61	1.77 ± 0.16		6.51 ± 1.09
18:1*n*-7+*n*-9	20.27 ± 1.80	11.98 ± 0.95	**	15.23 ± 1.21	15.19 ± 1.22		26.50 ± 2.28	21.34 ± 1.51	*	35.97 ± 0.43
18:2*n*-6	5.85 ± 1.62	2.26 ± 0.08	*	4.41 ± 0.19	2.74 ± 0.41		6.82 ± 0.38	7.45 ± 0.21	*	16.86 ± 0.19
18:3*n*-3	2.16 ± 0.22	2.38 ± 0.48		8.95 ± 0.70	7.85 ± 0.57	*	6.96 ± 0.41	8.87 ± 0.51	**	2.85 ± 0.07
18:4*n*-3	1.54 ± 0.61	3.61 ± 0.69		9.72 ± 0.65	10.10 ± 0.72		3.70 ± 0.36	5.55 ± 0.62		0.62 ± 0.05
20:4*n*-6	2.43 ± 0.37	3.11 ± 0.27		n.d	n.d		14.08 ± 1.17	15.03 ± 0.22		0.47 ± 0.03
20:5*n*-3	17.77 ± 2.90	20.44 ± 1.00		0.61 ± 0.13	1.25 ± 1.14		7.66 ± 0.74	9.95 ± 0.39	**	2.13 ± 0.11
22:6*n*-3	8.75 ± 1.00	11.85 ± 1.01	**	n.d	n.d		n.d	n.d		4.59 ± 0.32
∑*n*-3	32.03 ± 3.62	40.07 ± 1.54	*	27.35 ± 1.87	27.61 ± 2.30		19.16 ± 1.54	25.24 ± 1.42	**	11.43 ± 0.51
∑*n*-6	9.02 ± 1.25	6.94 ± 0.46	*	5.00 ± 0.24	3.45 ± 0.44	**	22.42 ± 1.59	24.18 ± 0.07	*	18.09 ± 0.23
∑*n*-3/∑*n*-6	3.66 ± 0.98	5.79 ± 0.37	*	5.46 ± 0.25	8.04 ± 0.36	**	0.85 ± 0.03	1.04 ± 0.06	**	0.63 ± 0.03
∑SFA	19.52 ± 2.36	22.39 ±1.00	*	46.30 ± 3.35	48.78 ± 3.37		29.35 ± 3.48	26.02 ± 0.50		25.72 ± 1.42
∑MUFA	32.99 ± 0.92	19.95 ± 1.39	**	20.88 ± 1.62	20.07 ± 1.66		29.07 ± 2.32	24.42 ± 1.48	*	44.77 ± 0.81
∑PUFA	42.81 ± 2.65	48.48 ± 1.80	*	32.82 ± 1.94	31.19 ± 2.73		41.58 ± 3.08	49.43 ± 1.42	**	29.52 ± 0.64

nd: not detected; * $p<0.05$; **$p<0.001$

∑SFA: 14:0, 15:0, 16:0, 17:0; 18:0, 20:0, 21:0, 22:0, 24:0; **∑MUFA:** 15:1, 16:1, 16:1*n*-7, 16:1*n*-9, 17:1, 17:1*n*-9, 18:1*n*-7+*n*-9, 20:1, 20:1*n*-9, 20:1*n*-7, 22:1*n*-11, 22:1*n*-9, 24:1*n*-9; **∑PUFA:** 16:2, 16:2*n*-6, 16:3*n*-3, 16:4*n*-3, 18:2, 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 18:4*n*-3, 20:2, 20:2*n*-6, 20:3*n*-6, 20:3*n*-3, 20:4*n*-6, 20:4*n*-3, 20:5*n*-3, 22:4, 22:4, 22:5*n*-6, 22:5*n*-3, 22:6*n*-3

Table 5.2: Results of the permutational multivariate analysis of variance (PERMANOVA) of fatty acids and fatty acid classes of ascidians (Ascidiacea) and seaweeds (sea lettuce, *Ulva* spp. and bladderwrack, *Fucus* sp.) sampled in locations with *versus* without the influence of organic-rich effluents from fish farming activities (+Org or -Org, respectively). Significant differences were considered at $p<0.05$ (represented in bold); P(perm): p-values based on more than 999 permutations.

	Permanova +Org *vs* -Org	
	Fatty acids	**Fatty acids classes**
Ascidiacea	**0.006**	**0.011**
***Ulva* spp.**	**0.021**	0.341
***Fucus* sp.**	**0.013**	**0.013**

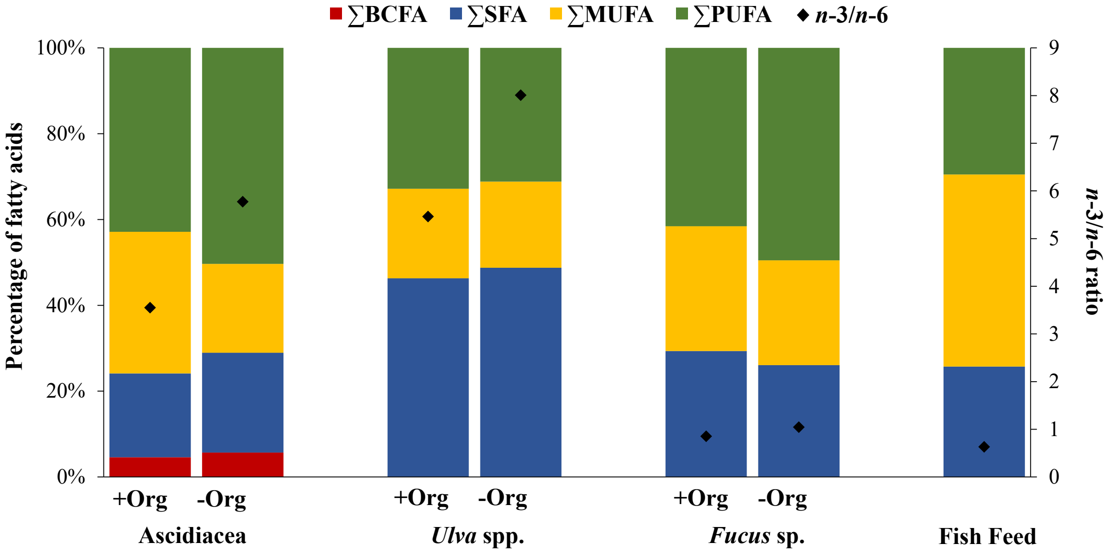

Figure 5.2: Fatty acid classes expressed as a percentage of the total pool of fatty acids (only values above 1% were considered) of ascidians (Ascidiacea) and seaweeds (sea lettuce, *Ulva* spp. and bladderwrack, *Fucus* sp.) sampled in locations with *versus* without the in-fluence of organic-rich effluents from fish farming activities (+Org or -Org, respectively), as well as the formulated fish feed (FF) most commonly supplied in fish farming activities in the study location. and their respective *n*-3/*n*-6 ratios. BCFA: branched fatty acids, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Branched FAs (BCFA) represented the least abundant FA class identified in specimens sampled from both locations (4.6% for +Org; 5.5% for -Org) (Figure 5.2). Saturated FAs (SFA) and PUFA registered higher values in specimens from -Org (22.3% and 48.5%, respectively). In addition, monounsaturated FAs (MUFA) values were higher at +Org (33% for +Org and 20.7% for -Org)

(Figure 5.2). Similarity Percentage Species Contributions (SIMPER) analysis (Table 5.3A) showed that the FA profiles of Ascidiacea originating from the two locations displayed an average dissimilarity of 10.6%, with more than 50% cumulative dissimilarities being explained by the following FAs: eicosenoic acid 20:1*n*-9, 18:1*n*-7+*n*-9, linoleic acid – LA 18:2*n*-6, and stearidonic acid - SDA 18:4*n*-3.

5.3.2 Seaweeds

The total of lipid content *Ulva* spp. of 1.4% for -Org and 2.4% for +Org and for *Fucus* sp. 3.1% for -Org and 3.5% for +Org was calculated. A total of 17 and 24 different FAs were identified for *Ulva* spp. and *Fucus* sp., respectively (Table SI 5.3) (Table 5.1). The FAs palmitic acid 16:0 and 18:1*n*-7+*n*-9 were dominant in both seaweeds (37.7% for +Org and 38.1% for -Org; 15.2% for +Org and 15.2% for -Org, respectively). However, some contrasts worth highlighting were also recorded, such as the relative abundance of arachidonic acid (AA) 20:4*n*-6 and EPA in *Fucus* sp. (14.1% for +Org and 15.0% for -Org; 7.7% for +Org and 10.0% for -Org; respectively) that where either non detected or present at trace levels (respectively) in *Ulva* spp.

Statistically significant differences were detected in 18:3*n*-3 for *Ulva* spp. ($p=0.025$), while for *Fucus* sp. FAs 18:1*n*-7+*n*-9, 18:2*n*-6, 18:3*n*-3, and EPA all differed significantly ($p=0.003$, $p=0.013$, $p<0.001$, $p<0.001$, respectively). PERMANOVA test showed statistical differences in the mean FA profiles of seaweeds originating from the two sampling locations ($p=0.021$ for *Ulva* spp.; $p=0.013$ for *Fucus* sp.), yet only significant differences were seen in the FA classes of *Fucus* sp. ($p=0.013$) (Table 5.2), with significant differences being recorded between MUFA and PUFA ($p=0.005$, $p<0.001$, respectively) of specimens of this brown seaweed originating from the two sampling locations (Table 5.1).

The *n*-3/*n*-6 ratio also exhibited significant differences between both sampling locations ($p<0.001$ for *Ulva* spp., $p<0.001$ for *Fucus* sp.) (Table 5.1), with higher values being recorded for seaweeds at -Org. The prevailing FA class in *Ulva* spp. was SFA (46.3% for +Org and 48.8% for -Org) (Figure 5.2), while PUFA registered higher values for *Fucus* sp. (41.6% for +Org; 49.4% for -Org). The MDS plot (Figure 5.3) revealed a distinct separation between the two seaweeds and the two sampling sites, with similarity values of 59% grouping both FA profiles. SIMPER analysis (Table 5.3A) revealed that the FA profiles of *Ulva* spp. and *Fucus* sp. display comparable values of dissimilarities between +Org and -Org (5.29% and 5.48%, respectively), with FA 18:0 contributing to the most for such differences.

5.3.3 Fish Feed

The total of lipid content of 14.3% was calculated. A total of 26 FAs were identified for fish feed (Table SI 5.3) (Table 5.1). MUFA was the most abundant FA class for fish feed (44.8%) (Figure 5.2) with a major contribution of FA 18:1*n*-7+*n*-9 (36.0%) (Table 5.1). SFA and PUFA presented similar values (25.7% and 29.5%, respectively). The *n*-3/*n*-6 ratio obtained was 0.63, indicating higher amounts of *n*-6 FAs. The MDS plot (Figure 5.3) revealed that the FA profile of fish feed is more similar to the FA profile of Ascidiacea from +Org than from -Org. SIMPER analysis of the FA profiles of fish feed and Ascidiacea (Table 5.3B) revealed higher dissimilarities with specimens originating from -Org. For Ascidiacea, EPA was the main responsible for such differences.

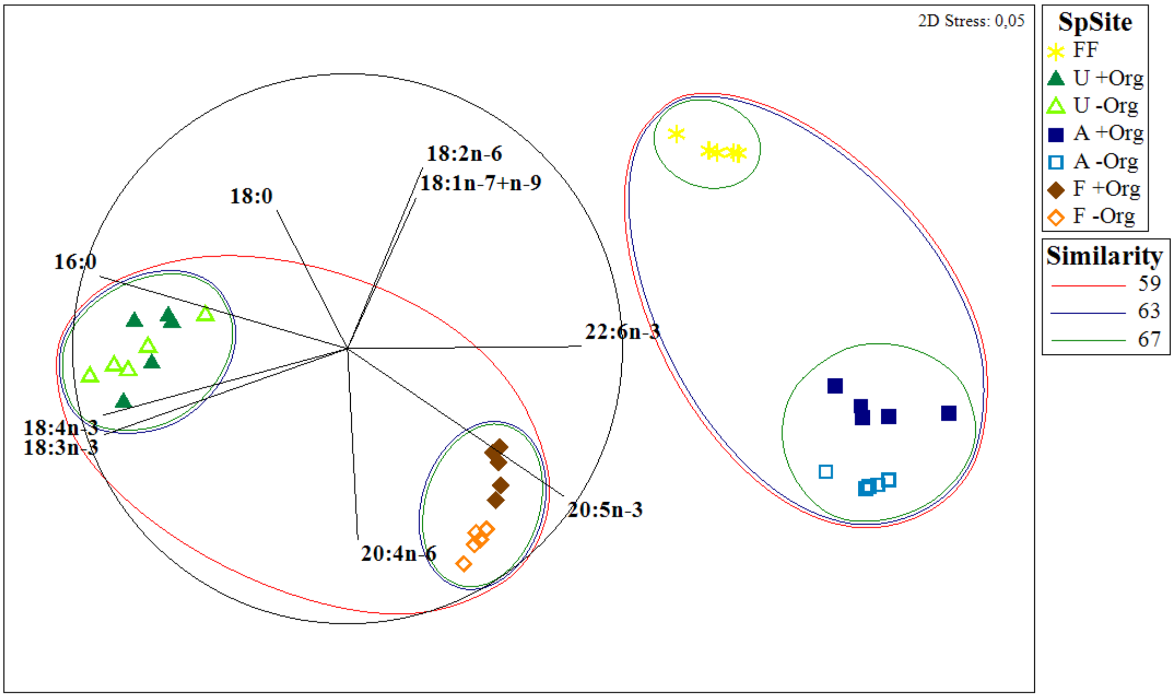

Figure 5.3: Multidimensional scaling (MDS) ordination plot comparing the fatty acid profiles between specimens of ascidians (Ascidiacea) (A) and seaweeds (sea lettuce, *Ulva* spp. (U) and bladderwrack, *Fucus* sp. (F)) sampled in locations with *versus* without the influence of organic-rich effluents from fish farming activities (+Org or -Org, respectively) and the formulated fish feed (FF) most supplied in fish farming activities in the study location.

Table 5.3: Summary of SIMPER analysis listing the fatty acids that most contributed to discriminate: A) ascidians (Ascidiacea) and seaweeds (sea lettuce, *Ulva* spp. and bladderwrack, *Fucus* sp.) sampled in locations with *versus* without the influence of organic-rich effluents from fish farming activities (+Org or -Org, respectively); and B) ascidians from +Org or -Org with the formulated fish feed (FF) most commonly supplied in fish farming activities in the study location. Cut-off percentage: 50%.

Dissimilarities

A)	**Ascidiacea**				***Ulva* spp.**				***Fucus* sp.**			
	+Org *vs* -Org				**+Org *vs* -Org**				**+Org *vs* -Org**			
	10.62%				**5.29%**				**5.48%**			
-Org		+Org	-Org	Contrib%		+Org	-Org	Contrib%		+Org	-Org	Contrib%
vs	20:1*n*-9	2.21	1.03	15.81	18:0	2.48	2.93	23.19	18:0	2.05	1.33	22.78
+Org	18:1*n*-7+*n*-9	4.50	3.46	13.92	18:2*n*-6	2.10	1.65	14.23	18:1*n*-7+*n*-9	5.14	4.62	16.65
	18:2*n*-6	2.40	1.50	11.94	22:5*n*-3	1.54	1.91	11.59	18:4*n*-3	1.92	2.35	13.56
	18:4*n*-3	1.22	1.89	8.98	20:5*n*-3	0.78	1.04	9.76				

B)	**Ascidiacea**							
	+Org *vs* FF				**-Org *vs* FF**			
	31.06%				**36.35%**			
		+Org	FF	Contrib%		-Org	FF	Contrib%
Org	20:5*n*-3	4.21	1.46	13.91	20:5*n*-3	4.52	1.46	13.33
vs	18:2*n*-6	2.40	4.11	8.65	18:2*n*-6	1.50	4.11	11.34
FF	20:4*n*-6	1.55	0	7.87	18:1*n*-7+*n*-9	3.46	6.00	11.06
	18:1*n*-7+*n*-9	4.5	6.00	7.58	18:4*n*-3	1.89	0	8.24
	22:1*n*-11	0	1.36	7.05	20:4*n*-6	1.76	0	7.68
	18:4*n*-3	1.22	0	6.18				

5.4 Discussion

To the authors' best knowledge, the present study is the first approach reported in the scientific literature to screen for health-promoting FAs in ascidians grown under the influence of fish farming organic-rich effluents. Out of the total pool of FA identified in Ascidiacea (42 FA), only 4 of these biomolecules (16:0, 18:1*n*-7+*n*-9, EPA, and DHA) represented average values above 10% of the total pool of FA.

These findings share similarities with those reported from previous works screening the FA profile of ascidians (Jeong et al., 1996; Maoufoud et al., 2009; Sri Kumaran and Bragadeeswaran, 2014; Zhao and Li, 2016). The FAs 18:1*n*-7+*n*-9 and 18:2*n*-6 also displayed higher values in +Org, near twice the ones recorded for -Org. Considering that these FAs accounted for 53% of the fish aquafeed FA pool, it is likely that ascidians may selectively retain these FAs in their tissues. The higher levels of *n*-3 FAs present in the -Org resulted in a higher *n*-3/*n*-6 ratio, with FAs 18:4*n*-3, EPA, and DHA being the main contributors to this trend. This finding is consistent with Monmai et al. (2018), as these authors verified that in the edible ascidian *Halocynthia aurantium* *n*-3 FA was present in much higher levels than *n*-6 FA. Likewise, Zhao and Li (2016) documented that tunics and inner body tissues of ascidians *Halocynthia roretzi*, *Styela plicata*, *Ascidia* sp., and *Ciona intestinalis* presented higher levels of *n*-3 FAs.

Ulva spp. and *Fucus* sp. presented some similarities in their FA profiles, with 16:0 and 18:1*n*-7+*n*-9 displaying the highest relative abundances in the total pool of FA recorded in both locations. This finding is in line with previous studies (Kim et al., 1996; Schmid et al., 2014; Morais et al., 2021). Our results on the profiling of unsaturated FAs (MUFA+PUFA) are fully aligned with those reported by Herbreteau et al. (1997), who reported the FA composition of five species of seaweeds and verified that unsaturated FAs accounted for more than 50% of the total pool of FA, with this proportion reaching up to 75% for *Fucus* sp. Silva et al. (2013) focused on ten brown seaweeds also verifying important amounts of unsaturated FAs. In addition, our study recorded 46% to 49% of SFA in *Ulva* spp., unlike Lopes et al. (2020) who have reported about half of these values for the same seaweed species (≈24%). Yet, the values of FA classes reported for *Fucus* sp. by Lopes et al. (2020) are very much in line with the ones reported in the present work. Several studies (Silva et al., 2013; Kendel et al., 2015; Lopes et al., 2020) have mentioned that despite lipid content representing a minor fraction of seaweeds, it features levels of *n*-3 PUFAs worth being investigated.

Our results validated the presence of EPA in *Fucus* sp., but not DHA, and no traces of either of these FA were detected in *Ulva* spp. These latter values correlate fairly well with Pereira et al., (2012) with *Ulva* spp. also presenting higher proportions of FA 18:3*n*-3, thus, further supporting the idea that seaweeds do display an *n*-3/*n*-6 “healthy” ratio. Several studies (Vliet and Katan, 1990; Simopoulos, 2002; Candela et al., 2011; Strobel et al., 2012; Sprague et al., 2016) have reported an increase in the use of *n*-6 PUFA-rich land-based ingredients and oils in aquafeed formulations sometimes leading to an inverted *n*-3/*n*-6 ratio in fish aquafeeds.

Under organic-rich effluents, the biochemical profile of extractive species will most likely be shaped by the prevalence of these ingredients (Hodar et al., 2020). However, the availability of natural nutrients (Pedersen and Borum, 1997), sampling location, and season (Silva et al., 2013), amongst other factors, must be taken into consideration when profiling the FA of marine species, as they too can modulate their biochemical profile and findings being reported results must be interpreted with care. Kim et al. (1996) demonstrated how temperature, salinity, light, and nitrogen levels influence the level and profile of lipids present in the brown seaweed *Fucus serratus*. Similar findings were reported by Glencross (2009) who emphasized how the hydrological source is a primary factor weighing on the differences in FA requirements. This trend can extend to a multitude of marine organisms of interest for production under an IMTA framework, such as polychaetes (Luis and Passos, 1995; García-Alonso et al., 2008), isopods (Prato et al., 2012), bivalves (Ezgeta-Balić et al., 2012; Chetoui et al., 2019) and several fish species (Zlatanos and Laskaridis, 2007).

In conclusion, the present study demonstrated that Ascidiacea presented high values of EPA (17.8% in +Org; 20.4% in -Org) and DHA (8.8% in +Org; 11.9% in -Org) and can be considered as a potential new bioresource for *n*-3 long-chain FAs. The organic-rich effluent originating from fish farming systems can indeed shape the lipid profile of extractive species being employed in IMTA frameworks, whether as a consequence of direct consumption of available organic nutrients in dissolved and particulate form, as in the case of ascidians, or indirectly from *de novo* FA synthesis as in the case of seaweeds uptaking dissolved inorganic nutrients.

The use of extractive species to maximize the use of ingredients present in formulated aquafeeds employed to farm marine finfish and shrimp can be considered as a pathway towards more sustainable and efficient aquaculture practices, holding the potential to generate biomass with the potential to deliver important biomolecules for multiple biotechnological applications (Vieira et al., 2020). Our findings clearly point towards the need to further investigate the biochemical profile, particularly the FA profile of extractive species used in IMTA systems, as an approach to sequester valuable health-promoting FAs that will otherwise be lost to the aquatic environment through the effluents of fish farms.

Chapter 6

Final Considerations
and
Future Perspectives

6. Final Considerations and Future Perspectives

As the aquaculture industry continues to grow, it is rapidly becoming the major provider of aquatic products for mankind (FAO, 2022). In recent years, the key priority has been to make aquaculture productions responsible, sustainable, and environmentally friendly, and has increased greatly. In addition, an increased pressure to search for alternative and innovative ingredients for aquaculture feed is crucial. The goal is to promote diversification by identifying new protein sources through exploiting, repurposing, and developing all available resources. This pursuit will reduce the amount of fish-based ingredients contained in aquaculture feeds while preserving the important human health benefits of farmed seafood. Alongside this requirement for sustainability, the concept of Integrated Multi-Trophic Aquaculture (IMTA) is a promising solution for the development of aquaculture enterprises. This concept has been shown to support the principles of ecosystem-based management, reducing the impacts of nutrient waste, and increasing production efficiency and investment profitability by using organisms from different trophic levels.

Ascidians have been the focus of this research, given their unique features in terms of high filtration and fast growth rates and their poorly exploited added value concerning alternative ingredients for more sustainable aquaculture production. For these reasons, this thesis aimed to investigate the full potential of ascidians with special attention to their ability as extractive species in an IMTA framework.

Overall, the set of objectives initially established was accomplished revealing the following main findings:

- The systematic review revealed that ascidians, solitary and colonial, display great variability. However, in general, all ascidians present high filtration and fast-growth rates. These traits allow them to be excellent candidates as extractive species, where they operate well under an IMTA framework, especially with sea cucumbers and fish. Competition for resources and space with other filter-feeders, namely bivalves, can exist. Ascidians disclosed a rich source of essential fatty acids (EPA and DHA), granting them enormous possibilities as premium ingredients for aquafeed formulations and dietary supplements.
- The experiment performed demonstrated that IMTA organic-rich effluents can condition the lipid profile of ascidians and seaweeds, through direct assimilation of organic matter or indirectly from *de novo* FA synthesis. Ascidians displayed high relative abundance values of EPA (18 to 20%) and DHA (9 to 12%). Overall, even under the influence of organic-rich effluents from fish farming activities, ascidians and seaweeds are a valuable source of health-promoting fatty acids and can be considered as a potential new bioresource for n-3 long-chain fatty acids. These remarks confirm their potential for sustainable farming practices,

such as for IMTA systems.

- A groundbreaker survey took place in the coastal lagoon of Ria de Aveiro, where the presence, distribution, abundance, and biomass of ascidians were investigated. Two species, *Clavelina lepadiformis* and *Ascidiella aspersa*, were reported for the first time in the coastal waters of mainland Portugal. The establishment and success of the ascidian community are most highly influenced by the hydrodynamics, seasonality, and by nutrient-enriched waters present at each location. A preference for aquaculture environment locations was observed.
- The innovative customized 3-D star-shaped artificial substrate unit employed demonstrated to be an efficient support structure for short- or long-term monitorization of ecosystem shifts in the ascidian community and can be used to research fouling organisms in general. This structure may be used in aquaculture facilities to monitor biofouling activity and help producers act beforehand with mitigation programs. Furthermore, it can also be an important feature for environmental monitoring concerning global climate change and tracking the risk of invasive species. The presence or absence of specific species present in the 3-D structure may indicate variations in the salinity and temperature values, pH, oxygen, nutrient levels, etc.

The achievements detailed above can be seen as a direct contribution towards the improvement of aquaculture, efficiency, and sustainable production. A well-balanced inclusion of ascidians as extractive species into IMTA systems can contribute to the eco-intensification of marine aquaculture and provide alternative ingredients for the formulation of aquafeeds, as well as for human consumption. Ultimately, this study contributes to seven of the Sustainable Development Goals defined by the FAO for 2030 that are associated with the marine aquaculture industry: SDG1 no poverty; SDG2 zero hunger; SDG3 promoting health and well-being; SDG12 responsible consumption and production; SDG13 climate change; SDG14 life below water; SDG17 partnership for the goals. Further research on ascidians can provide an opportunity to contribute to the many initiatives created by the European Union (EU), from the Farm to Fork Strategy, the EU's Blue Growth Strategy, namely in the blue-biotechnology sector to the Biodiversity Strategy, all with common goals to ensure food sustainability provided from agriculture, fisheries and aquaculture contribute to the growth for Europe.

Subsequent to this thesis, several other questions are to be considered and should be brought to our attention in future research, namely:

- Develop a protocol aiming at maximizing the efficiency and profitability of ascidians in an IMTA framework and ensure the best combinations of co-extractive species and therefore, research for more co-extractive species that may interact positively with ascidians in an IMTA framework.

- ✶ Foster joint initiatives enhancing collaborations with fish farmers, aquaculture companies, and stakeholders to advertise the potential of ascidians and their added value and hopefully take a few steps forward in utilizing the full advantage of these organisms.
- ✶ In a more experimental approach, perform a lab-scale experiment to study multiple configurations of a dietary replacement of fish meal with ascidian biomass to elucidate the level of fatty acid incorporated and evaluate the growth performance of fed species (e.g., marine fin fish and shrimp).
- ✶ Enhance science-based knowledge of the ascidian community to further understand their behavior and to implement site-specific mitigation measures, if necessary. More specifically, in the Ria de Aveiro, this may be achieved by developing programs with expanded temporal and spatial monitoring. The development of an identification guide for ascidians in Portugal would be original and may promote student and public awareness.
- ✶ The importance of publishing first-time observed species enables updated datasets, making this information more valuable and accessible to the general public. Sharing this information in various online platforms, such as GBIF, also contributes to the global knowledge of ascidian diversity, promotes conservation and/or management solutions for each ecosystem, and allows for an interconnected world with new opportunities and collaboration with institutions and organizations.

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9. Supporting Information

Table SI 2.1: References retrieved for the systematic literature review, with no year restriction, using the databases Thomson Reuters Web of Science (Core Collection) (Topic) and Scopus (Article title, Abstract, Keywords) with Filtration AND (tunicate OR ascidian OR 'sea squirt*'); Aquaculture AND (tunicate OR ascidian OR 'sea squirt*'); Fatty acid* AND (tunicate OR ascidian OR 'sea squirt*') as the keyword search.

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Table SI 2.2: List of all species addressed in the scope of the present review. Scientific names are according to the World Register of Marine Species–available at http://www.marinespecies.org/ *Currently not accepted; **Misspelled.

Order	Family	Species	Type	Accepted
Aplousobranchia	Clavelinidae	*Clavelina lepadiformis*	Colonial	
	Didemnidae	*Didemnum molle*	Colonial	
		Didemnum moseleyi	Colonial	
		*Didemnum psammathodes**	Colonial	*Didemnum psammatodes*
		Didemnum vexillum	Colonial	
		Diplosoma sp.	Colonial	
		Leptoclinides uniorbis	Colonial	
		*Lissoclinum voeltzkowi**	Colonial	*Lissoclinum timorense*
		Polysyncraton sp.	Colonial	
	Polycitoridae	*Cystodytes violatinctus*	Colonial	
		Eudistoma bituminis	Colonial	
		Eudistoma viride	Colonial	
Phlebobranchia	Ascidiidae	*Phallusia julinea*	Solitary	
		Phallusia mammillata	Solitary	
		Phallusia nigra	Solitary	
	Cionidae	*Ciona intestinalis*	Solitary	
		Ciona robusta	Solitary	
		Ciona savignyi	Solitary	
	Corellidae	*Corella eumyota*	Solitary	
		Corella parallelogramma	Solitary	

	Octacnemidae	*Megalodicopia hians*	Solitary	
	Perophoridae	*Ecteinascidia turbinate***	Colonial	*Ecteinascidia turbinata*
	Ascidiidae	*Ascidia challengeri*	Solitary	
		Ascidia virginea	Solitary	
		Ascidiella aspersa	Solitary	
		Ascidiella scabra	Solitary	
Stolidobranchia	Molgulidae	*Molgula manhattensis*	Solitary	
		Molgula occidentalis	Solitary	
		Molgula pedunculata	Solitary	
	Pyuridae	*Boltenia echinata*	Solitary	
		*Cynthia savignyi**	Solitary	*Pyura microcosmus*
		Cynthia squamulata/***	Solitary	*Pyura squamulosa*
		Halocynthia aurantium	Solitary	
		Halocynthia papillosa	Solitary	
		Halocynthia pyriformis	Solitary	
		Halocynthia roretzi	Solitary	
		Halocynthia spinosa	Solitary	
		Herdmania momus	Solitary	
		Microcosmus exasperatus	Solitary	
		Microcosmus sabatieri	Solitary	
		Microcosmus savignyi	Solitary	
		*Microcosmus sulcatus**	Solitary	*Microcosmus vulgaris*
		Pyura chilensis	Solitary	
		Pyura microcosmus	Solitary	
		Pyura tessellata	Solitary	

Table SI 2.3: Summary of the fatty acid profiles addressed in the 28 publications selected regarding question 3 [For what?]. T: tunic; IB: inner body; TAG: triacylglycerol; PL: Phospholipid; SCF-CO_2: supercritical fluid; EtOAc: ethyl acetate; NA: not applicable.

Species	**Fatty acids**											**Reference**
	16:0	**18:0**	**20:0**	**16:1(*n*-7)**	**18:1**	**18:1(*n*-9)**	**20:1(*n*-9)**	**18:3(*n*-6)**	**20:4(*n*-6)**	**20:5(*n*-3)**	**22:6(*n*-3)**	
Ascidia sp.	20.94 (T) 17.35 (IB)	12.71 (T) 9.8 (IB)	0.47 (T) 0.28 (IB)		25.41 (T) 20.06 (IB)				2.35 (T) 2.08 (IB)	6.82 (T) 18.8 (IB)	2.59 (T) 11.01 (IB)	1.
Botryllus schlosseri	20.8	6.1	1.1	3.1		6.3	1.7	2.1	1.6	10.5	15.5	2.
Ciona intestinalis	0.08 (SCF-CO_2) 0.46 (EtOAc)	6.54 (SCF-CO_2) 10.42 (EtOAc)			16.78 (SCF-CO_2) 20.27 (EtOAc)					21.01 (SCF-CO_2) 18.24 (EtOAc)	8.70 (SCF-CO_2) 6.59 (EtOAc)	3.
	high amounts									high amounts	high amounts	4.
	16.99 (T) 16.25 (IB)	3.57 (T) 3.49 (IB)	1.82 (T) 1.30 (IB)	8.55 (T) 7.28 (IB)		12.61 (T) 8.98 (IB)	10.56 (T) 10.72 (IB)			24.13 (T) 25.73 (IB)	8.69 (T) 12.04 (IB)	5.
	11.67 (T) 18.15 (IB)	6.25 (T) 5.79 (IB)	1.67 (T) 0.96 (IB)		14.58 (T) 11.23 (IB)				0.42 (T) 0.54 (IB)	24.58 (T) 22.04 (IB)	12.92 (T) 12.56 (IB)	1.
Cynthia savignyi	23.6	5.9	1.5		11.7					12.5	6.4	6.
Cynthia squamulata	18.9	5.4	1.3		8.9					11.1	6.4	6.
Cystodytes violatinctus	21.2	24.8	3.1			20						7.
Didemnum molle	30.09-31.04	1.85-2.32	0.16-0.27	26.08-21.01		2.72-3.49	0.09-0.15		3.53-5.82	8.05-8.19	0.71-1.18	8.
Didemnum psammathodes	11.63	4.31	0.74	0.71		6.13	0.31	1.33	0.14	10.34	8.14	9.
Eudistoma bituminis	40	16.4	16.5									7.
Eudistoma sp.	15.9	8.2	0.6	9.7		9.4			7.8	8.9	3.0	10.
Eudistoma viride	10.72	6.22	1.55	0.18		7.12	0.19	1.01		11.10	7.12	9.

Halocynthia aurantium	16:0 + 18:0: 53.17				12.18				34.65		11.
Halocynthia roretzi	10.5	5.6	0.7			3.1	0.5		22.0	17.7	12.
	44.6	10.7			26.5				43.6	20.1	13.
	10.06 (T)	16.35 (T)	1.26 (T)		8.18 (T)			1.26 (T)	30.82 (T)	13.84 (T)	1.
	9.01 (IB)	6.37 (IB)	1.13 (IB)		11.98 (IB)			0.86 (IB)	28.22 (IB)	23.08 (IB)	
Leptoclinides uniorbis	4.9	3.7	2.3	12.0		5.8		6.0	16.7	2.5	10.
Megalodicopia hians		4.47 (Branched)		34.21 (Monoens)	30.7 (Even sat)		6.73 (Odd sat)	1.58 (Diens)	22.32 (PUFA)		14.
Microcosmus sulcatus	31.6	6.3	0.46	0.91		8.2	0.11	0.21	0.15	0.06	15.
Phallusia sp.	29.1 (TAG)	11.10 (TAG)			18.3 (TAG)			2.0 (TAG)	1.8 (TAG)		16.
	18.4 (PL)	16.1 (PL)			33.3 (PL)				10.4 (PL)		
Styela clava	0.03 (SCF-CO_2)	0.86 (SCF-CO_2)			7.55 (SCF-CO_2)				18.58 (SCF-CO_2)	8.45 (SCF-CO_2)	3.
	3.28 (EtOAc)	0.22 (EtOAc)			4.55 (EtOAc)				9.82 (EtOAc)	9.65 (EtOAc)	
Styela plicata	15.31 (T)	11.22 (T)	0.34 (T)		15.65 (T)			0.34 (T)	25.85 (T)	5.78 (T)	1.
	24.66 (IB)	15.66 (IB)	2.12 (IB)		19.17 (IB)			0.39 (IB)	11.08 (IB)	6.89 (IB)	
Styela sp.	25.4 (TAG)	8.3 (TAG)			18.4 (TAG)			3.7 (TAG)	10.7 (TAG)		16.
	22.4 (PL)	9.7 (PL)			11.8 (PL)			5.7 (PL)	10.3 (PL)	7.0 (PL)	

1. Zhao & Li (2016)
2. Carballeira et al. (1995)
3. Jiang et al. (2005)
4. Hassanzadeh (2014)
5. Zhao et al. (2015)
6. Maoufoud et al. (2009)
7. Viracaoundin et al. (2003)
8. Johns et al. (1981)
9. Sri Kumaran & Bragadeeswaran (2014)
10. Dagorn et al. (2010)
11. Monmai et al. (2018)
12. Vysotskii et al. (1992)
13. Jeong et al. (1996)
14. Ogawa et al. (2005)
15. Zlatanos et al. (2009)
16. Slantchev et al. (2002)

Table SI 3.1: Values of the environmental parameters (Temperature: Temp (ºC), Dissolved oxygen: DO (mg/L), pH, Salinity) registered at each sampled location throughout the duration of the experiment, in the Ria de Aveiro. D: day. TG: Tide Gauge; FH: Fishing Harbour; M: Marina; OF: Oyster Farm; IMTA: Integrated Multi-Trophic Aquaculture.

		D0	**D92**	**D127**	**D155**	**D191**	**D226**	**D260**	**D295**	Average
Temp (ºC)	**TG**	14.0	14.8	15.7	15.3	16.0	16.8	15.7	14.7	15.4
	FH	14.6	15.2	16.8	17.2	19.0	18.6	16.2	13.2	16.4
	M	14.3	15.4	17.6	16.4	18.6	17.1	17.3	15.2	16.5
	OF	16.2	16.8	18.6	18.8	23.2	19.9	14.7	13.0	17.7
	IMTA	14.4	16.5	22.6	23.0	20.7	18.8	15.5	13.0	18.1
DO (mg/L)	**TG**	8.3	7.0	7.6	6.7	7.3	7.5	8.1	8.3	7.6
	FH	8.1	7.3	6.9	7.9	5.9	6.8	7.5	8.4	7.4
	M	7.6	6.9	7.5	6.4	7.5	6.8	7.8	8.1	7.3
	OF	9.5	8.1	7.4	8.4	8.5	9.0	6.5	9.8	8.4
	IMTA	7.91	6.3	6.0	6.5	5.8	6.5	6.4	7.7	6.6
pH	**TG**	7.8	8.3	8.0	7.9	7.3	8.1	7.7	7.8	7.9
	FH	7.8	8.4	8.0	8.0	7.6	8.0	7.5	7.6	7.9
	M	7.6	8.3	8.1	7.9	7.5	8.0	7.3	7.8	7.8
	OF	7.73	8.5	8.0	8.1	8.2	8.3	7.2	7.9	8.0
	IMTA	7.65	8.3	7.8	7.7	7.4	7.8	7.1	7.4	7.6
Salinity	**TG**	36.4	32.7	34.4	34.5	33.7	34.0	34.4	24.6	33.1
	FH	29.0	33.2	34.3	34.4	32.6	32.3	32.6	26.4	31.9
	M	20.9	24.8	34.0	34.4	30.7	33.6	29.1	22.2	28.7
	OF	25.3	29.6	30.6	32.5	31.3	31.4	30.3	24.7	29.5
	IMTA	30.4	30.2	33.0	34.3	33.8	33.0	30.0	20.8	30.7

Table SI 3.2: Ecological indices for the ascidian community present in Ria de Aveiro. H = Shannon Index; Hmax = maximum diversity possible; pi = proportion of individuals found in the ith species; ln = natural logarithm; S = total number of species; Ds = Diversity Index; n = number of individuals for each species; N = total number of all individuals; DMg = Margalef's index.

Index	Formula	Reference	Classification
Species Richness	$S_r = S$		
Pielou's Evenness	$J = \frac{H'}{H'max} = \frac{H'}{lnS}$	(Pielou, 1969)	0 low diversity 1 high diversity
Shannon's Diversity	$H' = -\sum_{i=1}^{s} p_i * lnp_i$	(Shannon and Weaver, 1963)	0 low diversity ≥5 high diversity
Simpson's Diversity	$D_s = 1 - \sum \left(\frac{n}{N}\right)^2$	(Simpson, 1949)	1 low diversity 0 high diversity
Margalef's Richness	$D_{Mg} = \frac{(S-1)}{ln(N)}$	(Margalef, 1968)	<2 low diversity >5 high diversity

Table SI 3.3: Pairwise test analyses. A) differences between the total abundance of ascidians collected from each location and B) differences between the sampling days within each location. In bold are represented significant differences. D: day. TG: Tide Gauge; FH: Fishing Harbour; M: Marina; OF: Oyster Farm; IMTA: Integrated Multi-Trophic Aquaculture.

A)

	Ascidiella aspersa	***Ciona intestinalis***	***Clavelina lepadiformis***	***Microcosmus* sp.**	***Molgula* sp. 1**	***Molgula* sp.2**	***Styela plicata***	***Styela* sp.**
TG, FH	**0.001**	0.095	**0.04**	**0.004**	**0.001**	**0.001**	-	0.217
TG, M	0.491	0.387	**0.012**	**0.001**	**0.001**	**0.001**	-	**0.022**
TG, OF	0.464	0.067	**0.015**	**0.001**	**0.001**	**0.011**	**0.001**	-
TG, IMTA	**0.001**	**0.001**	**0.013**	**0.002**	**0.001**	**0.001**	**0.001**	-
FH, M	**0.001**	0.541	0.406	0.157	0.075	**0.001**	-	0.632
FH, OF	**0.01**	0.546	0.412	**0.001**	**0.001**	**0.001**	**0.001**	0.183
FH, IMTA	**0.001**	**0.001**	0.402	0.657	**0.001**	**0.001**	**0.001**	0.2
M, OF	0.191	0.258	-	**0.001**	**0.001**	**0.001**	**0.001**	**0.022**
M, IMTA	**0.001**	**0.001**	-	0.145	**0.001**	-	**0.001**	**0.029**
OF, IMTA	**0.001**	**0.001**	-	**0.001**	**0.001**	**0.001**	0.194	-

B)

	TG	**FH**	**M**	**OF**	**IMTA**
Sampling Days	P(MC)	P(perm)	P(MC)	P(perm)	P(perm)
D92, D127	-	**0.012**	**0.012**	**0.002**	**0.001**
D92, D155	**0.035**	**0.01**	**0.001**	**0.001**	**0.001**
D92, D191	0.108	**0.014**	**0.029**	**0.001**	**0.002**
D92, D226	**0.001**	**0.007**	**0.003**	**0.001**	**0.001**
D92, D260	0.358	**0.001**	**0.001**	**0.001**	**0.001**
D92, D295	0.199	**0.003**	**0.008**	**0.001**	**0.001**
D127, D155	**0.047**	0.076	0.081	**0.004**	**0.006**
D127, D191	0.114	**0.026**	**0.035**	**0.001**	**0.011**
D127, D226	**0.001**	**0.008**	**0.002**	**0.001**	**0.005**
D127, D260	0.345	**0.009**	**0.001**	**0.001**	**0.009**
D127, D295	0.237	**0.008**	**0.001**	**0.001**	**0.013**
D155, D191	0.074	**0.035**	**0.011**	0.673	**0.005**
D155, D226	**0.005**	**0.011**	**0.011**	**0.005**	**0.01**
D155, D260	**0.044**	**0.011**	**0.001**	**0.011**	**0.008**
D155, D295	0.051	**0.021**	**0.001**	**0.012**	**0.007**
D191, D226	**0.001**	**0.027**	0.781	0.119	**0.011**
D191, D260	0.231	**0.009**	0.34	**0.011**	**0.021**
D191, D295	0.206	0.152	**0.004**	**0.007**	**0.01**
D226, D260	**0.001**	0.082	0.522	**0.013**	**0.006**
D226, D295	**0.003**	0.215	**0.001**	**0.011**	**0.007**
D260, D295	0.359	0.082	**0.001**	0.415	**0.025**

Table SI 3.4: Values of the ecological indicators calculated for each sampled location in the Ria de Aveiro. TG: Tide Gauge; FH: Fishery Harbour; M: Marina; OF: Oyster Farm; IMTA: Integrated Multi-Trophic Aquaculture.

	Shannon's Index	**Simpson's Index**	**Pielou's Index**	**Margalef's Index**
TG	1.35	0.68	0.75	1.12
FH	1.21	0.64	0.58	1.34
M	0.85	0.49	0.53	1.00
OF	1.00	0.56	0.51	0.99
IMTA	1.21	0.62	0.62	0.94

Table SI 3.5: List of recorded ascidian species from Portugal (mainland, Azores and Madeira Islands). Superscript number 1 indicates that the previous ascidian species name is no longer accepted, and superscript number 2 indicates that the previous ascidian species name was misspelled.

Species	**Type**	**Location**			**Reference**
Abyssascidia millari	Solitary	Azores		Mainland	Ramos-Esplá 1988
Adagnesia charcoti	Solitary	Azores			Ramos-Esplá 1988
Adagnesia rimosa^{2}	Solitary		Madeira		Ramos-Esplá 1988
Agnezia atlantica^{1}	Solitary		Madeira		Ramos-Esplá 1988
Alloeocarpa loculosa	Colonial	Azores			Monniot 1974; Ramos-Esplá 1988; Cardigos et al. 2006; Borges et al. 2010
Aplidiopsis atlanticus	Colonial	Azores			Ramos-Esplá 1988
Aplidium albicans	Colonial			Mainland	Ramos-Esplá 1988
Aplidium appendiculatum	Colonial	Azores			Borges et al. 2010
Aplidium bermudae	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Aplidium densum	Colonial			Mainland	Ramos-Esplá 1988
Aplidium elegans^{1}	Colonial			Mainland	Ramos-Esplá 1988
Aplidium enigmaticum	Colonial	Azores	Madeira	Mainland	Ramos-Esplá 1988
Aplidium nordmanni^{2}	Colonial			Mainland	Ramos-Esplá 1988
Aplidium pallidum	Colonial			Mainland	Ramos-Esplá 1988
Aplidium proliferum	Colonial			Mainland	Ramos-Esplá 1988
Aplidium punctum	Colonial			Mainland	Ramos-Esplá 1988
Aplidium sagresensis	Colonial			Mainland	Ramos-Esplá et al. 1993
Aplidium sp.	Colonial			Mainland	Peck et al. 2015
Araneum sigma	Solitary	Azores		Mainland	Ramos-Esplá 1988
Asajirus indicus^{1}	Solitary	Azores		Mainland	Ramos-Esplá 1988
Ascidia conchilega	Solitary			Mainland	Ramos et al. 2015
Ascidia fistulosa	Solitary	Azores			Borges et al. 2010
Ascidia interrupta	Solitary	Azores			Monniot & Monniot 1994
Ascidia mentula	Solitary		Madeira		Wirtz 1995
Ascidia molguloides	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988; Borges et al. 2010
Ascidia muricata	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988; Borges et al. 2010
Ascidia tritonis	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988
Ascidiella aspersa	Solitary		Madeira		Ramos-Esplá 1988
Bathypyura celata^{2}	Solitary	Azores	Madeira	Mainland	Ramos-Esplá 1988
Bathystyeloides dubius	Solitary		Madeira	Mainland	Ramos-Esplá 1988
Bathystyeloides enderbyanus	Solitary	Azores	Madeira	Mainland	Ramos-Esplá 1988
Bolteniopsis prenanti	Solitary	Azores			Monniot 1974
Bolteniopsis sessilis	Solitary	Azores			Ramos-Esplá 1988
Botrylles	Colonial	Azores			Monniot 1974

Botrylloides leachii[2]	Colonial	Azores[a]	Madeira[b]		Wirtz 1995[b]; Borges et al. 2010[a]
Botrylloides violaceus	Colonial			Mainland	Nagar et al. 2010
Botryllus schlosseri	Colonial	Azores[a]	Madeira[b]	Mainland[c]	Monniot 1974[a]; Saldanha et al. 1974[b,c]; Ramos-Esplá 1988[c]; Ben-Shlomo et al. 2006[c]; Cardigos et al. 2006[a]; Borges et al. 2010[a]; Canning-Clode et al. 2013[b]; Canning-Clode et al. 2008[b]; Azevedo et al. 2020[c]
Ciona intestinalis	Solitary			Mainland	Azevedo et al. 2020
Ciona sp.	Solitary			Mainland	Ramos et al. 2015
Clavelina dellavallei	Colonial		Madeira		Wirtz 1995; Wirtz 1998
Clavelina lepadiformis	Colonial	Azores[a]	Madeira[b]		Monniot 1974[a]; Ramos-Esplá 1988[a]; Wirtz 1995[b]; Wirtz 1998[b]; Cardigos et al. 2006[a]; Borges et al. 2010[a]; Canning-Clode et al. 2013[b]
Clavelina oblonga	Colonial	Azores			Monniot 1974; Ramos-Esplá 1988; Monniot & Monniot 1994; Cardigos et al. 2006; Borges et al. 2010
Cnemidocarpa bathyphila[2]	Solitary	Azores	Madeira		Ramos-Esplá 1988
Cnemidocarpa bythia[2]	Solitary	Azores	Madeira	Mainland	Ramos-Esplá 1988
Cnemidocarpa devia[2]	Solitary			Mainland	Ramos-Esplá 1988
Cnemidocarpa digonas[2]	Solitary		Madeira	Mainland	Ramos-Esplá 1988
Cnemidocarpa platybranchia[2]	Solitary		Madeira	Mainland	Ramos-Esplá 1988
Corella eumyota	Solitary			Mainland	Nagar et al. 2010; Sofia Ruiz 2015
Corynascidia translucida[1]	Solitary			Mainland	Ramos-Esplá 1988
Cratostigma singularis	Solitary	Azores			Ramos-Esplá 1988
Culeolus suhmi	Solitary		Madeira	Mainland	Ramos-Esplá 1988
Cystodytes dellechiajei	Colonial	Azores[a]	Madeira[b]		Ramos-Esplá 1988[a]; Monniot & Monniot 1994[a]; Wirtz 1995[b]; Cardigos et al. 2006[a]; Borges et al. 2010[a]
Cystodytes guinensis	Colonial	Azores			Monniot & Monniot 1994
Cystodytes planus	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Dendrodoa grossularia	Solitary			Mainland	Ramos-Esplá 1988; Azevedo et al. 2020
Diazona violacea	Colonial			Mainland	Ramos-Esplá 1988; Ramos et al. 2015
Dicarpa pacifica	Solitary			Mainland	Ramos-Esplá 1988
Dicarpa simplex	Solitary		Madeira	Mainland	Ramos-Esplá 1988
Didemnum candidum	Colonial	Azores			Borges et al. 2010
Didemnum coriaceum	Colonial			Mainland	Ramos-Esplá 1988
Didemnum lahillei[2]	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Didemnum maculosum	Colonial	Azores[a]	Madeira[b]	Mainland[c]	Ramos-Esplá 1988[a, b, c]; Wirtz 1995[b];
Didemnum vexillum	Colonial			Mainland	Azevedo et al. 2020
Diplosoma listerianum	Colonial	Azores[a]	Madeira[b]	Mainland[c]	Ramos-Esplá 1988[c]; Wirtz 1995[b]; Borges et al. 2010[a]
Diplosoma sp.	Colonial		Madeira		Canning-Clode et al. 2008
Distaplia corolla	Colonial	Azores[a]	Madeira[b]		Monniot 1974[a]; Ramos-Esplá 1988[a]; Wirtz 1995[b]; Cardigos et al. 2006[a]; Wirzt 2006[b]; Borges et al. 2010[a]; Canning-Clode et al. 2013[b];
Distaplia magnilarva	Colonial	Azores			Borges et al. 2010
Distaplia rosea	Colonial	Azores		Mainland	Ramos-Esplá 1988
Distomus hupferi	Colonial	Azores			Monniot 1974; Ramos-Esplá 1988; Borges et al. 2010
Distomus variolosus[2]	Colonial			Mainland	Ramos-Esplá 1988
Ecteinascidia herdmani[2]	Colonial	Azores			Monniot 1974; Ramos-Esplá 1988; Borges et al. 2010
Eudistoma angolanum	Colonial	Azores[a]	Madeira[b]		Ramos-Esplá 1988[a]; Wirtz 1994[b]; Wirtz 1995[b]; Cardigos et al. 2006[a]; Borges et al. 2010[a]
Eudistoma clarum	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Eugyrosis borealis[3]			Madeira		Ramos-Esplá 1988
Halocynthia papillosa[2]	Solitary			Mainland	Ramos-Esplá 1988
Hemistyela pilosa[1]	Solitary		Madeira	Mainland	Ramos-Esplá 1988

Heterostigma separ	Solitary			Mainland	Ramos-Esplá 1988
Leptoclinides faeroensis	Colonial	Azores			Borges et al. 2010
Lissoclinum fragile	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Lissoclinum perforatum	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Lissoclinum rubrum	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Microcosmus exasperatus	Solitary	Azores			Ramos-Esplá 1988; Borges et al. 2010
Microcosmus hernius	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988
Microcosmus nudistigma	Solitary			Mainland	Ramos-Esplá 1988
Microcosmus polymorphus	Solitary			Mainland	Ramos-Esplá 1988
Microcosmus sabatieri	Solitary			Mainland	Ramos-Esplá 1988
Microcosmus squamiger	Solitary	Azores[a]	Madeira[b]	Mainland[c]	Turon et al. 2007[c]; Borges et al. 2010[a]; Canning-Clode et al. 2013[b]; Sofia Ruiz 2015[c]
Minipera papillosa[2]	Solitary		Madeira		Ramos-Esplá 1988
Minipera pedunculata	Solitary			Mainland	Ramos-Esplá 1988
Molgula azorensis[1]	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988
Molgula complanata	Solitary	Azores			Borges et al. 2010
Molgula manhattensis	Solitary			Mainland	Ramos-Esplá 1988
Molgula occidentalis	Solitary			Mainland	Sofia Ruiz 2015
Molgula plana	Solitary	Azores			Monniot 1971; Monniot 1974; Ramos-Esplá 1988; Cardigos et al. 2006; Borges et al. 2010
Molgula sp.	Solitary			Mainland	Peck et al. 2015
Molguloides crenatum	Solitary			Mainland	Ramos-Esplá 1988
Morchellium appendiculatum[1]	Colonial	Azores			Ramos-Esplá 1988
Octacnemus ingolfi[2]	Solitary	Azoresa		Mainland[c]	Ramos-Esplá 1988[a, c]; Borges et al. 2010[a]
Octacnemus zarcoi	Solitary		Madeira		Ramos-Esplá 1988
Oligotrema lyra[1]	Solitary	Azores	Madeira	Mainland	Ramos-Esplá 1988
Oligotrema unigonas[1]	Solitary		Madeira	Mainland	Ramos-Esplá 1988
Pareugyrioides chardyi[2]	Solitary		Madeira		Ramos-Esplá 1988
Perophora viridis	Colonial	Azores			Monniot 1974; Ramos-Esplá 1988; Borges et al. 2010
Phallusia mammillata	Solitary			Mainland	Wirtz 1994[b]; Wirtz 1995[b]; Oliveira et al. 2009[c]
Polycarpa comata	Solitary			Mainland	Ramos-Esplá 1988
Polycarpa errans	Solitary			Mainland	Ramos-Esplá 1988
Polycarpa fibrosa	Solitary			Mainland	Ramos-Esplá 1988
Polycarpa gracilis	Solitary			Mainland	Ramos-Esplá 1988
Polycarpa pomaria	Solitary	Azores			Monniot 1974
Polycarpa pseudoalbatrossi	Solitary		Madeira	Mainland	Ramos-Esplá 1988
Polycarpa pusilla	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988
Polycarpa scuba	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988; Borges et al. 2010
Polycarpa tenera	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988; Borges et al. 2010
Polycarpa violacea	Solitary			Mainland	Ramos-Esplá 1988
Polyclinum aurantium	Colonial	Azores			Monniot 1974; Ramos-Esplá 1988; Cardigos et al. 2006; Borges et al. 2010
Polysyncraton asterix	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Polysyncraton lacazei	Colonial			Mainland	Ramos-Esplá 1988
Proagnesia depressa	Solitary	Azores	Madeira	Mainland	Ramos-Esplá 1988
Protoholozoa pigra	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Protomolgula bythia	Solitary			Mainland	Ramos-Esplá 1988
Pseudodiazona abyssa	Colonial		Madeira	Mainland	Ramos-Esplá 1988
Pycnoclavella taureanensis	Colonial		Madeira		Wirtz 1995; Wirtz 1998
Pyura tessellata	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988; Cardigos et al. 2006; Borges et al. 2010
Ritterella glareosa	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Situla lanosa	Solitary	Azores		Mainland	Ramos-Esplá 1988
Styela canopus	Solitary		Madeira[b]	Mainland[c]	Ramos-Esplá 1988[c]; Canning-Clode et al. 2013[b]; Sofia Ruiz 2015[c]
Styela chaini	Solitary		Madeira		Ramos-Esplá 1988

Styela charcoti	Solitary		Madeira	Mainland	Ramos-Esplá 1988
Styela clava	Solitary		Madeira[b]	Mainland	Ramos-Esplá 1988[b, c]; Davis & Davis 2005[c]; Nagar et al. 2010[c]
Styela crinita	Solitary	Azores	Madeira	Mainland	Ramos-Esplá 1988
Styela loculosa	Solitary	Azores	Madeira	Mainland	Ramos-Esplá 1988
Styela plicata	Solitary			Mainland	Borges et al. 2010[a]; Nagar et al. 2010[c]; Chaninho et al. 2015[a]; Sofia Ruiz 2015[c]
Styela rustica[1]	Solitary			Mainland	Ramos-Esplá 1988
Styela sigma[1]	Solitary	Azores			Monniot 1974; Ramos-Esplá 1988
Styela similis	Solitary	Azores	Madeira	Mainland	Ramos-Esplá 1988
Sycozoa melopepona	Colonial	Azores			Ramos-Esplá 1988; Borges et al. 2010
Synoicum duboscqui	Colonial			Mainland	Ramos-Esplá 1988
Synoicum pulmonaria	Colonial			Mainland	Ramos-Esplá 1988
Tantillulum molle	Solitary		Madeira		Ramos-Esplá 1988
Tylobranchion nordgaardi	Colonial	Azores			Monniot 1974; Ramos-Esplá 1988

Table SI 5.1: Summary of the environmental parameters measured at the time of sampling in locations with *versus* without the influence of organic-rich waters from fish farming activities (+Org or -Org, respectively). Values are expressed as a percentage and are averages of three replicates (n=6) ± SD.

Oxygen (mg/L)		**Salinity**		**Temperature (ºC)**		**pH**	
+Org	**-Org**	**+Org**	**-Org**	**+Org**	**-Org**	**+Org**	**-Org**
10.1	15.9	33.9	25.7	16.7	16.4	8.10	9.14
9.9	12.2	33.8	25.5	16.5	17.1	8.16	9.06
8.3	13.2	33.2	33.5	15.4	17.0	8.20	8.95
8.3	9.4	33.8	25.6	15.4	16.9	8.22	9.07
13.6	13.0	33.2	33.5	16.7	17.2	8.58	9.29
12.7	12.7	33.9	25.8	16.6	17.0	8.61	9.10
Average ± SD		Average ± SD		Average ± SD		Average ± SD	
10.5 ± 2.2	12.7 ± 2.1	33.6 ± 0.3	28.3 ± 4.1	16.2 ± 0.6	16.9 ± 0.3	8.3 ± 0.2	9.1 ± 0.1

Table SI 5.2: Nutritional composition of the formulated fish feed provided to the fish at the fish farming location (+Org).

SORGAL, SOCIEDADE DE ÓLEOS E RAÇÕES, SA

Standard orange 4

Seabass; Gilthead Seabream; Complete Animal Feed; From 50g to 150g

Analytical Constituents

Crude Protein 43.0%; Crude Fat 17.0%; Crude Ash 10.0%; Crude Fiber 3.0%; Calcium 1.9%; Phosphorus 1.4%; Sodium 0.4%

Additives (per kg of feed)

Vitamins, pro-vitamins, and chemically well-defined substances have a similar effect:

3a672a - Vitamin A 5.000 UL; E671 - Vitamin D3 1.000 UL; 3a700 – All rac alfa-tocoferyl acetate 200 UL; 3a312 – Vitamin C (Ascorbil Monophospahte) 100 mg;

Compounds of trace elements

E1 – Fe - ferrous chelate of Glycine 2.0 mg; **3b202 – I –** Calcium iodate anhydrous 1.1 mg; **E4 – Cu** – Cupric chelate of amino acids, hydrate 12.0 mg; **E5 – Mn** – Manganese and amino acids chelate, hydrate 32.0 mg; **3b606 – Zn** – Zinc and amino acids chelate, hydrate 48.0 mg; **3b815 – Se** – L-Selenomethionine 0.1 mg

Amino acids, their salts, and analogues

3.2.2 - L – Lysine monohydrochloride 2.5 g; **3a301** – DL Methionine 3.0 g

Antioxidants

E310 – 0.39 mg; **E320 + E321** – 100 mg

Anticaking agent

E562 – 395 mg; **1m558** – 382 mg

Ingredients

10.4.2 Fish meal; **1.11.1** Wheat; **9.4.1** Processed animal protein (non-ruminants)

9.11.1 Feather meal; **2.18.4** Soy (bean) meal, dehulled; **10.4.6** Fish Oil; **2.19.4** Sunflower seed meal, dehulled; **3.7.4** Horse bean, dehulled; **9.7.1** Non-ruminant blood meal; **9.2.1** Poultry fat; **10.4.4** Fish protein, hydrolyzed; **1.2.8** Corn gluten meal; **12.1.4** Bacterial protein from *Corynebacterium glutamicum*; **11.3.3** Monocalcium phosphate; **12.1.5** Brewer's yeast; **12.2** Other fermentation by-products

Contains fish meal, non-ruminant blood products, and processed animal products – not to be used with ruminants. Not to be used with farming animals, except aquaculture feeds and fur animals.

Store cool and dry, away from direct sunlight

Table SI 5.3: Fatty acid profile of ascidians (Ascidiacea) and seaweeds (sea lettuce, *Ulva* spp. and bladderwrack, *Fucus* sp.) sampled in locations with *versus* without the influence of organic-rich effluents from fish farming activities (+Org or -Org, respectively), as well as the formulated fish feed (FF) most commonly supplied in fish farming activities in the study location. Values are expressed as a percentage of the total pool of fatty acids and are averages of five replicates (n=5) ± SD. BCFA: Branched fatty acids, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. n.d: not detected.

	Ascidiacea		***Ulva* spp.**		***Fucus* sp.**		**Fish Feed**
	+Org	-Org	+Org	-Org	+Org	-Org	
iso 14:0	0.16 ± 0.02	0.09 ± 0.02	n.d	n.d	n.d	n.d	n.d
14:0	0.94 ± 0.17	1.45 ± 0.12	0.68 ± 0.22	0.64 ± 0.17	8.04 ± 0.64	8.47 ± 0.27	1.53 ± 0.35
iso 15:0	1.44 ± 0.20	2.14 ± 0.15	n.d	n.d	n.d	n.d	n.d
anteiso 15:0	0.74 ± 0.13	0.27 ± 0.03	n.d	n.d	n.d	n.d	n.d
15:0	1.07 ± 0.18	0.61 ± 0.06	n.d	n.d	n.d	n.d	n.d
15:1	0.28 ± 0.06	0.34 ± 0.04	n.d	n.d	n.d	n.d	n.d
iso 16:0	0.59 ± 0.07	0.84 ± 0.04	n.d	n.d	n.d	n.d	n.d
anteiso 16:0	0.12 ± 0.03	0.05 ± 0.01	n.d	n.d	n.d	n.d	n.d
16:0	11.50 ± 1.31	12.56 ± 0.67	37.74 ± 1.14	38.05 ± 1.86	16.17 ± 1.29	15.03 ± 0.62	17.25 ± 0.68
16:1	0.36 ± 0.09	0.26 ± 0.05	n.d	n.d	n.d	n.d	n.d
16:1*n*-7	0.22 ± 0.03	0.38 ± 0.03	2.32 ± 0.43	2.21 ± 0.40	1.03 ± 0.04	1.46 ± 0.09	0.34 ± 0.04
16:1*n*-9	5.78 ± 0.62	5.37 ± 0.29	3.33 ± 0.27	2.67 ± 0.27	0.25 ± 0.04	0.29 ± 0.03	3.62 ± 0.18
16:2	n.d	n.d	0.47 ± 0.13	0.13 ± 0.01	n.d	n.d	n.d
16:2*n*-6	0.13 ± 0.03	0.16 ± 0.03	n.d	n.d	0.05 ± 0.01	0.30 ± 0.08	0.10 ± 0.02
16:3*n*-3	n.d	n.d	n.d	n.d	n.d	n.d	n.d
16:4*n*-3	n.d	n.d	5.18 ± 0.33	4.27 ± 0.67	0.59 ± 0.06	0.59 ± 0.05	n.d
iso 17:0	0.69 ± 0.08	1.51 ± 0.11	n.d	n.d	n.d	n.d	n.d
anteiso 17:0	0.45 ± 0.05	0.48 ± 0.02	n.d	n.d	n.d	n.d	n.d
17:0	0.64 ± 0.06	1.23 ± 0.12	n.d	n.d	n.d	n.d	n.d
17:1	0.26 ± 0.06	0.11 ± 0.01	n.d	n.d	n.d	n.d	n.d
17:1*n*-9	0.56 ± 0.10	0.28 ± 0.02	n.d	n.d	n.d	n.d	n.d
methyl-heptadecanoate	0.40 ± 0.09	0.10 ± 0.01	n.d	n.d	n.d	n.d	n.d
18:0	4.87 ± 1.23	5.89 ± 0.53	6.58 ± 3.99	8.70 ± 2.29	4.34 ± 1.61	1.77 ± 0.16	6.51 ± 1.09
18:1*n*-7+*n*-9	20.27 ± 1.80	11.98 ± 0.95	15.23 ± 1.21	15.19 ± 1.22	26.50 ± 2.28	21.34 ± 1.51	35.97 ± 0.43
18:2	1.19 ± 0.09	0.62 ± 0.09	n.d	n.d	n.d	n.d	n.d
18:2*n*-6	5.85 ± 1.62	2.26 ± 0.08	4.41 ± 0.19	2.74 ± 0.41	6.82 ± 0.38	7.45 ± 0.21	16.86 ± 0.19
18:3*n*-6	0.07 ± 0.02	0.29 ± 0.03	0.59 ± 0.07	0.71 ± 0.05	0.48 ± 0.03	0.46 ± 0.02	0.18 ± 0.02
18:3*n*-3	2.16 ± 0.22	2.38 ± 0.48	8.95 ± 0.70	7.85 ± 0.57	6.96 ± 0.41	8.87 ± 0.51	2.85 ± 0.07
18:4*n*-3	1.54 ± 0.61	3.61 ± 0.69	9.72 ± 0.65	10.1 ± 0.72	3.70 ± 0.36	5.55 ± 0.62	0.62 ± 0.05
20:0	0.41 ± 0.04	0.53 ± 0.04	n.d	n.d	0.36 ± 0.03	0.31 ± 0.04	0.27 ± 0.03

20:1	0.37 ± 0.17	0.16 ± 0.04	n.d	n.d	n.d	n.d	n.d
20:1*n*-9	4.90 ± 0.57	1.06 ± 0.08	n.d	n.d	0.31 ± 0.04	0.26 ± 0.03	2.38 ± 0.06
20:1*n*-7	n.d	n.d	n.d	n.d	n.d	n.d	0.22 ± 0.02
20:2	0.20 ± 0.04	0.16 ± 0.01	n.d	n.d	n.d	n.d	n.d
20:2*n*-6	n.d	n.d	n.d	n.d	0.33 ± 0.01	0.31 ± 0.06	0.49 ± 0.03
20:3*n*-6	0.23 ± 0.04	0.35 ± 0.04	n.d	n.d	0.66 ± 0.04	0.64 ± 0.09	n.d
20:3*n*-3	n.d	n.d	n.d	n.d	n.d	n.d	0.18 ± 0.03
20:4*n*-6	2.43 ± 0.37	3.11 ± 0.27	n.d	n.d	14.08 ± 1.17	15.03 ± 0.22	0.47 ± 0.03
20:4*n*-3	0.74 ± 0.06	0.76 ± 0.17	0.51 ± 0.07	0.50 ± 0.03	0.23 ± 0.02	0.28 ± 0.05	0.36 ± 0.03
20:5*n*-3	17.77 ± 2.90	20.44 ± 1.00	0.61 ± 0.13	1.25 ± 1.14	7.66 ± 0.74	9.95 ± 0.39	2.13 ± 0.11
21:0	0.09 ± 0.01	0.12 ± 0.02	n.d	n.d	n.d	n.d	0.05 ± 0.02
22:0	n.d	n.d	1.31 ± 0.21	1.39 ± 0.26	0.12 ± 0.02	0.12 ± 0.01	0.11 ± 0.02
22:1*n*-11	n.d	n.d	n.d	n.d	n.d	n.d	1.94 ± 0.20
22:1*n*-9	n.d	n.d	n.d	n.d	0.37 ± 0.03	0.35 ± 0.05	0.29 ± 0.05
22:4	0.24 ± 0.04	0.41 ± 0.05	n.d	n.d	n.d	n.d	n.d
22:4	0.13 ± 0.02	0.29 ± 0.04	n.d	n.d	n.d	n.d	n.d
22:5*n*-6	0.31 ± 0.05	0.76 ± 0.12	n.d	n.d	n.d	n.d	n.d
22:5*n*-3	1.07 ± 0.10	1.03 ± 0.07	2.38 ± 0.31	3.64 ± 0.47	n.d	n.d	0.69 ± 0.06
unknown	n.d	0.58 ± 0.23	n.d	n.d	n.d	n.d	n.d
22:6*n*-3	8.75 ± 1.00	11.85 ± 1.01	n.d	n.d	n.d	n.d	4.59 ± 0.32
24:0	n.d	n.d	n.d	n.d	0.33 ± 0.05	0.31 ± 0.03	n.d
24:1*n*-9	n.d	n.d	n.d	n.d	0.62 ± 0.05	0.72 ± 0.07	n.d
∑*n*-3	32.03 ± 3.62	40.07 ± 1.54	27.35 ± 1.87	27.61 ± 2.30	19.16 ± 1.54	25.24 ± 1.42	11.43 ± 0.51
∑*n*-6	9.02 ± 1.25	6.94 ± 0.46	5.00 ± 0.24	3.45 ± 0.44	22.42 ± 1.59	24.18 ± 0.07	18.09 ± 0.23
∑*n*-3/∑*n*-6	3.66 ± 0.98	5.79 ± 0.37	5.46 ± 0.25	8.04 ± 0.36	0.85 ± 0.03	1.04 ± 0.06	0.63 ± 0.03
∑BCFA	4.59 ± 0.53	5.48 ± 0.33	n.d	n.d	n.d	n.d	n.d
∑SFA	19.52 ± 2.36	22.39 ±1.00	46.30 ± 3.35	48.78 ± 3.37	29.35 ± 3.48	26.02 ± 0.50	25.72 ± 1.42
∑MUFA	32.99 ± 0.92	19.95 ± 1.39	20.88 ± 1.62	20.07 ± 1.66	29.07 ± 2.32	24.42 ± 1.48	44.77 ± 0.81
∑PUFA	42.81 ± 2.65	48.48 ± 1.80	32.82 ± 1.94	31.19 ± 2.73	41.58 ± 3.08	49.43 ± 1.42	29.52 ± 0.64