Universidade de Aveiro Departamento de Biologia
2020

Marco Freire Custódio

Integração da produção de halófitas para a eco-intensificação da aquacultura costeira

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciências, Tecnologia e Gestão do Mar, 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 Carlos Sebastian Villasante Larramendi, Professor Adjunto do Departamento de Economia Aplicada da Universidade de Santiago de Compostela e do Doutor Ricardo Jorge Guerra Calado, Investigador Principal do Departamento de Biologia da Universidade de Aveiro e CESAM - Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro

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palavras-chave Aquacultura, aquacultura multi-trófica integrada, hidroponia, halófitas, *Halimione portulacoides*, fitoremediação, nutrientes, lípidos, serviços ecossistémicos, soluções baseadas na natureza, inovação alimentar, sustentabilidade

resumo

As atividades aquícolas são uma parte integrante dos ecossistemas onde ocorrem, tendo um impacto inevitável no funcionamento dos mesmos. Certos modos de produção podem ter um impacto positivo acrescido na capacidade dos ecossistemas em providenciar serviços de regulação e manutenção, para além dos serviços mais óbvios de aprovisionamento de biomassa vegetal e animal. É o caso da Aquacultura Multi-Trófica Integrada (IMTA, do inglês *Integrated Multi-trophic Aquaculture*), uma estrutura de produção aquícola mais sustentável que pode ser definida como a produção otimizada de organismos aquáticos de dois ou mais grupos funcionais (com funções ecossistémicas complementares), ligados troficamente através de fluxos de energia e de nutrientes. Um desses grupos funcionais, que produzem serviços chave de fitoremediação, são as plantas halófitas. Capazes de suportar salinidades elevadas, as halófitas podem ser facilmente integradas em sistemas de IMTA em águas salinas como espécies extrativas com valor económico, podendo ser usada para a nutrição humana. A presente tese tem como objetivo principal testar a eficiência da halófita *Halimione portulacoides* (L.) Aellen na remoção de nutrientes (DIN e DIP, respetivamente do inglês *dissolved inorganic nitrogen* e *dissolved inorganic phosphorus*) presentes em soluções hidropónicas que simulam condições reais de efluentes de aquacultura, para avaliar a sua aptidão como espécie extrativa para a IMTA costeira. A produtividade e o potencial de valorização da planta são também demonstrados. O primeiro passo foi perceber o estado-da-arte relativamente ao uso de halófitas na remediação de efluentes aquícolas através de uma revisão sistemática da literatura. De seguida foram executados dois ensaios de crescimento em sistema hidropónico para perceber a capacidade extrativa e a produtividade do *H. portulacoides*. O primeiro ensaio consistiu num estudo exploratório da resposta da planta sob diferentes concentrações de DIN e DIP representativas de efluentes de aquacultura semi-intensiva, intensiva e super-intensiva. O segundo ensaio foi desenhado de forma a providenciar dados adicionais sobre a influência da densidade de plantação e da iluminação no crescimento e na eficiência de remediação da planta. A partir da biomassa produzida foram analisados e caracterizados o perfil nutricional e o lipidoma da biomassa edível. Adicionalmente, foi realizado um inquérito estruturado a uma amostra de consumidores portugueses para avaliar as suas preferências e disponibilidade-a-pagar por halófitas embaladas e prontas a consumir e determinar potencias segmentos de consumidores destes novos produtos. Por fim, uma segunda revisão da literatura é apresentada onde se discute a utilidade de avaliar e valorar os serviços dos ecossistemas no contexto da aquacultura de modo a capturar o valor multidimensional de certos tipos de produção e promover práticas sustentáveis como o IMTA. Os resultados obtidos demonstram que as halófitas são plantas ainda subvalorizadas com imenso potencial no contexto da indústria alimentar no general, e da aquacultura em particular. No caso específico do *H. portulacoides*, as condições para a sua produção em hidroponia foram exploradas e, em condições nutricionais não-limitantes, as unidades hidropónicas apresentaram uma produtividade de 54 – 73 g m^{-2} day^{-1}, e eficiências de extração até 70% do DIN e 50% do DIP. A densidade de plantação pode ser ajustada de modo a aumentar a produtividade e capacidade extrativa das unidades hidropónicas. Além disso, o perfil nutricional das folhas é análogo ao de outras halófitas comestíveis e vegetais verdes e apresentou um perfil mineral baixo em sódio, apresentando-se como um pontencial substituto do sal. A análise do lipidoma polar permitiu identificar 175 espécies presentes no extrato lipídico das folhas. O questionário aos consumidores demonstrou que a disponibilidade média a pagar por uma embalagem de 50 g de *Salicórnia* pronta-a-consumir é de 2,10 €. O género feminino e o 'consumidor aventureiro' (baseado no instrumento de segmentação *Food Related-Lifestyle*) são dois segmentos de consumidores chave para os vegetais salgados. No geral, o *H. portulacoides* apresenta um bom desempenho de crescimento e extração de DIN e DIP em condições hidropónicas salinas (salinidade 20) e, portanto, é considerada uma espécie extrativa adequada para a IMTA costeira e apresenta elevado potencial para valoração económica. O conhecimento científico obtido fornece um ponto de partida sólido para o cultivo e ampliação da produção hidropónica de *H. portulacoides*.

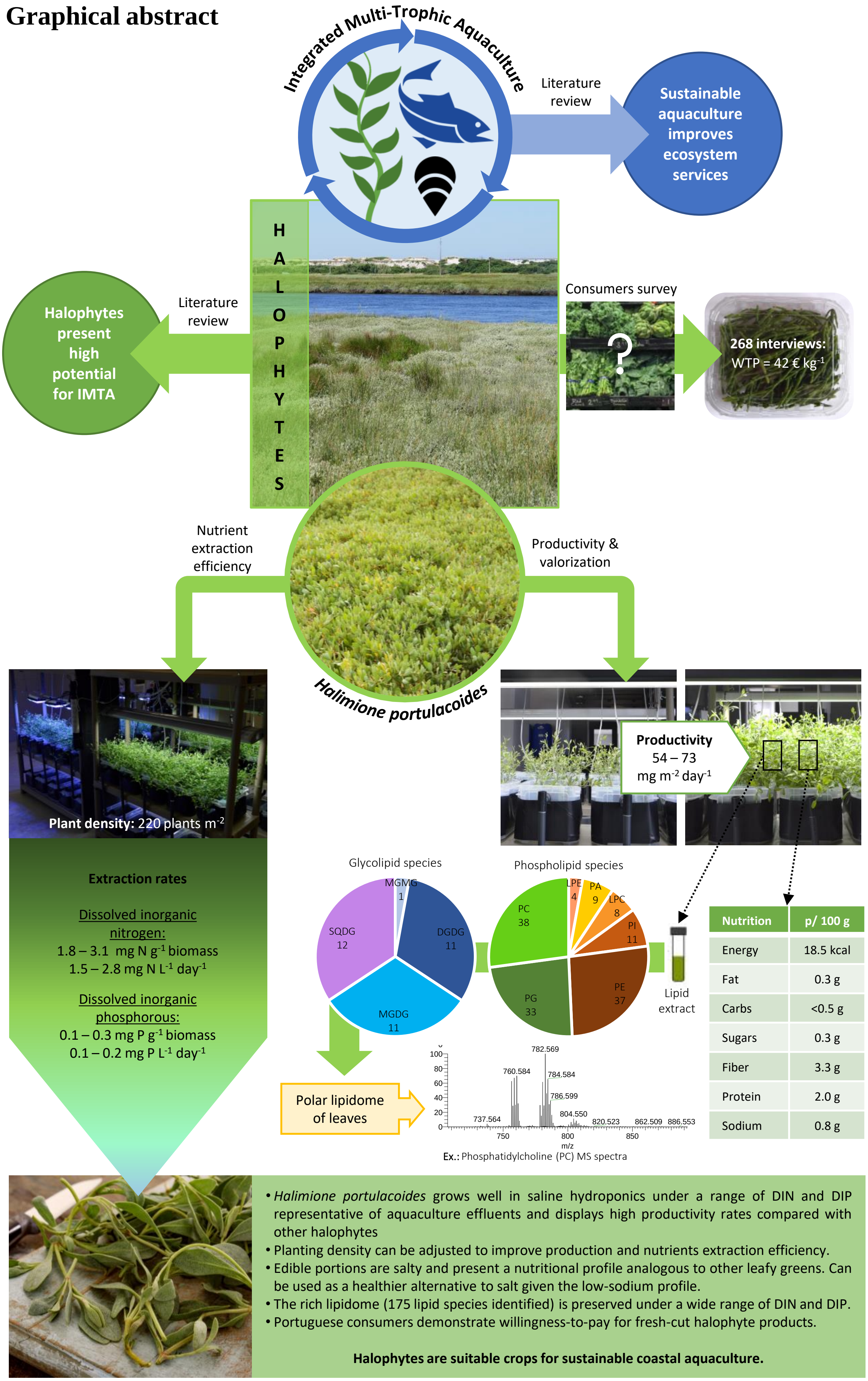
keywords

Aquaculture, integrated multi-trophic aquaculture, hydroponics, halophytes, *Halimione portulacoides*, phytoremediation, nutrients, lipids, ecosystem services, nature-based solutions, food innovation, sustainability

abstract

Aquaculture activities are connected to some degree with the ecosystems on which they occur and from which they depend to operate, having an inevitable impact on their functioning. Certain modes of aquaculture production can have a positive impact on the capacity of ecosystems to deliver regulation and maintenance ecosystem services, besides the obvious provisioning services of biomass from aquatic plants and animals. This is certainly the case of Integrated Multi-Trophic Aquaculture (IMTA), a sustainable aquatic production framework, which can be defined as the enhanced production of aquatic organisms of two or more functional groups (with complementary ecosystem functions), that are trophically connected by demonstrated nutrient flows. One of these functional groups, with key ecosystem services of phytoremediation for IMTA, is halophyte plants. Capable of withstanding high salinities, halophytes can be easily integrated into saltwater based IMTA as extractive species and be developed into new valuable and nutritious crops with multiple uses in different industries. The main objective of this thesis was to test the capacity of the halophyte *Halimione portulacoides* to extract dissolved inorganic nitrogen (DIN) and phosphorous (DIP) from saline hydroponic solutions that mimic the conditions of real aquaculture effluents, to evaluate its suitability as an extractive species for coastal IMTA. The productivity and the valorization potential of the plant were also evaluated. The first step was to understand the state-of-the-art regarding the use of halophytes to remediate aquaculture effluents through a systematic review of the literature. Afterward, two hydroponic grow-out studies were designed and performed to understand the extractive capacity and production of *H. portulacoides*. The first trial was an exploratory study on the performance of this plant under different levels of DIN and DIP concentrations, mimicking those of semi-intensive, intensive, and super-intensive aquaculture effluents. The second trial was designed to further understand the influence of hydroponic production variables (plant density and artificial illumination) in the performance of *H. portulacoides* under non-limited nutrient conditions. From the biomass produced, further analyses were performed to characterize the leaves' nutritional profile and lipidomic profile. Additionally, a structured survey was performed to a sample of Portuguese consumers to assess their preferences and willingness-to-pay for fresh-cut halophyte products and provide insight into potential consumer segments for these new products. At last, a second literature review was performed to understand the value of employing the ecosystem services framework to capture the multidimensional value of certain modes of aquaculture to foster more sustainable practices such as IMTA. The present work revealed that halophytes are undervalued crops with tremendous potential in the context of food production in general, and sustainable aquaculture in particular. In the specific case of *H. portulacoides*, the subject of this research, the conditions for its hydroponic production were explored. Under non-limited DIN and DIP conditions, hydroponic units displayed good productivity, varying between 54 – 73 g m^{-2} day^{-1}, and extraction efficiencies up to 70% DIN and 50% DIP. Moreover, plant density can be optimized to improve the productivity and extractive capacity of hydroponic units. The nutritional profile of leaves is comparable to that of other edible halophytes and leafy greens and presents a low-sodium profile. A lipidomic analysis identified 175 polar lipid species present in the lipidic extract of the leaves. A survey of Portuguese consumers showed that average willingness-to-pay for a 50 g fresh-cut *Salicornia* package is 2.10 €. Female consumers and the 'adventurous consumer' (based on the Food-Related Lifestyle scale) are two major consumer segments for salty vegetables. Overall, the results obtained indicate that *H. portulacoides* performs well under saline hydroponic conditions, with efficient DIN and DIP extraction and high biomass production and is, therefore, a suitable extractive species for coastal IMTA and other hydroponic applications, with a high potential for economic valorization. The scientific knowledge obtained provides a solid starting point for *H. portulacoides* hydroponic production and scale-up towards commercial production.

Graphical abstract



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Chapter 1

Introduction

1. Introduction

1.1. Sustainable development and marine aquaculture

Aquaculture is an increasingly important activity in terms of the services it provides to humans, including nutrition and economic development, and will become the main driver of change in the fishery production sector in the present decade (FAO, 2016). The industry is undergoing a consistent growth in production output since the 1980s and contributes, at present, to almost half (47%) of global fisheries production (FAO, 2018). Even though rates have slowed down from 9.5% in the 1990s to 5.8% between 2001-2016, models predict aquaculture will surpass capture fisheries in terms of total production during the next decade, reaching 2/3 of total fish production by 2030 (World Bank, 2013). In 2016, global aquaculture produced around 80 million tons of fish (including mollusks, crustaceans, and other animals) and 30 million tons of aquatic plants (FAO, 2018).

The rapid intensification of aquaculture is raising several concerns on its sustainability across the economic, ecological, and social landscapes, especially concerning its use of resources and potential impacts in contexts of climate change, biodiversity loss, economic disparity, and social inequality (Piketty & Saez, 2014; IPBES, 2019). The United Nations adopted the Sustainable Development Goals (SDGs) for 2030 (General Assembly resolution A/RES/70/1) to guide governments and decision-makers towards meaningful efforts to tackle pressing sustainability issues affecting the global society and the planet, and aquaculture must play its role in pushing the agenda and accomplishing those goals (FAO, 2018).

Aquaculture activities are, directly or indirectly, connected to aquatic ecosystems and are, therefore, relevant to the achievement of SDG 14 "life below water". In recent years, an Ecosystem-Approach to Aquaculture (EAA) has been conceptualized and mainstreamed for sustainable management of the industry that promotes healthy aquatic ecosystems (Brugère et al., 2019; FAO, 2010). Besides SDG 14, marine aquaculture is relevant to seven other SDGs, namely SDG 1 "no poverty (e.g. inclusive access to fisheries resources), SDG 2 "zero hunger" (e.g. food provision to humans), SDG 3 "good health and well-being (e.g. better nutrition and livelihoods), SDG 5 "gender equality" (e.g. women empowerment), SDG 8 "decent work and economic growth (e.g. economic opportunities), SDG 12 "responsible consumption and production (e.g. waste reduction), SDG 13 "climate action" (e.g. reduction of environmental impacts).

The demand for seafood worldwide will continue to grow in time, as recent projections estimate an increase in the world population from 7.7 billion people in 2019 to 9.7 billion by 2050 (United Nations, 2019). This need to produce more food for the human population is even more pressing, as hunger still afflicts 1 in every 9 people in the world today (FAO et al., 2019). The state of marine ecosystems is also a major global concern, with approximately 33% of fish stocks already overexploited (60% are maximally fished) and, across the globe, anthropogenic pressures continue to disrupt the structures and functions of marine and coastal ecosystems (Halpern et al., 2008; IOC/UNESCO et al., 2011).

As marine aquaculture intensifies its actions, it becomes an increasingly important driver of change with potential negative impacts in the ecosystem's capacity to deliver ecosystem services (ES) (see Chapter 7). For instance, the vast conversion of mangroves into shrimp farms in some countries of Southeast Asia is inflicting socioeconomic costs in local coastal communities due to a deterioration of ecosystems' capacity to deliver important ES, such as coastal protection, sediment retention, and habitats provision for a diversity of marine species, including those of halieutic value (Brander et al., 2012; Richards & Friess, 2016). Another example is the massive development in salmon farming, in countries like Norway and Chile, which is intensifying concerns regarding its impact in surrounding ecosystems, such as the dissemination of infections (e.g. virus, parasites), genetic contamination through escapees, deposition of organic matter below cages and eutrophication (Quiñones et al., 2019; Taranger et al., 2015).

A consensus points towards the production of aquafeeds as one of the major sources of environmental impact fostered by aquaculture, thus justifying why increasing feed-use efficiency has become a priority (Bohnes et al., 2019). Improvements in biomass production per unit of feed input can decrease feeds' environmental impacts which can be achieved through more sustainable sourcing of feed ingredients and enhanced conversion rates (e.g. higher digestibility and nutrients retention) by cultured species (Bohnes et al., 2019; Naylor et al., 2009). Additionally, impacts can be mitigated by recovering the fraction of nutrients inevitably lost from uneaten feeds and fish metabolic end products (e.g. feces and respiratory metabolites) through the co-cultivation of extractive organisms, an approach termed Integrated Multi-Trophic Aquaculture (IMTA) (Chopin et al., 2008).

Technological developments and the design of more sustainable production models, such as IMTA and Recirculating Aquaculture Systems (RAS) (Badiola et al., 2012; Falconer et al., 2019), as well as better site selection through integrated modeling approaches (Falconer et al., 2013; Stelzenmüller et al., 2017), aim at balancing out the negative impacts of aquaculture. Moreover, the introduction and promotion of low-trophic species in marine and coastal aquaculture value-chains can further contribute towards a more sustainable food and feed production (SAPEA, 2017; Sun et al., 2018).

1.2. Marine aquaculture effluents, a nutrient-rich resource

Nitrogen (N) and phosphorus (P) are two major nutrients present in formulated feeds given to fed aquaculture species (e.g., fish and shrimp) and a fraction of both is normally wasted as particulate and dissolved matter in the water. Previous estimations indicate N losses in fish-farms can represent up to 60 to 80% of its total input from feeds, and P losses can represent up to 70 to 85% of P inputs (Islam, 2005; Wang et al., 2012). Unfortunately, nutrient-rich marine aquaculture effluents, due to their relatively high levels of salinity, cannot be reutilized as fertilizer for the vast majority of crops, which are salt-sensitive (Machado & Serralheiro, 2017; Zörb et al., 2019).

A consequential environmental burden resulting from the unutilized nutrients present in saline aquaculture effluents is their building up downstream of production sites, which can promote aquatic eutrophication as well as changes in benthic chemistry and disturbance of ecological interactions in the surrounding ecosystems (Bannister et al., 2014; Sanz-Lázaro et al., 2011; Sarà et al., 2011; Troell et al., 2009; Valdemarsen et al., 2012). As previously referred, this loss of nutrients can also generate considerable negative externalities due to an additional demand for aquafeeds (Bohnes et al., 2019).

Besides being hindered by economic constraints imposed by the relatively inefficient use of aquafeeds, aquaculture farmers must also comply with regulations and best management practices regarding aquaculture effluents, further increasing the economic burden of nutrients loss. In the European Union, the Water Framework Directive (2000/60/EC) and the Marine Strategy Framework Directive (2008/56/EC) impose mechanisms to prevent water pollution, which include payment schemes for the load of N released in effluents, and reduce overall environmental impacts of maritime activities. Comprehensive guidance to facilitate the implementation of both directives in the context of the development of sustainable aquaculture has been laid out in the guidance document SWD (2016)178 final (European Commission, 2016). The obligation to comply with legal requirements and reduce the costs associated with nutrient discharges, along with economic and environmental benefits of recovering wasted nutrients, highlight the significance of developing cost-effective wastewater treatment for aquaculture.

Many different forms of wastewater treatment have been experimented with and implemented in the context of aquaculture, especially in intensive systems such as RAS (van Rijn, 2013). Solid matter is traditionally removed using gravitational and mechanical methods: heavy particles are separated from process water in settling tanks through gravity and fine/suspended particles can be removed using a diversity of mechanical methods such as rotating screen filters, expandable bead filters and foam fractionators (Cripps & Bergheim, 2000; Ebeling & Timmons, 2012). Concerning dissolved matter, inorganic N is of particular concern, as some of its chemical forms are toxic to fish and other cultured organisms (e.g. invertebrates) and must be dealt with efficiently (Ebeling & Timmons, 2012; Romano & Zeng, 2013). Bacterial biofilters are a common method that harnesses the nitrification capacity of certain bacteria. The first stage of this process is performed by ammonia-oxidizing bacteria (e.g. *Nitrosomonas*, *Nitrosococcus*, *Nitrospira*, *Nitrosolobus,* and *Nitrosovibrio*) which convert ammonia (NH_3 and NH_4^+) to nitrite (NO_2^-). The following stage is mediated by nitrite-oxidizing bacteria (e.g. *Nitrobacter*, *Nitrococcus*, *Nitrospira,* and *Nitrospina*), which convert NO_2^- to nitrate (NO_3^-), an N-form with low toxicity to fish (Rurangwa & Verdegem, 2015; Tomasso, 1994; Wongkiew et al., 2017). However, since nitrification processes do not remove N from the system, NO_3^- can easily build-up and values as high as 400-500 mg NO_3-N L^{-1} can occur in closed systems (van Rijn, 1996). Partial water exchanges can partially solve the problem but, due to regulations on nitrogen discharges imposed by EU directives, other cost-effective methods must be available to producers to remove dissolved N from the system. Phosphorous is also an important nutritional loss from aquafeeds and is mostly associated with solid excretions, which can be recovered by collecting sludge and processing it in digestors (Goddek et al., 2016a; Schneider et al., 2005). However, the dissolved inorganic fraction, which typically exists in the form of ionic orthophosphates ($H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}), also represents a significant waste that holds the potential to be reutilized (Yavuzcan Yildiz et al., 2017).

The present thesis focusses on the use of a nature-based solution to reduce the loss and accumulation of dissolved N and P in aquaculture process water and effluents, more specifically by harnessing the extractive capacity of plants in the context of IMTA production in marine/brackish waters. Conceptually, IMTA (Figure 1.1) is defined as the co-production of aquatic species in close proximity, where connectivity is seen in terms of ecosystemic functionalities, and systems can have diverse configurations and trophic levels of integration to extract from the culture water the different forms of nutrients generated, for instance, from feed losses and the metabolism of the different organisms (e.g. excretion, respiration) (Buck et al., 2018; Gunning et al., 2016). A more recent utilitarian definition of IMTA was proposed within the scope of the INTEGRATE Project, which states that IMTA is the "enhanced production of aquatic organisms (with or without terrestrial organisms) of two or more functional groups, that are trophically connected by demonstrated nutrient flows and whose biomass is fully or partially removed by harvesting to facilitate ecological balance" (Dunbar et al., 2020). Common low trophic groups being integrated into IMTA are i) filter feeders (e.g. bivalves, sponges, small crustaceans), which filtrate suspended particulate matter; ii) deposit feeders (e.g. sea cucumbers, polychaetes, sea urchins), which feed on deposited particulate matter; and iii) primary producers (e.g. plants, algae), which extract the dissolved matter (Chopin et al., 2012; Troell et al., 2009).

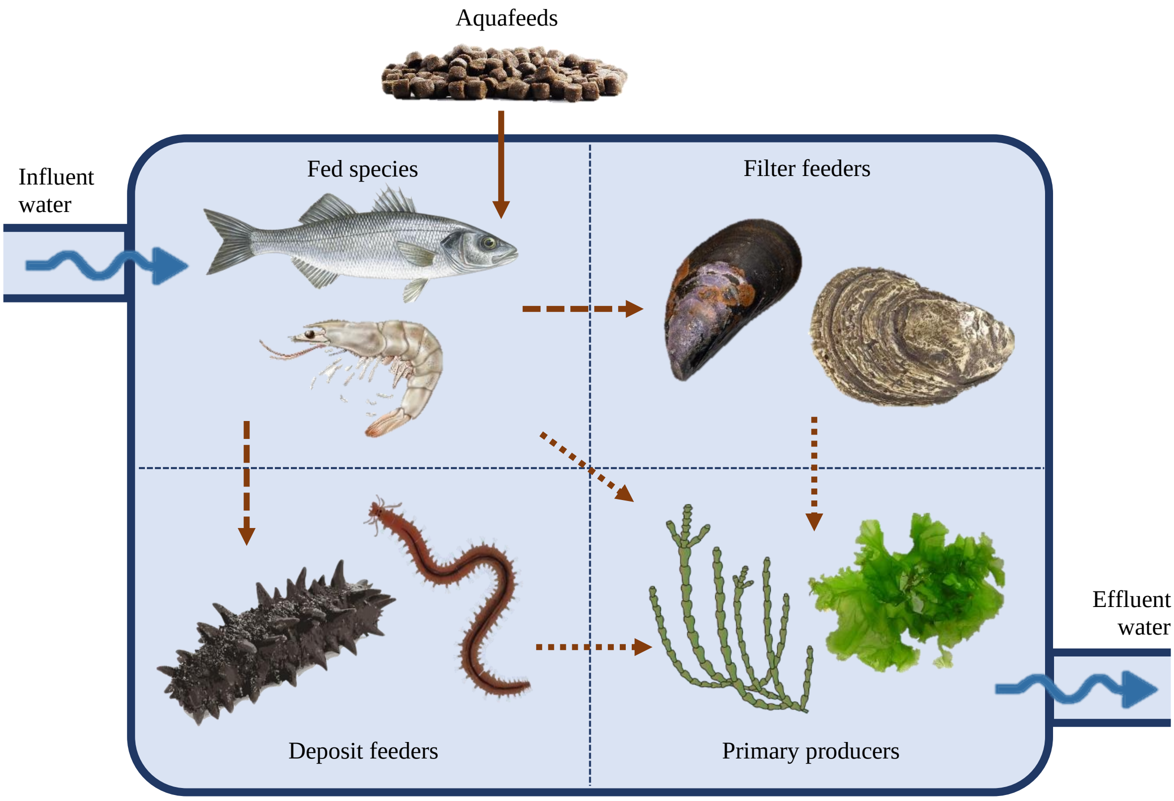

Figure 1.1: Conceptual representation of an IMTA system and nutrient flows

Yearly scientific publications addressing IMTA have been increasing at a steady rate over the last decade (Figure 1.2) and several extractive species have been studied, both in terms of their extraction capacity and growth performance. Some examples of such extractive species, in the context of saltwater-based marine and coastal IMTA, are: oysters (*Crassostrea* sp.) (Biswas et al., 2020; Ferreira et al., 2012; Omont et al., 2020), mussels (*Mytilus* sp.) (Cranford et al., 2013; Sanz-Lazaro and Sanchez-Jerez, 2017; Sterling et al., 2016), sea urchins (*Paracentrotus lividus*) (Israel et al., 2019; Shpigel et al., 2018), sea cucumbers (e.g. *Holothuria* sp., *Apostichopus* sp., *Cucumaria* sp.) (Israel et al., 2019; Yu et al., 2012; Zamora et al., 2018), bristle worms (*Hediste diversicolor*) (Carvalho et al., 2007; Marques et al., 2017), seaweeds/macroalgae (e.g. *Saccharina latissimia, Ulva lactuca, Gracilaria lemaneiformis*) (Duan et al., 2019; Holdt & Edwards, 2014; Marinho et al., 2015; Nardelli et al., 2019; Shpigel et al., 2019), amphipods (e.g. *Jassa* sp., *Monocorophium* sp., *Gammarus* sp.) (Fernandez-Gonzalez et al., 2018; Jiménez-Prada et al., 2018), microalgae (e.g. *Isochrysis* sp., *Tetraselmis* sp., *Phaeodactylum* sp.) (Andreotti et al., 2017; Li et al., 2019; Milhazes-Cunha & Otero, 2017) and halophytes (e.g. *Salicornia* sp., *Sarcocornia* sp., *Halimione portulacoides*) (Buhmann et al., 2015; Marques et al., 2017; Pinheiro et al., 2020; Webb et al., 2013). Some of these extractive species are represented in Figure 1.3.

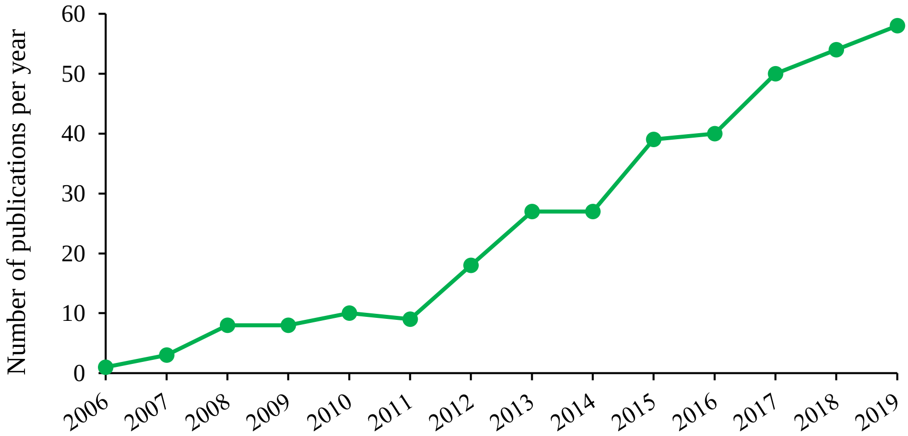

Figure 1.2: Number of publications per year retrieved using 'Integrated Multi-Trophic Aquaculture' as search topic in Scopus between 2006 and 2019 (available at https://tinyurl.com/y7jdgoyg, accessed on 30/4/2020)

Figure 1.3: Examples of extractive species subject to scientific research for Integrated Multi-Trophic Aquaculture: *Crassostrea* sp. (A); *Mytilus* sp. (B); *Paracentrotus lividus* (C); *Holothuria* sp. (D); *Hediste diversicolor* (E); *Gammarus* sp. (F); *Ulva lactuca* (G); *Tetraselmis* sp. (H); *Salicornia* sp. (I) (pictures licensed under Free Media License Agreement by Canva, Pty Ltd).

In the specific case of halophytes, the trophic group of interest for this thesis, two main support systems have been used to remediate aquaculture wastewater and support their growth, namely constructed wetlands, and hydroponic systems (see Chapter 2). The performance of plant extraction units usually depends on several factors, such as system configuration (Goddek et al., 2019; Vymazal, 2010), type of substrate (Boxman et al., 2017; Buhmann et al., 2015; Hunter et al., 2001; Lennard & Leonard, 2006), associated microbial communities (Lee & Lee, 2015; Ma et al., 2018; Rajan et al., 2018), water flow and retention time (García et al., 2010; Rousseau et al., 2008; Vera et al., 2016; Wu et al., 2015). Successful integration will greatly depend on the capacity of a plant extraction unit to remove N and P at a time-efficient rate and produce harvestable biomass with economic value.

The correct vertical integration of low-trophic groups with fed-aquaculture species can create sustainable integrated systems, where nutritional inputs are maximally assimilated by organisms and converted into valuable biomass for food, fodder, and raw materials with potential applications in other industries. Establishing which systems design and species are the most efficient for specific culture conditions (e.g. temperature, salinity, location) is essential for the successful implementation of IMTA as a profitable, sustainable, and socially acceptable business for the future

1.3. Halophytes

Halophytes are a broad group of higher plants mainly characterized by their capacity to complete their life-cycle under saline environments with salt concentrations (> 200 mmol L^{-1}) toxic to the vast majority of plants (Flowers et al., 1986). They display unique physiologic and anatomic adaptations to survive in these environments and different mechanisms help them prevent the accumulation of Na^{+} and Cl^{-} in cells cytoplasm (Flowers & Colmer, 2015, 2008; Grigore et al., 2014; Shabala, 2013). These include uptake regulation and ion exclusion at the root level, cellular compartmentalization in specialized vacuoles and excretion of ions (salt bladders) and production of organic osmolytes (e.g. betaines, polyphenols, glutamine, proline, glycerol) in the cytoplasm to counter external osmotic pressure and maintain cell functions and integrity.

Given these characteristics, halophytes can be irrigated with saline water, circumventing one of the major constraints regarding glycophytes (salt-sensitive plants) cultivation, which represent the bulk of agricultural plants: dependence of freshwater resources, which are increasingly limited in many parts of the world (Elliott et al., 2014; FAO, 2011). Since saline water is ubiquitous across coastal areas, halophytes could become important crops, promoting diversification and economic development in these areas, while also removing some of the pressure from freshwater reserves (Glenn et al., 2013; Panta et al., 2014; Ventura et al., 2015). A few halophytes are already being cultivated at commercial scale in some countries such as Israel, Mexico, The Netherlands, and France, with *Salicornia* spp. being the ones receiving most of the attention (Loconsole et al., 2019; Ventura et al., 2015; Ventura

& Sagi, 2013). In the context of coastal IMTA, these can become important co-products suitable for cultivation in brackish waters (Gunning et al., 2016).

In the present thesis, the species *Halimione portulacoides* (L.) Aellen (Figure 1.3), commonly known as sea purslane, is studied as a potential halophyte for coastal IMTA. This species is a C3 evergreen eudicot from order Caryophyllales, family Amaranthaceae, sub-family Chenopodioideae, and can be found almost exclusively in coastal lagoons, in mid-high marsh areas (Sousa et al., 2017). It is an autochthonous halophyte to European and North American Atlantic coasts, Mediterranean coasts, and Austral Africa, being abundant across Portuguese saltmarshes (Castroviejo et al., 1990). Leaves from *H. portulacoides* are suitable for human consumption (raw or cooked) and bioactive secondary metabolites found in its tissues are known to have nutraceutical and pharmaceutical value (Maciel et al., 2018; Rodrigues et al., 2014; Zengin et al., 2018). The potential of *H. portulacoides* for saline wastewater remediation has been previously studied and results suggest it might be a suitable candidate extractive species for IMTA (Buhmann et al., 2015; Marques et al., 2017). The main features of *H. portulacoides* that inspired its further exploration as a candidate for coastal IMTA in the present thesis are:

i. Autochthonous species to Portuguese saltmarshes (native plant) with ample distribution in other geographical areas (abundant resource).
ii. Perennial life-history (continuous year-round production).
iii. Edible leaves, suitable for human consumption (nutritional and economic value).
iv. Accumulation of bioactive compounds in edible and non-edible tissues (added-value product).
v. Shortage of studies in the context of IMTA compared with other halophytes (research opportunity).

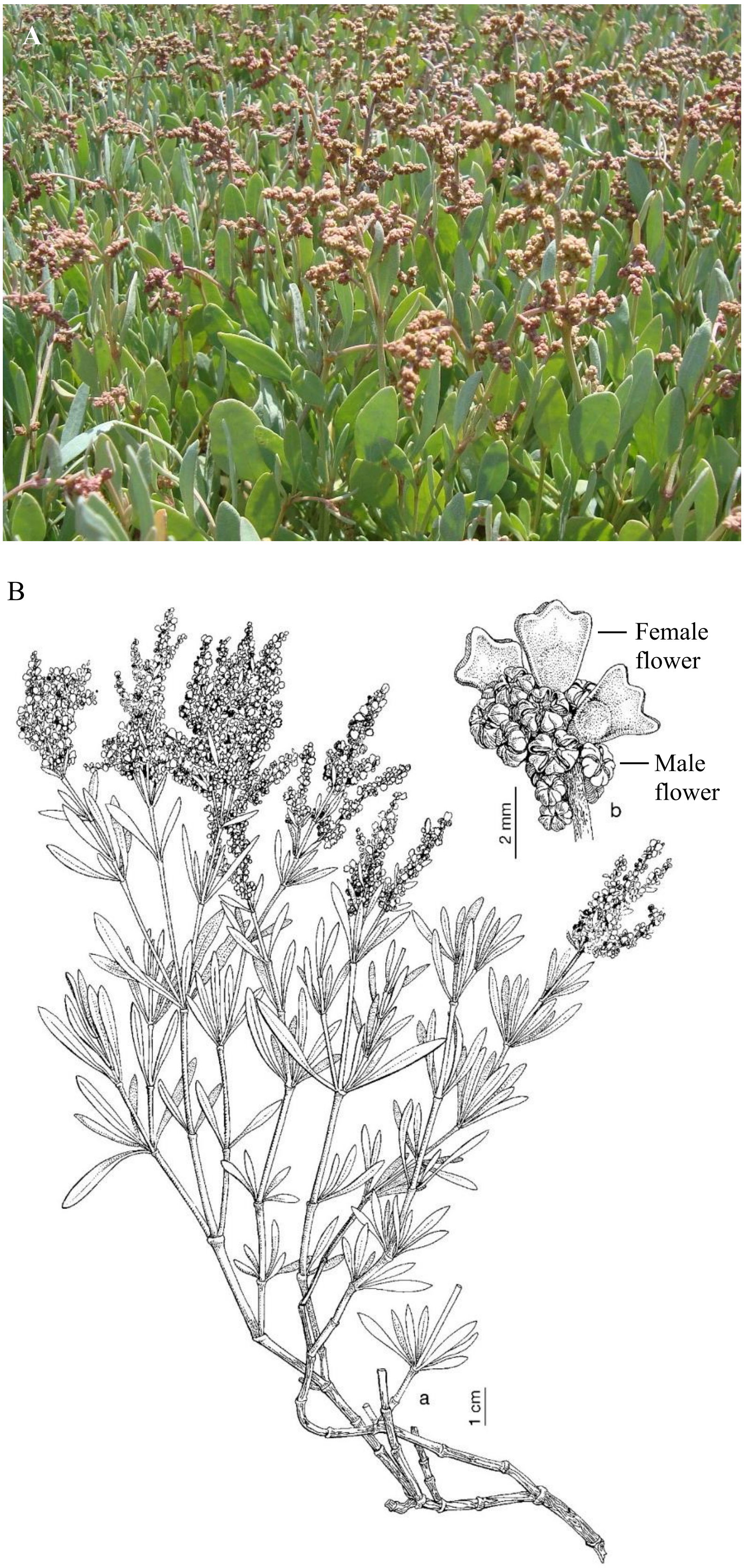

Figure 1.4: Flowering *Halimione portulacoides* plants (A) and corresponding scientific illustration adapted from Castroviejo et al. (1990) (B): a) floriferous branch; b) inflorescence.

1.4. Scope, objectives, and thesis outline

Nutrient waste produced in aquaculture farms is a growing societal concern due to potential negative impacts in aquatic ecosystems resulting from excess nutrient accumulation in water and sediment. Simultaneously, aquaculture producers incur an economic loss due to unused nutrients that represent a cost to the production. Given aquaculture activities are expected to continue their global expansion and intensification, integrative and circular production systems that do not rely exclusively on the carrying capacity of aquaculture sites and promote better nutrient-use efficiency and waste valorization in the context of sustainable development and circular economy are necessary. Aquaculture is expected to contribute to the maintenance and provision of ecosystem services and different organisms as well as different modes of farming them can either decrease or increase that contribution. Halophytes are recognized as having a vital role in salt-marsh ecosystems, promoting nutrient cycling and phytoremediation among other ecosystem services, and their integration in aquaculture systems to extract dissolved nutrients from saline wastewater is a relatively new nature-based solution for the industry that needs to be better explored. In that context, the present thesis aims to answer the following question:

Are halophytes suitable crops for sustainable coastal aquaculture?

This research follows a transdisciplinary approach to answer this question. The objectives are i) to understand how halophytes are being integrated into food systems and aquaculture, in particular; ii) to evaluate the hydroponic performance of *H. portulacoides*, a species with potential for IMTA, in terms of its productivity and capacity to extract dissolved inorganic N and P present in saline effluents; and iii) to assess halophytes' economic potential, from a product (valorization of the biomass) and consumer (willingness-to-pay for halophytes) perspectives, and elaborate on their viability as future crops.

To address the proposed objectives, the research was divided into five tasks and Chapters 2 to 6 feature the most relevant findings from each task. Chapter 7 provides an overview of the application of the ecosystem services framework to help support the practices discussed throughout this thesis and Chapter 8 summarizes a general conclusion and future perspectives:

- **Chapter 2** – Critical analysis of peer-reviewed literature addressing the potential of halophytes to remediate nutrient-rich effluents from marine and coastal aquaculture, followed by a special focus on *H. portulacoides*, with emphasis on its biology, ecology, and biochemistry.
- **Chapter 3** – Evaluation of the integration potential of *H. portulacoides* in coastal aquaculture settings (brackish waters) by assessing its growth performance and nutrient extraction capacity using hydroponic units. Plants were grown under different combinations (3 nutrient treatments) of dissolved inorganic N (DIN) and dissolved inorganic P (DIP) that mimic the range of nutrient availability in aquaculture effluents, as reported from the scientific literature. Hoagland's solution was used as a control treatment. Hydroponic units under non-limited conditions of N and P yielded 63 - 73 g m^{-2} of total biomass per day and were able to extract between 1.8 – 3.1 mg DIN-N g^{-1} of biomass produced and 0.1 – 0.3 mg DIP-P g^{-1}. The nutritional profile of edible leaves (crude protein, crude lipids, carbon hydrates, sugars, sodium, ash, dietary fiber, energy, and moisture contents) was characterized, being comparable to that of analogous products. The right trade-off between extraction efficiency and plant productivity is key to an effective halophyte extraction unit for IMTA.
- **Chapter 4** – Characterization of the polar lipidome of *H. portulacoides* to determine potential channels for biomass valorization and assess potential changes in the lipidome imposed by the availability of N and P. Analyzed samples were obtained from leaves' biomass produced in the experiment detailed in Chapter 2. Lipids were extracted using the Blight & Dyer method, phospholipids (PLs) and glycolipids (GLs) were quantified and the lipidomic profile of the extract was analyzed using hydrophilic interaction liquid chromatography (HILIC) coupled with mass spectrometry (MS) and tandem MS (MS/MS). The data generated were analyzed using bioinformatic tools (MZmaine 2.32) and statistical analysis (chemometric methods). 175 polar lipid species were identified: 140 PLs and 35 GLs. At the lowest concentration of N and P tested, leaves showed a decrease in PLs and GLs per unit of leaves dry mass and the GLs fraction of the lipidome changed significantly compared to other nutrient treatments. To add value to these new vegetable products

it is relevant to characterize the lipidome of halophyte crops and define the culture conditions that yield a consistent product, from the perspective of its lipidic profile.

- **Chapter 5** – Evaluation of the effect of planting density and artificial lighting on the indoor hydroponic performance of *H. portulacoides* in terms of growth, nutrient extraction efficiency, and concentration of photosynthetic pigments. These two horticultural variables can influence plant development, biomass allocation, and nutrient uptake and the goal was to determine how these influence *H. portulacoides* to optimize hydroponic cultivation for IMTA. A 2x2 factorial design was employed to understand the effect of variables 'plant density' (low density *vs* high density) and 'artificial lighting' (fluorescent light vs LEDs). The concentrations of N and P that were chosen for the nutrient solution guaranteed a non-limited availability of those nutrients. High-density units (220 plants m^{-2}) produced more biomass per unit of area (54 – 57 g m^{-2} day^{-1}) than low-density units (110 plants m^{-2}; 34 – 37 g m^{-2} day^{-1}) and displayed better extraction efficiencies of both DIN and DIP. The light source did not affect the parameters being surveyed. Plant density can be optimized to improve both productivity and nutrient extraction efficiency of hydroponic units and the most energy-efficient lighting source is likely to be the better option for commercial production.
- **Chapter 6** – Assessment of the potential of halophytes as a fresh vegetable product based on consumers' insights using a survey-based approach targeting the inhabitants of the city of Aveiro. The survey main objective was to determine habits of vegetable consumption, willingness-to-pay for fresh halophyte vegetables, and consumer segmentation based on demographic and psychographic (food-related lifestyle) variables. *Salicornia* sp. cultivated locally was used as a proxy for halophyte products and willingness-to-pay (WTP) for a package with 50 g of fresh shoots was elicited. A total of 268 responses were gathered at point-of-purchase locations (fresh produce market and mall supermarket). Results suggest that 30% of the inhabitants of Aveiro had some knowledge about Salicornia but other halophytes were mostly unknown. On average, consumers in Aveiro are willing-to-pay 2.1 € for a 50 g package (= 42 € kg^{-1}, fresh weight), with women being willing-to-pay more than men for the same product, as well as consumers who report diversifying their vegetable consumption. Three food-related lifestyle clusters were obtained ('adventurous,

'conservative', and 'careless' consumer) but no differences were detected in WTP between the groups. The consumer insights obtained can help guide marketing strategies to promote the consumption and integration of halophytes in the Portuguese diet.

- **Chapter 7** – Review of the literature about the use of the ecosystem services (ES) framework and existing ES valuation methods to inform on the ES produced and disturbed by aquaculture activities. The potential of the framework to be implemented under ecosystem-based management, to promote sustainable aquaculture practices, is discussed. The fact that certain species/trophic-groups, as well as production models, hold the potential to increase the capacity of ecosystems to deliver ES is highlighted.
- **Chapter 8** - A summary discussion of the research results and general conclusions are provided along with guidelines and perspectives for future research and commercial production of halophytes and IMTA.

Chapter 2

Unravelling the potential of halophytes for marine Integrated Multi-Trophic Aquaculture – a perspective on performance, opportunities and challenges

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2. Unraveling the potential of halophytes for marine Integrated Multi-Trophic Aquaculture – a perspective on performance, opportunities and challenges

Abstract

The present study critically analyses peer-reviewed literature addressing the potential of halophytes to remediate nutrient-rich effluents from marine and coastal aquaculture, as well as the potential for their economic valorization, from human consumption to an untapped source of valuable secondary metabolites with pharmaceutical potential. The growing body of evidence discussed in this review supports the perspective that halophytes can become a new source of nutrition and other high-value compounds and be easily incorporated into saltwater-based Integrated Multi-Trophic Aquaculture (IMTA) systems. In this context, halophytes act as extractors of dissolved inorganic nutrients, primarily nitrogen and phosphate usually wasted in marine aquaculture farms. Phytoremediation using halophytes has been proven to be an efficient solution and several ways exist to couple this practice with land-based marine aquaculture systems, namely through constructed wetlands and aquaponics. Focusing research on ecosystem-based approaches to aquaculture production will provide valuable data for producers and policymakers to improve decision-making towards sustainable development of this economic sector. Eco-intensification of aquaculture through IMTA will potentially increase the overall productivity and resilience of the sector and halophytes, in particular, are on the verge of becoming key-players for the diversification and promotion of land-based IMTA. This work specifically documents the uncharted potential of *Halimione portulacoides*, an important halophyte in European salt-marsh ecosystems, as a new extractive species for IMTA.

Keywords: Sustainable aquaculture; bioremediation; dissolved nutrients; coastal IMTA; saltwater aquaponics; blue growth; circular economy

2.1. Introduction

Aquaculture has experienced a fast and steady growth over the last decades, achieving a 7.5% annual growth rate between 1990 and 2009, significantly surpassing all other livestock sectors (Troell et al., 2014). Part of such rapid development is explained by the overexploitation of fish stocks that limits the supply of wild marine fish (FAO, 2016), leaving aquaculture as the only alternative to meet an ever-growing demand for seafood. Nonetheless, the fast development of the industry, which already supplies 50% of global seafood, has brought concerns about the extent of its environmental impact (FAO, 2016). Organic waste produced in fish-farms negatively impact aquatic ecosystems by modifying water biochemistry and ecological interactions (Troell et al., 2014). Particulate organic matter and dissolved inorganic nutrients, especially nitrogen and phosphorous forms, can promote water eutrophication and dramatically change sediment chemistry and associated benthic biodiversity (Bannister et al., 2014; Sanz-Lázaro et al., 2011; Sarà et al., 2011; Valdemarsen et al., 2012). In this way, new integrative, non-linear production methods are necessary to reduce the ecological impact of fish-farms. To promote such measures, the EU (through the Marine Strategy Framework Directive, Water Framework Directive, Circular Economy strategy, and the Blue Growth strategy) demands new approaches towards sustainable aquaculture practices and waste management and re-utilization (European Commission, 2012a, 2015; European Environment Agency, 2016; Science for Environment Policy, 2015).

Integrated Multi-Trophic Aquaculture (IMTA) systems have been recently studied and endorsed by scientists as a real sustainable solution for the industry (Abreu et al., 2011; Barrington et al., 2010; Chopin, 2015, 2017; Fang et al., 2016; Granada et al., 2016; Troell et al., 2009) Conceptually, IMTA is based on an ecosystem approach framework, where nutrients wasted on one trophic level, in particulate and dissolved forms, are redirected to downstream trophic levels to be filtered and/or extracted by capable organisms and utilized for growth. By performing this way, waste is reduced, productivity is increased (Hughes & Black, 2016) and the overall resilience of the global food system is improved (Troell et al., 2014). The integration of additional trophic levels greatly depends on the type of aquaculture systems in terms of production intensity and water salinity. Freshwater aquaculture allows for the integration of salt-sensitive extractive species such as vegetables commonly farmed in agriculture, often by coupling fish-rearing systems with hydroponics, an activity known as aquaponics (Graber & Junge, 2009; dos Santos, 2016; Somerville et al., 2014). However, a major portion (~ 5/6) of European aquaculture is marine and coastal water-based (FAO, 2016) and extractive species need to be salt-tolerant to remediate saline effluents. Important research already exists concerning the use of organisms such as shellfish and seaweeds on marine IMTA (Chopin, 2015; Neori et al., 2004; Troell et al., 2009), yet an underrated group of salt-tolerant plants could take IMTA to another level – halophytes.

This paper aims to contextualize the importance of halophytes on a new era of sustainable aquaculture and, particularly, elaborate on the potential of *Halimione portulacoides* (L.) Aellen (Figure 2.1), a low C3 shrub from the Chenopodiaceae family and Caryophyllales order, as a bioremediation plant and valuable co-product for IMTA. This view is supported by both biological and ecological traits, as demonstrated through a critical survey of available peer-reviewed literature. This species was chosen due to its wide geographic distribution, namely in European salt-marshes where it colonizes low and mid-marsh areas (Castroviejo, 1990; Waisel, 1972); it is also a key species characterizing the *Mediterranean and thermo-Atlantic halophilous scrubs* habitat, classified in the scope of EU Habitats Directive (Council Directive 92/43/EEC) and protected in several EU Natura 2000 sites (European Commission DGEnv., 2013); the background knowledge on the species ecology by some of the authors and ample presence in the Portuguese salt-marshes (e.g. Sousa et al., 2008, 2010, 2011; Válega et al., 2008a, 2008b), particularly in the Aveiro region; and the fact that it is a perennial and evergreen halophyte removes the need for manipulation of the life-cycle, as happens with annual plants (e.g. *Salicornia europaea* s.l.). Plus, the species has the potential for integration and valorization in the context of the aquaculture sector in regions where it naturally occurs, being suitable for IMTA solutions compatible with Marine Protected Areas (Chopin, 2017). Within this context, *H. portulacoides* could diversify the offer of autochthonous halophytes within the market of sea vegetables.

Figure 2.1: *Halimione portulacoides*: aerial view of a specimen (A) and closeup of the leaves (edible part) (B), showing their succulence and epidermal bladders. Location: section of the Aveiro Lagoon at Gafanha da Boa-Hora, Aveiro, Portugal (40° 32' 55.9'' N, 8° 46' 0)

2.2. Halophytes - the new players in sustainable marine aquaculture

Halophytes are salt-tolerant plants that complete their life cycle in saline environments, to which they are highly adapted (Flowers & Colmer, 2008; Glenn et al., 1999; Panta et al., 2014). A generally accepted definition for halophytes sets a salt concentration tolerance of at least 200 mM NaCl if the remaining environmental conditions are within the natural environment (Flowers et al., 1986). These unconventional crop plants have been overlooked by the food production sector, which mainly produces salt-sensitive vegetable species, i.e. glycophytes, which depend on freshwater irrigation for optimum yields. Nonetheless, humans in coastal communities within Europe and North America have consumed edible halophytes for centuries. For example, the salty leaves of 'sea purslane' (the common name given to plants from the sister genera *Atriplex* and *Halimione*; please see Kadereit et al., 2010) have been appreciated in some European countries and, nowadays, are collected from the wild by professional foragers and sold in specialized online platforms (e.g. online on Farmdrop and Fine Food Specialist, UK), local restaurants and gourmet cuisine (Barreira et al., 2017). The most recent case of emergent success is *Salicornia* L. spp., which have shown high levels of omega-3 polyunsaturated fatty acids and β-carotene antioxidants, and are already being produced on commercial scale agriculture operations in the USA and Europe (Boer, 2008; Lu et al., 2010; Panta et al., 2014; Ventura & Sagi, 2013; Ventura et al., 2015). Moreover, halophytes can also be used as bioenergy sources (Abideen et al., 2011; Boeing, 2014; Sharma et al., 2016; Ventura et al., 2015) and nutraceutical products, such as mineral-rich herbal salts (Kim & Kim, 2013).

Halophytic species developed remarkable physiological traits to succeed in high saline environments where the majority (> 90%) of plant species would perish (Flowers et al., 2010). These adaptations allow for the retention of water, protection of enzymatic machinery and maintenance of homeostasis (Ksouri et al., 2011; Flowers & Colmer, 2008; Flowers et al., 2010). A panoply of metabolites are biosynthesized by these plants (Aquino et al., 2011; Maciel et al., 2016) and many display bioactivity against oxidative stress, microbes, inflammations and tumors (Rodrigues et al., 2014; Ksouri et al., 2011; Buhmann & Papenbrock, 2013a; Boughalleb & Denden, 2011), which emphasizes their potential to be used by the pharmaceutical industry.

The integration of halophytes with economic potential in marine aquaculture systems to remediate nutrient-rich effluents and process water has received growing attention by research groups interested in sustainable aquaculture and a developing body of knowledge is already available, indicating promising results (Buhmann & Papenbrock, 2013b; De Lange et al., 2013; Shpigel et al., 2013, Waller et al., 2015). Halophytes can be integrated into IMTA systems through modules that allow for sustained plant growth and water (re)circulation, and the two main structures used for that purpose are usually constructed wetlands (CWs) and aquaponics systems. CWs have proven to be efficient at removing a wide range of organic and inorganic substances from different wastewater sources (Imfeld et al., 2009; Verhoeven & Meuleman, 1999; Vymazal, 2010; Vymazal, 2011; Shelef et al., 2013) including aquaculture (Carballeira et al., 2016; De Lange et al., 2013; Turcios & Papenbrock, 2014). Aquaponics systems, on the other hand, have been mostly experimented on freshwater setups (dos Santos, 2016; Somerville et al., 2014). Both systems have the potential to be used as growth modules for halophytes and support their integration in marine aquaculture activities (Turcios & Papenbrock, 2014).

2.3. Survey of scientific literature

A stepwise review of available scientific literature reporting the utilization of halophytes for remediation of marine aquaculture waters was performed, followed by a special focus on *Halimione portulacoides*, with emphasis on its biology, ecology, and biochemistry. The different steps of the process carried out for the selection of relevant literature are outlined in

Figure 2.2. A first assessment was conducted using Science Direct (SD) and Scopus (S) digital databases by searching for specific keywords within the title, abstract, and keywords sections of papers available online by November 2016. The search term "*Atriplex portulacoides"* was included in the assessment as it is a homotypic synonym of *H. portulacoides* and some authors opted for that name in their publications. On a subsequent assessment, the abstracts of all publications were surveyed, and the final selection of articles was imported into Mendeley^{TM} (n = 44). All these papers were fully read, from which 35 peer-reviewed articles were selected as the most relevant for the present review (the complete list of the selected publications is provided in Annex - Table A2.1). Selected articles had to fulfill the following criteria: i) include experiments using halophytes as extractive species for saltwater aquaculture effluents and ii) address halophytes growing in CWs and/or aquaponics/hydroponics systems or iii) focus the research on *H. portulacoides* biology, ecology and/or biochemistry.

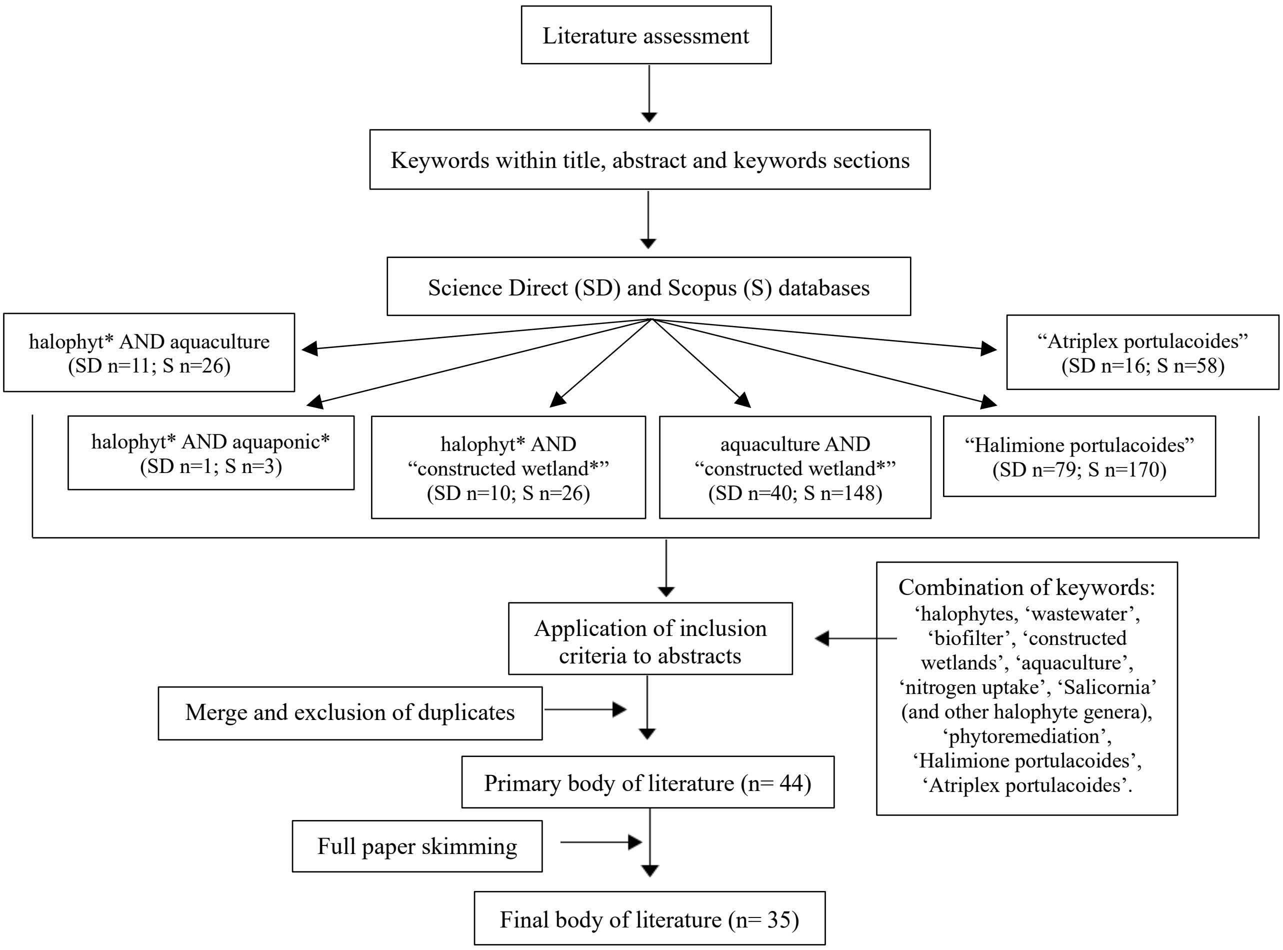


Figure 2.2: Process employed for the selection of relevant literature

2.4. Halophytes in aquaculture: facts and figures

Concerning the use of halophytes as biofilters for aquaculture, 15 original research articles and 4 reviews were selected, where the integration and performance of several species were evaluated and discussed. The criteria for species selection, where referred, were based on local availability, salinity tolerance, and economic potential. In total, 22 halophyte species (17 genera) were tested, and full species names, the number of aquaculture remediation studies per plant species, and references are represented in Figure 2.3.

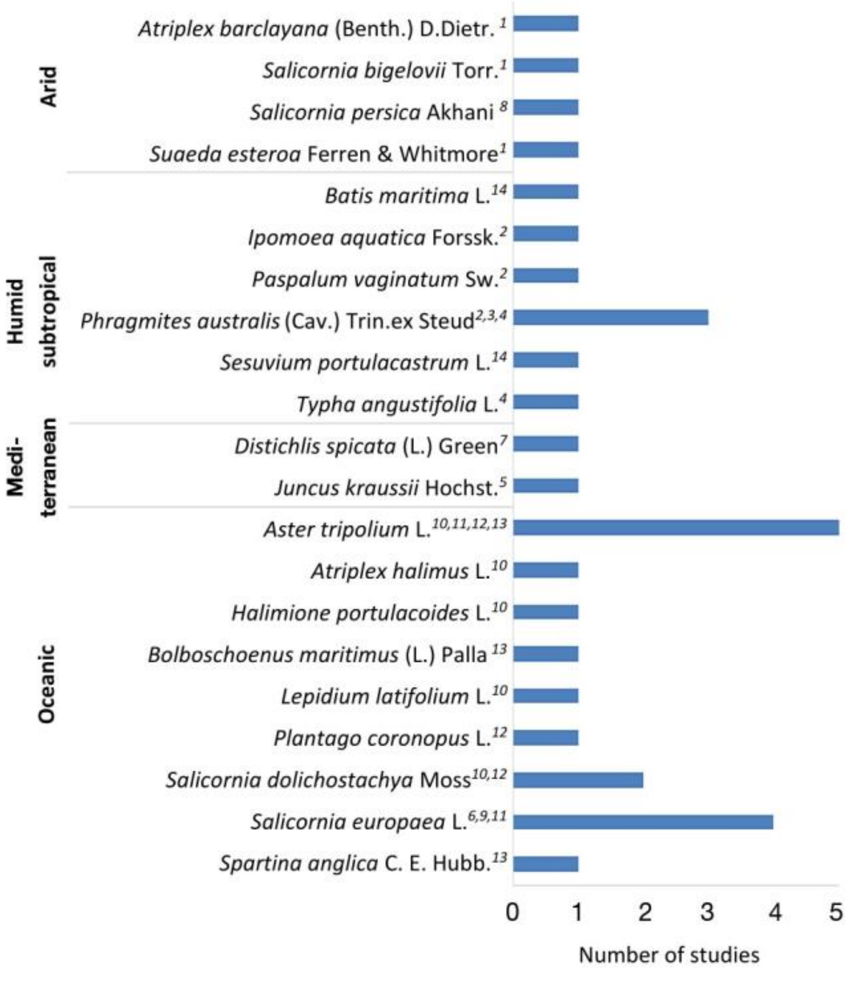


Figure 2.3: Number of studies per halophyte species where phytoremediation was tested, and growth performances evaluated upon irrigation with saline aquaculture wastewater. The geographic location where each experimental trial took place was ranked following the Köppen climate classification. [1]Brown et al. (1999); [2]Lin et al. (2002b); [3]Lin et al. (2003); [4]Lin et al. (2005); [5]Lymbery et al. (2006); [6]Webb et al. (2012); [7]Lymbery et al. (2013); [8]Shpigel et al. (2013); [9]Webb et al. (2013); [10]Buhmann et al. (2015); [11]Quintã et al. (2015a,b); [12]Waller et al. (2015); [13]De Lange & Paulissen (2016); [14]Boxman et al. (2017). Notes: (i) *Aster tripolium* as *Tripolium pannonicum* (Jacq.) Dobrocz. in Buhmann et al. (2015) and Waller et al. (2015); (ii) *Halimione portulacoides* as *Atriplex portulacoides* L. in Buhmann et al. (2015); (iii) *Bolboschoenus maritimus* as *Scirpus maritimus* L. in De Lange & Paulissen (2016)

The most studied halophyte to date was *Aster tripolium* (5 studies; including the homotypic synonym *Tripolium pannonicum*), followed by *Salicornia europaea* (4 studies), *Phragmites australis* (3 studies), and *Salicornia dolichostachya* (2 studies). All the other species have been addressed only once. The growing modules for halophyte plants were either hydroponics-based or substrate-based and it appears that the choice of medium depends on the type of intensification being employed for the production of the target fish-species (semi-intensive *vs.* intensive/RAS), as well as halophyte species and biofilter main purpose (wastewater treatment *vs.* plant biomass production) (Buhmann & Papenbrock, 2013b; Buhmann et al., 2015; Chen & Wong, 2016). Farmed species originating the effluents included different fish, shrimp, and, in some studies, artificial solutions mimicking the organic load of aquaculture effluents were used (Buhmann et al., 2015; De Lange & Paulissen, 2016; Quintã et al., 2015a). Farmed species included *Chanos chanos* Forssk., 1775 (Lin et al., 2002b), *Dicentrarchus labrax* L., 1758 (Quintã et al., 2015b; Waller et al., 2015), *Oncorhynchus mykiss* Walbaum, 1792 (Lymbery et al., 2006, 2013), *Oreochromis* sp. Günther, 1889 (Brown et al., 1999), *Penaeus vannamei* Boone, 1931 (Lin et al., 2003, 2005; Webb et al., 2012, 2013), *Solea senegalensis* Kaup, 1858 (Webb et al., 2012), *Sparus aurata* L., 1758 (Shpigel et al., 2013) and *Xiphophorus* sp. Heckel, 1848 (Boxman et al., 2017). Effluents originating from the culture of freshwater species were salinized by adding NaCl before the irrigation of halophytes. The experiments were performed in diverse geographic regions and climates (Figure 2.4): the arid climates of southern Israel (Shpigel et al., 2013) and southwestern USA (Brown et al., 1999), the humid subtropical regions of Taiwan (Lin et al., 2002b, 2003, 2005) and southeastern USA (Boxman et al., 2017), the oceanic climate of northwestern Europe (Buhmann et al., 2015; De Lange & Paulissen, 2016; Quintã et al., 2015a; Waller et al., 2015; Webb et al., 2012, 2013) and the Mediterranean climate of southwestern Australia (Lymbery et al., 2006, 2013). Yet, the diversity of studies is still low and additional studies with endemic species in different climate regions are needed. Concerning the economic valorization of plants biomass, researchers referred to the potential of some species to be used as food for human consumption (e.g. *Salicornia* spp., *A. tripolium,* and *Halimione portulacoides)* (Buhmann et al., 2015; Isca et al., 2014; Lu et al., 2010; Quintã et al., 2015a; Webb et al., 2012), as forage for livestock (e.g. *Suaeda esteroa* and *Distichlis spicata*) (Brown et al. 1999; Lymbery et al. 2013; Panta et al. 2014), as oil sources (e.g. *Salicornia* spp. seeds) (Brown et al., 1999; Sharma et al., 2016; Weber et al., 2007) and as sources of extracts with pharmacologic applications (Buhmann & Papenbrock, 2013a; Ksouri et al., 2011).

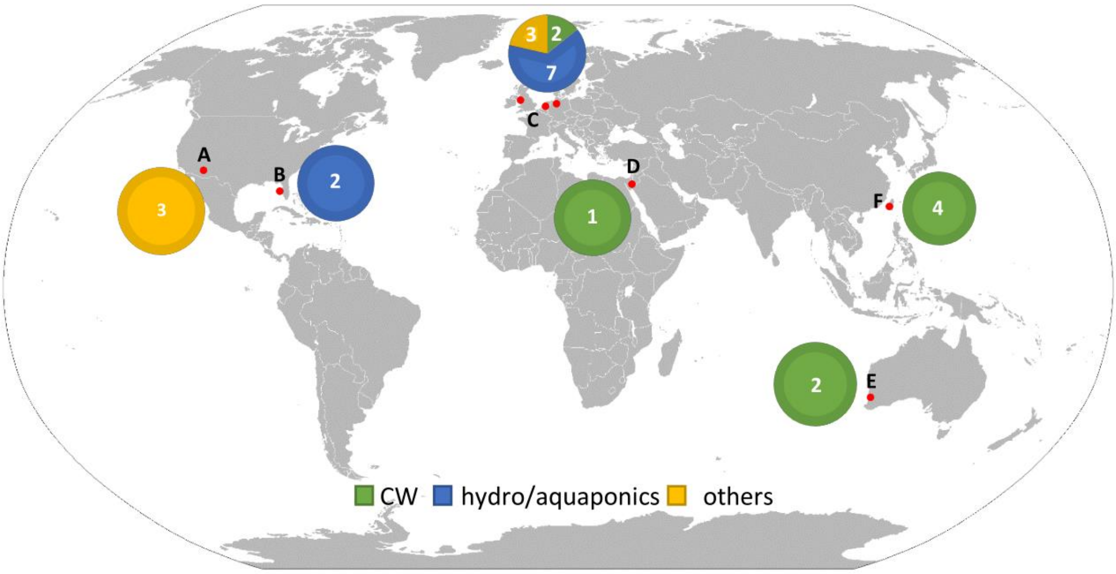


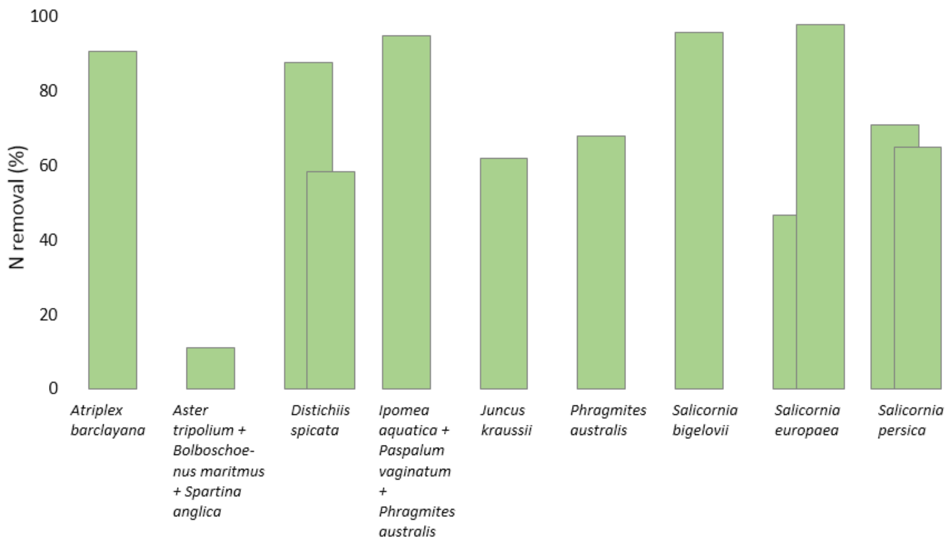
Figure 2.4: Geographic locations of previous experiments using halophytes as aquaculture effluent remediators (red dots). Pie charts represent plant-growing systems (CW: constructed wetland; hydro/aquaponics and others [pot-planted or lysimeter]) used in each region in relation to the number of species tested (white numbers). Regions and species (from left to right): (A) South Arizona (USA) − *Atriplex barclayana; Salicornia bigelovii* and *Suaeda esteroa*; (B) Florida (USA) − *Batis maritima* and *Sesuvium portulacastrum*; (C) Northern Europe − hydro/aquaponics: *Aster tripolium, Atriplex halimus, Halimione portulacoides, Lepidium latifolium, Plantago coronopus, Salicornia dolichostachya* and *Salicornia europaea*; CW: *A. tripolium* and *S. europaea*; others: *A. tripolium, Bolboschoenus maritimus* and *Spartina anglica*; (D) Israel − *Salicornia persica*; (E) Southwestern Australia − *Distichlis spicata* and *Juncus kraussii*; (F) Taiwan − *Ipomoea aquatica, Paspalum vaginatum, Phragmites australis* and *Typha angustifolia*. Map editing software: ArcGIS

In Table 2.1, data from experiments using CWs is displayed concerning the performance of different halophytes in removing N and P from wastewater. Due to the reduced number of experiments involving hydroponic/aquaponic setups (Boxman et al., 2017; Buhmann et al., 2015; Quintã et al., 2015a and Waller et al., 2015), out of which only two included N and P removal efficiencies, and due to several differences in surveyed variables to allow a direct comparison of the data with those reported from CW setups, studies addressing hydroponic/aquaponic setups were not included in Table 2.1. For easier comparison between experiments using CWs and whenever possible, values reported on the different studies were converted to a common unit. Due to the variability in environmental and biological factors between experimental conditions (e.g. salinity, substrate, nutrient concentration, water volume, retention time, duration of the experiment, plant density, age of plants and climatic conditions such as temperature and light) results cannot be directly compared. However, despite the existing variability in terms of nutrient removal, which seems to depend on systems design, flow regime, nutrients concentration and species (Buhmann & Papenbrock, 2013b), not all setups *per se* are equally effective for nutrient removal, taking into account the specific objectives established for each CWs. N removal capacity attained around 90% and over in four of the studies that were surveyed (Brown et al., 1999; Lin et al., 2002; Lymbery et al., 2013; Webb et al., 2012) and only one experiment reported a low N removal capacity (11%) (Lange et al., 2016). While in some of the studies P removal was close to 100% (e.g. Brown et al., 1999), in one of the experiments reported, P removal was solely 13% (Shpigel et al., 2013). Figure 2.5 illustrates different halophyte species performance, in terms of N and P removal efficiency attained under different experimental conditions (based on data summarized in Table 2.1). Although results should not be directly compared, the key point is to highlight the phytoremediation service provided by halophytes in CWs as most of them fulfilled the objectives under the tested conditions.

Table 2.1: Performance of constructed wetlands (CWs) using halophytes to remove nitrogen (N) and phosphate (P) from marine aquaculture effluents. TDIN: total dissolved inorganic nitrogen, DIP: dissolved inorganic phosphate, TDN: total dissolved nitrogen, TAN: total ammonium nitrogen, PO4-P: orthophosphate, TN: total nitrogen, TP: total phosphate, NO3-N: nitrates. Entries with 2 values indicate separate remediation experiments (different N and P concentrations, system design, or plants) within the same study.

Species	**Effluent origin**	**Salinity**	**Substrate**	**Time (days)**	**Effluent N concentration**	**N removal (%)**	**Effluent P concentration**	**P removal (%)**	**Reference**
Salicornia europaea	Shrimp *(P vannamei)*	-	Quarry sand	84	2 g m^{-2} day^{-1} TDIN	47	0.81 g m^{-2} day^{-1} DIP	67	Webb et al. (2013)
Salicornia europaea	Shrimp, sole and turbot	10 - 29	Quarry sand + Limestone	58	1.5 - 5.4 mg L^{-1}	98	1.05 - 2.79 mg L^{-1}	36 - 89	Webb et al. (2012)
Salicornia persica	Gilthead seabream (*S. aurata*)	35	Gravel stone	90	11.1 g m^{-2} day^{-1} TDN 10.5 g m^{-2} day^{-1} TDN	71 65	1.6 g m^{-2} day^{-1} 1.5 g m^{-2} day^{-1}	12 13	Shpigel et al. (2013)
Ipomea aquatica + *Paspalum vaginatum* + *Phragmites australis*	Milkfish (*C. chanos*)	5	River gravel + local soil	35	0.6 g m^{-2} day^{-1} TDIN	95	0.9 g m^{-2} day^{-1} P-PO4	71	Lin et al. (2002b)
Phragmites australis	Shrimp *(P. vannamei)*	-	Local soil + river gravel	80	0.21 mg L^{-1} TAN 0.41 mg L^{-1} NO3-N	57 68	8.45 mg L^{-1} PO4-P	5.4	Lin et al. (2003)
Salicornia bigelovii *Atriplex barclayana*	Hybrid tilapia	35 (added)	Lysimeter (with soil)	120	77.2 mg L^{-1} TN	95.8 90.7	25.27 mg L^{-1} TP	99.5 99.7	Brown et al. (1999)
Aster tripolium + *Bolboschoenus maritimus* + *Spartina anglica*	Artificial effluent	12.9	Original soil (cores planted on pots)	63	15 mg L^{-1} TN	11	2.5 mg L^{-1} TP	35	De Lange & Paulissen (2016)
Juncus kraussii	Rainbow trout (*O. mykiss*)	24 (added)	Basalt gravel	38	3.8 mg L^{-1} TN	62	1.27 mg L^{-1} TP	77	Lymbery et al. (2006)
Distichlis spicata	Rainbow trout (*O. mykiss*)	15 (added)	Washed quartz sand	231	5.0 mg L^{-1} TN 1.0 mg L^{-1} TN	87.7 58.3	1.0 mg L^{-1} TP 0.2 mg L^{-1} TP	91.2 84.5	Lymbery et al. (2013)

A



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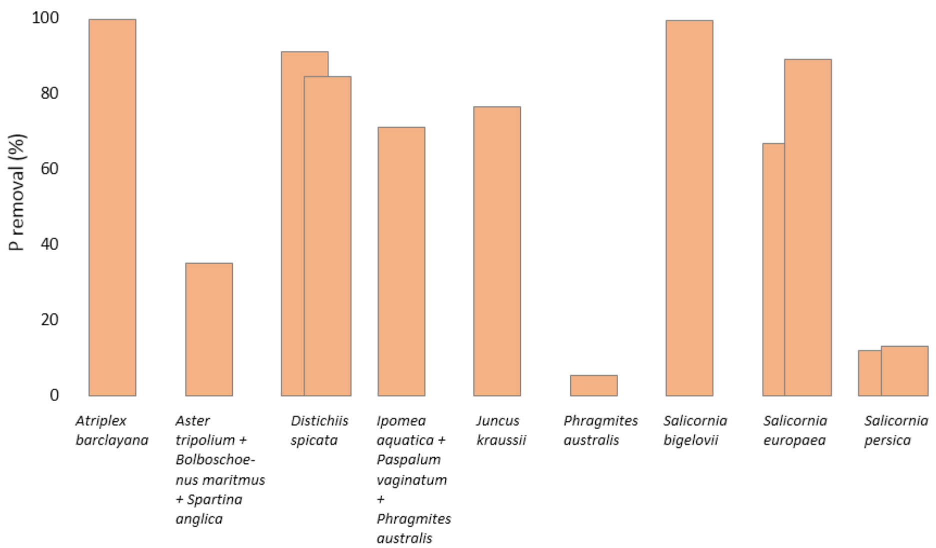


Figure 2.5: Average percentage of N removal (A) and P removal (B) from saline aquaculture effluents by halophyte species planted in constructed wetlands (CWs; according to data reported in Table 1). Overlapping bars correspond to different studies using the same species

2.5. Challenges and opportunities for integrating halophytes in IMTA

The eutrophication of water bodies has become a major issue of modern aquaculture due to the intensification and expansion of production and increased use of high-protein pelleted feeds (Edwards, 2015). It was estimated that in conventional aquaculture, fish assimilate solely 25-40 % of the whole N and P available in their diets (Lupatsch & Kissil, 1998; Wang et al., 2012), while the rest is wasted into effluent water through feed lixiviation and fish excretion/metabolism. Yet, nutrient-rich wastes could be redirected to trophic levels capable of assimilating these nutrients and convert them into biomass with economic value, while simultaneously reducing water pollution. Given that food waste is an increasing concern in Europe, the potential of this waste redirection can help the implementation of the EU plans for a circular economy, in which the challenge of transition towards the reduction of waste and sustainable resource efficiency are key to develop a competitive EU economy (European Commission, 2015). Technological improvements and adaptation processes of fish farms could transform aquaculture production, creating new windows of opportunity for a sustainable Blue Growth of European coastal areas (European Commission, 2012a).

The integration of CWs and aquaponics systems to grow halophytes in IMTA are relatively new concepts that deserve scientific scrutiny to evaluate their potential for large-scale application. In the case of CWs, plants function as a solid biological filter where nitrification and denitrification processes occur and nutrients are restrained and extracted from the effluent by both the plants, soil microorganisms, and substrate (Shpigel et al., 2013; Webb et al., 2012, 2013). In aquaponics, the system necessarily requires an independent upstream biofilter to promote nitrification processes. This is essential in 'free-floating' configurations [e.g. floating-rafts (aka deep-water culture) and nutrient-film technique], meanwhile systems using inert growth-media, such as expanded clay, allow nitrifying bacteria to establish in the solid surface (Boxman et al., 2017; Buhmann et al., 2015; Waller et al., 2015). Buhmann & Papenbrock (2013b) reviewed some studies that utilized halophytes CWs as biofilters, showing promising results yet referring to the need for more comprehensive research. To date, the halophytes that received most of the attention, including in agriculture studies, were *Salicornia* and *Sarcocornia* A.J. Scott, which exhibited promising results in terms of growth rates and phytoremediation (Brown et al., 1999; Katschnig et al., 2013; Ventura et al., 2011a, 2011b; Ventura & Sagi, 2013; Webb et al., 2012, 2013). Other species, including *Aster tripolium, Plantago coronopus, Lepidium latifolium, Halimione portulacoides,* and *Atriplex* *halimus* also demonstrated good potential, as already summarized above. Nonetheless, great variability in results is undeniable and these are most likely due to variable experimental conditions, species-specific traits, systems design, and the lack of standardized research methods. More data needs to be generated under standardized conditions to evaluate which are the most suitable halophytes for IMTA to achieve a more cohesive and robust body of knowledge.

The variables that need to be studied concerning the selection of the best halophyte plants for IMTA are salinity tolerance, macro- and micro-nutrients requirements, light, and hydraulic regimens, plant density, as well as the potential for economic valorization (Buhmann & Papenbrock, 2013b; Buhmann et al., 2015; Verhoeven & Meuleman, 1999; Vymazal, 2010). For example, to investigate the relevance of plant density, *Salicornia europaea* was grown at 10,000 and 200 plants m^{-2} in CWs, with no significant differences in nutrient removal; up to 85% of total dissolved inorganic nitrogen (TDIN) was removed, with a maximum removal rate of 1.5 g N m^{-2} d^{-1} (Webb et al., 2013). Previous observations have shown even higher removal rates (up to 100%) of TDIN (Brown et al., 1999; Webb et al., 2012). Nonetheless, some studies state that most N removal results from microbial processes and, to a lesser degree, from plant uptake (Hadad et al., 2006; Lin et al., 2002a). Nonetheless, studies with certain species of halophytes advocate otherwise (Webb et al., 2012, 2013). Recently, Quintã et al. (2015b) concluded that hydroponically grown *S. europaea* and *A. tripolium* could assimilate dissolved organic nitrogen (DON, specifically alanine-N and trialanine-N), suggesting that DON removal should also be taken into consideration in phytoremediation of wastewater. Other authors also concluded that some halophytes species, namely *Phragmites australis* and *Spartina alterniflora*, seem to directly assimilate both inorganic and organic forms of N (Mozdzer et al., 2010). In terms of dissolved inorganic phosphates (DIP), a CW employing *S. europaea* was able to perform a removal of up to 89%; yet, it is commonly accepted that plants play a small role in phosphates removal, as it is assumed that most of the elimination recorded is achieved through adsorption to the substrate (Lüderitz & Gerlach, 2002; Webb et al., 2012, 2013).

While CWs and aquaponics systems can both be used to remediate wastewater and grow halophytic cash-crops, they differ on their applicability and purpose. The primary concern of CWs is usually wastewater treatment where the interplay of many biological and chemical processes results in high removal rates of N and P, but where only a fraction of these nutrients is uptaken by plants (Turcios & Papenbrock, 2014). On the other hand, aquaponics main objective is to maximize plant production (Goddek et al., 2015), which is usually the main source of revenue in freshwater aquaponics. Growing halophytes hydroponically would be a reasonable choice for intensive fish-farming using RAS (Buhmann et al., 2015; Waller et al. 2015). These systems can provide high concentrations of N for plant growth, but parallel nitrifying biofilters are usually necessary to produce the necessary nitrate-N, more easily absorbed by plants (Jensen, 1985; Stewart et al., 1973). To retain most of N in nitrate-N form, anoxic conditions need to be minimized to avoid denitrification, which might occur at very low oxygen concentration (<10%), with the consequent release of N in its atmospheric form (Verhoeven & Meuleman, 1999). Since aquaponics systems are typically well aerated and new optimized aquaponics systems are being designed (Goddek et al., 2016b; Kloas et al., 2015), this issue may be easily addressed. In CWs, denitrification processes are more likely to happen due to the fundamental characteristics of the system, which create more oxic-anoxic interactions throughout the sediment profile, enhancing the coupling between nitrification and denitrification. For that reason, if the main goal is water remediation, CWs are the most cost-effective choice and can be used in both open and closed aquaculture systems. Eventually, as highlighted by Chen and Wong (2016), a hybrid approach comprised of both types of growing systems would allow taking advantage of both mechanisms, maximizing nutrient removal, and plant biomass production.

Regarding biomass yields in both systems, variability is also evident. Using hydroponics growing systems, Boxman et al. (2017) tested the performance of *Sesuvium portulacastrum* and *Batis maritima* for 30 days (initial density of 24 plants m^{-2}) and obtained average yields of 0.53 and 0.32 kg m^{-2}, respectively. Waller et al. (2015) grew *Salicornia dolichostachya, A. tripolium,* and *P. coronopus* for 35 days (initial density of 39 plants m^{-2}) with final average yields of 2.70, 1.25 and 0.83 kg m^{-2}, correspondingly. In a CW, Webb et al. (2013) obtained average yields of *Salicornia europaea* after 21 days (initial density of 200 plants m^{-2}) of 2.2 kg m^{-2}. Yield variability might be explained by initial planting densities, availability of physical space for growth and grow-out time to harvest; yet, species-specific variability is certainly a factor to consider.

The inclusion of halophytes in marine IMTA has been certainly overlooked until recent years due to the lack of a tangible market for its commercialization, when compared with seaweeds, which are commonly studied and used as extractive species in IMTA (Abreu et al., 2011; Chopin, 2015; Fang et al., 2016). In fact, seaweeds demand is increasing around the world and the commercial seaweed market is expected to reach USD 22.13 billion by 2024 (Grand View Research, 2016). Another important factor that makes macroalgae more practical and widely chosen for IMTA is that marine IMTA has been mostly implemented in offshore settings (Chopin, 2012; Fang et al., 2016; Troell et al., 2009). As we move towards the implementation of an increasing number of land-based marine IMTA systems (e.g. saltwater aquaponics, RAS coupled with constructed wetlands) which have numerous advantages relative to off-shore settings (Gunning et al., 2016), halophytes can be progressively introduced as an extractive species with commercial and socio-ecologic interest for those systems. A few localized niche markets already exist for halophytes (e.g. gourmet cuisine) and their distinctive nutritional and biochemical composition can further boost their marketability in the future (Barreira et al., 2017; Sharma et al., 2016).

2.6. The potential of *Halimione portulacoide*s

To our knowledge, by November 2016, only one study evaluated the potential of *Halimione portulacoides* as an extractive species for IMTA. Buhmann et al. (2015) used a hydroponics system and an artificial effluent characterized by a salinity of 15 ppt, 50 mg NO_3-N L^{-1} and 9.8 mg PO_4–P L^{-1} to investigate the plant's performance. Under the experimental conditions, *H. portulacoides* was able to retain 30% of N and 18% of P in the shoots and roots and the average decrease of nitrate-N in the effluent was 29 mg L^{-1} and phosphate-P was 5 mg L^{-1}, over 5 weeks. Moreover, a more recent study by Marques et al. (2017), published after the literature survey was completed, evaluated the capacity of *H. portulacoides* to extract DIN from an intensive RAS farm effluent. The average decrease in DIN was 65%. In both studies, the plant was considered a suitable candidate for the remediation of aquaculture effluents.

A total of 16 studies addressed *H. portulacoides* physiology (n=4), phytoremediation (n=8), primary productivity (n=1), and secondary metabolites (n=3), which contribute to highlight the potential of this halophyte species for IMTA (Figure 2.6). This species is widely distributed throughout salt-marsh ecosystems of the Mediterranean, Irano-Turanian and West Euro-Siberian, North American, and South African regions (Castroviejo, 1990; Waisel, 1972). It plays an important role in the ecosystem services provided by coastal wetlands, namely in nutrient cycling and phytoremediation processes (Sousa et al., 2010, 2011;

Válega et al., 2008a). Its distribution is correlated with good soil drainage and it tolerates frequent short inundations as occurs in the intertidal zones where it thrives (Jensen 1985). It can cope and grow within a wide concentration range of dissolved NaCl in the water, from zero to full-strength seawater (~500 $mol.m^{-3}$) and over (up to 1000 $mol.m^{-3}$) (Jensen, 1985; Redondo-Gómez et al., 2007). Specialized vacuoles within leaves are responsible for compartmentalizing Na^+ and Cl^- which were further excreted through epidermal bladders, protecting the metabolic machinery from salt-induced stress (Benzarti et al., 2012, 2015; Redondo-Gómez et al., 2007; Shabala et al., 2014). Within the above-mentioned spectrum of salinity, optimal growth was found at 85-200 $mol.m^{-3}$ NaCl and a gradual depression was observed between 410-690 $mol.m^{-3}$ NaCl (Jensen, 1985; Redondo-Gómez et al., 2007). Nonetheless, growth is stimulated at higher NaCl concentrations with increasing concentrations of dissolved nitrate-N (Jensen, 1985). At supra-optimal salinity levels, Cl^- directly competes with NO_3^- uptake (Benzarti et al., 2015), explaining the positive impact of higher nitrate-N concentration at higher salinities. Moreover, decreased stomatal conductance is also observed with increasing Na^+ and Cl^- concentrations (Flowers & Colmer, 2015; Redondo-Gómez et al., 2007), a mechanism that prevents water loss and modulates water transport to reduce net uptake of salts to the shoots (Ayala & O'Leary, 1995; Katschnig et al., 2013; Khan et al., 2001). In terms of primary production, Neves et al. (2007) conducted field-studies in the south of Portugal and determined that mean aboveground biomass production was 598 g m^{-2} yr^{-1}, with maximum values registered in spring, reaching 1077g m^{-2} yr^{-1}.

In terms of biochemical composition, Vilela et al. (2014) screened for lipophilic and phenolic compounds with potential bioactivity and found that lipophilic fractions of leaves and stems are mainly composed of long-chain aliphatic acids and alcohols and smaller quantities of sterols. Also, they identified 13 phenolic compounds with a higher concentration in the leaves [4.6 g kg^{-1} dry matter (DM)], from which 3.1 g kg^{-1} DM were sulfated flavonoids. A rare triterpenic ketone with pharmaceutical properties (Hill et al., 2015) was found at high concentrations (2.8 g kg^{-1} DM) in the roots, namely the molecule hop-17(21)-en-3-one (Vilela et al., 2014). Rodrigues et al. (2014) looked at the bioactivity of *H. portulacoides* extracts and found high radical scavenging activity (IC50 = 0.9 mg mL^{-1}) against ABTS and a decrease in nitric oxide production after incubation of macrophages with lipopolysaccharide and a chloroform extract (IC50 = 109 µg mL^{-1}), indicative of anti-inflammatory properties. More recently, two new bioactive compounds designated as 'portulasoid' and 'septanoecdysone' were isolated from the plant (Ben Nejma et al., 2015).

This species has also been studied for its high regeneration potential and its remarkable metals phytoremediation capacities, which include stabilization, at the root level, of toxic inorganic substances and extraction and retention of several compounds in aboveground biomass (Andrades-Moreno et al., 2013; Cambrollé et al., 2012b, 2012a; Sousa et al., 2008, 2010, 2011; Válega et al., 2008a, 2008b). These processes occur without compromising key metabolic sites and reinforce its role as an ecological buffer, helping maintain the homeostasis of the salt-marsh ecosystem. The physiological adaptations to salt-marsh environments and phytoremediation potential of *H. portulacoides* make this species a good candidate to mitigate potential negative impacts promoted by marine aquaculture effluents, as demonstrated so far. By being exposed to numerous abiotic stresses, these plants are expected to cope with multiple stress-inducing factors that fluctuate on a short-term scale, reinforcing their suitability for IMTA (Lutts & Lefèvre, 2015; Walker et al., 2014). Moreover, *H. portulacoides* is widely distributed geographically, with apparent good productivity (Neves et al., 2007) and can be easily propagated through cuttings, and therefore its use at a large scale is not dependent on wild populations for the harvesting of seeds (Sousa et al., 2010).

Additionally, halophytes have shown a positive correlation between increasing salinity and production of secondary metabolites (Aquino et al., 2011; Benzarti et al., 2012; Buhmann & Papenbrock, 2013a) and enhanced production of phenols and flavonoids during the flowering period (Jallali et al., 2012; Medini et al., 2011), allowing for the manipulation of such molecules within the plant. The leaves of *H. portulacoides* have high average levels of sulfated flavonoids (Vilela et al., 2014), therefore being a potential source of these compounds of pharmacologic interest (Correia-da-Silva et al., 2014). For instance, *Flaveria bidentis* (L.) Kuntze is recognized as a good source of sulfated flavonoids, namely isorhamnetin 3-sulfate, with about 744 mg kg^{-1} DM (Xie et al., 2012), solely 1/4 of the content exhibited by *H. portulacoides*. Moreover, long-chain chloroalkanes were also recorded in leaf waxes (Grossi & Raphel, 2003) and volatile organic compounds in root exudates (Oliveira et al., 2012). A rare bioactive triterpenic ketone extracted from the roots of this halophyte (Vilela et al., 2014) further elevates the pharmacological interest of this species and future biochemical studies using omics-approaches will likely reveal new bioactive compounds of interest. Furthermore, by presenting edible leaves and tips, this halophyte may actively contribute to the diversification and expansion of the sea vegetable market.

The potential of *H. portulacoides* to be used as a halophyte biofilter is undeniable, yet little information is available relative to its use and performance. To explore its suitability, additional data is required on its planting density, hydraulic regimes, growth medium, nutrient requirements and availability, and how these affect growth performance, nutrient uptake, phytoremediation efficiency, and biochemical composition. Both CWs and hydroponics modules should be tested to find out which growing system is better for the species.

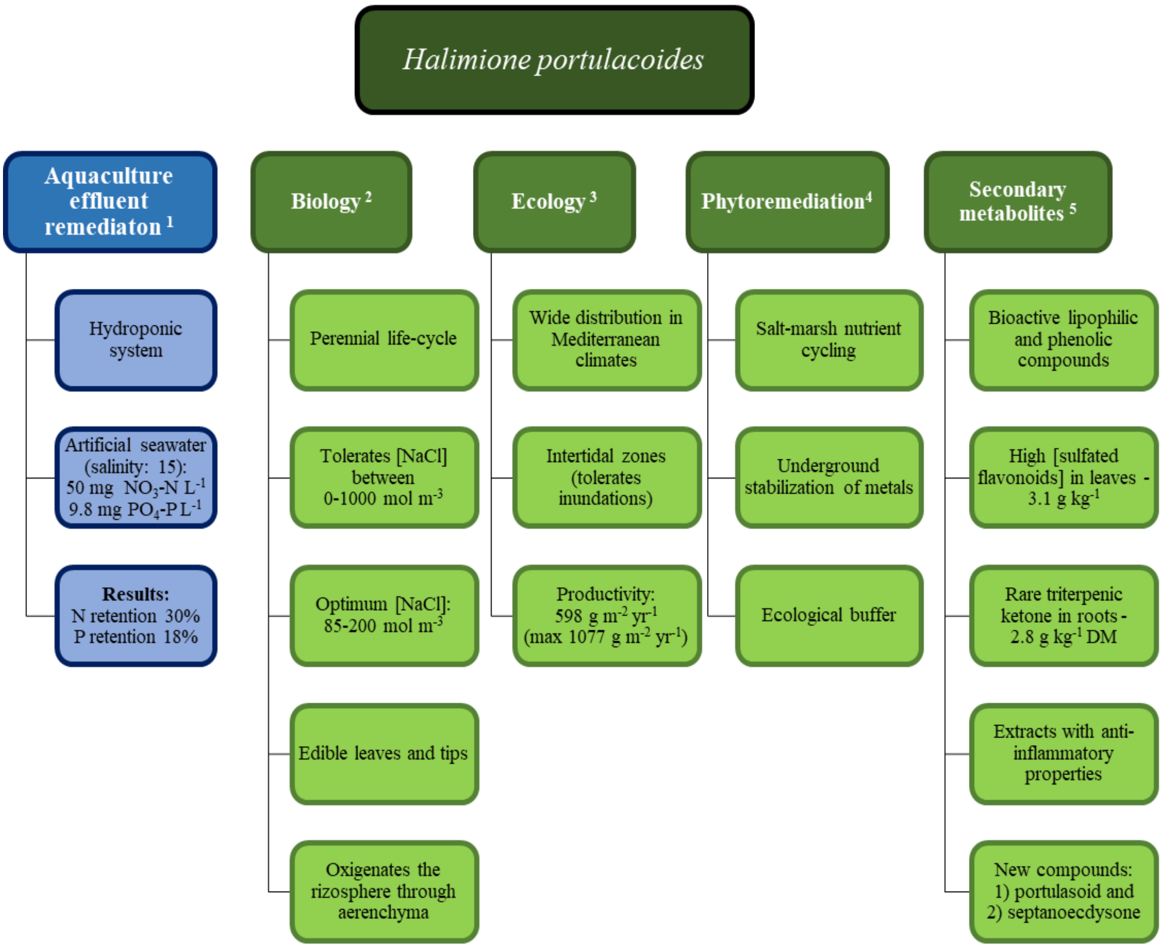


Figure 2.6: Summary of *Halimione portulacoides* relevant characteristics found in the scientific literature. [1]Buhmann et al. (2015); [2]Neves et al. (2007), Waisel (1972); [3]Jensen (1985), Redondo-Gómez et al. (2007); [4]Andrades-Moreno et al. (2013), Cambrollé et al. (2012a,b), Sousa et al. (2010, 2011), Válega et al. (2008a); [5]Ben Nejma et al. (2015), Hill & Connolly (2015), Rodrigues et al. (2014), Vilela et al. (2014).

2.7. Present setbacks and future opportunities

Aquaculture continues to be the fastest-growing industry in the animal food-producing sector and its sustainability has been a major source of discussion (FAO, 2016; Troell et al., 2014). In many regions of the world, including several southern European countries, a significant part of the aquaculture industry is based on semi-intensive farming practices, which are in their essence more sustainable than intensive/super-intensive productions (Bunting, 2013; Edwards, 2015). Nonetheless, economic issues are a setback to the expansion of those production models, usually related to the price of the end-product (which competes in the market with intensively produced ones), the slower capital return, and stakeholders' perception (FAO, 2016). These limit investment and result in the lack of innovation in systems design and process optimization. Additionally, the promotion of public awareness and political support of these production systems are needed (Bostock et al., 2016; Feucht & Zander, 2015).

One of the main challenges faced by these aquaculture practices is how to increase their competitiveness while maintaining their more ecologic modes of seafood production. A new focus on product differentiation and certification, highlighting its origin, sustainability, quality, and health benefits, will likely be the only pathway to balance economic and environmental tradeoffs of semi-intensive aquaculture and drive investment. In this context, future studies using *Halimione portulacoides* as an aquatic biofilter will generate valuable insights on the integration of halophytes in IMTA, contributing to the diversification of aquaculture and sustainable food production.

Besides the technical and biological features of IMTA, research also needs to address social and economic aspects. For IMTA to attain its true potential it needs to be socially accepted and satisfying key stakeholders will be paramount for business success and resilience (Alexander et al., 2016; Chopin, 2017). The relatively low number of studies exploring these questions usually addressed consumer's perspective and it is now evident that they lack knowledge on aquaculture species and production methods, including IMTA (Barrington et al., 2010; Shuve et al., 2009). Yet, they do recognize socio-economic benefits from aquaculture and are concerned with sustainability issues (Barrington et al., 2010; Fernandez-Polanco & Luna, 2012; Whitmarsh & Palmieri 2009). Aquaponics, for example, is regarded as the fittest land-based IMTA for sustainable urban farming (Specht et al., 2014) and a European consumer's survey about that mode of production found a positive attitude towards local products (Milicic et al., 2017). In the same study, willingness to pay regarding food was mostly based on price and whether the products are free of antibiotics, pesticides, and herbicides. These type of studies provides valuable guidance concerning marketing efforts that, in this specific case, should be directed towards local shops and restaurants, emphasizing sustainable and organic-based food production (Goddek et al., 2015). A recent study about European stakeholder's perspective on IMTA, which included industry actors, policy-makers, fishermen and other users of the marine environment, found they positively discriminated IMTA in terms of environmental benefits, creation of new income streams and improvement of the overall negative public image of aquaculture (Alexander et al., 2016). Moreover, IMTA systems can incorporate additional sources of profit, including tourism and educational activities. Junge et al. (2017) outlined that a multi-disciplinary approach to aquaponics is essential to its success and additional actors, others then biologists and engineers, (such as designers, architects, social and health/nutritional scientists) would be important propellers for the socio-economic valorization of the activity. More multi-dimensional valuation studies are needed to assess not only the economic potential of IMTA in general and halophytes in particular but also the ecological and social benefits they can provide to fully understand the scope of IMTA in the future of aquaculture.

Chapter 3

Testing the hydroponic performance of the edible halophyte *Halimione portulacoides*, a potential extractive species for coastal Integrated Multi-Trophic Aquaculture

Submitted for publication

3. Testing the hydroponic performance of the edible halophyte *Halimione portulacoides*, a potential extractive species for coastal Integrated Multi-Trophic Aquaculture

Abstract

Sea purslane *Halimione portulacoides* (L.) Aellen is a candidate extractive species for coastal Integrated Multi-Trophic Aquaculture (IMTA) to recycle the dissolved inorganic nitrogen (DIN) and phosphorus (DIP) wasted by excretive species. To test its suitability, saline aquaculture effluents were simulated in the laboratory using a hydroponics approach to cultivate the plants. Nutrient extraction efficiency, growth performance, and nutritional profile were assessed under a range of DIN and DIP concentrations representing three different aquaculture intensification regimes and using a Hoagland's solution as a control. Over 10 weeks, hydroponic units under non-limited N and P conditions displayed daily extraction rates between 1.5 – 2.8 mg DIN-N L^{-1} day^{-1} and 0.1 – 0.2 mg DIP-P L^{-1} day^{-1} and yielded between 63 - 73 g m^{-2} day^{-1} of *H. portulacoides* biomass. Relatively to biomass produced, *H. portulacoides* extracted between 2.6 – 4.2 mg DIN-N g^{-1} and 0.1 – 0.4 mg DIP-P g^{-1}. The treatment with low-input of DIN and DIP (6.4 mg N L^{-1} and 0.7 mg P L^{-1}) induced some degree of nutrient limitation, as suggested by the extremely high extraction efficiencies of DIN extraction (99 %) in parallel with lower productivity. The nutritional profile of *H. portulacoides* leaves is comparable to that of other edible halophytes and leafy greens and could be a low-sodium alternative to salt in its lyophilized form. From the present study, we conclude that the edible halophyte *H. portulacoides* can be highly productive in hydroponics using saline water irrigation with non-limiting concentrations of DIN and DIP and is, therefore, a suitable extractive species for coastal IMTA in brackish waters.

Keywords: sustainable aquaculture, aquaponics, nutrients, phytoremediation, halophytes

3.1. Introduction

In the European Union, the aquaculture industry must support good ecological status and sustainable economic growth (Science for Environment Policy, 2015) which led to the development of more sustainable aquaculture production models such as Integrated Multi-Trophic Aquaculture (IMTA) systems (Chopin et al., 2012). These systems entail the integration of low trophic groups to recover the relatively high amounts of nutrients wasted in different physical (particulate and dissolved) and chemical forms (organic and inorganic) during the culture cycle of artificially fed organisms (e.g. fish, shrimp) (Chopin et al., 2008).

Many aquaculture fish-species have protein retention efficiencies below 30% (Fry et al., 2018; Ytrestøyl et al., 2015) and previous estimations suggest total nitrogen (N) losses in fish-farms can reach percentages as high as 60 - 80% of total N-input from aquafeeds, while total phosphorus (P) losses can reach 70 - 85% of total P-input (Islam, 2005; Wang et al., 2012). Besides promoting eutrophication, the accumulation of nutrient buildup in the ecosystem can also shift benthic chemistry and disturb ecological interactions (Bannister et al., 2014; Sanz-Lázaro et al., 2011; Sarà et al., 2011; Troell et al., 2009; Valdemarsen et al., 2012), especially if the dilution/carrying capacity of the ecosystem is compromised (Guillen et al., 2019). These lost nutrients, in both particulate and dissolved forms, can be used by non-fed extractive organisms, namely filter-feeders (e.g. bivalves), bottom-feeders (e.g. polychaetes) and primary producers (e.g. plants), through IMTA. Several publications have already addressed IMTA from different scientific perspectives, demonstrating its environmental and economic benefits (e.g. Abreu et al., 2011; Barrington et al., 2010; Buck et al., 2018; Chopin, 2015; Fang et al., 2016; Granada et al., 2016; Hughes & Black, 2016; Kleitou et al., 2018; Knowler et al., 2020; Li et al., 2019).

Several low trophic-level species have already been investigated in terms of their productivity and extraction capacity under IMTA and halophytes are particularly interesting due to their ability to thrive in saline environments (Flowers et al., 1986; Flowers & Colmer, 2008), some with recognized agricultural uses (Panta et al., 2014), which makes them potentially suitable extractive species for IMTA in brackish waters (Gunning et al., 2016). Previous research indicates consistent positive outcomes in terms of productivity and nutrients extraction capacity, using either constructed wetlands or hydroponics/aquaponics systems as halophytes extraction units for IMTA (Custódio et al., 2017).

Still mostly unknown to the general public, some halophyte species are suitable for human consumption (Barreira et al., 2017; Loconsole et al., 2019) and are a rich source of bioactive secondary metabolites with commercial applications (e.g. nutra-, pharma- and cosmeceuticals) (Buhmann & Papenbrock, 2013a; Ksouri et al., 2012; Maciel et al., 2016; Patel et al., 2019; Rodrigues et al., 2014). These features indicate the existence of a rather untapped economic potential that can prompt the integration of the most suitable halophyte species into IMTA production frameworks.

The main objective of the present study is to evaluate the productivity, nutritional profile and nutrient extraction capacity of *Halimione portulacoides* (L.) Aellen, a common edible halophyte of European saltmarshes, in saline hydroponic conditions to understand its horticultural potential for IMTA in brackish waters. To mimic real aquaculture effluents, namely from semi-intensive, intensive and super-intensive aquaculture systems, different combinations of dissolved inorganic nitrogen and phosphorous concentrations were tested, based on values reported in the literature.

3.2. Material and methods

The effect of the different concentrations of dissolved inorganic nitrogen (DIN) and phosphorous (DIP) on *H. portulacoides* growth performance was assessed by quantifying the whole plant biomass, the belowground and aboveground biomasses stems length and number of leaves produced. The nutrient extraction efficiency was assessed by measuring the decrease of NH_4-N, NO_x-N, and PO_4-P concentrations in the hydroponic solution. The nutritional profile of the *H. portulacoides* leaves, the edible organs, was analyzed by a certified laboratory (Mérieux NutriSciences®, Vila Nova de Gaia, Portugal).

3.2.1. Plant material

Halimione portulacoides stems were collected in Ria de Aveiro coastal lagoon (40°38'04.1"N 8°39'40.0"W) in April 2017. Stems were cut-off from healthy fully-grown plants and brought to the laboratory to produce grafts. Grafts with 4 nodes were placed in polyethylene containers with Hoagland's solution under natural conditions of light and temperature to promote root development. Elemental nutrient concentrations in the solution were as follows: 40 mg Ca L^{-1}, 60 mg K L^{-1}, 16 mg Mg L^{-1}, 56 mg N L^{-1}, 16 mg P L^{-1}, 0.28

mg B L^{-1}, 0.03 mg Cu L^{-1}, 1.12 mg Fe L^{-1}, 0.11 mg Mn L^{-1}, 0.34 mg Mo L^{-1}, 0.13 mg Zn L^{-1}.

After three months, in July 2017, rooted grafts underwent a week of acclimation to controlled indoor conditions and salinity before the start of the experiment. Plants were progressively adapted to a salinity of 20 ppt by adding 0.5, 1.0, 1.5, and 2.0% artificial sea salt to the Hoagland's solution consecutively and every two days. Grafts with similar weights were randomly selected and distributed across the experimental hydroponic units.

3.2.2. Experimental setup

Opaque polypropylene boxes (interior volume: 270 x 170 x 170 mm) were used for the hydroponic units and the indoor grow-out experiment lasted 10 weeks. The nutrient extraction efficiency was assessed from weeks 2 to 10 (9 weeks). Experimental hydroponic units were designed to be a deep-water culture type hydroponics. Each unit had a 30 mm thick extruded polystyrene raft floating on the water column. Rafts were perforated with 10 holes (20 mm wide) to insert plants. Plants were fixed in place with natural cotton. An overflow inlet was created (at 110 mm from the bottom) to keep the water volume at 5 L in each unit and the water column was continuously aerated by an air stone connected to a small aerator. Units were refilled with reverse osmosis water to compensate for evapotranspiration as needed. The basis for the nutrient solution was artificial seawater with a salinity of 20 ppt, prepared by dissolving commercial Red Sea salt (Red Sea, Cheddar, UK) in tap water purified by reverse osmosis (V2Pure 360 RO System, TMC, Hertfordshirem UK). The photoperiod was 14 h light: 10h dark and hydroponic units were illuminated by tubular fluorescent white lamps (Philips 54W/830 Min Bipin T5 HO ALTO UNP) delivering an average photosynthetically active radiation (PAR) of ~320 µmol m^{-2} s^{-1} (canopy top), checked twice a week with a spherical micro quantum sensor (US-SQS/L, Heinz Walz, Pfullingen, Germany). Water temperature and pH were measured with a multi-parameter portable meter (ProfiLine pH/Cond 3320, WTW, Weilheim, Germany) and dissolved oxygen was measured with a portable oxygen meter (Oxi 3310, WTW, Weilheim, Germany).

The experimental design consisted of 4 nutrient solutions (including a control solution) and 5 replicates per treatment, in a total of 20 hydroponic units. Two hundred randomly selected plant grafts were distributed across the experimental units, in a total of 10 plants per unit. Plant density was equivalent to 220 plants m^{-2}. The control solution was the modified

Hoagland's solution used for graft development as described above, which guaranteed non-limited nutritional conditions (Control = 56 mg N L^{-1} and 15.5 mg P L^{-1}). The three treatment solutions consisted of different combined concentrations of N and P to represent the wide range of values recorded across the fish-farming intensification continuum, i.e., super-intensive, intensive and semi-intensive land-based marine fish farms. Hoagland's solution, prepared with saline water, was used as a control as it ensures that plants were not limited by nutrients, corresponding in this way to optimal nutritional conditions. The published literature on the remediation of saline aquaculture effluents by halophytes was consulted to select realistic N: P combinations corresponding to semi-intensive (low [N, P]), intensive (medium [N, P]) and super-intensive (high [N, P]) effluents (Buhmann et al., 2015; Lin et al., 2005; Quintã et al., 2015a; Waller et al., 2015; Webb et al., 2013). Treatment labels and theoretical concentrations of N and P chosen as the treatment solutions were: $[N,P]_{low}$ = 6 mg N L^{-1} and 0.8 mg P L^{-1}; $[N,P]_{med}$ = 20 mg N L^{-1} and 3.0 mg P L^{-1}; $[N,P]_{high}$= 100 mg N L^{-1} and 6.0 mg P L^{-1}. All other macro- and micro-nutrients were kept equal across treatments. The detailed elemental composition of experimental treatments is presented in Table A 3.1 in Annex

3.2.3. Hydroponic media analysis

Retention times (RTs; the time wastewater remains in a remediation tank) used in remediation studies are highly variable, spanning from a couple of hours to several days, depending on the desired efficiencies (Toet et al., 2005). In constructed wetlands, higher RTs are positively correlated with higher N and P extraction efficiencies and time recommendations for significant extraction of contaminants are between 3 - 10 days (García et al., 2010; Vera et al., 2016; Wu et al., 2015). Nutrient extraction studies with halophytes in the context of IMTA have used a wide range of RTs, from 12 hours (Marques et al., 2017) to 5 weeks (Buhmann et al., 2015). After taking into consideration the range of RTs used in previous studies and the above recommendations, one week (7 days) was considered a reasonable and operationalizable RT for IMTA to allow for substantial extraction efficiencies.

Hydroponic media samples were collected from the hydroponic units at the end of every extraction period to obtain final N and P concentrations. Initial media samples were collected from each treatment-solution batch to determine the initial N and P concentrations and calculate weekly mass-balances. Each sample was filtered (Whatman GF/C, 1.2 μm pore size) and stored at -20 °C before analysis.

A Skalar San^{++} Continuous Flow Analyzer (Skalar Analytical, Breda, The Netherlands) was used to determine dissolved ammonium (NH_4-N), nitrogen oxides (NO_x-N) and orthophosphate (PO_4-P) concentrations in media samples, using Skalar's standard automated methods for NH_4-N (Modified Berthelot reaction for ammonia determination), NO_x-N (Total UV digestible nitrogen/ nitrate + nitrite/ nitrite) and PO_4-P (Total UV digestible phosphate/ orthophosphate). Dissolved inorganic nitrogen (DIN-N) was calculated as the sum of NH_4-N and NO_x-N and dissolved inorganic phosphorus (DIP-P) corresponded to PO_4-P.

3.2.4. Growth performance

Halophyte grafts were identified, individually photographed, and weighed before being distributed throughout the hydroponic units. Twenty randomly selected rooted grafts from the bunch not selected for the experiment were used to establish the initial weight condition for above- and belowground biomass. At the end of the experiment, plants were again individually photographed, separated into above- and belowground parts, and weighed. Aboveground biomass was further separated into edible (leaves) and non-edible biomass (stems) since the edible biomass was to be analyzed for its nutritional profile. Leaves were pooled per experimental unit and stored at -80 °C until further analysis. Photos were analyzed with an image processing software (ImageJ 1.51) to measure stems and count leaves.

3.2.5. Nutritional profile analysis

Nutritional analysis was carried out on homogeneous samples of the pooled biomass of leaves from each experimental unit to determine the nutritional profile of *H. portulacoides* leaves and assess any potential changes promoted by the availability of N and P. The parameters analyzed were ash, carbohydrates, crude protein, dietary fiber, energy, fat, moisture, sodium, and sugars. All values are presented in grams per 100g of wet weight (WW), except for energy which is presented as kJ per 100g of WW. Nutritional parameters were analyzed by a certified laboratory, following internal analytical procedures (Mérieux NutriSciences, Vila Nova de Gaia, Portugal).

3.2.6. Statistical analysis

Statistical analysis was performed using R v3.4.3 (64-bit) software in combination with R Studio. Data were checked for normality (Shapiro-Wilk test) and homogeneity of variance

(Levene's test) to inform about the appropriate test. One-way ANOVA was used to compare average growth, nutritional and extraction measurements. *Post-hoc* Tukey's HSD test for individual means comparison was performed when significance was observed. Non-parametric Kruskal-Wallis test was used whenever data failed to meet ANOVA assumptions, followed by a Wilcoxon signed-rank test for pairwise comparison if statistical significance was detected. Repeated measures ANOVA was used to assess changes in average extraction efficiencies (%) in time. The Geenhouse-Geisser correction was employed when the sphericity assumption was violated. Bonferroni *posthoc* test was used for pairwise comparison. Significant differences were considered at $p < 0,05$ in all statistical tests.

3.3. Results

3.3.1. Environmental conditions

Average water temperature in hydroponic units was 22.2 ± 1.3 °C, salinity was 20.3 ± 0.3 ppt, dissolved oxygen was 7.2 ± 0.5 mg L^{-1} and PAR (measured at the top of the canopy) was 317.5 ± 52.9 µmol m^{-2} s^{-1}. The average pH, measured at the end of the extraction period, was 7.7 ± 0.1 during the first five weeks. At the end of the week, the pH dropped to values between 6.6 - 6.8 in all treatments, which coincided with an abrupt increase in room temperature overnight due to a failure in the ventilation system. This event resulted in a consequent increase in water temperature of ~2.4 °C above average values registered in the week prior (Table A 3.2). Plants were exposed to an increase in water temperature for a maximum of 16 hours, since solutions were renewed in the following morning, corresponding to the end of a remediation period. From this point on, $[N,P]_{high}$ units consistently displayed an acidic pH ($6 < pH < 7$), contrarily to the other treatments which displayed values > 7 in the following weeks (Table A 3.2). Average pH values in each treatment condition during the entire experimental period were: $[N,P]_{low}$ = 7.5 ± 0.3; $[N,P]_{med}$ = 7.5 ± 0.3; $[N,P]_{high}$ = 6.9 ± 0.8 and Control = 7.4 ± 0.4.

3.3.2. DIN and DIP extraction efficiency

The initial concentrations of DIN-N and DIP-P measured in each treatment solution were as follows: $[N,P]_{low}$ = 6.38 ± 0.15 mg DIN-N L^{-1} and 0.68 ± 0.04 mg DIP-P L^{-1}; $[N,P]_{med}$ = 20.83 ± 0.53 mg DIN-N L^{-1} and 2.76 ± 0.20 mg DIP-P L^{-1}; $[N,P]_{high}$= 101.47 ± 2.66 mg DIN-

N L^{-1} and 5.08 ± 0.24 mg DIP-P L^{-1}; and Control (modified Hoagland's solution) = 55.58 ± 5.99 mg DIN-N L^{-1} and 11.85 ± 0.94 mg DIP-P L^{-1}.

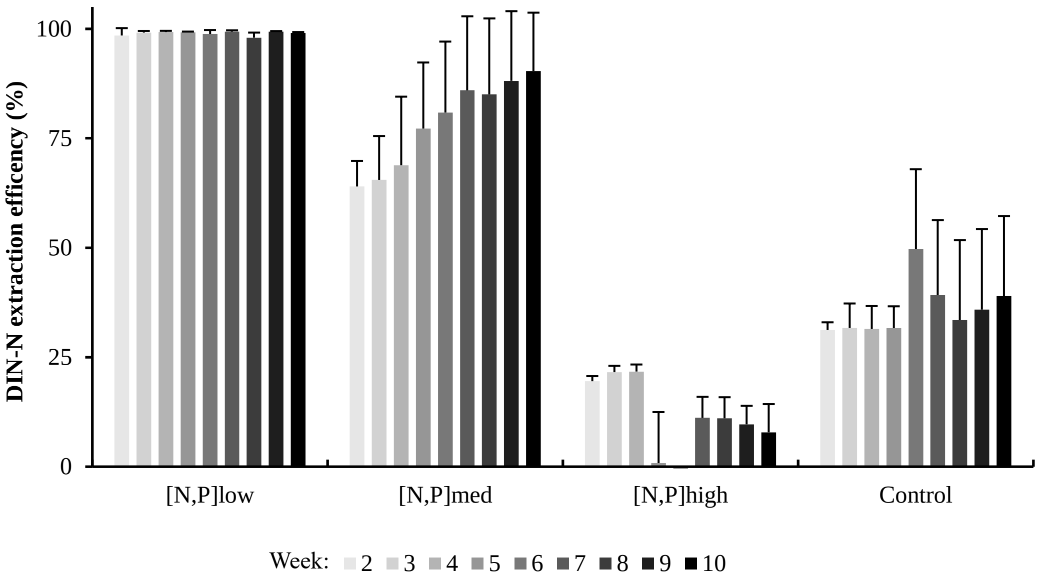
DIN-N extraction efficiencies in each hydroponic unit were measured at the end of each extraction period (Figure 3.1A) and a repeated-measures ANOVA determined that 'treatment', 'time', and the interaction of both factors had a significant main effect ($p < 0.001$) in extraction efficiencies. *Post-hoc* tests revealed that units under $[N,P]_{low}$ and $[N,P]_{med}$ were significantly more efficient on average ($p < 0.004$) than both $[N,P]_{high}$ and Control, a direct result of the lower concentration of DIN-N present in the former treatments. Since an interaction effect of 'treatment' and 'time' was present, statistical analyses were performed to determine differences in efficiency i) between treatments at each time point and ii) within treatments across time-points. Non-parametric Kruskal-Wallis test was used for i) and at all time-points 'treatment' had a significant main effect in DIN-N extraction efficiency ($p < 0.01$). Pairwise comparisons revealed that plants in Control were less efficient ($p < 0.05$) than $[N,P]_{low}$ in weeks 2 - 5 and plants under $[N,P]_{high}$ were less efficient ($p < 0.05$) than both $[N,P]_{low}$ and $[N,P]_{med}$ in weeks 2 - 10. Moreover, changes in DIN-N extraction efficiency across time within each treatment condition were assessed and showed that plants under $[N,P]_{low}$ and Control did not display significant changes in their efficiency over time ($p = 0.35$ and $p = 0.15$, respectively. On the other hand, the factor 'time' significantly affected ($p = 0.01$) the extraction efficiencies of plants under $[N,P]_{med}$ and $[N,P]_{high}$. The pairwise comparison revealed that $[N,P]_{med}$ units were more efficient ($p < 0.05$) in week 9 compared with week 2, and $[N,P]_{high}$ units were less efficient ($p < 0.05$) in week 6 compared with all other weeks, except week 5.

In terms of the total levels of DIN-N extracted (Figure 3.2A), the Control units extracted the most, with a total of 882.4 ± 284.8 mg (= 2.8 ± 0.9 mg L^{-1} day^{-1}) extracted, followed by $[N,P]_{med}$ units which extracted a total of 736.8 ± 125.9 mg (= 2.3 ± 0.4 mg L^{-1} day^{-1}). The $[N,P]_{high}$ units extracted significantly less DIN-N than Control ($p = 0.01$), with a total 483.8 ± 179.5 mg (= 1.5 ± 0.6 mg L^{-1} day^{-1}). The $[N,P]_{low}$ units removed 284.3 ± 0.5 mg, practically the total amount of supplied DIN-N, indicating the onset of N-limitation during the extraction period and cannot be compared with the other treatments. Overall $[N,P]_{low}$ units extracted, on average, 99 % of the total N input, $[N,P]_{med}$ units extracted 79 %, $[N,P]_{high}$ units 11 % and Control units 35 %. The normalization of total DIN-N extracted by the total biomass produced (Figure 3.3A) suggests that certain nutritional conditions might promote higher extraction rates per unit of biomass. $[N,P]_{med}$ and Control units extracted on average 4.2 (± 0.3) and 4.2 (± 0.6) mg DIN-N g^{-1} of biomass gain respectively, which was significantly more ($p < 0.05$) than $[N,P]_{high}$ (2.5 ± 0.7 mg DIN-N g^{-1}). $[N,P]_{low}$ also displayed lower rates but due to the total depletion of DIN-N during the extraction period.

Regarding DIP-P extraction results, repeated measures ANOVA determined that 'treatment' and the interaction of 'treatment' with 'time' had a significant main effect ($p < 0.001$) in the extraction efficiencies of DIP-P (Figure 3.1B). *Post-hoc* tests revealed that all treatments significantly differed between each other in terms of average extraction efficiency ($p < 0.04$), with $[N,P]_{low}$ and $[N,P]_{med}$ displaying the highest efficiencies (associated with the lower concentrations of DIP-P in those treatments compared with $[N,P]_{high}$ and Control). Kruskal-Wallis test, performed at each time-point, revealed that 'treatment' had a significant effect in the extraction efficiencies ($p < 0.01$) and pairwise comparisons showed that Control removed significantly less DIP-P ($p < 0.05$) than $[N,P]_{low}$ at all time-points and $[N,P]_{med}$ from weeks 2 - 6. Units under $[N,P]_{high}$ removed significantly less ($p < 0.05$) than $[N,P]_{low}$ from weeks 5 - 10. Moreover, 'time' had a significant main effect ($p < 0.02$) in extraction efficiencies within all treatments. Pairwise comparisons revealed that the extraction efficiency in $[N,P]_{low}$ units was significantly lower ($p < 0.05$) only in week 2 compared with the other weeks, and $[N,P]_{med}$ did not display significant changes on efficiencies between extraction periods. $[N,P]_{high}$ units were significantly less efficient ($p < 0.05$) in week 8 compared with weeks 4 and 9, and Control units were less efficient ($p < 0.05$) in week 2 compared with week 9.

In terms of the total quantities of DIP-P extracted (Figure 3.2B), the Control units extracted a total of 29.3 ± 22.6 mg (= 0.09 ± 0.07 mg L^{-1} day^{-1}), which was significantly less ($p < 0.01$) than $[N,P]_{med}$, with a total of 65.3 ± 11.2 mg (= 0.21 ± 0.04 mg L^{-1} day^{-1}) extracted. $[N,P]_{high}$ units extracted 46.6 ± 8.5 mg (0.15 ± 0.03 mg L^{-1} day^{-1}) and $[N,P]_{low}$ extracted 26.1 ± 2.9 mg (0.08 ± 0.01 mg L^{-1} day^{-1}), which was close to total input suggesting possible P-limitation during the experimental period. Overall $[N,P]_{low}$ units extracted, on average, 85 % of the total P input, $[N,P]_{med}$ units extracted 52 %, $[N,P]_{high}$ units 20 % and Control units 5 %. After normalizing total DIP-P extracted by the total biomass produced (Figure 3.3B), $[N,P]_{med}$ emerged as the condition with the highest rate of DIP-P extracted, 0.37 (± 0.03) mg DIP-P g^{-1}, significantly higher ($p < 0.05$) than the other treatments.

A



B

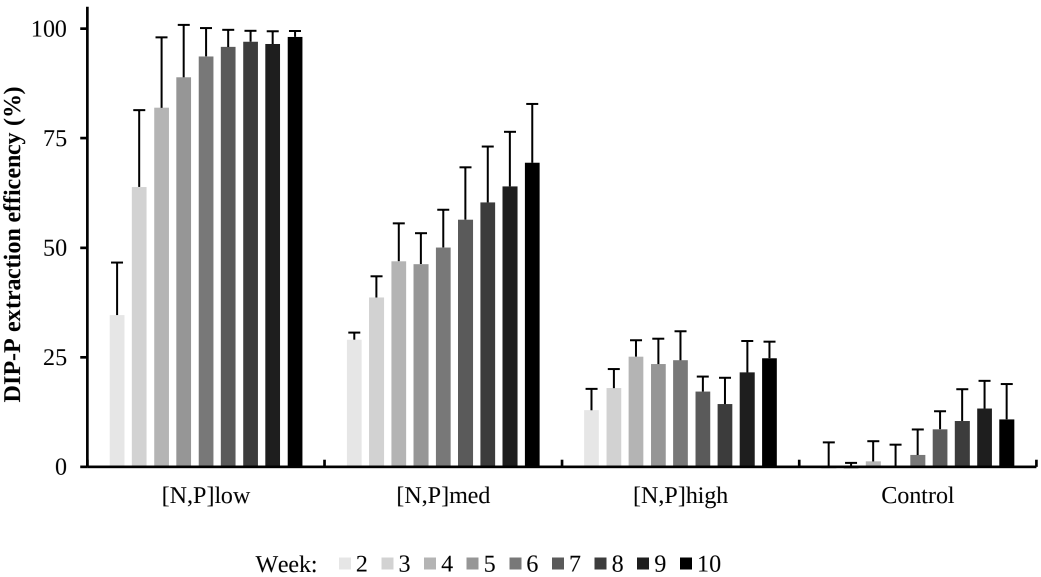


Figure 3.1: Weekly extraction efficiencies of DIN-N (A) and DIP-P (B). Bars represent standard deviations. Test-statistic: Repeated measures ANOVA (statistical results in 'Results' section). Treatments: $[N,P]_{low}$ = 6 mg N L^{-1} & 0.8 mg P L^{-1}; $[N,P]_{med}$ = 20 mg N L^{-1} & 3.0 mg P L^{-1}; $[N,P]_{high}$= 100 mg N L^{-1} & 6.0 mg P L^{-1}; Control = 56 mg N L^{-1} & 15.5 mg P L^{-}1.

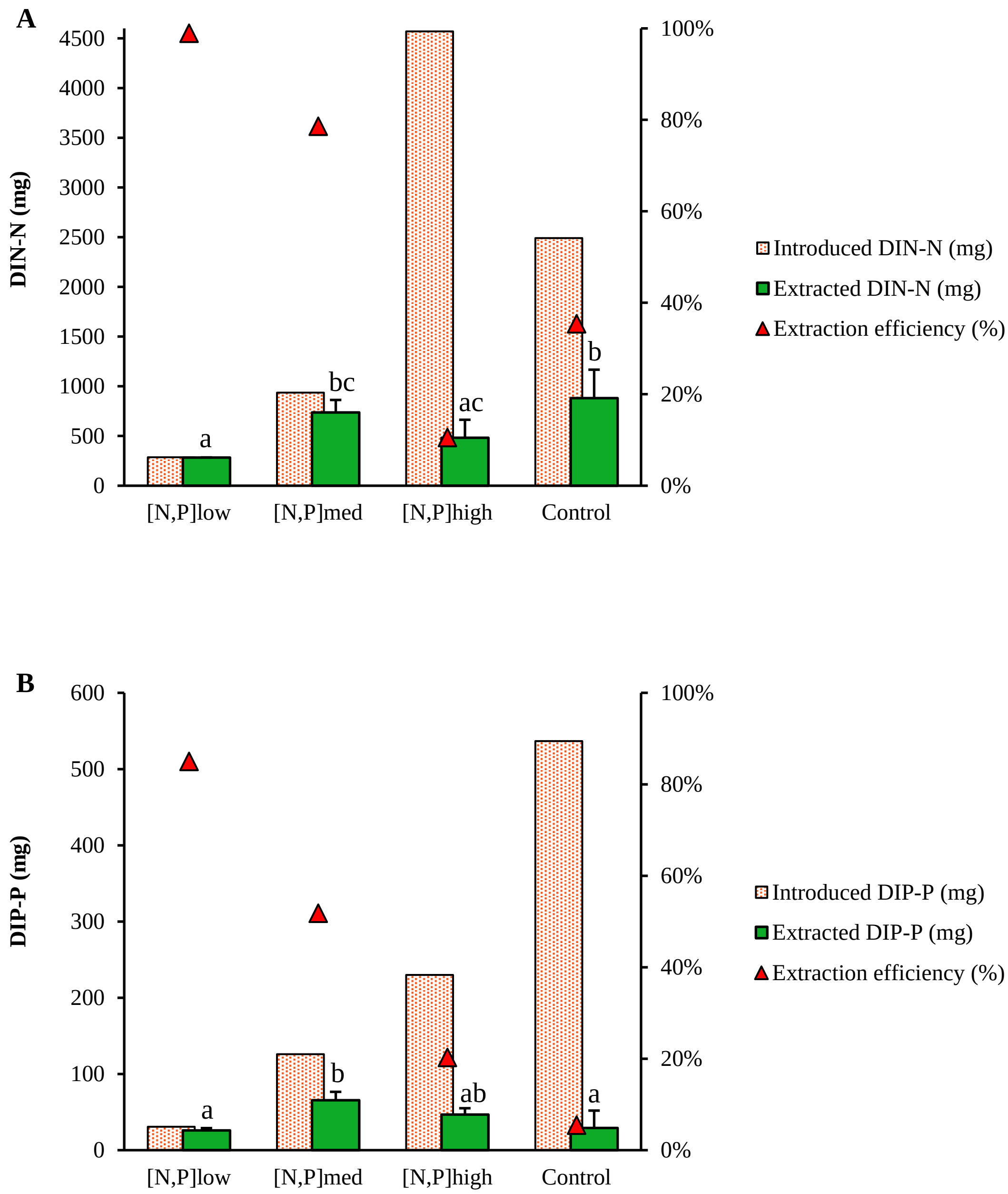


Figure 3.2: Total extracted DIN-N (A) and DIP-P (B) from week 2 to week 10 (9 weeks). Vertical bars represent the standard deviations. Test-statistic: One-way ANOVA & Tukey HSD's test for pairwise comparison, with different letters showing significant differences in 'Extracted DIN-N' between treatments ($p < 0,05$). Treatments: $[N,P]_{low}$ = 6 mg N L^{-1} & 0.8 mg P L^{-1}; $[N,P]_{med}$ = 20 mg N L^{-1} & 3.0 mg P L^{-1}; $[N,P]_{high}$= 100 mg N L^{-1} & 6.0 mg P L^{-1}; Control = 56 mg N L^{-1} & 15.5 mg P L^{-1}.

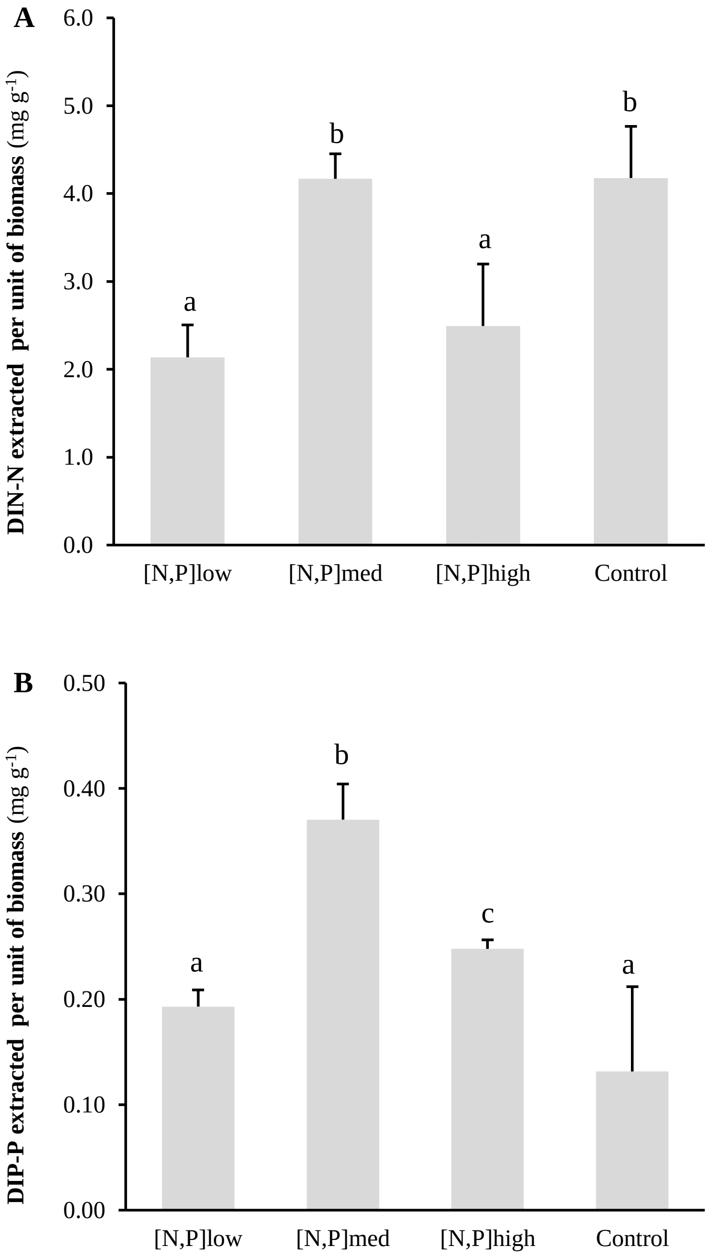


Figure 3.3: Extracted DIN-N (A) and DIP-P (B) per unit of biomass produced. Vertical bars represent the standard deviations. Test-statistic: One-way ANOVA & pairwise Tukey HSD's test (DIN-N) and non-parametric Kruskal-Wallis test & pairwise Wilcoxon signed-rank test (DIP-P), with different letters showing significant differences between treatments ($p < 0,05$). Treatments: $[N,P]_{low}$ = 6 mg N L^{-1} & 0.8 mg P L^{-1}; $[N,P]_{med}$ = 20 mg N L^{-1} & 3.0 mg P L^{-1}; $[N,P]_{high}$= 100 mg N L^{-1} & 6.0 mg P L^{-1}; Control = 56 mg N L^{-1} & 15.5 mg P L^{-1}.

3.3.3. Growth performance

Growth parameters were determined for each hydroponic unit by pooling measurements from each individual (n = 10). Each group started the experimental grow-out period with average initial biomass between 44.6 and 49.3 g per hydroponic unit (Table 3.1). At week 10, the Control displayed the highest total biomass (279.4 ± 44.7 g), which was significantly higher ($p = 0.02$) than $[N,P]_{low}$ (195.9 ± 28.7 g) (Table 3.1).

Vegetative development over the experimental period can be visualized in Figure 3.4. The $[N, P]_{low}$ units yielded a significantly lower aboveground biomass (155.2 ± 16.7 g) compared with Control (245.4 ± 40.4 g; $p = 0.003$), $[N, P]_{med}$ (216.3 ± 36.5; $p = 0.048$) as well as $[N, P]_{high}$ (228.6 ± 35.4 g; $p = 0.015$). The belowground biomass and the root: shoot ratio of plants growing under $[N, P]_{low}$ were higher than the other treatments, but only root: shoot differences statistically significant ($p < 0.0001$). Results suggest the higher ratio resulted from a lower aboveground development rather than a higher belowground development. The number of leaves was lowest in $[N,P]_{low,}$ but differences were not significant. The total sum of stems length was significantly lower in $[N,P]_{low}$ compared with Control ($p = 0.002$) and the other treatments ($p < 0.05$).

Table 3.1: Growth performance of *Halimione portulacoides* hydroponic units (mean ± standard deviation). Values presented are pooled measurements of the individual plants in each hydroponic unit. Test-statistic: One-way ANOVA & Tukey HSD's test for pairwise comparison, different letters indicate statistically significant differences between treatments: $p < 0,05$. Treatments: $[N,P]_{low}$ = 6 mg N L^{-1} & 0.8 mg P L^{-1}; $[N,P]_{med}$ = 20 mg N L^{-1} & 3.0 mg P L^{-1}; $[N,P]_{high}$= 100 mg N L^{-1} & 6.0 mg P L^{-1}; Control = 56 mg N L^{-1} & 15.5 mg P L^{-1}.

			$[N,P]_{low}$	$[N,P]_{med}$	$[N,P]_{high}$	Control
Initial biomass		g $unit^{-1}$	44.6 ± 6.0	47.4 ± 6.3	49.3 ± 6.2	48.6 ± 2.1
Final biomass	Total	g $unit^{-1}$	195.9 ± 28.7 ^{a}	245.5 ± 39.7 ^{ab}	257.6 ± 40.8 ^{ab}	279.4 ± 44.7 ^{b}
	Aboveground	g $unit^{-1}$	155.2 ± 16.7 ^{a}	216.3 ± 36.5 ^{b}	228.6 ± 35.4 ^{b}	245.4 ± 40.4 ^{b}
	Belowground	g $unit^{-1}$	40.3 ± 12.9	28.9 ± 4.5	29.0 ± 5.5	32.0 ± 6.7
Total productivity		g m^{-2} day^{-1}	48.0 ± 7.9 ^{a}	62.9 ± 13.7 ^{ab}	66.1 ± 11.2 ^{ab}	73.3 ± 14.5 ^{b}
Root: shoot ratio			0.26 ± 0.06 ^{a}	0.13 ± 0.02 ^{b}	0.13 ± 0.01 ^{b}	0.13 ± 0.02 ^{b}
Leaves count		n $unit^{-1}$	1658 ± 167	1879 ± 103	2008 ± 190	1958 ± 277
Stems length (sum)		m $unit^{-1}$	1.83 ± 0.12 ^{a}	2.29 ± 0.26 ^{b}	2.39 ± 0.22 ^{b}	2.51 ± 0.32 ^{b}

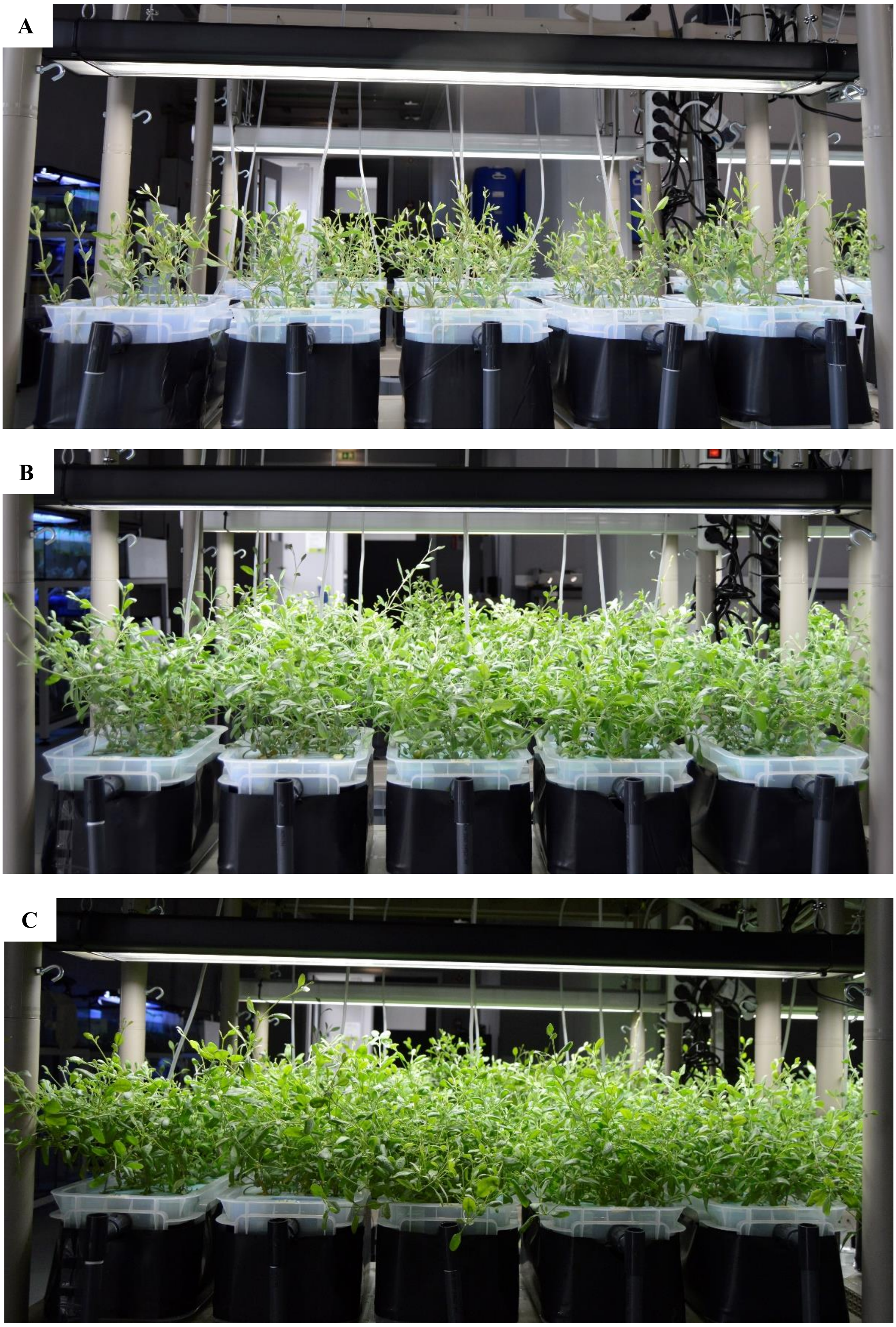


Figure 3.4: Overview of experimental hydroponic units at week 1 (A), week 5 (B) and week 10 (C).

3.3.4. The nutritional profile of leaves

To the authors' best knowledge, the nutritional profile of *H. portulacoides* edible leaves was analyzed for the first time in the present study. Results from the nutritional analysis are summarized in Table 3.2. The fresh leaves of *H. portulacoides* displayed a water content of 90 % and their average nutritional profile (Control condition) was as follows: ash = 3.5 g $100g^{-1}$, carbohydrates = <0.05 g $100g^{-1}$, dietary fibers = 3.3 g $100g^{-1}$, fat = 0.3 g $100g^{-1}$, protein = 2.0 g $100g^{-1}$, sodium = 0.8 g $100g^{-1}$ and sugars = 0.3 g $100g^{-1}$. Moreover, 100 g of fresh leaves yield 76.5 kJ (or 18.5 kcal) of energy.

Protein and sodium concentrations in the leaves of *H. portulacoides* were significantly affected by the experimental conditions. Protein was significantly lower ($p < 0.001$) in the leaves of $[N,P]_{low}$ treated plants (1.5 g $100g^{-1}$ wet weight (WW)), compared to the Control and the other two treatments (2.0 – 2.1 g $100g^{-1}$ WW). Sodium concentration was significantly lower ($p < 0.02$) in the leaves of $[N,P]_{high}$ treated plants (0.6 g $100g^{-1}$ WW), compared with Control and the other treatments (0.8 g $100g^{-1}$ WW). The possibility of a dilution effect in the total edible aboveground biomass was assessed by calculating the total amount of protein and sodium in each unit. The protein content in the total fresh edible biomass collected from $[N,P]_{low}$ units (2.0 ± 0.3 g) was also significantly lower ($p < 0.01$) than Control (4.3 ± 0.9 g), $[N,P]_{med}$ (3.5 ± 0.6 g) and $[N,P]_{high}$ (4.0 ± 0.5 g). The sodium content in the total fresh edible biomass of $[N,P]_{high}$ units did not differ in absolute quantities (1.1 ± 0.1 g) compared with the other treatments, contrary to its concentration values, suggesting a dilution of sodium. Only $[N,P]_{low}$ units (1.0 ± 0.2 g) were significantly lower ($p = 0.01$) than Control (1.6 ± 0.4 g). $[N,P]_{med}$ displayed a total of 1.4 ± 0.3 g of sodium in its edible biomass.

Halimione portulacoides grown hydroponically indoors displayed a distinct visual phenotype compared to its wild counterparts (Figure 3.5). Specimens of *H. portulacoides* grown indoors are greener than conspecific plants in the wild and both their leaves and stems show a more delicate phenotype, as they appear thinner and less lignified.

Table 3.2: Nutritional parameters from *Halimione portulacoides* leaves. Test-statistic: One-way ANOVA & Tukey HSD's test for pairwise comparison, different letters indicate statistically significant differences between treatments: $p < 0,05$. Treatments: $[N,P]_{low}$ = 6 mg N L^{-1} & 0.8 mg P L^{-1}; $[N,P]_{med}$ = 20 mg N L^{-1} & 3.0 mg P L^{-1}; $[N,P]_{high}$= 100 mg N L^{-1} & 6.0 mg P L^{-1}; Control = 56 mg N L^{-1} & 15.5 mg P L^{-1}.

		$[N,P]_{low}$	$[N,P]_{med}$	$[N,P]_{high}$	**Control**
Ash (inorganic matter)	g $100g^{-1}$ WW	3.63 ± 0.15	3.64 ± 0.13	3.50 ± 0.13	3.53 ± 0.08
Carbohydrates	g $100g^{-1}$ WW	<0.5*	<0.5*	<0.5*	<0.5*
Dietary fiber	g $100g^{-1}$ WW	2.70 ± 0.68	2.76 ± 0.43	2.84 ± 0.54	3.30 ± 0.30
Energy	kJ $100g^{-1}$ WW	75.7 ± 14.6	73.4 ± 10.9	77.3 ± 13.4	76.5 ± 5.8
Fat†	g $100g^{-1}$ WW	0.32 ± 0.05	0.26 ± 0.04	0.30 ± 0.14	0.33 ± 0.07
Moisture	g $100g^{-1}$ WW	90.7 ± 0.8	90.7 ± 0.4	90.5 ± 0.8	90.4 ± 0.2
Protein	g $100g^{-1}$ WW	1.50 ± 0.04 [b]	1.97 ± 0.13 [a]	2.09 ± 0.10 [a]	2.01 ± 0.12 [a]
Sodium	g $100g^{-1}$ WW	0.77 ± 0.10 [a]	0.76 ± 0.02 [a]	0.61 ± 0.08 [b]	0.75 ± 0.04 [a]
Sugars	g $100g^{-1}$ WW	0.30 ± 0.10	0.36 ± 0.05	0.36 ± 0.05	0.30 ± 0.07

WW – wet weight.
* Below equipment detection limit.
† Non-parametric Kruskal-Wallis test (normality assumption violated)
[a,b] Different letters indicate statistically significant differences between treatments ($p < 0.05$).

Figure 3.5: *Halimione portulacoides* visual characteristics in indoor hydroponics (A; present experiment) and in the wild (B; Aveiro lagoon).

3.4. Discussion

3.4.1. Extraction efficiency and productivity

Most of the studies available in the literature which assessed halophytes' extraction capacity in the context of IMTA have been performed using constructed wetlands or aquaponics (using inert media) and only a few have used soilless hydroponic systems (Custódio et al., 2017). Nonetheless, a general observation (regardless of the plant extraction module employed) is the difficulty to reliably compare extraction efficiencies from different studies due to a great diversity of production variables potentially affecting performance.

Using hydroponic modules, Marques et al. (2017) exposed *H. portulacoides* to real aquaculture effluents with DIN and DIP concentrations very similar to those of $[N,P]_{low}$ conditions (salinity of 20 ppt; 9 mg DIN-N L^{-1} and 0.3 mg DIP-P L^{-1}), but using a much smaller RT (12 hours) than the present study, achieving a 65% reduction in DIN, yet DIP increased by 27% probably due to mineralization of organic matter. Under the same conditions of inorganic N and P, in the present study, *H. portulacoides* removed 99% of DIN and 85% of DIP with an RT of one week. In another experiment, Buhmann et al. (2015) exposed *H. portulacoides* to N and P concentrations similar to the Control treatment using a much longer RT (5 weeks) and achieved a 58% reduction in DIN and a 51% reduction in DIP. Under similar nutrient conditions, *H. portulacoides* in the present study extracted at best around 20% of DIN and 10% of DIP. Other species have shown limited extraction capacity when RTs are not high enough given the high amount of nutrients in the water. For instance, Waller et al. (2015) irrigated *Aster tripolium* L. and *Salicornia dolichostachya* Moss with an artificial effluent with 19 mg N L^{-1} and 3 mg P L^{-1} (similar to $[N,P]_{med}$) employing an RT of 1 day but DIP extraction efficiencies were practically 0%, and only S. *dolichostachya* was capable of effectively removing DIN with a 20% efficiency. These different results, even when using the same species, show the importance of choosing, for instance, the appropriate hydraulic retention times depending on the availability of nutrients to promote a substantial extraction of N and P, since the extraction capacity rate of plants can become saturated as observed in the present study.

By testing a wide range of N and P concentrations in this study, it was possible to estimate the extraction capacity of *H. portulacoides* units and, excluding the $[N,P]_{low}$ results (due to evidence of nutrient limitation), daily extraction rates varied between 1.5 – 2.8 mg DIN-N L^{-1} day^{-1} and 0.1 – 0.2 mg DIP-P L^{-1} day^{-1}. Relatively to biomass production, *H.*

portulacoides extracted between 1.8 – 3.1 mg DIN-N g^{-1} and 0.1 – 0.3 mg DIP-P g^{-1} of biomass produced.

Regarding growth, *H. portulacoides* was affected by the available concentrations of DIN and DIP in the solution, as differences in biomass allocation and a reduction in vegetative growth were observed in $[N,P]_{low}$ units compared to the other conditions. Plants in $[N,P]_{low}$ units favored root development instead of vegetative development and showed a decline in total productivity. Prioritizing the allocation of resources towards increasing root area at the expense of aerial growth to boost nutrient uptake is typically observed in plants exposed to nutrient-limited conditions (Ågren & Franklin, 2003; Bonifas et al., 2005; Gedroc et al., 1996; Levang-Brilz & Biondini, 2003) and the evidence from this study suggest plants in $[N.P]_{low}$ were nutrient-limited. Because N and P are both essential elements involved in numerous biological and physiological processes in plants (e.g. genetic material, transfer of energy), the low availability of those limiting nutrients will constrain plant development (Hopkins and Huner, 2008). In the other experimental conditions, *H. portulacoides* displayed similar productivity and aerial development suggesting that plants were not nutrient-limited at concentrations of at least 20 mg DIN-N L^{-1} and 3 mg DIP-P L^{-1}.

Under non-limited nutrient conditions, *H. portulacoides* displayed a productivity range between 63 – 73 g m^{-2} day^{-1} (88% is aboveground biomass). To compare the productivity of *H. portulacoides* with other studies, yields reported in other publications are converted to *g* m^{-2} $day^{-,}$ calculated from the published data. In other hydroponic studies, *H. portulacoides* displayed total productivity values substantially lower (< 35 g m^{-2} day^{-1}) (Buhmann et al., 2015) and substantially higher (112 g m^{-2} day^{-1}) (Marques et al., 2017) than the present study. Again, reliable comparisons are difficult to make since the experimental conditions in the different studies were considerably different, including the availability of nutrients, hydraulic factors (e.g. RTs), and light (e.g. PAR), which affect plant development. In the wild, *H. portulacoides* aboveground productivity at the peak of the growing season (Spring), productivity was measured at 3 g m^{-2} day^{-1} in a saltmarsh in the south of Portugal (Neves et al., 2007). Overall, the productivity of *H. portulacoides* seems to fluctuate considerably, due to environmental conditions and ecological factors such as nutrient availability and competition for resources (Emery et al., 2001; Morzaria-Luna & Zedler, 2014), which also explain the differences in productivity between natural and controlled hydroponic conditions.

Other halophytes with economic potential have also been studied in the context of hydroponics/aquaponics to assess their suitability for IMTA (Custódio et al., 2017). Boxman et al. (2017) tested the productivity of *Sesuvium portulacastrum* (L.) L. and *Batis maritima* L. irrigated with platyfish (*Xiphophorus* sp.) aquaculture effluents (~ 6 - 12 mg DIN-N L^{-1}) and obtained yields of 17.7 and 10.7 g m^{-2} day^{-1} respectively. Irrigated with a red drum (*Sciaenops ocellatus*) effluent (~ 10 - 70 mg DIN-N L^{-1}), *S. portulacastrum* and *B. maritima* displayed total productivity of 3.5 and 1.1 g m^{-2} day^{-1} of dry weight respectively (Boxman et al., 2018). These were substantially poorer performances compared with *H. portulacoides* in this study. *Aster tripolium* and *S. dolichostachya* irrigated with a European seabass (*Dicentrachus labrax*) effluent (~ 19 mg DIN-N L^{-1} and 3 mg DIP-P L^{-1}) displayed total productivities of 35.0 g m^{-2} day^{-1} for *A. tripolium* and 86.0 g m^{-2} day^{-1} for S. *dolichostachya* (Waller et al., 2015). *Sarcocornia ambigua* (Michx.) M.A.Alonso & M.B.Crespo irrigated with a Pacific white shrimp (*Litopenaeus vannamei*) effluent (~ 22 mg DIN-N L^{-1} and 5 mg DIP-P L^{-1}) produced 112.3 g m^{-2} day^{-1} (Pinheiro et al., 2017). Differences in growth performance between studies using different species can be associated with their different life-cycles and species-specific physiological adaptations which are modulated in different manners by the combination of biotic and abiotic factors (Crain et al., 2004; Silvestri et al., 2005; Veldkornet et al., 2016) but also by broadly different experimental conditions (e.g. location, RT, PAR, salinity, planting density, space available for growth, grow-out time, etc.). Therefore, the specificity of extraction units should be taken into consideration when comparing the performance of plants.

Deciding on the appropriate trade-off between nutrient extraction efficiency and productivity is paramount for an extraction unit to be effective. As observed in this study, an effluent with relatively low availability of nutrients, despite allowing for potentially high extraction efficiencies, will decrease total productivity if RTs are too long. The connectivity between IMTA functional groups (excretive species and extractive species) must be intentionally managed to optimize productivity while maximizing nutrient uptake which is the main purpose of multi-trophic integration. Moreover, the development of an IMTA technical standard is necessary for research and commercial purposes to allow for reliable comparisons between systems and enable the social and economic potential of IMTA.

3.4.2. Nutritional profile

The nutritional composition of the edible parts (a blend of leaves and tips) was described for the first time in *H. portulacoides*. Even though secondary metabolites with antioxidant properties have been previously measured (Boestfleisch & Papenbrock, 2017), to our best knowledge no previous publications are presenting the proximate composition. The nutritional profiling of plants under the different treatments demonstrate that the concentration of some nutritional compounds is affected by the availability of nutrients in the solution.

Protein content was significantly lower in $[N,P]_{low}$ treated plants and a decrease in protein is a typical symptom of N-limitation (Geary et al., 2015; Hopkins & Huner, 2008), which further confirms the state of nutrient limitation of *H. portulacoides* in that condition. Sodium content was found at lower concentrations in $[N,P]_{high}$ treated plants, but a dilution effect (in the total aboveground biomass) could partially explain this observation since the absolute values of sodium in $[N,P]_{high}$ were not significantly different from other treatments. Nonetheless, lower accumulation of sodium in plant tissues, when N is available at very high concentrations, has been previously observed in glycophytes (ryegrass and barley) and the halophyte *Spartina alterniflora* Loisel. (Hessini et al., 2009; Kant et al., 2007; Sagi et al., 1997).

Halimione portulacoides leaves can be consumed either as a fresh product or processed as biosalt, an approach already employed for other commercially available halophytes (Feng et al., 2013; Loconsole et al., 2019). The nutritional profile of leaves, both in their fresh and dried format, is described and compared with analogous products such as other halophyte species (*Salicornia* spp.), two leafy greens, a seaweed, and regular table salt (Table 3.3). The reference nutritional composition for *H. portulacoides* is assumed to be the one resulting from plants irrigated with the control solution.

In its raw format, *H. portulacoides* leaves present the lowest carbohydrates content and the highest dietary fiber content compared with the other products. In all products, sugars and fat contents are < 0.6% and protein content ranges between 1.5% (*S. bigelovii*) and 2.9% (kelp). In terms of sodium, *S. bigelovii* has the highest percentage (1%), *H. portulacoides* comes in second (0.8%) followed by kelp (0.2%). The remaining products have residual amounts of sodium. In its dry format, *H. portulacoides* has more inorganic matter (ash), lipids, and protein contents than *Salicornia* spp. In terms of sodium, dried *S. ramosissima* has the highest amount (9.0%), followed by *H. portulacoides* (7.8%) and *S. perennis* (6.4%).

Regular table salt content in sodium is 4 to 5 times higher than in dehydrated halophytes, therefore these plants could be used as low-sodium alternatives to salt for culinary purposes. Nonetheless, sodium is still present in relatively higher amounts in halophytes than other plants and, following the World Health Organization recommendation of < 2 g day^{-1} of sodium (WHO, 2012), a healthy adult can consume a maximum of 270 g of fresh *H. portulacoides* leaves per day (25 g dried).

Halimione portulacoides grown hydroponically indoors displayed distinct morphological features (phenotypes) when compared to wild specimens, namely a greener pigmentation and thinner leaves and stems. Phenotypic plasticity might explain those differences, as plants must adapt to sometimes very different indoor conditions (Palacio-López et al., 2015). Since indoor conditions lack many of the natural environmental stimuli that shape the plant's "natural" phenotype, indoor plants can feature distinct morphological and physiological adaptations, such as higher specific leaf area and higher leaf N concentration, compared with their wild counterparts (Poorter et al., 2016). For example, *Arabidopsis thaliana* (L.) Heynh. grown indoor displayed larger leaves with different shapes and longer petioles, as well as 25-35% more total chlorophyll content and 30% fewer xanthophyll pigments than field-grown plants (Mishra et al., 2012). A major environmental stimulus that greatly dictates the morphology of plants is the wind, as it promotes shorter and thicker leaves and stems to reduce aerodynamic drag and increase mechanical strength (Onoda & Anten, 2011; Wu et al., 2016). Lack of wind stimulation promotes longer, thinner leaves and stems in indoor plants, as observed in *H. portulacoides.* From a product development perspective, phenotypic plasticity of plants can be advantageous to producers, as it provides the possibility to tailor sensory and functional traits (e.g. color, texture, secondary metabolites) of cultured plants and, as such, contribute to add more value to products (Marondedze et al., 2018).

Table 3.3: Nutritional profile of *Halimione portulacoides* leaves and other comparable food items.

	Ash g 100g^{-1}	Carbohydrates g 100g^{-1}	Dietary fiber g 100g^{-1}	Energy kJ 100g^{-1}	Fat g 100g^{-1}	Moisture g 100g^{-1}	Protein g 100g^{-1}	Sodium g 100g^{-1}	Sugars g 100g^{-1}	Ref.
Table salt	-	-	-	-	-	-	-	38.76	-	USDA 2019
Fresh product										
Halimione portulacoides	3.53 ± 0.08	<0.5	3.30 ± 0.30	76.5 ± 5.8	0.33 ± 0.07	90.4 ± 0.2	2.01 ± 0.12	0.75 ± 0.04	0.30 ± 0.07	present study
Salicornia bigelovii	4.36 ± 0.37	4.48 ± 0.46	0.83 ± 0.13 (crude fiber)	-	0.37 ± 0.01	88.4 ± 1.4	1.54 ± 0.10	1.00 ± 0.71	-	Lu et al. 2010
Kelp (seaweed)	-	9.57	1.3	179.9	0.56	81.58	1.68	0.23	0.6	USDA 2019
Spinach (Spinacia oleracea)	-	3.63	2.2	96.2	0.39	91.4	2.86	0.08	0.42	USDA 2019
Watercress (*Nasturtium officinale*)	1.20	1.29	0.5	46	0.10	95.11	2.30	0.04	0.20	USDA 2019
Dried product										
Halimione portulacoides	36.67 ± 1.05	-	34.22 ± 2.90	793.4 ± 59.9	3.40 ± 0.69	0	20.87 ± 1.14	7.82 ± 0.38	3.10 ± 0.69	present study
Salicornia ramosissima	29.2 ± 0.6	-	-	-	1.87 ± 0.18	0	5.20 ± 0.29	8.99 ± 0.05	-	Barreira et al. 2017
Sarcocornia perennis	23.3 ± 0.3	-	-	-	2.25 ± 0.05	0	6.9 ± 0.7	6.41 ± 0.09	-	Barreira et al. 2017

3.5. Conclusions

The capacity of *H. portulacoides* to extract a substantial amount of DIN and DIP from saline effluents was experimentally demonstrated in the present study. Moreover, *H. portulacoides* leaves present a nutritional profile very similar to that of some leafy greens and other commercial halophytes and with low amounts of sodium (-80%) compared with regular table salt, making it a suitable vegetable for human use with economic potential. The integration of *H. portulacoides* in aquaculture systems can, therefore, promote the eco-intensification of coastal aquaculture in brackish waters, decreasing the loss of dissolved nutrients to the environment and increasing biomass production per unit of feed input with little additional production costs. Promoting halophytes production through IMTA can help make aquaculture enterprises cleaner and more productive, competitive, and sustainable.

Chapter 4

Nutrient availability affects the polar lipidome of *Halimione portulacoides* leaves cultured in hydroponics

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4. Nutrient availability affects the polar lipidome of *Halimione portulacoides* leaves cultured in hydroponics

Abstract

Halophytes are increasingly regarded as suitable extractive species and co-products for coastal Integrated Multi-Trophic Aquaculture (IMTA) and studying their lipidome is a valid means towards their economic valorization. *Halimione portulacoides* (L.) Aellen edible leaves are rich in functional lipids with nutraceutical and pharmaceutical relevance and the present study aimed to investigate the extent to which its lipidome remains unchanged under a range of dissolved inorganic nitrogen (N) and phosphorus (P) concentrations typical of aquaculture effluents. Lipidomics analysis, done by hydrophilic interaction liquid chromatography coupled to high-resolution mass spectrometry, identified 175 lipid species in the lipid extract of leaves: 140 phospholipids (PLs) and 35 glycolipids (GLs). Plants irrigated with a saline solution with 20 – 100 mg DIN-N L^{-1} and 3 – 15.5 mg DIP-P L^{-1} under a 1-week hydraulic retention time displayed a relatively stable lipidome. At lower concentrations (6 mg DIN-N L^{-1} and 0.8 mg DIP-P L^{-1}), plants exhibited fewer PLs and GLs per unit of leaves dry weight and the GLs fraction of the lipidome changed significantly. This study reveals the importance of analyzing the lipidomic profile of halophytes under different nutritional regimens to establish nutrient-limitation thresholds and assure production conditions that deliver a final product with a consistent lipid profile.

4.1. Introduction

Halophyte plants display unique physiological and ecological adaptations to salt-marsh ecosystems, which allows them to live and thrive under a wide range of salt concentrations that most plants are unable to tolerate (Flowers et al., 1986; Flowers & Colmer, 2015, 2008; Panta et al., 2014). These plants have been investigated in several contexts, providing important insights on salt-tolerance mechanisms to improve salt-sensitive crops (Loescher et al., 2011; Mishra & Tanna, 2017; Zhang M. et al., 2018). Moreover, their potential as alternative crops has also been investigated for multiple applications (Abd El-Hack et al., 2018; Barreira et al., 2017; Buhmann & Papenbrock, 2013a; Maciel et al., 2016; Ventura and Sagi, 2013). In the context of aquaculture, recent studies have been testing the integration of halophytes production as an approach to extract nutrients from nutrient-rich saline effluents produced by fish-farming activities, which have been recently reviewed by Custódio et al. (2017). These investigations are typically performed in the context of Integrated Multi-Trophic Aquaculture (IMTA), a conceptual production model regarded as a more sustainable solution for the aquaculture industry (Barrington et al., 2010; Fang et al., 2016; Granada et al., 2016; Troell et al., 2009).

Entrepreneurs and society, in general, are only recently realizing the potential of halophytes as crops for the future and, besides the more obvious suitability of a handful of species for direct human and animal consumption (e.g. fresh/dried produce, plant meal), a particularly interesting market-positioning strategy for added-value could be the pharmaceutical and nutraceutical industries. Recent studies demonstrated that the leaves from certain halophytes are rich in bioactive molecules, such as phenols, flavonoids and other lipophilic compounds (Boughalleb & Denden, 2011; Buhmann & Papenbrock, 2013a; Ksouri et al., 2012, 2013; Maciel et al., 2018; Rodrigues et al., 2014; Zengin et al., 2018).

Marine lipids are regarded as an untapped pool of molecules with nutraceutical and pharmaceutical potential, especially those from marine macrophytes (da Costa et al., 2019; Da Costa et al., 2017; da Costa et al., 2015; Maciel et al., 2018, 2016; Melo et al., 2015). Glycolipids (GLs) and phospholipids (PLs) present in seaweeds (e.g. *Codium tomentosum* Stackhouse, *Gracilaria* spp., *Porphyra dioica* J. Brodie & L. M. Irvine) displayed antioxidant, anti-inflammatory and antibacterial properties and their fatty acid composition is rich in polyunsaturated aliphatic chains, which increase their functional properties for human health (Cortés-Sánchez et al., 2013; Horn & Benning, 2016; Ksouri et al., 2013; Küllenberg et al., 2012; Rodrigues et al., 2014). Several bioactive properties have been related to GLs and PLs (e.g. anti-inflammatory and anticarcinogenic) as well as enhanced human cognitive functions and motor performance (Burri et al., 2012; Chung et al., 1995; Cortés-Sánchez et al., 2013; Jäger et al., 2007; Lopes et al., 2014; Schneider et al., 2017; Sun et al., 2018). Halophytes, contrarily to algae, have been particularly overlooked on that regard, and the existing lipid characterizations have been mostly limited to fatty acids, non-polar lipids and sterols (Isca et al., 2014; Ksouri et al., 2012; Maciel et al., 2018; Sui et al., 2010). To date, only one publication attempted to describe the polar lipidome of two edible halophyte species (*Salicornia ramosissima* J. Woods and *Halimione portulacoides* (L.) Aellen), using liquid chromatography coupled with mass spectrometry (LC-MS) (Maciel et al., 2018). Fully exploring the lipidome of halophytes is a major step towards their valorization as relevant cash-crops for both agriculture and aquaculture. Besides it is also important to take into consideration the potential variations in their lipidomic profile in response to changes in environmental and metabolic conditions (Hou et al., 2016; Kostetsky et al., 2004; Maciel et al., 2016; Stengel et al., 2011; Sui et al., 2010). Understanding the circumstances and extent of those variations is essential to guarantee the supply of a consistent product when a stable lipid profile is a requisite.

The present study aimed to describe and assess potential shifts in the lipidome of sea purslane *H. portulacoides* leaves, grown hydroponically under different concentrations of dissolved inorganic nitrogen (DIN) and phosphorous (DIP). The concentrations used in this study aim to represent a wide range of possible values, as recorded in aquaculture effluents used in previous halophyte bioremediation studies under IMTA conditions (Buhmann et al., 2015; Lin et al., 2005; Quintã et al., 2015a; Waller et al., 2015; Webb et al., 2013). To understand if contrasting concentrations of DIN and DIP affect the polar lipidome of *H. portulacoides* leaves, the present study tested the following null hypothesis (H_0): 'There are no significant changes in the polar lipidome of *H. portulacoides* cultivated in low-, medium- and high-input of N and P'. Lipid profile was evaluated by state of the art lipidomics analysis using HILIC coupled with mass spectrometry (MS) and tandem mass spectrometry (MS/MS), bioinformatics tools, and statistical analysis.

4.2. Material and methods

4.2.1. Plant material

Halimione portulacoides stems were harvested on April 2017 at Ria de Aveiro (mainland Portugal) (40°38'04.1"N 8°39'40.0"W) and 500 grafts with 4 nodes each were cut, put into polyethylene containers, irrigated with a modified Hoagland's solution and placed under natural sunlight and temperature to promote root development. The elemental composition of the modified Hoagland's solution was: 60 mg K L^{-1}, 56 mg N L^{-}1, 40 mg Ca L^{-1}, 16 mg Mg L^{-1}, 16 mg P L^{-1}, 1.12 mg Fe L-1, 0.34 mg Mo L-1, 0.28 mg B L^{-1}, 0.13 mg Zn L^{-1}, 0.11 mg Mn L^{-1} and 0.03 mg Cu L^{-1}. After three months, in July 2017, rooted plants were transferred and acclimated to indoor conditions for two weeks. In the second week, plants were progressively exposed to a target water salinity of 20 ppt, with increments of 5 every second day, before the beginning of the experiment.

4.2.2. Growth trial

The hydroponics growth trial took place indoors at ECOMARE (Laboratory for Innovation and Sustainability of Marine Biological Resources of the University of Aveiro) facilities, for 10 weeks (from July to September under an artificial photoperiod of 14 light: 10 dark) to allow plants to develop harvestable aboveground biomass. The hydroponic units were made of opaque polypropylene material, with dimensions of 300 x 200 x 170 mm and a volume of 5 L of solution maintained through an overflow outlet. Twenty polystyrene floating-rafts were perforated with ten holes equally spaced between them (20 mm) and 200 three-months-old rooted grafts of *H. portulacoides* with similar weights were randomly distributed into 20 hydroponic units, at a density of 10 plants per unit. Plants were inserted in the holes by the roots and fixed in place at the lower level of the stem using natural cotton.

The experiment consisted of 4 treatment solutions (including a control) and 5 replicate units (n = 5). The basis for the treatment solutions was artificial seawater produced by mixing sea salts (Red Sea salt, Red Sea Aquatics, Cheddar, UK) with tap water purified by reverse-osmosis (V2Pure 360 RO System, TMC, Hertfordshirem UK) at a salinity of 20 ppt. At this salinity, the minerals Ca, Mg and K in the base solution are at a concentration of 235 - 248 mg L^{-1} Ca, 703 - 742 mg L^{-1} Mg, and 213 - 226 mg L^{-1} K, according to information provided by the manufacturer. The control solution was the modified Hoagland's solution described above. The low-, medium- and high-input treatments consisted of modified versions of the control, where only nitrogen (N) and phosphorous (P) were adjusted, to mimic aquaculture-like effluents. Nomenclature and concentrations of N and P are as follows: $[N,P]_{low}$ = [6 mg N L^{-1}, 0.8 mg P L^{-1}]; $[N,P]_{med}$ = [20 mg N L^{-1}, 3.0 mg P L^{-1}]; $[N,P]_{high}$= [100 mg N L^{-1}, 6.0 mg P L^{-1}]; Control = [56 mg N L^{-1}, 15.5 mg P L^{-1}]. For a detailed molecular and elemental composition of each treatment please see Table A 3.1 in the Annex section. The solutions within each unit were continuously aerated with a small aerator to keep oxygen levels high and units were refilled with reverse-osmosis water as needed, to compensate for evapotranspiration. The treatment solutions were renewed weekly, as the retention time for nutrient extraction was set to one week. At the end of the growth trial, the leaves of individual plants were cut out, pooled by hydroponic unit, and stored at -80 °C until further analysis. Water temperature and pH were measured regularly with a multi-parameter water quality meter and photosynthetically active radiation (PAR) was measured with a spherical micro quantum sensor (US-SQS/L, Heinz Walz GmbH, Pfullingen, Germany). Average values recorded at the end of each week, before the renewal of treatment solutions, are presented as annex Table A 3.2.

4.2.3. Analytical methods

4.2.3.1. Reagents

HPLC grade chloroform, methanol and acetonitrile were obtained from Fisher Scientific Ltd. (Loughborough, UK). Lipid internal standards 1,2-dimyristoyl-sn-glycero-3-phosphate (dMPA), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (dMPC), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (dMPE), 1,2-dimyristoyl-sn-glycero-3-phospho-(10-rac-glycerol) (dMPG), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylinositol (dPPI) and 1-nonadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Milli-Q water (Synergy, Millipore Corporation, Billerica, MA, USA) was produced when ultrapure water was necessary. All other reagents were purchased from major commercial sources.

4.2.3.2. Leaves lipid extraction

Total lipids were extracted according to the method proposed by Bligh and Dyer (1959), modified for seaweeds and halophytes (Maciel et al., 2018). 3.75 mL of chloroform: methanol (1:2, v/v) was added to 100 mg of freeze-dried and grounded leaves followed by 2-

minutes vortex stirring and 1-minute sonication. Samples were then incubated on an orbital shaker for 2.5 h, on ice. The homogenates were centrifuged at 2000 rpm for 10 min. The chloroform: methanol extraction followed by centrifugation was repeated twice to improve extraction efficiency. After extraction, 2.3 mL of ultrapure water was added to each supernatant, stirred on the vortex and centrifuged at 2000 rpm for 10 min. Two liquid phases originate and the inferior organic phase, which contains the lipids, was recovered and dried under a stream of nitrogen gas. Each dried extract was dissolved in 600 µL of chloroform and transferred to dark vials. Lipid extracts were dried under nitrogen gas, weighed (for 'total lipid' calculation), and stored at -20 °C before LC−MS analysis.

4.2.3.3. Quantification of phospholipids

Quantification of the total phospholipid content was achieved by using the protocol by Bartlett and Lewis (1970). First, in glass tubes, 125 µl of percloric acid (70% v/v) was added to dried lipid extracts and the mixtures were incubated for 40 minutes at 170 °C. In the meantime, standards were prepared, also in glass tubes, using 0,1 to 2 µg of phosphorous. After, 825 µl of ultrapure water, 125 µl of ammonium molybdate (2.5 % v/v) and 125 µl of ascorbic acid (10 % v/v) were added to each sample and standards. All tubes were then vortexed. Tubes were incubated in a water bath at 100 °C for 10 minutes and transferred to ice to cool down. The absorbance of samples and standards were measured at 797 nm using a microplate reader (Multiskan GO, Thermo Scientific, Hudson, NH, USA).

4.2.3.4. Quantification of glycolipids

Quantification of the total glycolipid content was achieved using the orcinol assay, as done in our lab (Da Costa et al., 2017; Cyberlipid). First, an orcinol solution (0.2% v/v in 70% sulfuric acid) was prepared and 1 mL was added to tubes with N_2-dried lipid extract samples. Tubes were heated at 80ºC for 20 minutes and transferred to ice to cool down. The absorbance of samples and standards were measured at 505 nm using a microplate reader (Multiskan GO, Thermo Scientific, Hudson, NH, USA). The concentration of glucose was calculated by comparing the data with those of glucose standards (between 0 - 50 µg prepared from an aqueous solution containing 2 mg mL^{-1} of glucose and following the same procedure as experimental samples).

4.2.3.5. Analysis of polar lipids by high-resolution LC-MS and MS/MS

The polar lipids from *H. portulacoides* leaves were analyzed by high−performance LC (HPLC) system (Thermo Scientific Accela, Thermo Fisher Scientific, USA) with an autosampler coupled online to the Q-Exactive® mass spectrometer with Orbitrap® technology following the method previously used for halophyte lipid analysis (Maciel et al., 2018). The solvent system consisted of two mobile phases: mobile phase A [acetonitrile:methanol:water 50:25:25 (v/v/v) with 1 mM ammonium acetate] and mobile phase B [acetonitrile:methanol 60:40 (v/v) with 1 mM ammonium acetate]. Initially, 0% of mobile phase A was held isocratically for 8 min, followed by a linear increase to 60% of A within 7 min and a maintenance period of 15 min, returning to the initial conditions within 10 min. A volume of 5 µL of each sample containing 20 µg of lipid extract, a volume of 4 µL of internal standards mix (dMPA - 0.02 µg $µg^{-1}$; dMPC - 0.005 µg $µg^{-1}$, dMPE - 0.005 µg $µg^{-1}$, dMPG - 0.003 µg $µg^{-1}$, dPPI - 0.02 µg $µg^{-1}$, LPC - 0.005 µg $µg^{-}$1) and 91 µL of mobile phase B were pipetted and introduced into the Ascentis®Si column (15 cm × 1 mm, 3 µm, Sigma-Aldrich) with a flow rate of 40 µL min^{-1} at 30 °C. The mass spectrometer with Orbitrap® technology was operated in simultaneous positive (electrospray voltage 3.0 kV) and negative (electrospray voltage −2.7 kV) modes at a resolution of 70,000 and AGC target of 1e6, the capillary temperature was 250 °C and the sheath gas flow was 15 U. In MS/MS experiments, a resolution of 17,500 and AGC target of 1e5 were used and the cycles consisted of one full scan mass spectrum and ten data-dependent MS/ MS scans, repeated continuously throughout the experiments with a dynamic exclusion of 60 s and intensity threshold of 1e4. Normalized collision energy™ (CE) ranged between 25, 30, and 35 eV. MZmine 2.27 software was used to process MS raw data and identify lipid species by mass accuracy from high-resolution MS data.

Thermo Xcalibur 3.0.63 software was used to analyze the chromatograms and MS/MS spectra, to confirm lipid species identity and discriminate their fatty-acid composition. The classes lysophosphatidylethanolamine (LPE), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and sulfoquinovosyldiacylglycerol (SQDG) were detected as anionized adducts of $[M\text{-}H]^{-}$; digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG) and monogalactosylmonoacylglycerol (MGMG) were detected as cationized adducts of $[N+NH_4]^{+}$; and lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) were detected as cationized adducts of $[M+H]^{+}$.

The FA composition of PCs was identified by analysis of the MS/MS of anionized adducts of acetate $[M+Ac]^-$, which detects the carboxylate anions $R\text{-}COO^-$ that allow the determination of the fatty acyl composition. The dataset with the peak intensities, normalized to internal standards, is available online at https://doi.org/10.1038/s41598-020-63551-1

4.2.4. Statistical analysis

Statistical analysis was performed using R (v3.4.3) in combination with RStudio (v1.1.463) and MetaboAnalyst (v4.0) (Chong et al., 2018). Before analysis, the lipidomic dataset was normalized by dividing the peak-intensity values of each molecular species with the peak-intensity of their respective internal standard. Secondly, datasets were created for each lipid class, where the relative abundance of each molecular species was computed for each replicate. These datasets are available online at https://doi.org/10.1038/s41598-020-63551-1

Before the multivariate analysis, data normalization procedures - *log-transformation* followed by *auto-scaling* - were employed to decrease the influence of high-concentration metabolites and increase the statistical strength of low-concentration metabolites. Both unsupervised (Principal Components Analysis - PCA) and supervised (Partial Least Squares Discriminant Analysis - PLS-DA) methods were used.

The univariate analysis consisted of the analysis of variance, using the non-parametric Kruskal-Wallis test, of species i) peak-intensities and ii) relative abundance within each class. *Post-hoc* Dunn's test was used for pairwise comparisons and the Benjamini-Hochberg method was used to control for type-I errors (Checa et al., 2015). Significant differences were assumed at a critical p-value < 0,05.

4.3. Results

4.3.1. Total lipids, glycolipids, and phospholipids quantification

Total lipid content was estimated by gravimetry and expressed as *g 100 g*$^{-1}$ of dry weight (DW) (Figure 4.1). Non-significant differences were detected between each treatment and the control (CT), with a tendency for increased lipid content in the leaves of *H. portulacoides* at higher concentrations of N and P in the solution: $[N,P]_{low}$ yielded 6,18 ± 0,99 g 100 g^{-1} of leaves dry weight (DW), followed by $[N,P]_{med}$, with 7.96 ± 2.05 g 100g^{-1} DW; and 9.23 ± 3.06 g 100g^{-1} DW for $[N,P]_{high}$. The total amount of lipid extract obtained from $[N,P]_{high}$ was similar to that recorded in the CT (9.44 ± 3.83 g 100g^{-1} DW).

The levels of GLs and PLs present in the lipid extracts of the leaves of *H. portulacoides* were also estimated (Figure 4.2-A), expressed as *µg mg^{-1}* of lipid extract. No significant differences were detected between treatments in neither GLs nor PLs contents. The overall average content of GLs was 455.87 ± 57.32 µg mg^{-1} of lipid extract and that of PLs was 175.49 ± 39.56 µg mg^{-1} of lipid extract.

Significant differences were recorded between treatment conditions regarding the concentration of GLs and PLs in the leaves of *H. portulacoides*, expressed as *mg g^{-1}* DW (Figure 4.2-B). The $[N, P]_{low}$ group had a significantly lower concentration of GLs (29.1 mg g^{-1} DW) than the CT (42.1 mg g^{-1} DW) and other treatments (38.2 – 39.1 mg g^{-1} DW), as well as a significantly lower concentration of PLs (9.7 mg g^{-1} DW) than the CT (17.5 mg g^{-1} DW) and other treatments (14.1 – 16.8 mg g^{-1} DW). The $[N, P]_{med}$ and $[N, P]_{high}$ groups did not differ from the CT in either type of lipids.

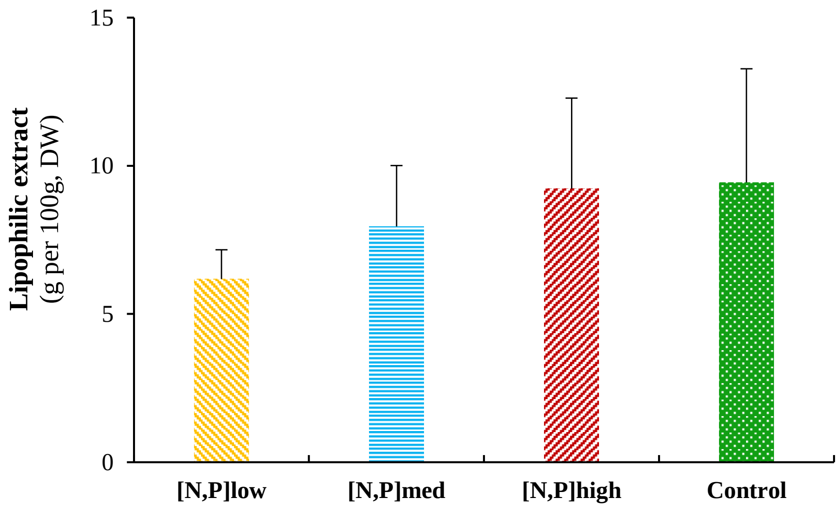


Figure 4.1: Total amount of the lipid extract of *Halimione portulacoides* leaves. Error bars represent standard deviations.

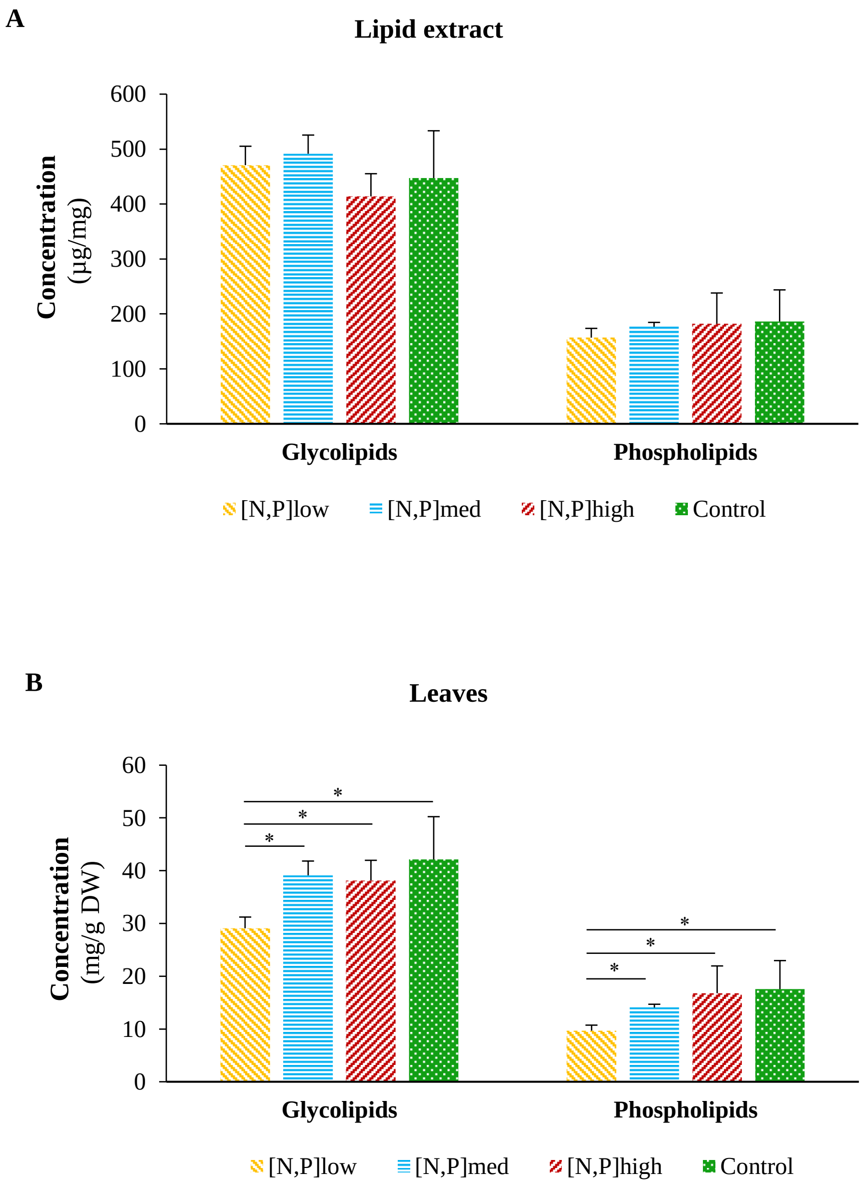


Figure 4.2: Glycolipids and phospholipids concentrations in leaves lipid extract (A) and in leaves dry mass (B) of *Halimione portulacoides*. Error bars represent standard deviations and horizontal lines with symbol * represent significant differences: * $p < 0,05$.

4.3.2. Lipidomic signature

A non-targeted lipidomics approach was used to evaluate the stability of the lipidome across treatment conditions. This approach provided a global profile of the polar lipid molecular species present in the extracts and potentially used as a lipid signature that characterizes states of N and P limitation and/or excess.

MS and MS/MS analysis allowed the accurate identification of 175 lipid species, namely 140 PLs (Table 4.1) and 35 GLs (Table 4.2), which were detected in all conditions. In a few cases, MS/MS spectra did not provide enough information to determine the fatty acyl composition, but the classes were confirmed through the identification of the polar head and are therefore included in Table 4.1. No lipids were found to be unique to any one condition. The lipid classes identified were previously recorded in wild specimens (Maciel et al., 2018) and are DGDGs, LPCs, LPEs, MGDGs, MGMG, PAs, PCs, PEs, PGs, PIs and SQDGs. The number of species identified per lipid class is represented in Figure 4.3.

After raw data processing and species identification, the dataset was analyzed using chemometric statistical methods to extract and interpret data from a biologically relevant perspective, looking at changes in the lipidome in general and within specific lipid groups (GLs and PLs). A PCA analysis was applied to a matrix with all lipid species, to highlight possible changes in the total lipidome imposed by treatments, from which scores plot (Figure 4.4-A) and loadings plot (Figure A4.1-A available in Annex) of the two principal components were obtained. PCA did not differentiate treatment conditions and there was a higher degree of variability within the CT group (and, to a lesser extent, in $[N,P]_{high}$) compared with the other treatments.

Following this observation, PLS-DA was used to maximize the separation between conditions and the projection plot (Figure 4.5-A) revealed some degree of discrimination between $[N,P]_{low}$ and both CT and $[N,P]_{high}$. There was no discrimination between CT and both $[N,P]_{med}$ and $[N,P]_{high}$. The Variable Importance in Projection (VIP) scores were used to rank variables in terms of their importance in the projection of the PLS model, and the top 20 variables are presented in Figure 4.5-D. Fifteen out of those twenty species presented higher concentrations in $[N,P]_{low}$ than CT and $[N,P]_{high}$. Nonetheless, within the top five species explaining the separation, three were at the lowest concentrations (PA 34:1, PA 36:3, PA 34:2) and two at highest concentrations (PI 36:6, DGDG 34:3) in $[N,P]_{low.}$

Table 4.1: Phospholipids molecular species identified by LC-MS and tandem MS (MS/MS) from total lipid extracts of *Halimione portulacoides* leaves.

[M+H]$^+$	**Lysophosphatidylcholine**
496.3396	LPC 16:0
518.3230	LPC 18:3
520.3387	LPC 18:2
522.3564	LPC 18:1
550.3869	LPC 20:1
552.4024	LPC 20:0
580.4350	LPC 22:0
608.4656	LPC 24:0
[M-H]$^-$	**Lysohosphatidylethanolamine**
452.2779	LPE 16:0
474.2621	LPE 18:3
476.2779	LPE 18:2
478.2938	LPE 18:1
[M-H]$^-$	**Phosphatidic acid**
667.4347	PA 34:4*
669.4505	PA 16:0/18:3
671.4647	PA 16:0/18:2
673.4816	PA 16:0/18:1
691.4335	PA 18:3/18:3
693.4498	PA 18:2/18:3
695.4647	PA 18:2/18:2; PA 18:1/18:3
697.4812	PA 18:1/18:2
699.4955	PA 18:1/18:1
[M+H]$^+$	**Phosphatidylcholine**
700.4889	PC 30:3*
728.5230	PC 16:0/16:3
730.5378	PC 16:0/16:2; PC 14:0/18:2
734.5684	PC 16:0/16:0
750.5072	PC 18:3/18:3
754.5373	PC 16:1/18:3; PC 16:2/18:2; PC 16:3/18:1
756.5538	PC 16:0/18:3; PC 16:1/18:2
758.5690	PC 16:0/18:2; PC 16:1/18:1
760.5829	PC 16:0/18:1
772.4896	PC 36:9*
776.5193	PC 36:7*
778.5370	PC 18:3/18:3
780.5529	PC 18:2/18:3
782.5682	PC 18:2/18:2; PC 18:1/18:3
784.5841	PC 18:1/18:2; PC 18:0/18:3
786.5997	PC 18:1/18:1; PC 18:0/18:2
800.5198	PC 38:9*
802.5347	PC 38:8*
804.5510	PC 38.7*
806.5667	PC 38:6*
808.5818	PC 18:2/20:3; PC 18:3/20:2
810.5978	PC 18:3/20:1; PC 18:2/20:2
812.6158	PC 18:2/20:1
814.6334	PC 18:1/20:1; PC 18:2/20:0
832.5815	PC 40:7*
834.5967	PC 40:6*
838.6321	PC 18:3/22:1
840.6473	PC 18:3/22:0; PC 18:2/22:1
842.6634	PC 18:2/22:0; PC 18:1/22:1
844.6785	PC 18:1/22:0
868.6785	PC 18:3/24:0; PC 18:2/24:1
870.6933	PC 18:2/24:0; PC 18:1/24:1
[M-H]$^-$	**Phosphatidylethanolamine**
684.4609	PE 16:0/16:3; PE 14:0/18:3
686.4753	PE 16:0/16:2; PE 14:0/18:2
688.4914	PE 16:0/16:1; PE 14:0/18:1
708.4596	PE 16:2/18:3; PE 16:3/18:2
710.4754	PE 16:1/18:3; PE 16:2/18:2
712.4916	PE 16:0/18:3; PE 16:1/18:2
714.5068	PE 16:0/18:2; PE 16:1/18:1
716.522	PE 16:0/18:1
734.4766	PE 18:3/18:3
736.4921	PE 18:2/18:3
738.5086	PE 18:2/18:2; PE 18:1/18:3
740.5227	PE 18:1/18:2
742.5387	PE 18:1/18:1
764.5219	PE 18:0/20:5
766.5389	PE 18:3/20:1; PE 18:2/20:2
768.5536	PE 18:2/20:1; PE 18:3/20:0; PE 18:1/20:2
770.5684	PE 18:1/20:1; PE 18:2/20:0
794.5699	PE 18:/22:1
796.5857	PE 18:3/22:0; PE 18:2/22:1
798.5998	PE 18:2/22:0
800.6154	PE 18:1/22:0
824.6159	PE 18:2/24:1; PE 18:3/24:0
826.6316	PE 18:2/24:0
[M-H]$^-$	**Phosphatidylglycerol**
693.4703	PG 14:0/16:0
719.486	PG 16:0/16:1; PG 14:0/18:1
721.5013	PG 16:0/16:0; PG 14:0/18:0
739.4554	PG 16:1/18:4; PG 16:2/18:3; PG 16:3/18:2
741.4701	PG 16:0/18:4; PG 16:1/18:3; PG 16:2/18:2; PG 16:3/18:1
743.4861	PG 16:0/18:3; PG 16:1/18:2; PG 16:2/18:1
745.5014	PG 16:1/18:1; PG 16:2/18:0; PG 16:0/18:2
747.5162	PG 16:0/18:1; PG 16:1/18:0
763.4543	PG 18:3/18:4
765.4716	PG 18:3/18:3; PG 18:2/18:4
767.4850	PG 18:2/18:3
769.5012	PG 18:2/18:2; PG 18:1/18:3; PG 16:1/20:3
771.5160	PG 18:1/18:2
773.5316	PG 18:1/18:1; PG 18:0/18:2; PG 16:0/20:2
775.5458	PG 16:0/20:1; PG18:0/18:1
[M-H]$^-$	**Phosphatidylinositol**
831.5016	PI 16:0/18:3
833.5165	PI 16:0/18:2
835.5318	PI 16:0/18:1
853.4849	PI 18:3/18:3
855.5002	PI 18:2/18:3
857.5163	PI 18:2/18:2; PI 18:1/18:3
859.5319	PI 18:1/18:2
861.5471	PI 18:1/18:1; PI 18:0/18:2
863.5607	PI 18:1/18:0

*confirmed *m/z* and class but missing fatty-acyl information to identify species

Table 4.2: Phospholipids molecular species identified by LC-MS and tandem MS (MS/MS) from total lipid extracts of *Halimione portulacoides* leaves.

$[M+NH_4]^+$	**Digalactosyldiacylglycerol**	$[M+NH_4]^+$	**Monogalactosylmonoacylglycerol**
910.6472	DGDG 16:0/16:0	532.3482	MGMG 18:3
926.5816	DGDG 18:3/16:3		
932.6296	DGDG 18:3/16:0; DGDG 18:2/16:1	$[M-H]^-$	**Sulfoquinovosyldiacylglycerol**
936.6576	DGDG 18:1/16:0	787.4660	SQDG 18:3/14:0; SQDG 16:3/16:0
954.6144	DGDG 18:3/18:3; DGDG 18:4/18:2	789.4800	SQDG 18:2/14:0
958.6440	DGDG 18:3/18:1; DGDG 18:2/18:2	791.4970	SQDG 16:1/16:0
960.6601	DGDG 18:3/18:0; DGDG 18:2/18:1	793.5120	SQDG 16:0/16:0
		813.4820	SQDG 18:3/16:1
$[M+NH_4]^+$	**Monogalactosyldiacylglycerol**	815.4970	SQDG 18:3/16:0
764.5308	MGDG 18:3/16:3; MGDG 18:4/16:2; MGDG 18:2/16:4	837.4800	SQDG 18:3/18:3
768.5630	MGDG 18:3/16:1	839.4970	SQDG 18:3/18:2; SQDG 20:2/16:3
770.5768	MGDG 18:3/16:0; MGDG 18:2/16:1; MGDG 18:0/16:3	843.5280	SQDG 18:3/18:0; SQDG 18:2/18:1
792.5615	MGDG 18:3/18:3; MGDG 18:4/18:2		
796.5910	MGDG 18:2/18:2; MGDG 18:3/18:1		

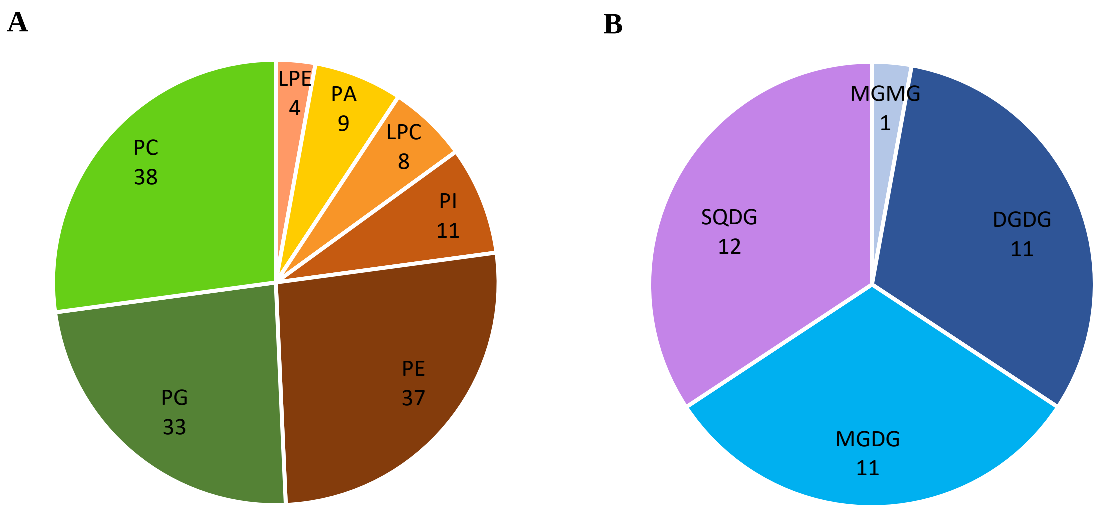


Figure 4.3: Number of phospholipids (A) and glycolipids (B) molecular species identified in the lipid extract of *Halimione portulacoides* leaves by MS/MS. DGDG – digalactosyldiacylglycerol; LPC – lysophosphatidylcholine; LPE – lysophosphatidylethanolamine; MGDG – monogalactosyldiacylglycerol; MGMG – monogalactosylmonoacylglycerol; PA – phosphatidic acid; PC – phosphatidylcholine; PG – phosphatidylglycerol; PE – phosphatidylethanolamine; PI – phosphatidylinositol; SQDG - sulfoquinovosyldiacylglycerol.

PCA – Total lipidome **PCA - Phospholipids** **PCA – Glycolipids**

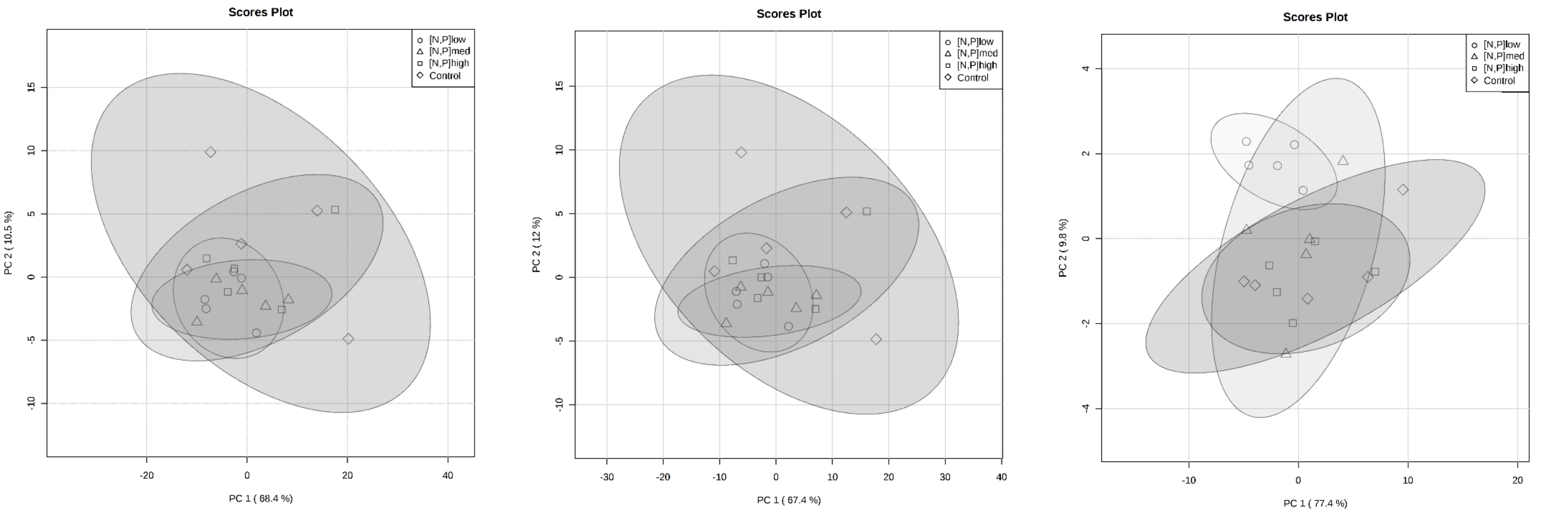


Figure 4.4: Principal component analysis (PCA) scores plot of total lipidome (A), phospholipids (B) and glycolipids (C) normalized peak-intensity, obtained from the lipid extracts from *Halimione portulacoides* leaves.

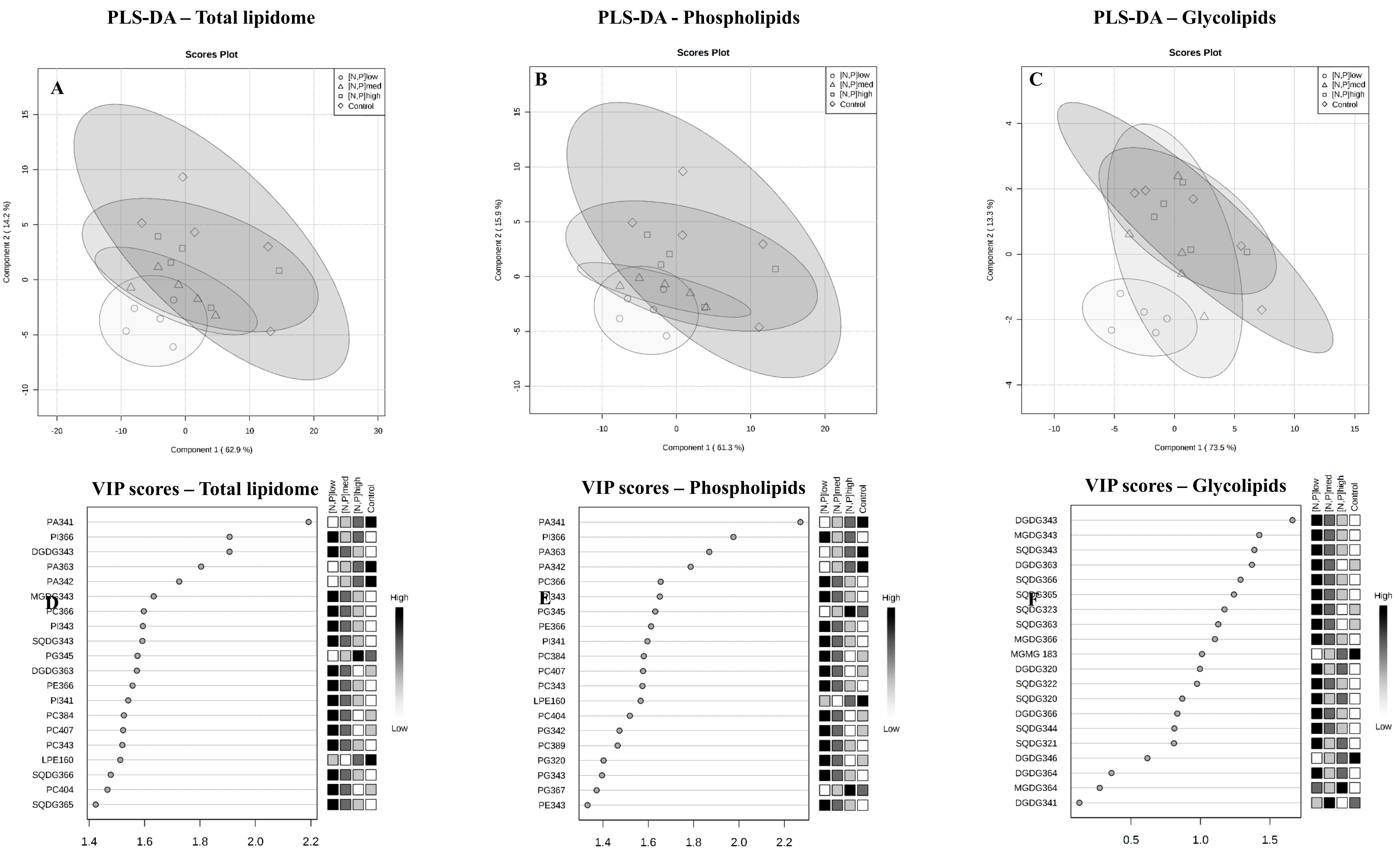


Figure 4.5: Partial least squares – discriminant analysis (PLS-DA) plots of total lipidome (A), phospholipids (B) and glycolipids (C) peak-intensity matrices detected in the lipid extract from *Halimione portulacoides* leaves. The Variable Importance in the Projection (VIP) scores of each PLS-DA model are displayed below each plot (top 20 variables): total lipidome (D), phospholipids (E) and glycolipids (F).

PCA and PLS-DA were also applied to matrices composed of only PL or GL molecular species, to decipher if any one of these two major lipid groups were changing more markedly than the other. The PCA of PLs (Figure 4.4-B; loadings in Annex as Figure A4.1-B) showed a very similar trend to the PCA plot of the total lipidome data, suggesting no clear discrimination between treatment conditions. Similarly, the PLS-DA (Figure 4.5-B) also resembles the one obtained with the total lipidome matrix, with the most important species influencing the model also being PA 34:1, PA 36:3, PA 34:2 and PI 36:6 (Figure 4.5-E).

The PCA of GLs (Figure 4.4-C; loadings in in Annex as Figure A4.1-C), on the other hand, evidences a clear separation between the $[N,P]_{low}$ group and both CT and $[N,P]_{high}$ groups. The PLS-DA model projection (Figure 4.5-C) further discriminates those groups, while the $[N,P]_{med}$ group intersects both low-input and high-input clusters. The species that most contributed to the separation was DGDG 34:3, followed by MGDG 34:3, SQDG 34:3, and DGDG 36:3, all of them at higher concentrations in $[N,P]_{low}$ than CT and $[N,P]_{high}$ (Figure 4.5-F). From the top fifteen GL species influencing the PLS-DA model, fourteen were at the highest concentration in $[N,P]_{low}$.

The univariate analysis of individual lipid species intensities (Figure A4.2 in Annex) displayed significant differences between certain treatment groups for PA 34:1, DGDG 34:3, DGDG 36:3, and PI 36:6, all of which were at the top of the VIP scores. MS/MS spectra from all conditions for some of the top VIP features can be consulted in the Annex section as Figure A4.3.

4.3.3. Polar lipid changes in each class

A univariate analysis was also performed regarding the average relative abundance of molecular species within each class of PLs and GLs. In the case of PLs, most of the significant differences in species relative abundance were observed between $[N,P]_{low}$ and either CT or $[N,P]_{high}$ or both conditions. These were observed in PCs (8 species), PEs (2), PGs (8), PIs (5), LPCs (2), and LPEs (2) (Figure A4.4 in Annex). In PAs, 5 species were significantly different in terms of relative abundance between CT and the other treatments. In the case of GLs, differences were mostly observed between $[N,P]_{low}$ and either CT or $[N,P]_{high}$ or both, in DGDGs (4 species), MGDGs (4) and SQDGs (5) (Figure A4.5 in Annex).

Some lipid species with similar fatty acyl chains were more abundant in $[N,P]_{low}$, such as PC, PE, PG, PI, DGDG and SQDG with 34 carbons and 3 double bonds (34:3) and the

PC, PE, PA and DGDG with 36 carbons and 6 double bonds (36:6). Regarding lyso-forms, the LPC 18:3 and LPE 18:3 were also more abundant in $[N,P]_{low}$. The lipid species that were lower in $[N,P]_{low}$ when compared with CT were more diversified in their fatty acyl composition and included several PLs (e.g. PC and PA 36:3, PC 32:2, PA 34:2, PI and PG 32:1), two lyso-PLs (LPC and LPE 16:0) and some GLs (e.g. MGDG 34:6, DGDG 34:6 and, SQDG 34:4). Other differences were observed between the $[N,P]_{low}$ and $[N,P]_{high}$ conditions: $[N,P]_{low}$ treatment resulted in the significantly lower relative abundance of PG 32:1 and 34:4, PI 34:2, SQDG 32:0, 32:1, 32:2 and 34:4, MGDG 34:3, 34:4, 36:4, DGDG 34:6 and 36:6; and higher relative abundance of PG 34:2, PI 34:1, SQDG 34:3, DGDG 34:3, 34:6 and 36:3.

4.4. Discussion

The present study evaluated the polar lipidome signature of leaves from hydroponically grown *H. portulacoides* under different concentrations of N and P. The aim was to describe and reveal possible shifts in the lipidomic profile of its leaves under a wide range of N and P concentrations that represent possible IMTA contexts.

The total lipids extracted from *H. portulacoides* leaves ranged, on average, between 6.2 – 9.4 g 100 g^{-1} DW, with higher amounts extracted from the high-input treatments, including CT (Hoagland's solution). A recent study by Patel et al. (2019) analyzed the total lipid content in the shoots (which can include leaves and other superior organs) of several halophytes and concluded that non-succulent halophytes (e.g. *Sporobolus virginicus* (L.) Kunth and *Aeluropus lagopoides* (L.) Trin. ex Thwaites) presented higher lipid content, between 5.5 - 7.2 g 100g^{-1} FW, followed by shrubby halophytes (e.g. *Atriplex nummularia* Lindl. and *Atriplex griffithii* Moq.) with 2.6 – 2.8 g 100 g^{-1} FW, and succulents (e.g. *Sesuvium portulacastrum* (L.) L. and *Salicornia brachiate* Miq.), with 1.5 – 1.8 g 100 g^{-1} FW. Regarding that *H. portulacoides* leaves have a moisture content of ~ 90% (Chapter 3), the equivalent amount of lipid extract in fresh leaves ranges from 0.8 – 0.9 g 100 g^{-1} FW, which is much lower than the values mentioned above for other species. Nonetheless, other studies reported values (both in DW and FW) within the same order of magnitude as *H. portulacoides,* which contradict the values reported above. For instance, *Salicornia bigelovii* Torr. was reported to have 0.4 g of total lipids 100g^{-1} FW (Lu et al., 2010); *S. ramosissima*, 1.9 g 100g^{-1} DW (Barreira et al., 2017); *Sarcocornia perennis* (Mill.) A. J. Scott, 2.3 g 100g^{-1} DW (Barreira et al., 2017) and *Crithmum maritimum* L., 1.5 – 2.2 g 100g^{-1} DW (Ben Hamed et al., 2005).

This discrepancy could be explained by the inclusion of seeds along with the shoots in Patel et al. (2019)**,** which would substantially increase total lipid content. Yet, total lipids in fertile shoot segments containing seeds were described in *Sarcocornia ambigua* (Michx.) M.A. Alonso & M.B. Crespo at concentrations between 1.4 – 5.2 g $100g^{-1}$ DW (Costa et al., 2014), in *Salicornia virginica* L. 2.4 – 3.6 g $100g^{-1}$ DW (Kulis et al., 2010) and in *Salicornia europaea* L. 3.5 – 7.1 g $100g^{-1}$ DW (Kulis et al., 2010), values that do not match up with the aforementioned concentrations reported for succulents. A misreport of DW as FW could also be a possible explanation for that inconsistency.

The PLs and GLs are two major lipid groups present in the total lipid extracts of halophyte leaves, carrying a wide array of fatty acids (FAs), from which α-linolenic acid (C18:3, *n*-3), palmitic acid (C16:0), linoleic acid (C18:2, *n*-6), and oleic acid (C18:1, *n*-9) are the most abundant (Duarte et al., 2018; Maciel et al., 2018; Ventura et al., 2011a). In this study, *H. portulacoides* displayed a profusion of lipid species with C16 and C18 chains and some species exhibited polyunsaturated fatty acids with up to four double-bonds (e.g. MGDG 18:2/16:4; PG 16:0/18:4 and PG 18:2/18:4). Polyunsaturated FAs have been largely associated with beneficial health effects in humans and animals (Harris, 2010; Husted & Bouzinova, 2016; Liu et al., 2017; Shahidi & Ambigaipalan, 2018; Simopoulos, 2011) and *H. portulacoides* leaves can be a good source for obtaining those FAs, given its relatively high lipid content compared with other halophytes.

The quantities of GLs and PLs in the lipid extract were comparable across treatments. The GLs constituted approximately 46% of the total extract, PLs constituted around 18% and, therefore, polar lipids constituted 64% of the total. When the concentrations were expressed in terms of dry weight (DW) of leaves, GLs and PLs turned out to be significantly lower in $[N,P]_{low}$ treatment. Higher percentages of polar lipids were previously observed in other halophytes, especially GLs. For instance, the total lipidic extract of chloroplast-enriched portions from *Salicornia perennans* Willd. was reported to have 67% GLs and 31% PL; *Limonium gmelinii* (Willd.) Kuntze, 60% GLs and 32% PLs; and *Artemisia santonicum* L., 80% GLs and 15% PLs (Rozentsvet et al., 2016). Other halophytes displayed lower percentages of GLs, such as *Halostachys caspica* C.A.Mey. and *Halocharis hispida* (Schrenk) Bunge, with 22 – 29% GLs and 16 – 17% PLs in their extract (Asilbekova et al., 2009). In terms of the amounts of GLs and PLs per unit of leaves, a previous study reported values for several halophyte species to range between 5 – 47 mg GLs g^{-1} DW and 2 – 17 mg PLs g^{-1}

DW (Rozentsvet et al., 2014). Concerning the present study, *H. portulacoides* from the high-input treatments displayed values very similar to the upper end of those ranges. Nonetheless, comparisons should be taken merely as an illustration of the range of possible concentrations in the edible portions of different species of halophytes.

Regarding the lipidome, the null hypothesis under test stated that *no changes occur in the polar lipidome of H. portulacoides cultivated in low-, medium- and high-input of N and P*. Since P is an important element of polar lipids, hydroponic conditions that would offer limited access of this element to the plant could promote alterations in the lipidome of leaves. A decrease in PLs in parallel with an increase in non-phosphorus GLs (e.g. SQDG and DGDG) and betaine lipids (in algae) was observed in plants and algae when exposed to conditions of P-limitation, as previously reported for *Arabidopsis* (Benning & Ohta, 2005; Hartel et al., 2000; Jouhet et al., 2004; Kelly & Dörmann, 2004), rice (Okazaki et al., 2013), oat (Andersson et al., 2003), soybean (Okazaki et al., 2017, 2013), periphyton (Bellinger & Mooy, 2012) and *Ulva* (Kumari et al., 2014). From the PCA results, it follows that the total lipidome signature of *H. portulacoides* leaves remained relatively unchanged across groups after long-term exposure to nutrient concentrations varying between 6 - 100 mg DIN-N L^{-1} and 0.8 - 15.5 mg DIP-P L^{-1}. However, a sequential overlap of treatment groups, from lowest to highest P-input ($[N,P]_{low} < [N,P]_{med} < [N,P]_{high} <$ CT), was evident in the PLS-DA projection. The $[N,P]_{low}$ group stood out as the group with the least amount of overlap with the other groups. When GLs were analyzed separately from PLs, both PCA and PLS-DA plots separated the $[N,P]_{low}$ group from both the CT and $[N,P]_{high}$ groups. These results suggest that the GL profile of the leaves is changing according to the availability of P, but its effect on the total polar lipidome is masked by the PL profile which remains relatively stable across treatments. Therefore, at low-input concentrations of P, the leaves of *H. portulacoides* display a low degree of lipidome remodeling associated with significant changes in GLs. In plants, GLs are typically found in chloroplast thylakoids, being their major lipid constituents, but under P-limited conditions, GLs (particularly DGDG) can partially replace PLs in extra-plastidial membranes (Kalisch et al., 2016). The upregulation of genes encoding GLs synthase (ex. DGD1, DGD2, MGD2/MGD3) which activate additional GLs biosynthetic pathways in plants under P-limited conditions (Hölzl & Dörmann, 2007; Kalisch et al., 2016; Moreau et al., 1998) could explain the changes observed in *H. portulacoides'* GL profile under the conditions of low P-input. Previous studies, in both plants and microalgae, demonstrated that the availability of N also affects the morphology and function of chloroplasts in superior plants and microalgae (Damiani et al., 2010; Goncalves et al., 2013; Liu et al., 2018; Tóth et al., 2002; Wang X., et al., 2016) and accumulation of GLs can be therefore observed under both N- and P-limitation. The GL species that most contributed to the discrimination of treatment groups in the PLS-DA models (i.e. MGDG 34:3, DGDG 34:3, SQDG 34:3, DGDG 36:3 and SQDG 36:6) also displayed a significantly higher relative abundance in $[N,P]_{low}$ than in either CT or $[N,P]_{high}$.

The PLs that allowed some discrimination between groups were PA 34:1, PA 36:3 PA 34:2, PI 36:6, and PC 36:6. The PAs displayed higher intensity as the input of P increased meanwhile PI 36:6 and PC 36:6 displayed an opposite pattern. This is also evident from PAs relative abundances, since PA 34:1, PA 34:2, and PA 36:3 were more abundant in CT than the other groups, while C36:6 species were generally in lower abundance in CT. In plants, PAs are precursors of PL and GL synthesis and also function as signal molecules of environmental stress (Dubots et al., 2012). The marked differences in the abundance of several PA species between CT and the other treatments could be related to the activation of different metabolic pathways mediated by the availability of P. Moreover, certain FA configurations were constantly associated with lipid species that displayed significant differences in relative abundance (e.g. C34:1, C34:2, C34:3, and C36:6). For instance, C34:3 displayed the highest abundance in $[N,P]_{low}$ across all classes of PLs (except PA) and GLs. In general, variations in relative abundance were observed most evidently between $[N,P]_{low}$ and both the CT and $[N,P]_{high}$ treatments, suggesting a possible metabolic adaptation from high-input to low-input conditions.

Following the observations discussed above, *H. portulacoides* was probably under some level of nutrient limitation under $[N,P]_{low}$. Firstly, they exhibited less PLs and GLs per unit weight of leaves. Secondly, GLs were suffering some degree of remodeling. Thirdly, the relative abundances of certain species in each class changed as a function of N and/or P availability, as suggested by their gradual increase (or decrease) from low-input to high-input of N and/or P. Nonetheless, one could argue about the extent of nutrient limitation that *H. portulacoides* was potentially exposed to under the $[N,P]_{low}$ treatment, by looking at how other plants behaved in similar conditions. For instance, wild specimens of *Arabidopsis thaliana* (L.) Heynh, exposed to 0.03 mM P (similar to $[N,P]_{low}$) during 12 days followed by 4 days without P, were considered P-starved as they exhibited significant decreases in shoot's PLs (PC, PE, PG, and PS) and significant increases in shoot's GLs (MGDG, DGDG, SQDG)(Pant et al., 2015). Some species of MGDG and DGDG were also found at markedly higher levels in soybean (*Glycine max* (L.) Merr.) leaves under P-limited conditions (Okazaki et al., 2017). In this experiment, *H. portulacoides* did not exhibit such patent changes in the lipidome in the low-input conditions, which indicates that plants were not starved. Note, however, that *H. portulacoides* is a perennial plant, and both *A. thaliana* and *G. max* are annual plants, and these different life-history strategies might affect nutrient utilization and threshold conditions for nutrient-limitation (Friedman & Rubin, 2015; Rennenberg & Schmidt, 2010). Another important fact to consider is that the impact of P-limitation might not affect leaves homogeneously. For instance, in *G. max* under P-limitation, there seems to be a mechanism of P-remobilization from older leaves, where differences in the lipidome between limited and non-limited conditions were substantial, to younger leaves, where the lipidome profile between different conditions was very similar (Okazaki et al., 2017). In the present study, there was no control regarding leaves' age, as the lipidome was representative of the total pool of leaves from *H. portulacoides*.

Plants, in general, display a range of responses to low P (generally referred to as P-starvation responses) that aim to minimize the negative effects of its scarcity in plants (e.g. decreased growth, increased root/shoot ratio, increased root-hair density, increased carboxylate exudation, P-remobilization) (Karthikeyan et al., 2014; Lambers et al., 2006; Plaxton & Tran, 2011; Ticconi et al., 2001; Yang & Finnegan, 2010). Under the conditions of the present experiment, the extent to which *H. portulacoides* underwent a starvation response under $[N,P]_{low}$ that affected the polar lipids of its leaves was defined by a decrease in total GLs and PLs and some degree of lipid remodeling detected in the GLs pool. The availability of P was still high enough in the low-input treatment to maintain the PLs pool relatively unchanged.

Halimione portulacoides appears to be a good candidate for IMTA in terms productivity and nutrient-extraction (Buhmann et al., 2015; Marques et al., 2017) and has the potential to become a valuable co-product with uses in human nutrition (Barreira et al., 2017) and for other applications (Custódio et al., 2017; Horn and Benning, 2016; Maciel et al., 2018). A note should be made, however, about the possibility of halophytes accumulating undesired compounds if these are present in effluents, like metals (Cabrita et al., 2019; Castro et al., 2009) and chemicals used for therapy and prophylaxis in aquaculture (Kümmerer, 2009).

This possibility must be taken into account when selecting halophytes for IMTA, since the accumulation of contaminants in edible plant organs can pose risks to human health (Rai et al., 2019) and species that do not accumulate contaminants or concentrate them mostly in non-edible tissues will be more appropriate from a product-safety perspective. The same concern has been put forward regarding other extractive species (e.g. seaweeds) and changes in regulatory frameworks are necessary to promote the safety of new products from IMTA (Alexander et al., 2015; Stévant et al., 2017).

4.5. Conclusion

Within an IMTA framework, it is fair to conclude that *H. portulacoides* is capable of maintaining a fully stable lipidome across a variety of N and P concentrations typical of aquaculture effluents, specifically 20 – 100 mg DIN-N L^{-1} and 3 – 15.5 mg DIP-P L^{-1}. At lower concentrations (e.g. $[N,P]_{low}$ values: 6 mg DIN-N L^{-1} and 0.8 mg DIP-P L^{-}1) the leaves' lipidome displays some changes, particularly regarding GLs, as well as a generalized decrease in the quantity of polar lipids in the leaves. These changes suggest a metabolic adaptation to the lower nutrient conditions and could be indicative of nutrient limitation. Data on growth performance support a scenario of nutrient-limited conditions in $[N,P]_{low}$, as *H. portulacoides* exposed to those same concentrations of N and P produced less biomass than those exposed to higher concentrations.

Determining which nutritional conditions can lead to nutrient-limitation scenarios is important information for future IMTA/halophyte producers, to guide nutrition strategies that guarantee a consistent end-product, especially under highly variable nutritional outputs which can occur in aquaculture activities. For researchers, this data can guide the establishment of reference nutritional concentrations for future studies targeting the production of *H. portulacoides*. Future lipidomic studies in *H. portulacoides* should also attempt to characterize and quantify seed oils, since these comprise a significant fraction of the aboveground biomass during the reproductive period of this species and could have valuable high-end applications, such as pharmaceuticals, biofuels, detergents, polymers, and cosmetics.

Fully characterizing the diversity of lipid species across *H. portulacoides* tissues and how they change along the production cycle and environmental conditions is of tremendous importance for the commercial exploration of its lipids. This will allow for strategic choices to be made on how to produce it and manipulate its life cycle so to maximize the delivery of value-added compounds with commercial applications and consequently increase its economic value.

Chapter 5

Optimization of plant density and lighting conditions for hydroponic production of *Halimione portulacoides*, an edible halophyte for saline farming

Submitted for publication

5. Optimization of plant density and lighting conditions for hydroponic production of *Halimione portulacoides*, an edible halophyte for saline farming

Abstract

Halimione portulacoides (L.) Aellen, an edible saltmarsh halophyte popularly known as sea purslane, is a candidate extractive species for coastal Integrated Multi-Trophic Aquaculture (IMTA) with further horticultural potential. Saline crops can be integrated into aquaculture systems to extract wasted dissolved inorganic nutrients from fish-farming effluents and be cultivated in salt-affected areas. The present study investigates how 'plant density' and 'artificial lighting' affect *H. portulacoides* production and nutrient extraction efficiency in saline hydroponics, providing important information for its potential use in IMTA systems (aquaponics) and hydroponic production. Plants were unaffected by the type of artificial light (white fluorescent lights *vs* blue LEDs) but high-density units (220 plants m^{-2}) produced more biomass per unit of area (54.0 – 56.6 g m^{-2} day^{-1}) than low-density units (110 plants m^{-2}; 34.4 – 37.1 g m^{-2} day^{-1}) and, in total, extracted more dissolved inorganic nitrogen (DIN) and phosphorus (DIP). Nonetheless, nitrogen extraction rates expressed in terms of biomass produced were higher in low-density units (5.8 – 6.4 mg g^{-1}) compared with high-density units (4.7 – 5.4 mg g^{-1}). Phosphorus extraction rates were comparable (~0.3 mg g^{-1}). LED lighting is a suitable option for cost-effective systems. Overall, sea purslane *H. portulacoides* can be successfully cultivated in saline hydroponics and plant density can be optimized to improve both productivity and nutrient extraction capacity of hydroponic production units.

Keywords: halophyte, saline agriculture, light-emitting diodes, hydroponics, aquaponics, nutrients

5.1. Introduction

Halophytes are plants characterized by a range of morphological and physiological features that allow them to thrive in saline environments (Flowers and Colmer, 2008) and studying their horticultural potential, particularly the edible ones, in the context of sustainable food systems has never been more timely (Fedoroff et al., 2010; Willett et al., 2019). Edible halophytes can produce food in saline conditions and can, therefore, promote food security in salt-affected areas and open the possibility for saltwater irrigation in coastal regions (Atzori et al., 2019; Cheeseman, 2015; Glenn et al., 1999; Panta et al., 2014; Ventura et al., 2015; Ventura & Sagi, 2013).

Edible halophytes are being advocated as new saline crops (e.g. *Salicornia* spp. and *Sarcocornia* spp.) capable of delivering food products with distinct organoleptic and functional properties, vegetable oils and bioactive compounds with numerous applications (e.g. Barreira et al., 2017; Buhmann & Papenbrock, 2013; Ksouri et al., 2012; Loconsole et al., 2019; Maciel et al., 2018; Sharma et al., 2016). Moreover, their relatively low-sodium content makes them suitable salt alternatives to reduce sodium intake in populations at risk (WHO, 2012; WHO, 2015). Portugal, for instance, is one of the first European countries to recognize halophytes as salt alternatives that must be explored in the context of healthy food habits, as stated in recent food policy actions (Despacho n.° 11418/2017) (Graça et al., 2018). Given halophytes' socioeconomic, nutritional, and health benefits, investigations on their horticultural potential must continue.

In the context of aquaculture farming, halophytes have been studied as a nature-based solution for the treatment of eutrophic saline aquaculture effluents through an integrated production framework commonly known as Integrated Multi-Trophic Aquaculture (IMTA) (Chopin et al., 2008). A growing body of research in IMTA already demonstrates the horticultural potential and remediation capacity of several halophyte species using constructed wetlands and hydroponic systems as cultivation/remediation units (Custódio et al., 2017). Leading experts in agriculture believe that the ability of the industry to meet future demands for food sustainably depends on the development of farming systems that use saline water and integrate nutrient flows (Fedoroff et al., 2010), and coupling saline farming with IMTA fits perfectly within this agriculture paradigm. Moreover, the horticultural production of perennial crops has been advocated as more sustainable than that of annual crops (Smaje, 2015; Vico et al., 2016) and, under this assumption, perennial plants such as the sea purslane should be prioritized in the development of new vegetable crops in the context of sustainable foods systems (Willett et al., 2019).

Even though halophytes can be successfully cultivated outdoors under natural conditions (e.g. Glenn et al., 2013; Ventura and Sagi, 2013; Marques et al., 2017), their production in controlled indoor environments is a possibility that must be explored to create the conditions to maximize productivity and improve nutritional quality and bio-security (Benke & Tomkins, 2017; Rouphael et al., 2018). Light is a major variable that modulates plant development (Gelderen et al., 2018; Ouzounis et al., 2015), and providing optimal light conditions is paramount in commercial plant production (Jones, 2018). Full control of light parameters (e.g. spectrum, irradiance, photosynthetically active radiation) can only be achieved through artificial lighting systems, with solid-state LED lighting as the most flexible and cost-efficient technology for sustainable indoor horticulture (Viršilė et al., 2017).

Different light spectra produced by LEDs have been shown to influence yields and the synthesis of bioactive metabolites (e.g. photosynthetic pigments) in conventional crops, particularly leafy greens (Alrifai et al., 2019; Hasan et al., 2017). Several lettuce varieties (*Lactuca sativa* L. cultivars), basil (*Ocimum basilicum* L.), lentil (*Lens culinaris* Medik.) and some microgreens species (e.g. *Brassica oleracea* L. cultivars) growing in soil-based substrates displayed species-specific changes in their productivity and pool of photosynthetic pigments resulting from different LEDs spectral bands, which generally outperformed other types of artificial lighting and natural lighting (e.g. Kook et al., 2013; Sabzalian et al., 2014; Długosz-Grochowska et al., 2016; Kyriacou et al., 2016; Amoozgar et al., 2017). Regarding halophytes indoor cultivation, data on the influence of light spectra in vegetative development is still very limited (He et al., 2017; Sanoubar et al., 2018; Weeplian et al., 2018).

Plant density is another key variable regulating individual growth in plants. For instance, high-density planting can induce competitive behavior and change individual development (Cha et al., 2016; Poorter et al., 2012; Truax et al., 2018). A practical example of the relevance of choosing the appropriate plant density in plant production is the case of maize (*Zea mays* L.). Generally speaking, individual-level productivity of maize typically decreases with increasing plant density, while population-level yields increase (Cazetta & Revoredo, 2018; Jiang et al., 2018; Zhai et al., 2018). Since most maize producers process the whole aboveground biomass to be used in the animal feed industry (Shah et al., 2016), total population productivity is more important and high-density planting should be therefore prioritized. On the other hand, if the development of specific organs is targeted (e.g. cobs, kernels), high-planting density can hinder productivity (Greveniotis et al., 2019; Sangoi, 2001). In the case of halophytes, some species have previously shown a reduction in individual growth as plant density increased (e.g. *Atriplex prostrata* Boucher) (Wang et al., 2005) while others seem to be relatively unaffected by plant density (e.g. *Batis maritima* L., *Cressa cretica* L., *Salicornia europaea* L., *Sesuvium portulacastrum* (L.) L.) (Boxman et al., 2017; Khan & Aziz, 1998; Webb et al., 2013). Since different plant species can have different biomass allocation strategies under crowding conditions (Poorter et al., 2012), experimental grow-out studies can provide valuable information about the planting densities that maximize yields of target organs (e.g. fruits, seeds or leaves).

Indoor hydroponic cultivation experiments using halophyte crops are still limited in the scientific literature and the present study is a contribution to fill in this research gap. The main goal of this work is to determine the conditions that benefit the hydroponic production of the sea purslane *Halimione portulacoides* (L.) Aellen, a prospective extractive species for saltwater-based IMTA, by understanding the influence of plant density and artificial lighting on vegetative development and nutrient extraction. Potential changes in the pool of photosynthetic pigments are also assessed. The information provided is particularly relevant to halophyte producers interested in sea purslane as a potential saline crop and aquaculture farmers interested in the integration and operationalization of halophyte production units in their aquaculture activities following an IMTA approach.

5.2. Material and methods

The effects of two plant densities and two artificial lighting sources on the growth performance of *H. portulacoides* were tested under controlled conditions using a two-level factorial design with five replicated hydroponic units per treatment. Measurements consisted of recording individual biomass gain (one plant per hydroponic unit) every week and total biomass yields (whole, below- and aboveground), number of leaves, and stems' length. Photosynthetic pigments were also quantified as a proxy for the status of the photosynthetic apparatus. Nutrient extraction efficiencies were obtained through measurements and mass balance calculations of ammonium (NH_4-N), oxidized forms of inorganic nitrogen (NO_x-N), and orthophosphate (PO_4-P) in the hydroponic solution, at the beginning and the end of the extraction cycle. The initial concentrations of inorganic nitrogen (N) and phosphorus (P) in each batch of experimental hydroponic were around 60 mg N L^{-1} (4.3 mM N) and 3 mg P L^{-1} (0.1 mM P). Results from a previous trial (Chapter 3) demonstrated that these concentrations are non-limiting to *H. portulacoides* within the conditions of this experiment, which excludes the possibility of nutrient limitation affecting outcome variables.

5.2.1. Plant material

Stems of *Halimione portulacoides* were collected from wild specimens in Ria de Aveiro (Portugal) coastal lagoon (40°38'04.1''N 8°39'40.0''W) in March 2018 and transported to the laboratory. Six-hundred grafts with 4 nodes each were obtained from those stems and placed in polyethylene containers with a Hoagland's nutrient solution to promote root development. The elemental composition of the nutrient solution was: 40 mg Ca L^{-1}, 60 mg K L^{-1}, 16 mg Mg L^{-1}, 56 mg N L^{-1}, 16 mg P L^{-1}, 0.28 mg B L^{-1}, 0.03 mg Cu L^{-1}, 1.12 mg Fe L^{-1}, 0.11 mg Mn L^{-1}, 0.34 mg Mo L^{-1}, 0.13 mg Zn L^{-1}. Grafts developed under natural light and temperature for 3 months and, in June 2018, rooted grafts were randomly distributed over the hydroponic units. The average initial biomass was 6.8 g $plant^{-1}$ in all treatments. Plants were acclimated to the new hydroponic indoor conditions and progressively adapted to a salinity of 20 ppt (0.5% increments of NaCl every second day) for one week, before the beginning of the grow-out trial.

5.2.2. Experimental setup

The grow-out trial took place over a period of 10 weeks on a deep-water culture hydroponics configuration using extruded polystyrene floating-rafts (Figure A5.1 in Annex). The hydroponic units were made of opaque polypropylene boxes (300 x 200 x 170 mm) with an overflow outlet to maintain water volume at 5 L. The base aquatic medium was artificial seawater prepared by dissolving commercial Red Sea salt (Red Sea, Cheddar, UK) in tap water purified by reverse osmosis (V2Pure 360 RO System, TMC, Hertfordshirem, UK) until achieving a salinity of 20 ppt. The experimental hydroponic media was a modified version of the Hoagland's solution described above, using non-limiting and realistic concentrations of dissolved inorganic N and P as measured in fish-farming effluents. The detailed nutrient composition of the experimental hydroponic solution is presented in Table A 5.1 (Annex).

The experiment consisted of a factorial design with two factors, each with two levels: i) plant density (levels: 110 plants m^{-2} *vs* 220 plants m^{-2}); and ii) artificial lighting (levels: fluorescent lights *vs* LEDs). Treatment conditions were labeled as follows: F110 = fluorescent lights & 110 plants m^{-2}; F220 = fluorescent lights & 220 plants m^{-2}; L110 = LEDs & 110 plants m^{-2}; L220 = LEDs & 220 plants m^{-2}. Each treatment condition was replicated five times (n = 5), in a total of 20 hydroponic units with a water surface area of 0.0455 m^2. Grafts were fixed to the floating rafts using natural cotton held at the base of the aerial portion. The hydroponic medium was continuously aerated to maintain aerobic conditions and hydroponic units were refilled, as needed, with tap water purified by reverse osmosis to compensate for evapotranspiration. The fluorescent light was provided by tubular fluorescent lamps (Philips 54W/830 Min Bipin T5 HO ALTO UNP), while LED light was provided by solid-state LED lighting tiles (AquaBeam 1500 Ultima NP Ocean Blue Light). The spectra of both artificial lighting sources are represented in Figure A5.2 (Annex section). The photoperiod was set at 14 h light: 10 h dark and photosynthetically active radiation (PAR) reaching the plants was adjusted at the beginning of every week for every artificial lighting source to deliver identical values. The PAR values reaching the top of the canopy of stocked plants were measured twice a week with a spherical micro quantum sensor (US-SQS/L, Heinz Walz, Pfullingen, Germany), averaging 371.0 ± 12.0 µmol photons m^{-2} s^{-1}.

Water temperature and pH were measured with a multi-parameter portable meter (ProfiLine pH/Cond 3320, WTW, Weilheim, Germany) and dissolved oxygen was measured with a portable oxygen meter (Oxi 3310, WTW, Weilheim, Germany). Measurements were performed twice a week (day 2 and day 7) and average weekly values are presented in Figure A5.3 (in Annex). Overall, hydroponic units displayed an average water temperature of 22.9 ± 0.7 °C, a pH of 7.8 ± 0.2 and a dissolved oxygen concentration of 6.7 ± 0.6 mg L^{-1}.

5.2.3. Growth performance

One-hundred and fifty grafts with similar weights were randomly selected and assigned to hydroponic units, individually identified, photographed, and weighed, before the beginning of the experiment. One plant per experimental unit was randomly chosen and its total weight was measured at the end of every experimental week to track individual growth overtime (5 plants per treatment). At the end of the experiment (week 10), each plant was individually photographed, divided into roots, stems, and leaves and weighed. The three plant organs were pooled per experimental unit and stored at -80 °C. Image-analysis software (ImageJ 1.51) was used to measure the length of stems and to count the leaves.

5.2.4. Nutrient extraction efficiency

The efficiency of *H. portulacoides* hydroponic units in the extraction of N and P from a nutritive saline solution with 60 mg N L^{-1} (4.3 mM N) and 3 mg P L^{-1} (0.1 mM P) was determined using a retention time (amount of time the solution remains in the remediation/extraction unit) of one week (7 days). The time frame allowed for nutrients to be taken up by plants is crucial to the performance of a nutrient extraction unit and appropriate duration can be highly variable and species-specific. Previous nutrient extraction studies with halophytes have used a wide range of retention times, from 12 hours to 5 weeks (e.g. Ventura et al., 2011a; Webb et al., 2013; Buhmann et al., 2015; Marques et al., 2017). In constructed wetlands, time recommendations for significant extraction of contaminants are between 3 - 10 days, with longer retention times achieving better extraction efficiencies of N and P (García et al., 2010; Wu et al., 2015; Vera et al., 2016). Given that, a one-week (7 days) retention time was considered appropriate for this study.

By the end of each extraction period, water samples from each hydroponic unit were collected and filtered (Whatman GF/C filters) to flasks and final concentrations of ammonium (NH_4-N), oxidized forms of inorganic nitrogen (NO_x-N) and orthophosphate (PO_4-P) in the hydroponic media were determined using a San^{++} Continuous Flow Analyzer (Skalar Analytical, Breda, The Netherlands) following Skalar standard automated methods (NH_4-N: modified Berthelot reaction for ammonia determination; NO_x-N: Total UV digestible nitrogen/ nitrate + nitrite/ nitrite; PO_4-P: Total UV digestible phosphate/orthophosphate). After sampling, the medium in each unit was renewed with a new batch of hydroponic treatment solution and real initial concentrations of NH_4-N, NO_x-N, and PO_4-P in every new batch were determined, as described above. Nutrient extraction efficiency was estimated, both weekly and in total, for dissolved inorganic nitrogen (DIN-N), calculated as the sum of NH_4-N and NO_x-N, and dissolved inorganic phosphorus (DIP-P), equivalent to PO_4-P.

5.2.5. Photosynthetic pigments

Samples from the frozen pool of leaves of each hydroponic unit were freeze-dried and pigments were extracted using 95% cold-buffered methanol (2% ammonium acetate). Before extraction, samples were grounded with a mortar and 2-3 mg of sample were weighed into Eppendorf tubes. Subsequently, 1 mL of extraction solvent was added to each tube, followed by 45 s sonication and 20 min incubation at –20 °C in the dark. The extracts obtained were filtered through 0.2 μm PTFE membrane filters and 50 μL were injected into an HPLC equipment with an SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan). The chromatographic separation of pigments was achieved using a Supelcosil C18 column (Sigma-Aldrich, St. Louis, MO, USA) following Cruz et al. (2014). Pigments were identified from absorbance spectra and retention times and concentrations were calculated using linear regression equations obtained from pure crystalline standards (DHI, Hørsolm, Denmark).

5.2.6. Statistical analysis

Statistical analysis was performed using R v3.4.3 (64-bit) with R Studio and statistically significant differences were considered at $p < 0,05$. A two-way ANOVA was performed to assess the effects of ‘plant density’ and ‘artificial lighting’ on outcome variables. Data were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Levene’s test) before analysis. Post-hoc Tukey’s HSD test for individual means comparison was performed when significance was observed. A repeated-measures ANOVA was used to assess treatment differences in cumulative biomass gain and N/P extraction efficiency across time points. The Geenhouse-Geisser correction was applied when the sphericity assumption was violated, and Bonferroni correction was used when performing multiple pairwise comparisons.

5.3. Results

5.3.1. Grow-out parameters and productivity

At the beginning of the experiment, the initial biomass per plant was 6.80 ± 0.04 g, and significant differences in grow-out parameters were detected after 10 weeks (Table 5.1). At the individual level, treatment F110 resulted in significantly higher ($p < 0.05$) aboveground biomass than L220 and a higher ($p < 0.05$) stem development than F220 and L220. At the population level (pooled individual weights), both F220 and L220 resulted in significantly higher ($p < 0.05$) aboveground and belowground biomass, number of leaves and stem development than both F110 and L110. Total biomass production in F220 was higher ($p < 0.05$) than both L110 and F110, meanwhile L220 only differed ($p<0.05$) from L110. The same trend was observed in total productivity (expressed as g m^{-2} day^{-1}).

Cumulative biomass gains over time (Figure 5.1) suggest a large variance in individual weight in each treatment and, as a result, main effects of neither 'plant density' nor 'artificial lighting' were detected. Only a within-subject effect of 'week' was observed [$F_{(76.4,1222.7)}$= 120.58, p= 2.88e^{-09}, generalized η^2= 0.76].

Table 5.1: Growth parameters of *Halimione portulacoides* hydroponic units (n = 5). Different letters indicate significant differences between treatments ($p < 0.05$). F110 = fluorescent lights & 110 plants m^{-2}; F220 = fluorescent lights & 220 plants m^{-2}; L110 = LEDs & 110 plants m^{-2}; L220 = LEDs & 220 plants m^{-2}.

			F110	F220	L110	L220
Initial biomass (individual)		g $plant^{-1}$	6.8 ± 0.3	6.8 ± 0.2	6.8 ± 0.1	6.8 ±0.1
Initial biomass (hydroponic unit)		g $unit^{-1}$	34.0 ± 1.4	68.5 ± 1.5	33.9 ± 0.5	67.6 ± 1.3
Individual performance						
Final biomass	Total	g $plant^{-1}$	30.2 ± 5.0	24.7 ± 4.5	28.4 ± 4.9	23.8 ± 3.6
	Aboveground	g $plant^{-1}$	25.7 ± 4.4 ^{a}	21.1 ± 4.0 ^{ab}	24.4 ± 4.5 ^{ab}	20.3 ± 3.2 ^{b}
	Belowground	g $plant^{-1}$	4.5 ± 0.7	3.6 ± 0.5	4.1 ± 0.4	3.5 ± 0.5
Leaves		n $plant^{-1}$	243 ± 36	205 ± 34	261 ± 33	218 ± 28
Stems		mm $plant^{-1}$	55.2 ± 7.0 ^{a}	41.2 ± 5.5 ^{b}	50.3 ± 8.3 ^{ab}	40.9 ± 3.8 ^{b}
Unit performance						
Final biomass	Total	g $unit^{-1}$	150.8 ± 25.2 ^{ac}	246.9 ± 44.9 ^{b}	142.2 ± 24.3 ^{c}	237.6 ± 36.4 ^{ab}
	Aboveground	g $unit^{-1}$	128.5 ± 21.8 ^{a}	210.7 ± 40.4 ^{b}	121.9 ± 22.5 ^{a}	202.9 ± 32.1 ^{b}
	Belowground	g $unit^{-1}$	22.3 ± 3.4 ^{a}	36.2 ± 5.3 ^{b}	20.3 ± 2.0 ^{a}	34.7 ± 4.6 ^{b}
Leaves		n $unit^{-1}$	1215 ± 178 ^{a}	2050 ± 344 ^{b}	1305 ± 167 ^{a}	2176 ± 284 ^{b}
Stems		mm $unit^{-1}$	276.0 ± 35.2 ^{a}	412.0 ± 54.9 ^{b}	251.5 ± 41.4 ^{a}	409.2 ± 38.4 ^{b}
Root: shoot ratio		-	0.17 ± 0.01	0.17 ± 0.02	0.17 ± 0.02	0.17 ± 0.01
Relative growth rate		mg g^{-1} day^{-1}	21.1 ± 2.1	18.1 ± 2.5	20.3 ± 2.3	17.8 ± 2.1
Productivity		g m^{-2} day^{-1}	37.1 ± 7.8 ^{ac}	56.6 ± 14.0 ^{b}	34.4 ± 7.6 ^{c}	54.0 ± 11.5 ^{ab}

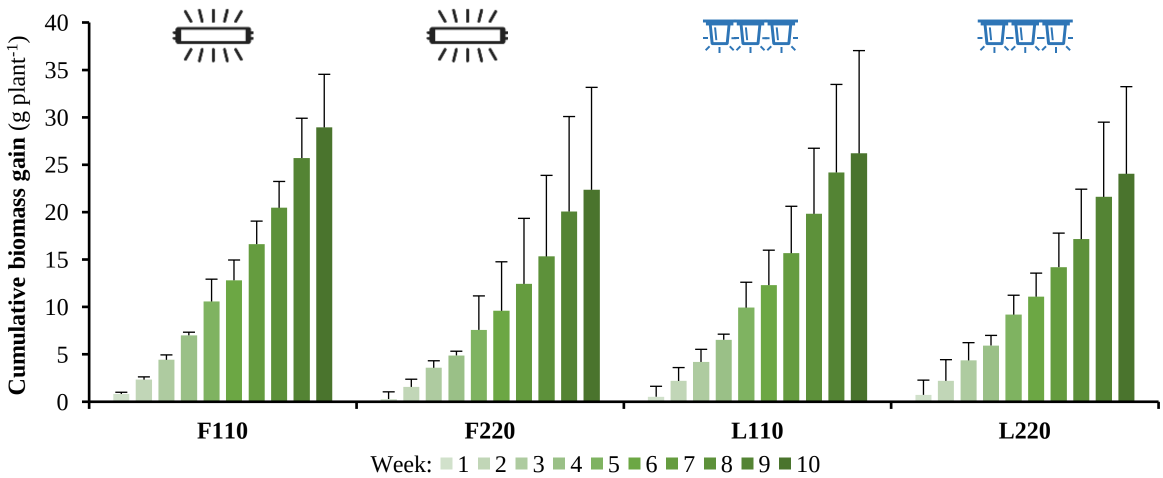


Figure 5.1: Individual-level cumulative weight gain. Bars represent standard deviations. F110 = fluorescent lights & 110 plants m^{-2}; F220 = fluorescent lights & 220 plants m^{-2}; L110 = LEDs & 110 plants m^{-2}; L220 = LEDs & 220 plants m^{-2}.

5.3.2. Extraction of dissolved inorganic N and P

Weekly initial concentrations of NH_4-N were 1.76 ± 0.12 mg N L^{-1} (0.13 ± 0.01 mM N), of NO_x-N were 61.50 ± 4.72 mg N L^{-1} (4.39 ± 0.34 mM N) and of DIN-N were 63.26 ± 4.82 mg DIN-N L^{-1} (4.52 ± 0.34 mM N). Orthophosphate concentrations were 3.09 ± 0.15 mg PO_4-P L^{-1} (0.10 ± 0.01 mM P).

DIN-N extraction efficiencies in each hydroponic unit were measured at the end of each extraction period (Figure 2A) and significant main effects of 'plant density' ($F_{(1,16)}$= 5.97, p= 0.027, generalized η^2= 0.11) and 'week' ($F_{(21.08,\ 337.24)}$ = 13.86, p=5.92e^{-08} , generalized η^2= 0.366) were detected by repeated measures ANOVA. An interaction effect of 'plant density: week' ($F_{(21.08,\ 337.24)}$ = 5.98, p=4.67e^{-04}, generalized η^2= 0.20) was also observed.

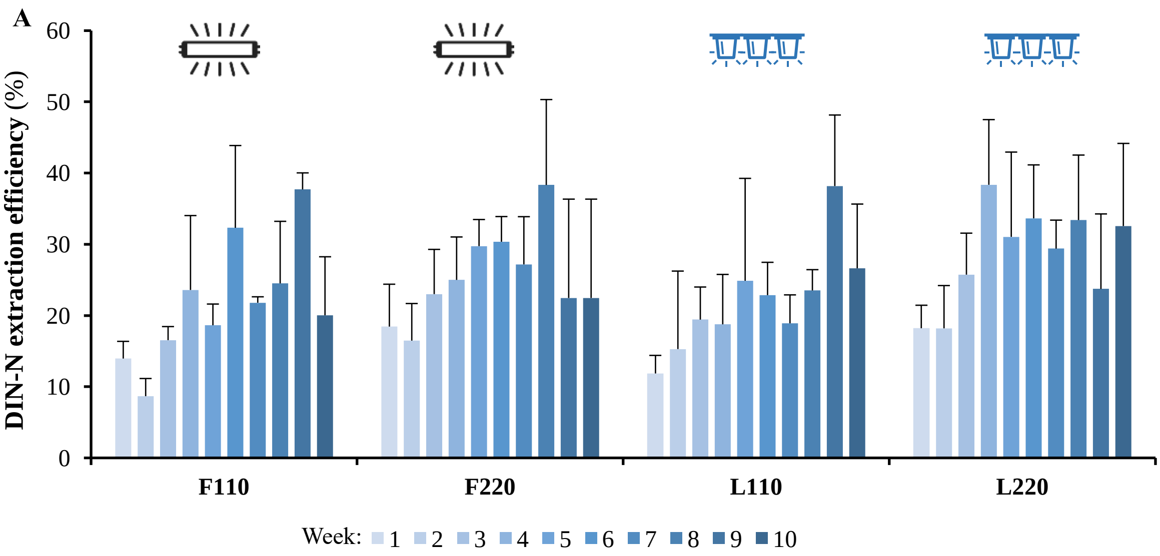
A main effect of 'plant density' ($p < 0.05$) was also observed regarding the total quantity of DIN-N extracted during the experiment (Figure 3A). High-density units extracted 2.5 (± 0.5) mg DIN-N L^{-1} day^{-1} (total = 875.3 ± 187.7 mg) and low-density units extracted 2.0 (± 0.3) mg DIN-N L^{-1} day^{-1} (total = 686.4 ± 94.2 mg). However, pairwise comparisons did not detect a treatment effect at the threshold p-value of 0.05. Overall, high-density units extracted more DIN-N on average than low-density units and extraction efficiencies were, according to each treatment, as follows: F110= 21.6 ± 1.8%; F220= 26.3 ± 6.9%; L110= 21.8 ± 4.1%; L220= 29.1 ± 5.1%.

After correcting the total amount of DIN-N removed per unit of biomass produced (Figure 3C), main effects of ‘plant density’ ($p < 0.001$) and ‘artificial lighting’ ($p < 0.05$) were detected: low-density units and LED units resulted in higher DIN-N extraction per unit of biomass produced. Pairwise comparisons show that F220 removed significantly less ($p < 0.01$) DIN-N per unit of biomass (4.7 ± 0.2 mg g^{-1}) than both F110 (6.0 ± 0.9 mg g^{-1}) and L110 (6.4 ± 0.6 mg g^{-1}).

Regarding the results of DIP-P extraction efficiency over time (Figure 2B), main effects were detected in ‘plant density’ [$F_{(1,16)}$= 14.25, p= 0.002, generalized η^2= 0.35] and ‘week’ ($F_{(30.2, 483.2)}$ = 111.60, p=1.37e^{-19} , generalized η^2= 0.738]. The interaction of ‘plant density: week’ also exerted a significant effect [$F_{(30.2, 483.2)}$ = 7.77, p=4.60e^{-04}, generalized η^2= 0.16].

The total amount of DIP-P removed (Figure 3B) was also affected by ‘plant density’ ($p < 0.01$) and pairwise comparisons indicated significantly lower values in F110 and L110 compared with both F220 ($p < 0.05$) and L220 ($p < 0.01$). High-density units extracted 0.16 (± 0.04) mg DIP-P L^{-1} day^{-1} (total = 56.3 ± 14.6 mg) and low-density units extracted 0.11 (± 0.01) mg DIP-P L^{-1} day^{-1} (total = 36.9 ± 5.2 mg). Overall, high-density units extracted more DIP-P in total than low-density units and extraction efficiencies recorded were as follows: F110= 24.6 ± 3.7%; F220= 35.5 ± 10.8%; L110= 23.1 ± 3.2%; L220= 37.4 ± 9.0%.

In terms of total DIP-P removed per unit of biomass (Figure 3D), neither main effects nor treatment effects were detected, and values ranged between 0.30 – 0.34 mg DIP-P per gram of biomass produced.



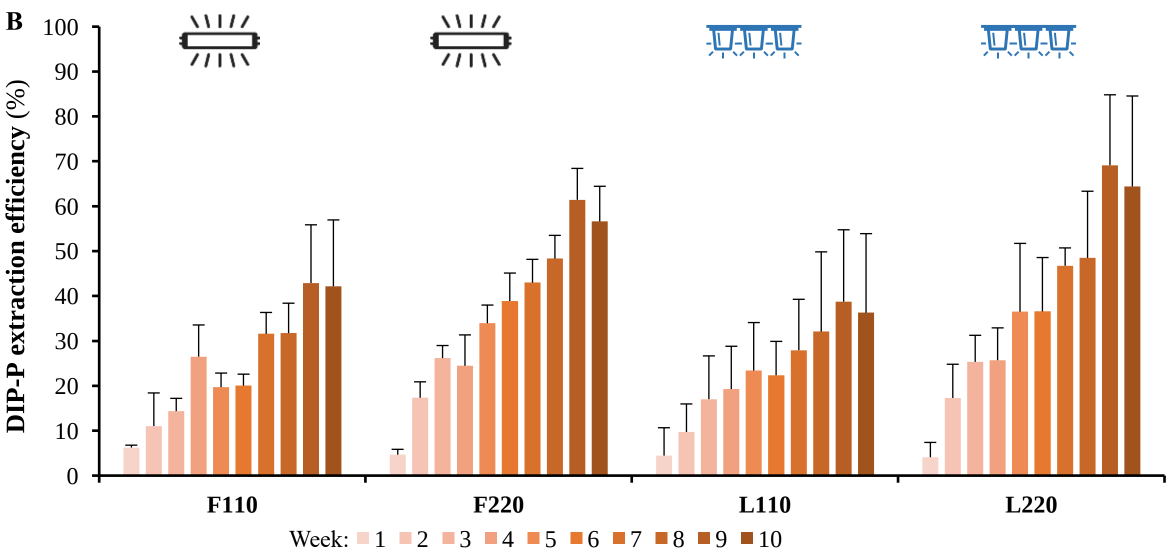


Figure 5.2: Extraction efficiency of DIN-N (A) and DIP-P (B). Bars represent standard deviations. F110 = fluorescent lights & 110 plants m^{-2}; F220 = fluorescent lights & 220 plants m^{-2}; L110 = LEDs & 110 plants m^{-2}; L220 = LEDs & 220 plants m^{-2}.

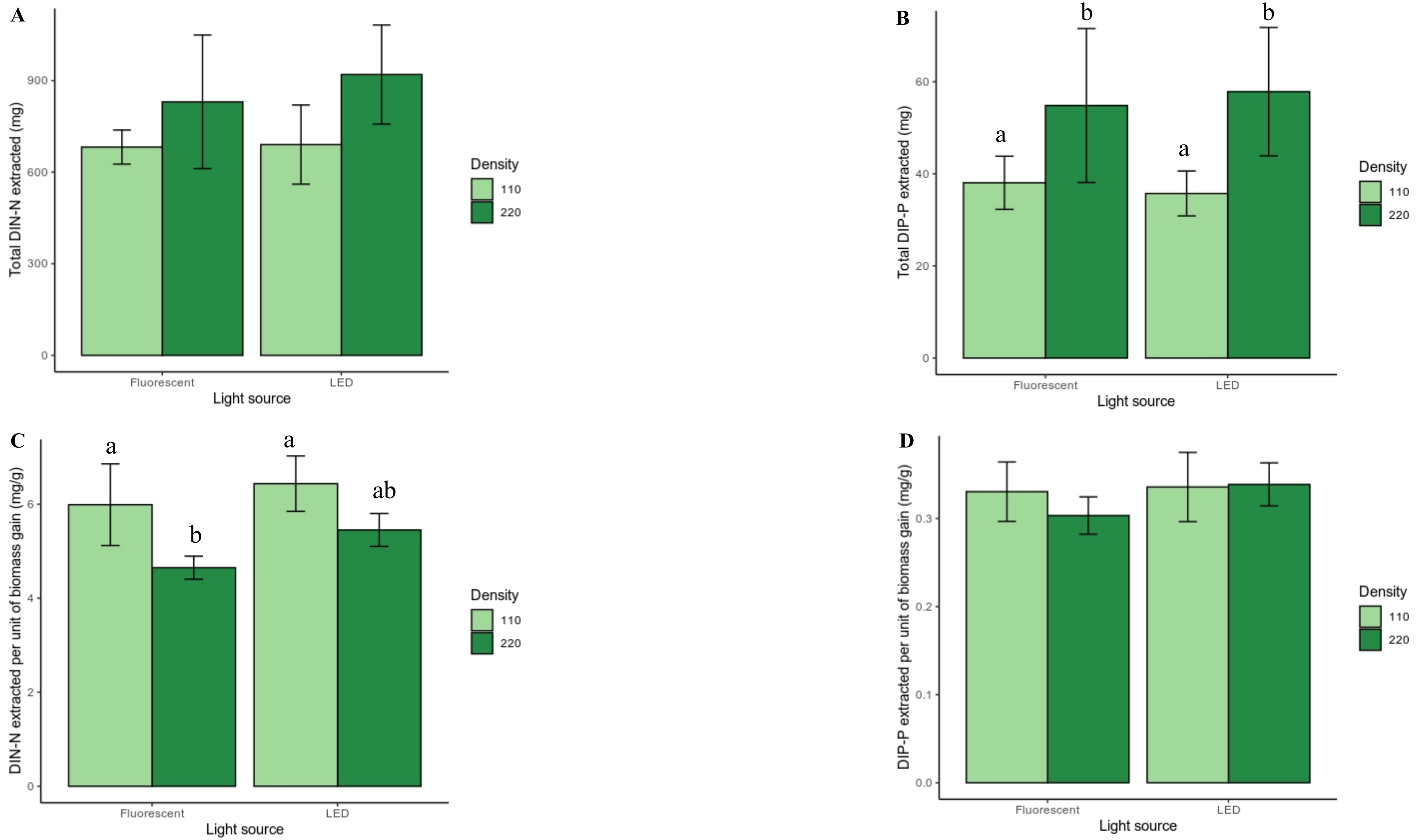


Figure 5.3: Total quantity of extracted DIN-N (A) and DIP-P (B) and relative quantity (as per biomass production) of extracted DIN-N (C) and DIP-P (D). Bars represent standard deviations. Different letters indicate significant differences between treatments ($p < 0.05$).

5.3.3. Photosynthetic pigments

The following photosynthetic pigments were quantified in *H. portulacoides* leaves: antheraxanthin (Ant), chlorophylls *a* and *b* (Chl *a*, Chl *b*), 9'*cis*-neoxanthin (*c*-Neo), lutein (Lut), violaxanthin (Viola), zeaxanthin (Zea) and β,β-carotene (ββ-Car). Pigment concentrations *per* leaf dry weight are summarized in Table 5.2 and statistical analysis suggests concentrations were not affected by treatments. A main effect ($p < 0.05$) of 'plant density' on the concentration of Ant and Zea was nonetheless detected: average concentrations of these two xanthophylls were higher in plants growing at low-density (Ant=38 – 40 $\mu g\ g^{-1}$ DW; Zea= 42 – 51 $\mu g\ g^{-1}$ DW) than plants in high-density units (Ant= 32 – 37 $\mu g\ g^{-1}$ DW; Zea= 36– 39 $\mu g\ g^{-1}$ DW).

Table 5.2: Photosynthetic pigments concentrations in *Halimione portulacoides* leaves (n = 5). F110 = fluorescent lights & 110 plants m^{-2}; F220 = fluorescent lights & 220 plants m^{-2}; L110 = LEDs & 110 plants m^{-2}; L220 = LEDs & 220 plants m^{-2}. Xanthophyll (Xant) = c-Neo + Ant + Lut + Viola + Zea.

		F110	**F220**	**L110**	**L220**
9'*c*-Neoxanthin	mg g^{-1} DW	0.292 ± 0.028	0.294 ± 0.030	0.287 ± 0.016	0.295 ± 0.031
Violaxanthin		0.415 ± 0.042	0.415 ± 0.048	0.427 ± 0.032	0.468 ± 0.026
Antheraxanthin		0.038 ± 0.003	0.037 ± 0.004	0.040 ± 0.007	0.032 ± 0.005
Lutein		0.875 ± 0.085	0.855 ± 0.105	0.801 ± 0.061	0.861 ± 0.071
Zeaxanthin		0.042 ± 0.006	0.039 ± 0.002	0.051 ± 0.013	0.036 ± 0.007
Chlorophyll *b*		2.243 ± 0.217	2.141 ± 0.190	2.126 ± 0.155	2.252 ± 0.236
Chlorophyll *a*		6.282 ± 0.533	5.950 ± 0.508	6.032 ± 0.451	6.269 ± 0.594
β,β-Carotene		0.335 ± 0.012	0.323 ± 0.038	0.320 ± 0.035	0.354 ± 0.043
Ratios					
Chl *b*: Chl *a*		0.357 ± 0.005	0.360 ± 0.002	0.352 ± 0.003	0.359 ± 0.008
β, β-Car: Chl *a*		0.053 ± 0.004	0.054 ± 0.002	0.053 ± 0.003	0.056 ± 0.003
Xant: Chl *a*		0.265 ± 0.003	0.275 ± 0.009	0.266 ± 0.005	0.270 ± 0.008

5.4. Discussion

The grow-out performance of *H. portulacoides* in hydroponics was generally affected by 'plant density'. Even though plants in high-density treatments displayed overall lower individual development, hydroponic units produced higher yields at high-density. Doubling plant density from 110 to 220 plants m^{-2} increased hydroponic units' productivity by 52 - 57%. Higher productivity values were previously measured by Marques et al. (2017) who reported an average of 112 g m^{-2} day^{-1} of total biomass in hydroponic units under considerably different culture conditions (Table 5.3). Oppositely, and also under different conditions, Buhmann et al. (2015) reported average productivity of 33 g m^{-2} day^{-1} (Table 5.3). These few studies already suggest contrasting productivity outcomes very likely influenced by culture conditions. The use of different ecotypes might also account for some of the differences observed in *H. portulacoides* (Duarte et al., 2018).

Plant density has been shown to have little effect on the performance of other halophytes (Table 5.3). For instance, Webb et al. (2013) observed that *S. europaea* displayed similar results between 200 and 10,000 plants m^{-2}. High morphological plasticity of *Salicornia* has been proposed as a possible explanation for this lack of a 'plant density' effect (Davy et al., 2001; Webb et al., 2013). Some *Salicornia* species seem to be nonetheless more productive than others under similar nutrient loadings. For instance, *S. europaea* (200 – 10,000 plants m^{-2}) produced between 105 – 124 g m^{-2} day^{-1} of harvestable biomass (Webb et al., 2013), meanwhile *Salicornia persica* Akhani (100 plants m^{-2}) (Shpigel et al., 2013), *Salicornia bigelovii* Torr. (260 plants m^{-2}) (Kong & Zheng, 2014) and *Salicornia dolichostachya* Moss. (38 plants m^{-2}) (Buhmann et al., 2015; Waller et al., 2015) produced up to 50 -70 g m^{-2} day^{-1}.

Salicornia species have an annual life cycle, in opposition to the perennial life cycle of *H. portulacoides*, which implies major differences in their life histories and growth strategies (Smaje, 2015). As such, comparisons between such contrasting species are only meaningful from a horticultural perspective, as some edible species display better horticultural traits (Table 5.3). Other edible perennial halophytes investigated in terms of their productivity under different plant densities were *S. portulacastrum* and *B. maritima* (92 *vs* 184 plants m^{-2}), both displaying no differences between density levels (Boxman et al., 2017). *Sarcocornia ambigua* (Michx.) M.A.Alonso & M.B.Crespo, a perennial halophyte morphologically very similar to *Salicornia*, displayed productivity values in aquaponics close to those seen in *S.*

europaea and *H. portulacoides* at a density of 100 plants m^{-2} (Pinheiro et al., 2017), yet in different conditions performance values as low as 11 g m^{-2} day^{-1} have been observed (Pinheiro et al., 2020)

Increasing plant density also affected DIN extraction efficiencies, with high-density units extracting more DIN in total and low-density units extracting more DIN per unit of biomass. The total amount of DIP removed from units was also affected, with high-density units removing more DIP, yet the amount of DIP extracted per unit of biomass was comparable between densities. In terms of percentages, best performing high-density treatments displayed average extraction efficiencies of 28% DIN + 36% DIP, differing from previous studies (see Table 5.3), where other ecotypes displayed extraction efficiencies of 50% DIN + 45% DIP (Buhmann et al., 2015) and 65% DIN + 0% DIP (Marques et al., 2017) under contrasting plant densities and culture conditions.

Regarding other species, Boxman et al. (2017) observed an improvement in DIN extraction efficiency by *B. maritima* planted at low-density compared with high-density units. Webb et al. (2013) reported similar extraction efficiencies after a 50-fold increase in *S. europaea* density, demonstrating that increasing plant density does not necessarily increase nutrient extraction capacity, suggesting the existence of optimal plant density thresholds. Most hydroponic studies mentioned above used some sort of inert solid substrate (e.g. quarry sand, coconut fiber, expanded clay, perlite) rather than a soilless hydroponic setting such as the present study, which can influence extraction efficiencies (Lüderitz & Gerlach, 2002).

Even though the extraction of nutrients from effluents in soilless hydroponics is mostly the result of plant uptake, their removal can be complemented by other processes (e.g. denitrification, adsorption), mediated by microorganisms present in the rhizosphere and biofilms, by aeration and water mixing (Wongkiew et al., 2017). The hydraulic retention time is an important factor enabling these processes (Endut et al., 2010) and is a major component of remediation processes to take into consideration.

Table 5.3: Growth and extractive performances of halophyte species under different plant densities. Note: values/indicators were retrieved, calculated or estimated from data reported in the referenced publications (e.g. methods, tables and graphics).

Species	Life cycle	Production system	Salinity (ppt)	Growth period (weeks)	Retention time	Initial N (mg L^{-1})	Initial P (mg L^{-1})	Plant density (plants m^{-2})	Yields (g m^{-2} day^{-1})	N extracted (%)	P extracted (%)	Reference
H. portulacoides												
	P	Hydro.	20	10	1 week	60	3	110	36	22	24	Present study
	"	"	"	"	"	"	"	220	55	28	36	"
	P	Aqua.	20	22	12 hours	8.6	0.4	-	112	65	0	(Marques et al., 2017)
Atriplex portulacoides (homotypic synonym)	P	Hydro.	15	5	5 weeks	50	9.8	38	33	50	45	(Buhmann et al., 2015)
Other halophytes												
Batis maritima	P	Aqua.	15	4	<2 hours	variable	-	92	11*	89	-	(Boxman et al., 2017)
"	"	"	"	"	"	"	-	184	11*	15	-	"
Salicornia bigelovii	A	Hydro.	12	4	1 week	278.3	36.7	260	73	-	-	(Kong and Zheng, 2014)
Salicornia dolichostachya	A	Hydro.	15	5	5 weeks	50	9.8	38	60	48	46	(Buhmann et al., 2015)
"	A	Aqua.	15	5	1 day	19.4	2.8	38	60	17	0	(Waller et al., 2015)
Salicronia europaea	A	C.W.	~28	3	2 days	~26	~10	20	105	48	70	(Webb et al., 2013)
"	"	"	"	"	"	"	"	10,000	124	45	64	"
Salicornia persica	A	C.W.	35	13	1.5 days	12.2	1.6	100	55	53	13	(Shpigel et al., 2013)
"	A	Hydro.	26	26	1 week	200	200	1000	87	-	-	(Ventura et al., 2011a)
Sarcocornia ambigua	P	Aqua.	36	10	No retention	22.3	5.3	100	110	-	-	(Pinheiro et al., 2017)
Sesuvium portulacastrum	P	Aqua.	15	4	<2 hours	variable	-	92	18*	18	-	(Boxman et al., 2017)
"	"	"	"	"	"	"	-	184	18*	70	-	"

A - annual, **P** – perennial, **Aqua**.- aquaponics, **Hydro**.- hydroponics, **C.W**.- constructed wetland
* total average (no differences between densities)

This study presents one of the first attempts to contrast LED and fluorescent artificial lighting in the horticultural production of a halophyte. Fluorescent lighting has been traditionally used in indoor plant production but LED lighting is now considered a more versatile and energy-efficient solution that offers additional improvements to indoor production, such as a higher degree of control over spectral outputs and radiation intensities (Janda et al., 2015; Schulze et al., 2014).

The two artificial lighting systems tested in this study displayed two very distinct light spectra that could have affected the hydroponic performance of *H. portulacoides*. Nonetheless, according to present results, this was not the case between white fluorescence and blue LED lighting. In contrast, previous studies demonstrated that some types of artificial lighting and associated light spectra do affect different stages of development of certain halophytes. For instance, a study on the differential effect of combined red: blue LEDs and fluorescent lighting concluded that germination rate and shoot development were higher in seedlings of *Atriplex halimus* L., *Atriplex hortensis* L., and *S. europaea* illuminated with red: blue LED (Sanoubar et al., 2018). The vegetative development of *A. hortensis* was also improved by the combination of red: blue LEDs (Izzo et al., 2019). *Mesembryanthemum crystallinum* L., an edible halophyte commonly known as ice plant, also displayed an improvement in vegetative development under red: blue LEDs compared to red or blue LEDs alone (He et al., 2017). A different study noted that red: white LEDs illumination resulted in the best hydroponic performance of *M. crystallinum* compared with other combinations of white, blue, red, and far-red LEDs (Weeplian et al., 2018). To the authors' best knowledge, these were the only artificial lighting studies performed to date targeting the production of edible halophyte plants.

The effect of LED lighting on the grow-out of major horticultural crops in hydroponics has been more intensively studied (Alrifai et al., 2019; Mitchell et al., 2015). The use of blue and red LEDs alone or combined seem to improve the quality and yields of several vegetables and fruits when compared with fluorescent lighting and, in some cases, sunlight (Hasan et al., 2017). In aquaponics, for instance, the productivity of kale (*B. oleracea*) and Swiss chard (*Beta vulgaris* L.*)* was higher under LEDs than under fluorescent lights (Oliver et al., 2018). Some studies refer that blue LEDs, used alone or in combination with red LEDs, stimulate leaf area enlargement and aboveground development of lettuce (*Lactuca sativa* L.) and other vegetables (e.g. Chinese cabbage, spinach, and coriander) (Amoozgar et al., 2017;

Viršilė et al., 2017). Table 5.4 presents a non-exhaustive summary of hydroponic studies looking at the differential effect of LEDs and fluorescent lighting in the vegetative growth of leafy greens, with contrasting results. For instance, *L. sativa* var. *'crispa'* displayed higher biomass under red LEDs compared to either blue or red: blue LEDs (Chen et al., 2014), but var. '*capitata*' revealed an opposite outcome, growing better under blue LEDs (Namgyel et al., 2018). Overall, there seems to exist a wide range of species sensitivity to light quality and across different developmental stages and between varieties of the same species (Bugbee, 2016). The effects of light quality in regulating growth processes and physiology in plants seem to be more complex than the effects of light intensity and photoperiod (Bian et al., 2015).

While the concentration of major photosynthetic pigments in *H. portulacoides* leaves was unaffected by artificial lighting, the concentrations of antheraxanthin and zeaxanthin suffered a main effect promoted by plant density, as values were lower in high-density units. Leaves in low-density units were potentially exposed to higher irradiance levels due to lower shading, leading to a higher overall accumulation of antheraxanthin and zeaxanthin as products of the photoprotective xanthophyll cycle (Demmig-Adams & Adams, 1996). Even though the type of illumination did not seem to affect photosynthetic pigments in *H. portulacoides*, an increase in the concentration of carotenoids (which include xantophylls and carotenes) has been previously observed in leafy vegetables exposed to blue LEDs (Alrifai et al., 2019; Metallo et al., 2018). For instance, several *L. sativa* varieties, cabbages (*Brassica rapa* L. and *B. oleracea* varieties) and water spinach (*Ipomoea aquatica* Forssk.) displayed an increase in the concentration of chlorophylls and other pigments when cultivated under blue LEDs alone or, in some cases, combined with red LEDs (Chen et al., 2014; Johkan et al., 2010; Kitayama et al., 2019; Kopsell et al., 2014; Meng et al., 2019, 2019; Metallo et al., 2018; Wang et al., 2016). Nonetheless, some *L. sativa* varieties displayed similar concentrations of photosynthetic pigments regardless of being exposed to fluorescent lights or red: blue LEDs (Lin et al., 2018, 2013). As PAR conditions were kept identical across treatments in these studies, LED light quality alone seems to stimulate the synthesis of certain photosynthetic pigments in some vegetable species.

The energetic efficiency of lighting systems must be taken into consideration when designing sustainable and cost-effective hydroponic systems, especially when plant productivity is unaffected by different lighting systems. The wattage of fluorescent lamps used in this experiment was 54 W, while that of LED units was 30 W. Operating on a 14:10 light: dark photoperiod, one fluorescent lamp consumed 0.76 kWh day^{-1} and one LED unit 0.42 kWh day^{-1}. Assuming electricity costs were around 0.16 € kWh^{-1} under the electricity contract that powered this experiment (https://www.edp.pt/particulares/energia/tarifarios/; excluding taxes), each fluorescent lighting system (two lamps) would cost 88.8 € $year^{-1}$, while an LED lighting system (three units) would cost 73.5 € $year^{-1}$ to operate. Replacing a fluorescent lighting system by an LED lighting system renders a reduction in lighting energy costs of 17%. Additionally, one must also consider that the lifespan and maintenance costs of LEDs are normally lower than that of fluorescent lights (Viršilė et al., 2017), which can ultimately drive mid and long-term operating costs further down. LEDs can eventually represent a higher initial investment cost but, given their higher energy-efficiencies, longer lifespans, and lower maintenance, are certainly more cost-effective (Singh et al., 2015). Because *H. portulacoides* displayed a similar hydroponic performance under both types of artificial lighting, LEDs can be considered as a more cost-effective lighting solution for the

Table 5.4: Hydroponic-based studies of vegetative grow-out performance and photosynthetic pigment accumulation of leafy greens under different LED spectra and fluorescent lighting. DWC- deep water culture; NFT- nutrient-film technique; FL- fluorescent lighting.

Species	Hydroponic technique	Grow-out (days)	Photoperiod L/D (h)	PAR (mol m^{-2} s^{-1})	Shoot biomass per plant (g)					Photosynthetic pigments*	Reference
					FL	LED Blue	LED Red	LED R+B	LED White		
Beta Vulgaris	Aquaponics	3 weeks	-	200	33.3	-	-	-	117.7	No differences	(Oliver et al., 2018)
Brocolli oleacea var. italica	DWC	20	16/8	250	51.0	-	-	71.8	-	Highest: LED R+B Lowest: FL	(Kopsell et al., 2014)
Ipomoea Aquatica	DWC	14	14/10	200	-	6.1	8.5	8.7	-	Highest: LED R+B, R Lowest: LED B	(Kitayama et al., 2019)
Lactuca sativa var. capitata	DWC	35	16/8	210	149.0	-	-	136.3	164.1 (+RB)	No differences	(Lin et al., 2013)
L. sativa var. capitata	NFT	35	16/8	-	-	69.7	51.0	64.5	-	-	(Namgyel et al., 2018)
L. sativa var. crispa	DWC	50	14/10	133	32.1	23.5	46.9	24.4	-	Highest: LED R+B Lowest: LED R	(Chen et al., 2014)
L. sativa var. Korea	NFT	3 weeks	16/8	150	29.5	-	-	21.2 - 42.6	-	No differences	(Lin et al., 2018)
L. sativa var. Ziwei	DWC	18	16/8	300	49.3	-	-	40.0	-	-	(Zhang X. et al., 2018)

* Concentration of chlorophylls (*a, b*) and carotenoids

5.5. Conclusions

As consumers become progressively aware of halophytes as suitable vegetables for human nutrition and industry players become more positive about their economic potential, the conditions for successful edible halophyte cultivation must continue to be addressed. By shedding light over the most suitable horticultural conditions to grow *H. portulacoides*, prospective producers will be better informed and more confident that their practical choices will translate into more sustainable and profitable production.

From a horticultural standpoint, *H. portulacoides* displayed productivity values in the higher end of those exhibited by other edible halophytes, which emphasizes its potential as an edible halophyte crop for hydroponics and IMTA frameworks. The present study determined that it is possible to improve the productivity and nutrient extraction capacity of *H. portulacoides* units by adjusting plant density. However, a potential biomass allocation trade-off in individual development should always be taken into consideration, as certain density thresholds might promote undesirable phenotypes and decrease the productivity of target organs. Ideally, density thresholds should be established if *H. portulacoides* commercial indoor production takes-off in the future. Regarding artificial lighting, *H. portulacoides* grows equally well under white fluorescent and blue LED lighting and, thus, the choice of the most suitable lighting system should be informed by their potential impact in other operational costs (cost-efficiency). Further research should continue to address which light spectra (single and combined) improve hydroponic performance and promotes the accumulation of bioactive compounds to enhance crop value as functional foods. It is also urgent to foster the design of experimental setups, as well as the reporting of experimental data, in a standardized way. This will allow a more consistent comparison between hydroponic studies and provide more reliable information on which halophytes are most suitable for commercial hydroponics.

Chapter 6

Halophytes as novel marine products – a consumers' perspective in Portugal and policy implications

Submitted for publication

6. Halophytes as novel marine products – a consumers' perspective in Portugal and policy implications

Abstract

Consumers today demand healthier and more sustainable seafood products that are tasty and convenient. Plant-based foods have been particularly sought for and the development of novel products in this category is expanding. Halophytes are emerging as a new category of marine vegetables with distinct organoleptic characteristics (e.g. salty) and functional properties. In Portugal, the promotion of halophytes as salt alternatives is already advocated up to the policy level but halophyte-based products are still uncommon. Consumers are normally skeptical towards new foods and acquiring consumer insights through survey has proven invaluable to inform marketing strategies that positively impact consumer acceptability. The objectives of this study were to examine consumers' perspective and willingness-to-pay (WTP) for fresh-cut halophyte vegetables and collect data for consumer segmentation to inform future marketing initiatives to introduce halophytes. Based on 268 in-person interviews at point-of-purchase locations in Aveiro, Portugal, the results showed that halophytes are still alien to the majority of consumers. A package with 50 grams of fresh-cut Salicornia was used to assess consumer preferences and average WTP was 2,10 €. Female respondents reported higher WTP and positive vegetable-intake diversification seems to predict higher WTP as well. Using the Food-Related Lifestyle instrument and cluster analysis, three consumer segments were identified ('adventurous', 'conservative', and 'careless' consumers) and the 'adventurous consumer' is arguably the most interesting segment to introduce edible halophytes. The findings of this study can inform consumer-based pricing and marketing strategies towards a successful introduction of these novel marine vegetables to Portuguese consumers and encourage similar approaches elsewhere.

Keywords: halophytes; contingent valuation; consumers; seafood; marketing; pricing

6.1. Introduction

The 2030 Agenda for Sustainable Development has set objectives for the contribution of fisheries and aquaculture towards nutrition and food security, to ensure sustainable economic, social, and environmental development (FAO, 2018). This global agenda arrives at a critical time in which the oceans are facing unprecedented cumulative pressures from human activities (Halpern et al., 2019; Hoegh-Guldberg et al., 2019). Therefore, negative impacts in marine living resources, marine habitats, and ecosystem functioning have increased dramatically over the past decades (IPBES, 2019; Rocha et al., 2015; Steffen et al., 2015).

In the wake of a new narrative to heal the oceans (Lubchenco & Gaines, 2019) and the onset of the UN Decade of Ocean Science for Sustainable Development (Claudet et al., 2020), new nature-based solutions in seafood production must be explored to satisfy an increasing global demand for marine products while maintaining oceans' health. However, innovative solutions are needed to overcome current impediments towards transformative changes in human diets and (sea)food production systems that are healthier and more sustainable (Willett et al., 2019).

Aquaculture's contribution to the global supply of aquatic food will likely intensify during the next decade, as most commercial fish stocks remain maximally sustainably fished or overfished (FAO, 2018), and is expected to deliver its function of seafood provision while adhering to the principles of sustainable development. In this context, halophytes present themselves as novel marine food products with a role to play in sustainable aquaculture (Custódio et al., 2017).

6.1.1. Halophytes for a sustainable aquaculture

Halophytes are salt-tolerant plants that survive and reproduce in environments with salt concentrations exceeding 200 mM of sodium chloride (Flowers & Colmer, 2008; Santos et al., 2016). These previously underutilized wild plants are emerging as new saline crops across the globe that can be used for human nutrition in a larger scale (Barreira et al., 2017; Loconsole et al., 2019; Petropoulos et al., 2018) and as raw material for the production of other goods such as biosalt (Feng et al., 2013), vegetable oil (Weber et al., 2007), biofuel (Sharma et al., 2016), and bioethanol (Abideen et al., 2011) and the extraction of bioactive secondary metabolites (Buhmann & Papenbrock, 2013; Ksouri et al., 2012; Maciel et al., 2018). Edible halophytes with economic potential include *Salicornia* spp. and *Sarcocornia* spp. (common names: glasswort, sea asparagus, samphire*), Halimione portulacoides* and *Sesuvium portulacastrum* (sea purslanes), *Aster tripolium* (sea aster), *Batis maritima* (saltwort), *Mesembryanthemum crystallinum* (ice plant), just to name a few. For a more extensive list of edible species check the works of Panta et al. (2014), Petropoulos et al. (2018) and Ventura et al. (2015).

The commercial production of halophytes can be established not only under agricultural settings (Ventura et al., 2015) but also under an integrated aquaculture framework known as Integrated Multi-Trophic Aquaculture (IMTA), which is characterized as the enhanced production of aquatic organisms, with complementary ecosystem functions, that are trophically connected by demonstrated nutrient flows (Buck et al., 2018; Chopin et al., 2008). Seafood consumers value an IMTA approach to aquaculture farming (Knowler et al, 2020; van Osch et al., 2017; Whitmarsh & Wattage, 2006; Yip et al., 2017) and, in the context of the European Union (EU), halophytes may easily become an environmentally and economically attractive functional group for IMTA to help boost an EU sector that is struggling to keep up with the global growth trends (Guillen et al., 2019). The diversification of aquaculture products using native species is advocated by the Food and Agriculture Organization of the United Nations (FAO) (FAO et al., 2017; Metian et al., 2019) and several halophytes considered good candidates for IMTA are also native to the European flora (Custódio et al., 2017), further supporting their study as novel marine (sea)food products to be added to the growing collection of organisms cultivated under IMTA.

6.1.2. Novel marine vegetables for humans

Halophytes can be sold as minimally processed fresh-cut vegetables in ready-to-use formats that are increasingly popular among consumers (Baselice et al., 2017; Sillani & Nassivera, 2015) and can be an important source of biosalt. Biosalt is characterized as being of vegetable origin with a low-sodium profile balanced with other minerals, rich in nutrients and bioactive substances, and helpful in the prevention of hypertension and other cardiovascular diseases (Feng et al., 2013), therefore halophyte consumption could have broader implications in human health.

Unhealthy salt consumption is a generalized pattern across the globe, as 181 out of 187 countries present estimated mean levels of sodium intake that exceed World Health Organization (WHO) recommendations (Mozaffarian et al., 2014). In Portugal, where the present study was performed, high salt-intake is the dietary risk-factor that most contributes to the burden of disease (Ministério da Saúde, 2016). The average citizen consumes an excess of 3 grams of salt per day above the maximum of 5 grams recommended by WHO (WHO, 2012), with an estimated 36 - 42% of the population suffering from hypertension (Ministério da Saúde, 2018; Polonia et al., 2014; A. P. Rodrigues et al., 2017). The elder population (> 65 years old) is of concern, featuring prevalence of hypertension of about 75% (Moreira et al., 2018; Polonia et al., 2014). Incidence of obesity is also positively correlated with high salt-intake (Ma et al., 2015), and recent estimates suggest that about 29% of the adult population is obese (Gaio et al., 2018) and 20% of adolescents are overweight or obese (Marques et al., 2018).

A moderate reduction in salt consumption can have major positive impacts on human health, significantly reducing blood pressure and reducing the risk of cardiovascular disease (Cook et al., 2014; He & MacGregor, 2011). However, taste seems to be a critical factor influencing dietary changes (Banerjee & Duflo, 2011), and low-salt foods are perceived to be unpleasant and tasteless (Walsh, 2007), which difficult salt-intake reduction. The introduction of halophyte products in diets typically high in sodium could offer a low-sodium salt-alternative and facilitate the reduction of sodium intake, in line with the recommendations by the WHO (WHO, 2012).

6.1.3. Transformative public policies towards healthy diets

The urgent need to promote healthy eating habits, paired with national and EU policy goals to facilitate healthy food environments (Graça et al., 2018; WHO & Regional Office for Europe, 2015), means that uncovering evidence on the value of salt-alternatives such as edible halophytes is critical. In 2012, Portugal implemented the first national food and nutrition policy - the National Programme for the Promotion of Healthy Eating (PNPAS), which was considered one of the eight priority programs to be carried out by the Ministry of Health (Gregório, 2018). Later, in 2017, the Integrated Strategy for the Promotion of Healthy Eating (EIPAS) policy was published as a Law to promote healthy food habits in the country (Despacho n.° 11418/2017) and in which several actions specifically mention halophytes (*Salicornia*) as salt alternatives that must be explored.

However, for halophytes to fully reveal their potential as new marine vegetables and deliver their health benefits, they must first and foremost be accepted by consumers (House, 2019). Consumer surveys are important methods to leverage the acceptance of new foods (Feldmann & Hamm, 2015; van Kleef et al., 2005) and were previously employed to assess European consumers' preferences regarding, for instance, duckweed (de Beukelaar et al., 2019), insects (Palmieri et al., 2019), and jellyfish (Torri et al., 2020) products. Concerning halophyte products, consumer studies are still lacking in the scientific literature.

The city of Aveiro, an historically and culturally distinguished region of marine-salt production (C. M. Rodrigues et al., 2011), is experiencing an introduction of Salicornia products by local specialty shops and was chosen to be the sampling location of the present study. Using a structured survey, the present work aimed to understand consumers' preferences regarding vegetable and halophytes consumption, their willingness-to-pay (WTP) for halophyte products, and identify potential consumer segments to facilitate the successful introduction of halophyte products and inform nationwide initiatives. Results from the present study can advise future halophyte and IMTA producers, sellers, and policymakers on pricing, marketing, and communication strategies to successfully introduce these new marine vegetables into consumers' diets and inspire the replication of this approach elsewhere.

6.2. Materials and methods

6.2.1. Survey

Consumer responses were collected via in-person interviews in the city of Aveiro, Portugal, at two point-of-purchase locations: a municipal market and a supermarket. A total of 268 consumers were successfully surveyed between April 30th to May 9th and between September 18th to 26th, 2019. Each interview lasted approximately 5 to 10 minutes and the questions were asked in Portuguese (see the complete questionnaire in English in the Annex section – Figure A6.1). A pre-test survey was executed on March 10th at a local market where 20 randomly selected people were interviewed. Based on the results of the pre-test, the duration and number of questions were reduced to decrease fatigue and increase willingness to participate and wording/sentences were reformulated to improve understanding of questions. The final questionnaire that supported the interviews was divided into three sections: (1) food-related habits questions; (2) product-related questions and WTP; and (3) sociodemographic questions.

In Section 1, two questions determined perceptions of vegetable consumption habits in terms of quantity and diversity and the remaining questions were based on the new version of the Food-Related Lifestyle (FRL) (Brunsø, 2018; Stancu et al., 2018), a psychographic-segmentation tool to determine what is meaningful to people when they engage in food-related activities (Brunsø et al., 1995). This tool is the best-validated instrument for international segmentation in the food domain and has been used cross-culturally in Western countries (Grunert, 2019). The new version of the FRL consists of three core dimensions/constructs denominated 'innovation', 'involvement', and 'responsibility and, according to (Stancu et al., 2018), they are supported by the literature in consumer behavior towards food choices concerning seafood (Carlucci et al., 2015; Reinders et al., 2016; Verbeke et al., 2007). In this study, each construct was determined using three psychometric items/questions (considered most adaptable to the Portuguese case study), instead of the original five, to reduce the duration of the questionnaire and minimize cognitive load. Participants were asked to rate their level of agreement with each item/question on a 7-point Likert scale labeled as 1= *totally disagree*, 2= *disagree*, 3= *somewhat disagree*, 4= *neither agree nor disagree*, 5= *somewhat agree,* 6= *agree* and 7= *totally agree*. Cronbach's alpha (CA) was used to assess the internal consistency of the measuring items from each construct (see Table 6.1). Composite variables were calculated for each construct as the sum of the scores of the items measuring a specific construct, divided by the number of items.

In Section 2, respondents were asked about their familiarity with halophytes, and their WTP for the test-product (50 grams of fresh *Salicornia*; Figure 6.1) was elicited using an open-ended Contingent Valuation (CV) question, a 'stated preference' method which provides direct hypothetical WTP estimates. Even though the approach has been previously criticized for generating hypothetical bias and overestimation of WTP (Breidert et al., 2006; Wertenbroch & Skiera, 2002), the open-ended CV has been shown to generate WTP estimates not different from other methods (e.g. conjoint analysis, experimental auction) and yields reliable estimates for food products (Grunert et al., 2009; Miller et al., 2011). Moreover, direct methods seem to result in more accurate estimates of hypothetical WTP than indirect methods (e.g. conjoint analysis) and are quicker to implement (Schmidt & Bijmolt, 2019) which is appropriate for face-to-face surveys. Before the elicitation of WTP, respondents were encouraged to examine the package and invited try the product.

In Section 3, sociodemographic variables were collected to characterize the sample, enable the profiling of consumer segments created from Section 1, and find potential demographic market segments for halophyte products based on WTP.

Table 6.1: Food-Related Lifestyle (FRL) dimensions and corresponding items.

Core dimension	Items	Cronbach's alpha (CA) (95% conf. int.)
1. Innovation	1.1. I love to try recipes from different countries 1.2. I like to try new foods that I have never tasted before 1.3. I look for ways to prepare unusual meals	0.72 – 0.80
2. Involvement †	2.1. Eating and food is an important part of my social life 2.2. Decisions on what to eat and drink are very important for me 2.3. Eating and drinking are a continuous source of joy for me	0.58 – 0.70
3. Responsibility	3.1. I try to choose food produced with minimal impact on the environment 3.2. I am concerned about the conditions under which the food I buy is produce 3.3. I try to choose food that is produced in a sustainable way	0.72 – 0.80

† Dropping item 2.2 improves C.A. (95% c.i. = 0.67 – 0.77)

Figure 6.1: Fresh-cut *Salicornia ramosissima* package (50 g) used for WTP elicitation.

6.2.2. Survey analysis and statistics

Descriptive statistics and frequency distributions were used to perform an exploratory analysis of the survey data and describe the sample. R v3.4.3 (64-bit), in combination with R Studio, was used for the statistical analysis.

Cluster analysis was performed using the 'k-means clustering', a partitioning clustering method commonly used in marketing analytics for consumer segmentation (France & Ghose, 2019). Composite FRL scores were used as the clustering variables to define FRL segments.

Missing data were detected and handled using the multiple imputation method 'MICE'. Only the variable corresponding to WTP responses was found to have missing data due to missing responses (6% NA, n= 17; Figure A6.2 in Annex). Missing WTP data points were imputed, after the removal of outliers (Figures A6.3 and A6.4).

To characterize FRL clusters resulting from the clustering analysis, the average values of continuous variables, and the proportions of categorical variables were computed and compared. Statistical differences of continuous variables were assessed using the non-parametric Kruskal-Wallis test and the Wilcoxon rank-sum test for pair-wise comparisons. Statistical differences in the proportions of categorical variables were assessed by Fisher's exact test. Statistical significance was assumed when $p < 0.05$.

6.3. Results

6.3.1. Sample characterization

The Aveiro region has a population size of 362598 inhabitants (2018, www.pordata.pt) and, assuming a confidence level of 95%, the margin of error of the sample (n= 268) is of 6.0%. In other words, sample statistics will be within 6 percentage points of the real population value 95% of the time. The characterization of the sample based on all responses is presented in Table 6.2.

Table 6.2: Frequency distribution and descriptive statistics of the responses in the total sample (n= 268)

Questions	**Option** (*categorical v.*)	**Statistic** frequency (%)	mean ± s.d.
Section 1 - Food-related questions			
What percentage of your day-to-day diet is composed of vegetable products?			47.2 ± 19.7
Do you diversify your vegetable intake in your day-to-day diet?	*No*	10.2	
	Yes	89.8	
I try to choose food produced with minimal impact on the environment *(Likert scale 1 - 7)*			5.3 ± 1.3
I love to try recipes from different countries *(Likert scale 1 - 7)*			5.3 ± 1.6
Eating and food are an important part of my social life *(Likert scale 1 - 7)*			5.6 ± 1.3

I am concerned about the conditions under which the food I buy is produced *(Likert scale 1 - 7)*			5.6 ± 1.3
I like to try new foods that I have never tasted before *(Likert scale 1 - 7)*			5.3 ± 1.4
Decisions on what to eat and drink are very important to me *(Likert scale 1 - 7)*			6.1 ± 1.0
I try to choose food that is produced in a sustainable way *(Likert scale 1 - 7)*			5.2 ± 1.4
I look for ways to prepare unusual meals *(Likert scale 1 - 7)*			4.3 ± 1.5
Eating and drinking are a continuous source of joy for me *(Likert scale 1 - 7)*			5.7 ± 1.4
Section 2 - Product-related questions and willingness-to-pay			
Do you know what halophyte plants are?	*No*	87.1	
	Yes	12.9	
Did you ever consume halophytes before (e.g. *Salicornia*)?	*No*	70.4	
	Yes	29.6	
Would you like to try this product [package with 50 g fresh *Salicornia*]?	*No*	28.4	
	Yes	71.6	
How much did you like the taste of this product (1-7) (n= 205)			5.8 ± 1.2
What is the maximum price in € you would be willing to pay for this product [package 50 g fresh *Salicornia*]? (n= 264)			2.1 ± 1.1
Section 3 - Sociodemographic questions			
What is your gender?	*Female*	57.5	
	Male	42.5	
How old are you?	*18-29*	22.8	
	30-39	18.3	
	40-49	17.2	
	50-59	16.8	
	>60	25.0	
What is your level of education?	*Secondary school or less*	47.0	
	University	53.0	

What is your employment status?	*Employee*	42.6	
	Self-employed	12.7	
	Unemployed	7.1	
	Retired	22.4	
	Student	11.6	
	Other	3.7	
What is your monthly income (€)?	*0-599*	27.2	
	600-1000	33.2	
	1001-2000	31.3	
	2001-3000	6.3	
	>3000	1.9	
What is the size of your household (number of members)?			2.7 ± 1.3

Briefly, the sample is slightly over-represented by female respondents (57.5%) and the most represented age groups are the elderly (≥ 60 years old, 25.0%) followed by young adults (18-29 years old, 22.8%). The sample is evenly distributed between secondary (47.0%) and higher educations (53.0%) and 59.0% of respondents have some sort of employment, either through hire (42.6%), self-employment (12.7%), or other formats (e.g. research grants) (3.7%). The non-employed respondents comprise 41.0% of the sample, distributed across retirees (22.4%), students (11.6%), and unemployed (7.1%). More than half of respondents earn below 1000 € per month (60.5 %) (the average salary in Portugal in 2018 was approximately 970 €; www.pordata.pt), out of which 27.2% received less than the 2019 minimum wage of 600 € per month (note that 80.8% of respondents in this category also belong to 'non-employed' categories). Respondents earning above 2000 € per month comprised 8.2% of the sample.

6.3.2. Identification and characterization of FRL segments

The cluster analysis was performed using the composite FRL scores from each construct and the average scores in the total sample were: ‘involvement’ = 5.8, ‘innovation’ = 5.0, and ‘responsibility’ = 5.4.

Before running the ‘k-means clustering’ algorithm, the number of clusters (k) to be computed must be chosen and the ‘within-cluster-sum-of-squares’ (WCSS) method was used to select the appropriate *k*, determined to be *k*= 3 (Figure A6.5 in Annex). The graphical representation of the ‘k-means clustering’ analysis is presented in Figure 6.2.

To validate the clusters, the silhouette coefficient was computed (Figure A6.6). This validation procedure measures how well each observation is clustered (cohesion) compared to the other clusters (separation). The average silhouette width was 0.3, indicating that most observations lie between two clusters and, therefore, compactness and separation of clusters in this analysis is relatively low.

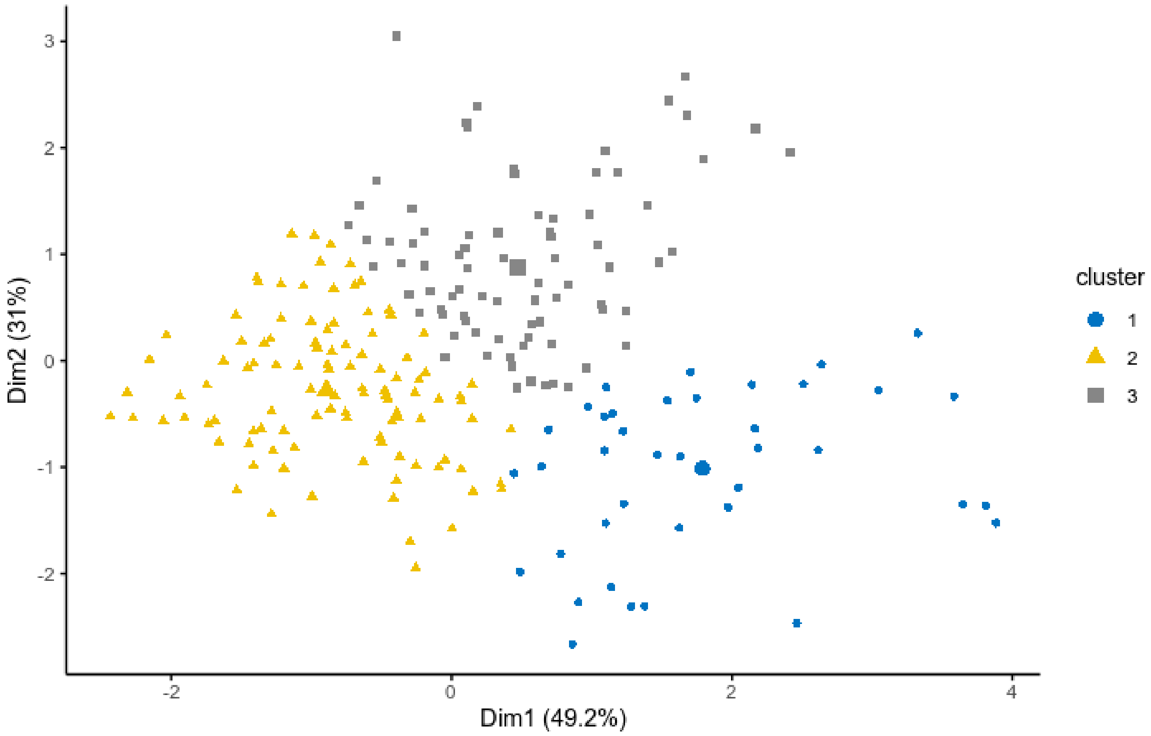


Figure 6.2: K-means clustering result (k= 3)

Cluster analysis grouped consumers according to their FRL scores and the three clusters significantly differed regarding the three constructs (Table 6.3). Cluster 2 (48.5% of respondents) is the most *innovative* and *responsible* segment, compared with the other clusters. Cluster 1 (21.6% of respondents) is the least *innovative* and *involved* and Cluster 3 (32.6% of respondents) is the least *responsible*. Clusters 2 and 3 are similar in terms of their *involvement* with food. Given the FRL segmentation attributes of each defined cluster, consumer segments will be designated as follows: Cluster 1 - 'conservative consumer', Cluster 2 - 'adventurous consumer', and Cluster 3 - 'careless consumer'. These three designations correspond roughly to the conservative, adventurous and careless segments referred to in the FRL literature (e.g. Karen Brunsø, 2018; Nie & Zepeda, 2011; Stancu et al., 2018; Szakály et al., 2012).

Compared with the total sample, the 'conservative consumer' is less *innovative* (score= 3.6) and *involved* (4.0) than the average consumer but equally *responsible* (5.5). The 'careless consumer' is more *involved* (6.0) than the average consumer, but less *innovative* (4.8) and *responsible* (4.2). The 'adventurous consumer' is more *involved* (6.1), *responsible* (6.1), and *innovative* (5.7) in all aspects of food than the average consumer. Regarding vegetable consumption, the 'careless consumer' incorporates fewer vegetables in its diet (38%) than the other segments (48 - 53%). In terms of reported diversification of vegetable intake, the highest rate of positive responses was observed in the 'adventurous consumer' (96% responded *yes*) and the highest rate of negative responses was observed in the 'careless consumer' (17.4% responded *no*).

Concerning demographic characteristics of FRL segments, the levels in categories 'age', 'employment status', and 'monthly income' were reduced by grouping certain levels together to increase observations for the Fisher's exact test to be executable (see Table 6.3 footnote). Differences between FRL segments were observed in the variables 'gender', 'age', and 'employment status'. The 'careless consumer' segment contains more male respondents, while the other segments contain more females. Regarding 'age', the 'conservative consumer' is more likely to be an elder (> 60), as the proportion of elders to young adults (18-29) and adults (30-59) is highest compared to the other clusters. The 'careless consumer' cluster has the highest proportion of young adults. In terms of 'employment status' ('employed' and 'non-employed'), the 'adventurous consumer' cluster has the highest proportion of employed respondents meanwhile the 'conservative consumer' cluster is highest in non-employed respondents (also influenced by the high number of retirees in the cluster).

Table 6.3: Characterization of the FRL consumer segments obtained by 'k-means clustering'. Test-statistic for numerical variables: Kruskal-Wallis test and Wilcoxon rank-sum test for pairwise comparison; for categorical variables: Fisher's exact test (differences in proportions)

Variable		Cluster 1 **Conservative consumer** n= 57; 21.6%	Cluster 2 **Adventurous consumer** n= 121; 48.5%	Cluster 3 **Careless consumer** n= 86; 32.6%
Continuous			Mean ± standard deviation	
Willingness to Pay for 50 g fresh Salicornia (€)		2.1 ± 1.3	2.2 ± 1.0	1.9 ± 0.9
Vegetables in the diet (%) *		48.3 ± 19.4 [a]	53.3 ± 18.5 [a]	38.1 ± 18.1 [b]
FRL dimension 1: Innovation * *(Likert scale: 1 - 7)*		3.6 ± 1.3 [a]	5.7 ± 0.7 [b]	4.8 ± 1.0 [c]
FRL dimension 2: Involvement * *(Likert scale: 1 - 7)*		4.0 ± 1.8 [a]	6.1 ± 0.8 [b]	6.0 ± 0.7 [b]
FRL dimension 3: Responsibility * *(Likert scale: 1 - 7)*		5.5 ± 0.8 [a]	6.1 ± 0.6 [b]	4.2 ± 0.8 [c]
Demographic continuous variables				
Household members		2.6 ± 1.5	2.7 ± 1.2	2.8 ± 1.4
Categorical			Proportion of counts	
Diversify vegetable intake *	Yes : No	50 : 7 [ab]	116 : 5 [a]	71 : 15 [b]
Knows what a halophyte is	Yes : No	7 : 50	77 : 103	9 : 77
Ate a halophyte before	Yes : No	19 : 38	35 : 86	24 : 62
Tried the product	Yes : No	39 : 18	90 : 31	60 : 26
Demographic categorical variables				
Gender *	Female : Male	39 : 18 [a]	76 : 45 [a]	37 : 49 [b]
Age † *	18-29 : 30-59	9 : 20 [ab]	21 : 77 [a]	29 : 41[b]
	18-29 : > 60	9 : 28 [a]	21 : 23 [ab]	29 : 16 [b]
	30-59 : > 60	20 : 28 [a]	77 : 23 [b]	41 : 16 [b]
Education	Sec. school : University	30 : 27	52 : 69	41 : 45
Employment status † *	Employed[1] : Non-employed[2]	26 : 31 [a]	79 : 42 [b]	50 : 36 [ab]
Monthly income †	< 1000€ : > 1000€	42 : 15	67 : 54	49 : 37

* Fisher's test shows the proportion of responses is different across lifestyle segments ($p < 0.05$)
[a,b,c] different letters represent significant difference between clusters ($p < 0.05$)
† number of categories reduced (merged)
[1] pooled categories: 'employee", 'self-employed' and 'other'
[2] pooled categories: 'unemployed", 'retired' and 'student'

6.3.3. Consumption and willingness-to-pay for halophytes

In general, most respondents had never previously heard the term 'halophyte', as only 13% were familiar with its definition. Nonetheless, about 30% answered that they had consumed a halophyte at least once before the survey (all reported *Salicornia* as the halophyte they had previously consumed). When asked if they wanted to test the product, 72% of respondents answered positively (39% of the respondents that responded negatively had already tried *Salicornia* before and were likely aware of their experience). Those who tested the product were asked their level of agreement with the sentence "I like the product" (on a Likert-scale of 1 to 7 as defined in the Material and Methods section) and 68% responded *agree* (score 6) and *totally agree* (score 7).

Consumers' WTP for a package with 50 grams of fresh *Salicornia* (discounting outliers) ranged between 0 € and 5.0 €, with the median value being 2.0 € and the average value 2.1 € (Figure 6.3). The WTP across categorical variables was also computed to determine if any particular category of consumers was willing to pay a higher price (Figure 6.4). The categories that displayed significant differences in WTP between category levels were 'gender', 'vegetable diversification', and 'product test'. Female consumers were willing to pay more (2.3 €) than males (1.8 €) and those consumers that reported diversifying their vegetable intake were willing to pay more (2.2 €) than those who do not diversify (1.4 €). Respondents who tested the product before WTP elicitation also reported willing to pay a higher price (2.2 €) than those who did not (1.9 €). Regarding the FRL consumer segments, WTP was not statistically different between them. The 'careless consumer' was willing to pay the least (1.9 €) and the 'adventurous consumer' was willing to pay the most (2.2 €).

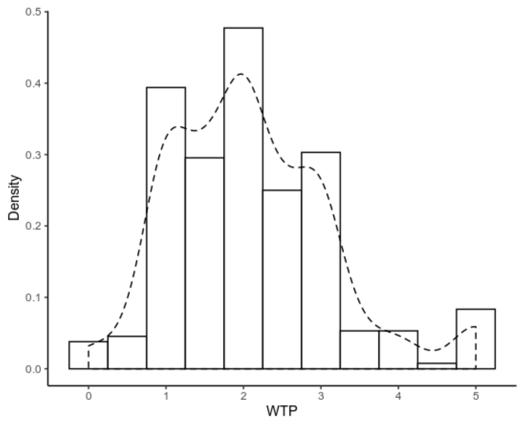


Figure 6.3: Density distribution of 'willingness-to-pay' responses (n= 264; after removal of outliers and multiple imputation of missing data). Histogram bin-width: 0,5.

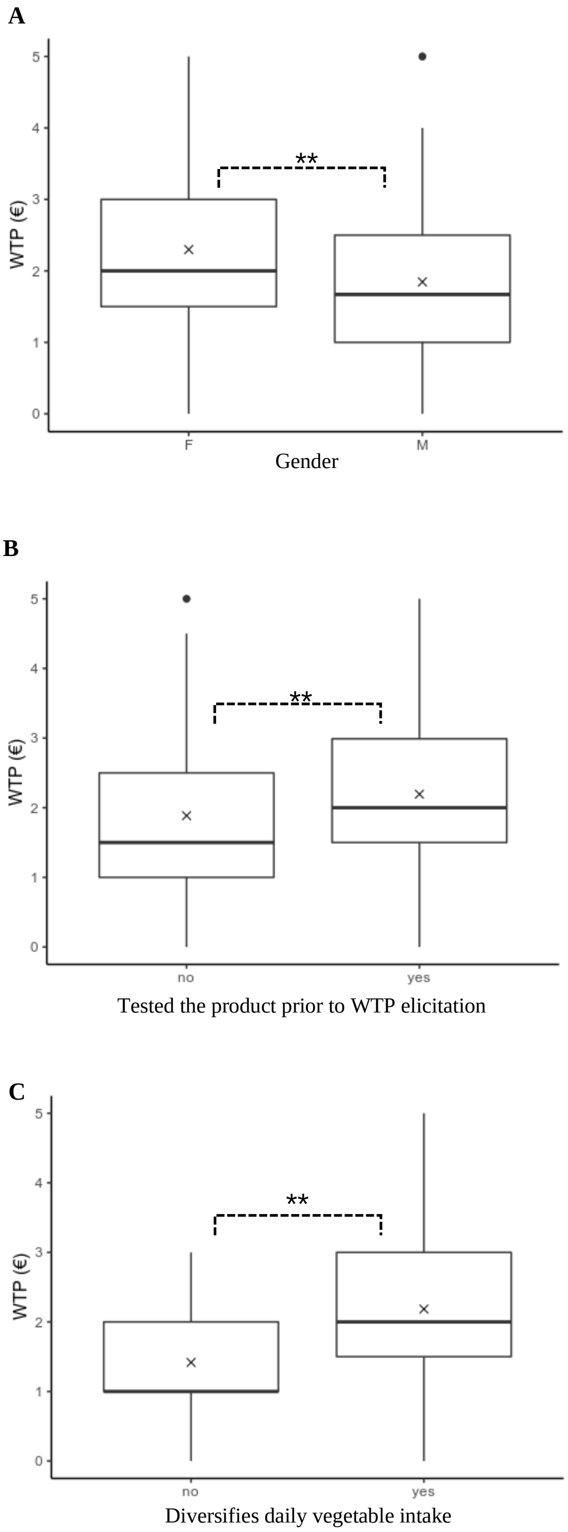


Figure 6.4: Boxplot representation of 'willingness-to-pay' distribution per category. Only categorical variables with statistically significant differences between levels are displayed (**: $p < 0.01$; ×: mean WTP): A) 'gender', B) 'product test, C) 'vegetable diversification'. Test-statistic: Kruskal-Wallis test and Wilcoxon rank sum test for pairwise comparison.

6.4. Discussion

6.4.1. The halophyte consumer

The findings of the present study suggest that halophytes are still mostly unknown to Portuguese consumers, although 30% of respondents surprisingly referred that they had already consumed *Salicornia*. This observation is probably the result of ongoing efforts to brand *Salicornia* as a local product of '*Ria de Aveiro*' (Aveiro's coastal lagoon), which has increased local awareness of this specific halophyte. The survey also revealed that about two-thirds of the participants that tested the product liked the product, suggesting a generally positive response to the taste of fresh-cut *Salicornia* by consumers.

The study elicited consumers' WTP to estimate the monetary value they would associate with halophytes sold as fresh-cut vegetable products. Consumers expressed an average WTP of 2.1 € for a package with 50 g of fresh-cut *Salicornia*, suggesting consumers are willing to pay (per kilogram) a maximum of 42 € kg^{-1}. Four respondents expressed 0 € as their WTP, which, according to the literature, could either represent a true zero or a protest response (Pennington et al., 2017). Given common practices, these responses were kept in the analysis as they represented only 1.5% of observations (and removing them could bias the results).

Female consumers were willing to pay more than total average WTP, and males were willing to pay less, suggesting gender differences in the value assigned to halophytes. Women could be an attractive segment for halophytes based not only on their higher WTP but also on their major decision-making role (for themselves and within households) in issues related to food. Previous studies have shown that women take more responsibility for food-related decisions (e.g. shopping, cooking) (Beardsworth et al., 2002; Flagg et al., 2014; Taillie, 2018), which highlights their importance for food-related communications and marketing. This was also observed in Portuguese consumers, as approximately one-third of men in a survey stated delegating their food choices to a second person (against only 9% of women) (Poinhos et al., 2009). Besides making more decisions, women also have a better knowledge of food and nutrition, display higher intakes of fruit and vegetables and lower intakes of salt and fat, are more conscious of their body-weight and are more likely to go on a diet, thus favoring healthy food-choices (Arganini et al., 2012; Conner et al., 2017; Neumark-Sztainer et al., 1999; Pingitore et al., 1997; Wardle et al., 2004). Moreover, women are also more sympathetic to novel food items and dietary changes (Beardsworth et al., 2002) and are, therefore, more, likely to try halophyte products. Women are thus considered a key segment that could more quickly incorporate halophytes into their diets (and their households), especially if their culinary uses and health benefits are efficiently communicated.

Consumers who reported diversifying their vegetable intake were also willing to pay more for fresh-cut *Salicornia* than those who did not, and scored higher in FRL 'innovation' and 'responsibility' traits, making them an arguably interesting segment for halophytes too. Certain personality types can be more inclined to try and accept halophytes as, for instance, people that score high in the Big Five personality trait 'openness' eat more plant-based foods (fruits and vegetables) (Conner et al., 2017; Mõttus et al., 2012), endorse healthier dietary habits (Brummett et al., 2008; Goldberg & Strycker, 2002; Mõttus et al., 2012), are more willing to try new foods and report higher WTP for organic food (Gustavsen & Hegnes, 2020).

Respondents who tested the product before WTP elicitation were also willing to pay more than those who did not. Exposure (e.g. sampling, testing) to a novel food product before decision-making has been previously shown to increase consumer acceptability (Lange et al., 2015; Lysák et al., 2019; Orjuela-Palacio et al., 2014), which could be used as a strategy to leverage the introduction of halophytes.

Nonetheless, hypothetical WTP is not always indicative of the price consumers are willing to pay in real purchase markets nor is higher WTP indicative of higher purchase intention (Barber et al., 2012; Zhang et al., 2018). Many different factors can influence consumer behavior and purchase intention of vegetable products, such as sensory appeal, social interactions, cost, time constraints, personal ideologies, and advertising (Pollard et al., 2002), making it difficult to predict attitude, intention, and purchase of a novel vegetable product. Moreover, familiarity with a product is related to the perception of safety and can influence the choice of a new food product, especially in more novelty-averse consumers (Laureati et al., 2014). The 'careless consumer', despite being somewhat *innovative* concerning food, consumes fewer vegetables and is less likely to diversify intake compared to other segments, which suggests a lower likelihood of purchasing new marine vegetables like halophytes. The 'conservative consumer, despite being somehow similar to the 'adventurous consumer' in terms of vegetable intake, features a low 'innovation' score, suggesting they are less likely to buy novel (unfamiliar) products. Considering the overall FRL consumer attributes extracted from this survey, the 'adventurous consumer' is probably the most interesting segment for halophyte vegetables. Considering its demographic characteristics, the 'adventurous consumer' is more likely to be an employed adult female, while the 'conservative consumer' is a non-employed (retiree) elder female and the 'careless consumer' a young/adult male.

6.4.2. Halophytes for transformative and healthy diets

Novel foods are being developed and introduced to the public regularly, as consumers demand food innovations that align with their needs and lifestyle. Consumers are increasingly attracted to food products that offer nutritional and functional qualities beneficial to their health (Bigliardi & Galati, 2013; Kyriacou & Rouphael, 2018). Consumers are also more concerned about the origin and environmental impact of their foods choice and value environmental responsibility in food labeling schemes (Tobi et al., 2019). Some studies showed that certain consumers are willing to pay more for food products perceived to be healthier and more sustainably produced (e.g. organic, locally-grown, low-waste production, low-carbon-footprint) (Barrington et al., 2010; Hemmerling et al., 2015; Husted et al., 2014; Nandi et al., 2017; Short et al., 2017; Zander & Feucht, 2018). Edible halophytes can deliver those important food attributes (Barreira et al., 2017; Ksouri et al., 2012), can be produced in sustainable aquaculture frameworks (Custódio et al., 2017), and can be used to promote healthier food-choices towards sodium-intake reduction. For instance, *Salicornia* has been reported to have 9 g of sodium per 100 g of dry weight (Barreira et al., 2017), approximately 77% less than regular table salt (= 39 g sodium $100g^{-1}$) (USDA, 2019).

As the global population continues to grow towards 9.7 billion people in 2050 (United Nations, 2019), the food production industry, policymakers, and society must work together towards meaningful transformative changes in food production and consumption. Important transformations must be put in place towards seafood and vegetable diversification (FAO et al., 2017; Nikalje et al., 2019), as many underutilized wild edible plants, including marine plants such as halophytes, present important socio-cultural, environmental, health, and economic benefits that must be captured (Bacchetta et al., 2016; Chivenge et al., 2015). A substantial shift towards mostly plant-based diets worldwide is paramount to support sustainable (sea)food systems and healthy diets (Willett et al., 2019) and halophytes can be an important addition to the array of plant-based food products available for human consumption, contributing to achieving the Sustainable Development Goals and provide socioeconomic opportunities to communities in developing and developed countries.

6.4.3. Public policy implications

Currently, no specific European legislation exists for halophytes production, although some regulations and recommendations apply to them. For instance, in the context of the Habitats Directive (92/43/EEC) and the Marine Strategy Framework Directive (2008/56/EC, CD 2017/848), the cultivation and integration of halophytes with coastal aquaculture is compatible with the conservation of coastal wetlands and biodiversity protection, helping reduce eutrophication in marine waters. Regarding the Novel Food Regulation (CE) Nº 2015/2283, novel foods should not be placed on the market or used as food for human consumption unless they are included in the Union list of novel foods[1] but very few halophyte species are included in the list (*Salicornia europea*, *Atriplex hortensis* and *Aster tripolium*). The compilation of an updated and complete list of halophyte species authorized as food in Europe would facilitate the introduction of new halophyte products to the market and increase public awareness of the use of halophytes as food.

In Portugal, there is also no specific legislation on halophytes production. However, the recent EIPAS policy (Despacho Nº 11418/2017) that aims at promoting healthy eating habits (Graça et al., 2018), is especially relevant to the context of this study[2]. In the EU, one of the priority interventions of the 'Action Plan for the Prevention and Control of Noncommunicable Diseases in the WHO European Region 2016–2025' (WHO, 2016) is the reduction of salt intake in the population, in line with WHO guidance, through the development, extension, and evaluation of salt reduction strategies, as also stated in the 'WHO European Food and Nutrition Action Plan 2015–2020' (WHO & Regional Office for Europe, 2015). Halophytes could be promoted as salt-alternatives at the broader EU level to

[1] Available at https://ec.europa.eu/food/safety/novel_food/catalogue/search/public/index.cfm)

[2] Under the "Strategic Area 3 – *Promote and develop literacy for healthy consumer food choice*", two actions explicitly state the relevance of *Salicornia* as a salt substitute to promote changes in salt consumption patterns: i) "6 - *Promote initiatives that value the consumption of proximity and of indigenous varieties and typical foods of the Mediterranean Diet. In particular, aromatic herbs and spices should be valued as salt substitutes and Salicornia as an alternative to salt.*"; and "11 - *Promote initiatives that make the population aware of the health impact of excessive salt consumption, as well as initiatives that encourage the use of salt substitutes such as aromatic herbs and spices and alternatives to salt such as Salicornia*.".

help reduce sodium consumption and incentivize member-states to create policies that promote halophytes production and their integration into IMTA frameworks.

The results presented here can effectively inform local, regional, and national initiatives aiming at promoting the consumption of Salicornia and help to open the door to the introduction of other edible halophytes. For instance, stated WTP estimates reflect perceived consumer value, allowing the creation of fresh-cut halophyte products that deliver the expected value at the target selling price. Moreover, targeting specific consumer segments that are more likely to accept these products (e.g. women, 'adventurous consumer') can positively influence the introduction of halophytes as new marine vegetables. In the meantime, many edible halophytes are showing potential for the most diverse applications and their nutritional role could expand from 'salt alternatives to functional foods and nutraceuticals (Ksouri et al., 2012; Patel et al., 2019; Petropoulos et al., 2018), potentially expanding their health and socioeconomic values within the (sea)food value chain.

6.5. Conclusions

Halophytes have been targeted by a multitude of scientific studies that highlight their utilization as novel marine vegetables for coastal IMTA, but such potential cannot be truly realized if there is little demand for halophyte products. Before starting the integration of halophytes in our (sea)food production systems it is relevant to assess consumers' perspective on these novel marine vegetables and gain further insights into the actual value assigned to them. In this context, the present study provides valuable information on the marketing potential and pricing strategies for novel halophyte products.

The introduction of fresh-cut marine vegetables like *Salicornia* in (sea)food markets is likely to appeal mostly to the 'adventurous consumer', especially at the beginning of market development, due to their higher disposition for innovation concerning food-related choices and higher appreciation for vegetable diversity in their diets. Women seem to be a key demographic segment for *Salicornia* since they demonstrate higher WTP compared to men and are more involved in food-related choices, as suggested in the scientific literature. A consumer-based pricing strategy using the WTP estimates obtained in this study, along with other strategies such as increased exposure (to improve familiarity) and effective communication of halophytes nutritional/health attributes and culinary uses could help leverage consumer acceptability and increase demand.

This paper focused on the Portuguese consumer but a wider European-level consumer survey could further contribute to estimating the (sea)food market potential of halophytes for the EU market and promote their incorporation in EU and member-States policies regarding halophyte aquaculture and their use as salt-alternatives and for other applications. Recognizing consumers' differences and preferences will be key to develop successful marketing campaigns that aim to bring halophytes to the expanding collection of herbs, fresh-cut vegetables, and plant-based products available to consumers, helping to promote healthier eating habits and the diversification of sustainable aquaculture production in the EU.

Chapter 7

Valuation of Ecosystem Services to promote sustainable aquaculture practices

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7. Valuation of Ecosystem Services to promote sustainable aquaculture practices

Abstract

Conceptual frameworks to assess and valuate Ecosystem Services (ES) are rapidly becoming important tools for ecosystem-based management, as they support transdisciplinary approaches to ecological economics and expand current asset boundaries to include natural and social capital. An important area where such ES assessment frameworks could become relevant management tools is aquaculture. Aquaculture activities are an interconnected part of the ecosystem in which they exist and, under certain circumstances, can support many of the same fundamental ES provided by nature. But, in most cases, aquaculture typically increases provisioning services at the expense of the other services (regulation & maintenance and cultural services). To understand the capacity of ES valuation methods to expose existing ES trade-offs in areas under aquaculture development, this study provides a literature review of publications that assessed and valuated ES delivered and/or impacted by aquaculture. In general, it seems that certain types of aquaculture do negatively impact overall ES delivery (e.g. intensive mangrove shrimp farming in Asia), yet certain modes of production (e.g. integrated multitrophic aquaculture) and cultured species (e.g. algae and certain bivalves) can have a positive impact on ES, not only improving provisioning services but also regulation and maintenance services and, potentially, cultural services. ES valuation methods provide important data that facilitate discussion among stakeholders and policymakers and should be included in marine and coastal management planning processes to foster a more sustainable aquaculture.

Keywords: blue growth, economic valuation, ecosystem approach to aquaculture, natural capital, sustainable aquaculture.

7.2. Introduction

7.2.1. The concept of Ecosystem Services

In the last 20 years, the Ecosystem Services (ES) concept has gained important visibility in environmental research and policymaking (e.g. Costanza et al., 2017). ES has been defined as the "benefits that people obtain from ecosystems" (MEA, 2005) and the "direct and indirect contribution of ecosystems to human well-being" (TEEB, 2010), supporting all domains of human society, from individual survival to the development of the global economy.

Despite major advances in developing and operationalizing the concept of ES for ecosystem-based management (EBM), researchers continue to debate and update existing conceptual frameworks for ES assessment, with the intent to create a comprehensive and overarching one. For instance, the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) has proposed the concept of 'nature's contributions to people', which builds upon the above definitions of ES and further recognizes the importance of transdisciplinary knowledge (e.g. indigenous and local knowledge) to understanding the links between people and nature (Díaz et al., 2015, 2018; Pascual et al., 2017).

Additionally, the creation of ES classification systems, as proposed in important publications (e.g. Millennium Ecosystem Assessment, The Economics of Ecosystems and Biodiversity and IPBES), is indispensable for measuring and assessing ES. The Common International Classifications for Ecosystem Services (CICES), in particular, aims to become a reference classification that provides a common language for interdisciplinary research, enabling users to move more easily between existing classification systems and avoid double counting when implementing the concept (Haines-Young & Potschin, 2017). For this reason, the CICES nomenclature is used in this review.

7.2.2. The importance of coastal ecosystems for human well-being

Marine and coastal ecosystems provide a wealth of benefits that span all three categories of ES identified in the last version (v5.1) of CICES: (i) provisioning, (ii) regulation and maintenance and (iii) cultural services (Haines-Young & Potschin, 2017; Lillebø et al., 2017). Coastal ES are used by over one-third of the human population inhabiting coastal areas and small islands. Remarkably, these areas comprise solely 4% of the world's total land (UNEP, 2006). Yet, due to intense human activities, these are exposed to several interconnected drivers of change, which contribute to their degradation and loss (de Groot et al., 2012). The main drivers contributing to this scenario include, among others, the development of aquaculture, overfishing, shipping (e.g. introduction of invasive species), land-based activities (e.g. nutrient loading from agriculture and urban development), coastal deforestation, shifting markets, climate change and globalization (Allison et al., 2009; Tröell et al., 2014; Villasante et al., 2012). Global fish stocks, in particular, are suffering a great deal due to anthropogenic drivers and several stocks are at risk of collapsing (e.g. Pauly & Zeller, 2016). Fish provide more than 3.1 billion people with ~20% of their average per capita intake of animal protein and, at present, more humans are consuming more fish (FAO 2016). Demand significantly increased during the last five decades, stemming from the rising living standards and prosperity of an ever-growing human population, both in developed and developing countries (Arrow et al. 2004; Steffen et al. 2011). As a solution to maintain the flow of this important provisioning service without collapsing the capacity of natural fishing stocks to deliver it, humans had to significantly develop aquaculture, which became itself an important driver of change in marine and coastal systems (Tröell et al. 2014).

7.2.3. An ecosystem-based approach to aquaculture management

In the period spanning from 1970 to 2008, aquaculture production increased, on average, 8.3% per year and this activity is now the fastest-growing food production industry, securing nearly 50% of the seafood supply worldwide (FAO, 2016). In light of such rapid growth, the sustainability of aquaculture has been a source of intense debate among experts. Opposing views point out several concerns such as lower water quality, eutrophication, coastal erosion, chemical accumulation, dependence on fish meal, biodiversity loss, and livelihood conflicts (e.g. Naylor et al., 2005; Olsen, 2011; Primavera, 1997; Tröell et al., 2014). Conversely, aquaculture advocates refer to it as likely the sole solution that may allow for the recovery of wild fish stocks, while simultaneously satisfying the ever-growing demand for seafood. Thus, aquaculture must be correctly planned and play a central role on EBM and conservation of marine and coastal areas (Froehlich et al., 2017; Le Gouvello et al., 2017; Long et al., 2015; Tacon et al., 2009).

To operationalize an EBM for aquaculture, FAO developed guidelines and defined the Ecosystem Approach to Aquaculture (EAA) as “a strategy for the integration of the activity within the wider ecosystem such that it promotes sustainable development, equity and resilience of interlinked social-ecological systems" (FAO, 2010). The EAA has three main objectives: ensure both (i) human and (ii) ecological well-being and (iii) facilitate the achievement of both in the context of other sectors and policies. Mainstreaming EAA in planning processes has raised awareness of the usefulness of holistic and participatory approaches in aquaculture and helped to steer the sector towards greater sustainability, yet the approach has had varying degrees of resonance and uptake with different user groups (Brugère et al., 2019).

In the EU context, aquaculture is one of the five maritime economic activities prioritized in the Blue Growth Strategy (European Commission, 2012a, 2017) and linking marine/coastal ES with the different blue economy sectors is key to accomplish a sustainable blue growth (Lillebø et al., 2017). Furthermore, United Nations Sustainable Development Goals (SDG) for 2030 acknowledges that sustainable aquaculture might contribute to support the sustainable use and conservation of oceans, seas and marine resources (SDG 14 – life below water) and offer ample opportunities to reduce hunger and foster well-being (SDG 2 – zero hunger; SDG 3 – good health and well-being). Like any other human activity, aquaculture evolves within complex environmental, social, economic, and cultural contexts, with each one of them having specific effects worth describing explicitly and systematically (Bostock et al., 2010). Aquaculture is an interconnected part of the ecosystem in which it occurs and can provide ES far beyond the provision of food (see Table 7.1) and recognizing the positive effects of certain modes of aquaculture is paramount. Given aquaculture's rapid expansion and intensification worldwide, reframing aquaculture trade-offs analysis through the lens of an ES framework can provide a novel and comprehensive analytical matrix of interactions with its multidimensional context, stimulate science-based EBM and promote sustainable solutions (Baulcomb, 2013; Bennett et al., 2009; Mach et al., 2015).

The present review synthesizes, to our best knowledge for the first time, the results from previous studies on the ES produced and/or affected by aquaculture. The evaluation of ES trade-offs between aquaculture development scenarios and conservation efforts are addressed through different valuation methods. It is our conviction that employing conceptual frameworks for the assessment and valuation of ES in the context of an EAA is key to environmental policymaking. This approach can support decision-making processes framed by the preservation of ecosystems, a conscientious regulation of the different components of the ES delivery chain – capacity, flow and demand – and the promotion of positive synergies between stakeholders and the marine/coastal environment. Overall, it can foster effective implementation of management options supporting the development of sustainable aquaculture practices.

Table 7.1: Examples of aquaculture Ecosystem Services

Section	**Example of services**
Provisioning services	Direct food provision *(e.g. aquatic plants and animals)*
	Indirect food provision *(e.g. habitat and organic enrichment for fisheries species)*
	Other non-food products *(e.g. agar, carrageenan, bivalve shells, ornamental fish)*
	Medicinal resources *(e.g. extracts from algae and marine invertebrates)*
Regulation & maintenance services	Bioremediation and water filtration *(e.g. filter-feeders, bottom-feeders and algae)*
	Wave attenuation/coastal protection *(e.g. offshore mussel farms, oyster reefs)*
	Carbon sequestration and storage *(e.g. bivalves and algae)*
	Buffer for ocean acidification *(e.g. algae)*
	Sediments stabilization *(e.g. constructed wetlands)*
	Habitat provision *(e.g. pseudo-reserves around farms)*
Cultural services	Spiritual and physical connection *(e.g. coastal communities, natural reserves)*
	Cultural symbols *(e.g. Koi carp aquaculture)*
	Sense of place *(e.g. employment opportunities, gender equity)*
	Livelihood *(e.g. alternative activity for fishing communities)*
	Tourism and recreation *(e.g. ecotourism, food tourism, sport fishing)*
	Education *(e.g. education-oriented activities)*
	Research *(e.g. pilot-scale experiments)*

Note: based on Alleway et al. (2018).

7.3. Valuation studies around the globe

7.3.1. A systematic review

The EAA has been increasingly discussed in recent years and the existing literature on the subject is still fragmented but emerging. Nonetheless, the literature survey (Figure 7.1) and selected publications that informed the present study (Table A 7.1 in Annex) provided an important insight into the relevance of the ES framework as a sustainable management tool for EBM in areas displaying high aquaculture potential.

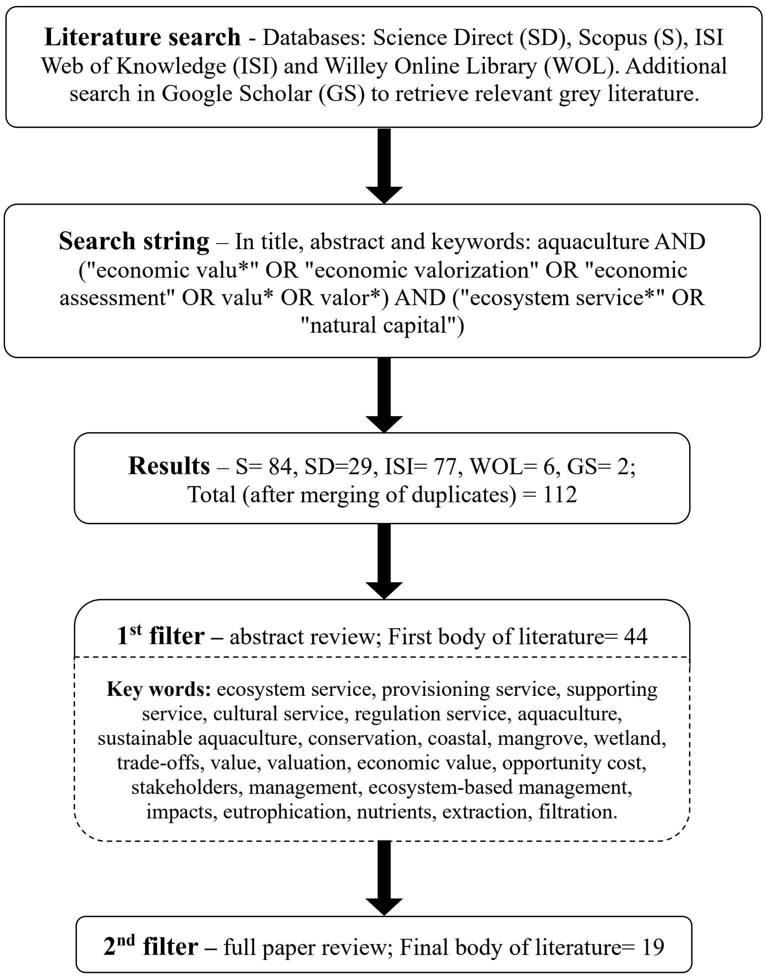

Figure 7.1: Schematic representation of the process employed for the selection of relevant literature.

Out of 19 relevant publications, only nine have tried to describe and valuate ES from aquatic ecosystems under aquaculture development using different valuation methods, as summarized in Table 7.2. The other 10 publications, which include three reviews, a Ph.D. thesis, and a BSc thesis clearly addressed the key role of aquaculture on the flow of ES, through some form of biological/environmental indicators and models but did not perform any type of valuation.

The geographical locations of these first assessments can be seen in Figure 7.2, with Southeastern Asia concentrating most of them, followed by Europe, China, and lastly the USA with a single study so far. It is worth observing that China, the country that most contributes to the global aquaculture production (>60%), is not necessarily using this approach to assess the impacts of aquaculture development (at least according to available publications in English). Some Southeast Asian and European countries are at the forefront of such an approach, even though their combined contribution to global aquaculture production is less than 20% (Figure 7.2). Due to the rapid development of shrimp farming in Asia, efforts to bring EBM to mangrove areas have increased in some countries. Growing demand from foreign markets and high economic revenues have been the major driving forces for the blind conversion of mangroves into shrimp farms, at the expense of other ES provided by these ecosystems, which have been evidently overlooked (Brander et al., 2012; Polidoro et al., 2010). Mangroves are recognized to be important ES providers, including provisioning (e.g. food, timber, fuelwood, charcoal), regulation and maintenance (e.g. floods buffer, storm and erosion protection, prevention of saltwater intrusion, spawning and nursery habitat, biodiversity) and cultural services (e.g. recreation, aesthetic, nonuse) (e.g. Brander et al., 2012). Unsurprisingly, the first case studies attempting to bring an ES assessment approach to aquaculture management have been done in Asia.

Table 7.2: Empirical studies using ES valuation methods to evaluate trade-offs between aquaculture and the environment.

Ecosystem type	Country	Aquaculture type	Species type	Source of evidence	ES assessed †	Valuation method	Reference
Coastal waters	China	Intensive	Shrimp	Survey and fieldwork	P, R, C	P - Market Price; R - Replacement cost method and contingent valuation; C - other methods.	Liu *et al*. (2010)
	China	Extensive to intensive	-	Survey	R	Contingent valuation method	Zhang *et al.* (2012)
Freshwater ponds	France	Extensive	Fish	Survey	P, R, C	Stated preferences	Blayac *et al*. (2014)
Mangroves	Indonesia	Semi-intensive	Shrimp	Survey and fieldwork	P, R	P - Market price; R - Replacement cost and benefit-transfer methods and carbon-credits	Malik *et al.* (2015)
	Philippines	Extensive to intensive	Fish	Fieldwork	R	Carbon credits	Thompson *et al*. (2014)
	Philippines	-	-	-	P, R, C	Post-Normal Science method	Farley *et al*. (2010)
	Thailand	Semi-intensive	Shrimp	Model (based on previous studies)	P, R	P - Market price, Surrogate price R - Production function and Replacement cost method	Barbier *et al.* (2008)
	Thailand	Semi-intensive and intensive	Shrimp	Model and fieldwork	P, R	Bayesian belief networks	Schmitt & Brugère (2013)
	Vietnam	Intensive	Shrimp	Survey	P, R, C	Contingent valuation method	McDonough *et al*. (2014)

† P- Provisioning services; R- Regulation & maintenance services; C- Cultural services.

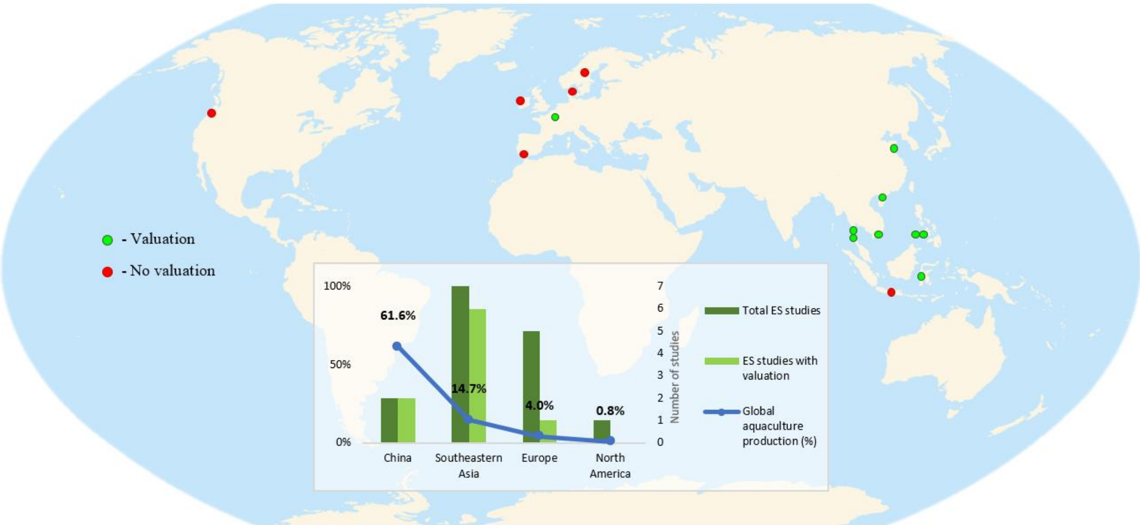

Figure 7.2: Global distribution of reviewed empirical studies. Review studies (4) have not been included as they are not location specific. The center chart shows each region contribution to global aquaculture production in 2014 (FAO, 2016) in relation to the number of aquaculture ES studies performed in those regions.

7.3.2. Case studies

The cost-benefit of shrimp aquaculture to society is widely debated and there are concerns about its environmental, social and economic costs, including externalities, as shrimp farming expands and intensifies in many countries (Knowler et al., 2009; Hatje et al., 2016; Hossain et al., 2013; Philcox et al., 2010; Primavera, 1997). Mangrove conservation is likely more beneficial to local communities, providing higher economic value (Primavera, 1997). McDonough et al. (2014), for example, used a Contingent Valuation Method (CVM) to assess stakeholder's stated preferences regarding mangrove ES in different aquaculture development scenarios. Participants demonstrated a preference for ES maintained at natural state (56–74%), followed by present state (21–35%), and only 6–9% of them chose the scenario for intense aquaculture development.

In Indonesia, Total Economic Value (TEV) of mangroves in South Sulawesi was determined, to understand the impacts of their conversion into shrimp-farms in terms of ES (Malik et al., 2015). The TEV is the sum of direct-use, indirect-use, and option values[1] and different monetary valuation methods (market and non-market based) exist to assess each component. For instance, direct-use value (e.g. fishery and forestry products) was estimated through the 'market price method', suitable for products traded in real markets. Indirect-use

[1]For a detailed discussion about the meaningfulness of the option value in the literature, see Perman et al. (2011);

value, namely coastal protection, seawater intrusion, nursery ground, and carbon sequestration, was assessed through 'replacement cost' and 'benefit transfer' methods, estimating people's willingness-to-pay (WTP) and/or cost of measures to avoid adverse effects stemming from lost services. The study concluded that TEV of intact mangroves (4000–8000 USD per ha) exceeded that of commercial aquaculture (3000 USD per ha), with indirect-use value accounting for most of the benefits.

In China, Zhang et al. (2012) assessed the ES of coastal aquaculture in the Shandong province, based on a CVM. The main factors influencing both WTP and willingness-to-accept (WTA)[2] were 'age', 'annual income', and 'education', demonstrating the importance of demographics and socioeconomic variables on ES valuation. The average WTP for marine protection was 561.8 CNY (90.2 USD – 31/12/12 exchange rate) and WTA compensation for marine pollution was 5175.5 CNY (830.6 USD). The 'free rider' effect was evident, as WTA is ~10 times higher than WTP. Such a gap is a consistently observed phenomenon regarding public goods and is explained by several cognitive biases inherent to human behavior which have been widely discussed (Horowitz & McConnell, 2002; Morewedge & Giblin, 2015). In Guangdong province, also in China, Liu et al. (2010) used several valuation methods (e.g. market price, replacement cost, contingent valuation) to expose the multiple costs and benefits of shrimp aquaculture within the mangrove ecosystem.

Furthermore, transdisciplinary approaches, that is, combining science-based knowledge with stakeholders/users' common knowledge, to ES valuation are being used, such as 'Bayesian belief networks' (Schmitt & Brugère, 2013) and the 'post-normal science' (PNS) methodology (Farley et al., 2010), with practical application in local mangrove aquaculture management decisions. Interestingly, the PNS method moves beyond the boundaries of conventional science-based knowledge to include alternative knowledge systems (e.g. folk knowledge, investigative journalism), a consideration that is being taken seriously by some recent conceptual frameworks on ES assessment (e.g. IPBES Conceptual Framework; Díaz et al., 2018; Pascual et al., 2017). PNS rationale lays in the need for urgent informed decisions with limited data, prioritizing open debates among stakeholders to peer-reviewed data, and analytical rigor (Turnpenny et al., 2011). So far, a consensus stems from these studies on mangrove aquaculture development in Asia: intact mangroves score highest for

[2]A discussion about the factors influencing the WTP and willingness-to-accept (WTA) can be consulted in Hanley et al. (1997).

all ES except food provision, which is usually higher in mangroves converted to shrimp farming. Nonetheless, conversion consistently goes together with lower delivery of all other ES. Decision-makers are advised to include mangrove ES assessments in their coastal EBM (van Oudenhoven et al., 2015).

On the single valuation study performed in Europe, which was applied to inland aquaculture in north-eastern France, Blayac et al. (2014) surveyed stakeholder's perception of ES delivered by an extensive freshwater fish polyculture in the Lorraine region. Participants included fish farmers, industry operators, institutions, service users, and residents. Results suggested that the demographic characteristics that most influence the perception of services are 'age' and 'education'. 'Age' has a positive effect on the preference for provisioning services and 'education' has a positive correlation with a preference for regulation and maintenance services. These results demonstrate again the relevance of the sociocultural context for ES valuation (Bennett et al., 2015; Díaz et al., 2018; Perrings et al., 2011).

By comparing the value of ES in different scenarios of conservation versus conversion to aquaculture, we can provide decision-makers with better data for EBM planning processes that entail an EAA. Decisions can fall either into total conservation, total conversion or integration. According to Barbier et al. (2008), ES delivered by coastal ecosystems should vary non-linearly with habitat variables such as area, as suggested by ecological theory (e.g. Petersen et al., 2003) Indeed, the socioecology of marine ES over space and time may be linear or non-linear, and may contain unexpected, even abrupt, ecological thresholds (Hughes et al., 2013) and social tipping points (Milkoreit et al., 2018; Villasante et al., 2017). Therefore, an optimal management solution will most likely be the integration of development and conservation measures (e.g. Knowler & Barbier, 2005). For example, a modeling case study of coastal protection service by coastal systems in Thailand established a relationship between mangrove area and measurements of wave attenuation (Barbier et al., 2008). Data suggested that a non-linear ecological function was a better fit and, thus, the aggregate value of shrimp farming and preserved habitat would find its highest value at a partial conversion state. Such outcomes can produce a more equitable distribution of value across stakeholders (e.g. investors, farmers, local communities, and ecologists).

7.4. Aquaculture can deliver key Ecosystem Services

7.4.1. Sustainable modes of aquaculture production

Some types of aquaculture are potentially more impactful on the supply of ES than others due to their high energy needs and ecological risk. Both fish- and shrimp farming are usually on top of the list, as they are typically fed with artificial feeds, which promote externalities (e.g. sourcing fish meal from fisheries) and nutrient pollution, and pose greater a threat to local biodiversity due to, for example, escapees, disease and chemical inputs. For instance, a global review on the impacts of tilapia production on the supply of ES suggested real ecological changes due to tilapia introduction in many countries, although social and economic benefits have also been reported (Deines et al., 2017). On the other side, certain modes of aquaculture production and cultured species can increase the local capacity and flow of several ES while simultaneously satisfying the demand for seafood, the primary objective of aquaculture.

Regarding production systems design, Integrated Multi-Trophic Aquaculture (IMTA) has been endorsed by scientists as a more sustainable mode of aquaculture than intensive monocultures, as that practice can enhance multiple ES (Buck et al., 2018; Chopin et al., 2012; Granada et al., 2016; Marques et al., 2017). In IMTA, nutrients wasted on artificially fed cultures (e.g. fish, shrimp), in both particulate and dissolved forms, are redirected to downstream trophic levels to nourish extractive species. Bottom feeders (e.g. sea cucumber, polychaetes) and filter-feeders (e.g. bivalves) feed on the wasted particulate fraction and other extractive species, such as seaweeds and macrophytes, utilize the dissolved nutrients for growth (Chopin et al., 2012). Such a system mimics natural trophic interactions, benefiting from ES supported by certain aquatic species to create a more sustainable and productive environment. Walton et al. (2015) assessed the potential ES delivered by sustainable aquaculture systems in wetlands from Doñana National Park, Spain, and concluded that properly designed dual-purpose farms could provide a suitable environment for ecological synergies to develop. Moreover, a review on the status of semi-intensive and extensive aquaculture in Southern European countries suggested that developing IMTA in degraded wetlands would potentially benefit stakeholders and improve ES in those areas (Anras et al., 2010). The European Commission has provided guidance on the integration of aquaculture activities within Natura 2000 sites, so they can also provide habitats for local species and boost biodiversity (European Commission, 2012b). Examples of successful coexistence exist in the Natural Park of La Brenne in France, the Sado Estuary in Portugal, the Bahía de Cadiz Natural Park in Spain, the Nesyt lake in the Czech Republic and several fishponds in Slovakia (European Commission, 2012b). It is also advised that the prospection of new suitable locations for aquaculture expansion should take into consideration the mapping of ES. Such a priori mapping will provide knowledge on the actual values delivered by the ecosystem into which an aquaculture activity would be established and identify major trade-offs between aquaculture and existing ES (Marciano, 2015).

7.4.2. Seaweeds and rooted macrophytes

Seaweed farming represents approximately 27% of global aquaculture production, generating around 27.5 million tons, which in 2014 alone were valued in 5.6 billion USD (FAO, 2016). Researchers working on seaweed aquaculture have been advocating in favor of its intensification due to important additional ES they support beyond the supply of biomass for nutrition, materials, and energy. Important regulation and maintenance ES include the extraction of dissolved inorganic nutrients and carbon, which decrease aquatic eutrophication and acidification of coastal waters (Chopin et al., 2012; Radulovich et al., 2015). Moreover, seaweed farms also provide habitat to many aquatic organisms, boosting biodiversity onsite and near the farm (Walls, 2017).

Recently, Kim et al. (2017) estimated that the total nitrogen (N) and carbon (C) extracted by the five most heavily cultured seaweed groups (*Eucheuma*, kelp, *Gracilaria*, *Porphyra* and *Sargassum*) added up to 65,000 tons of N year^{-1} and 760,000 tons of C year^{-1}. Yet, there still is a gap in the literature on the economic valuation of ES provided by seaweeds (Barbier, 2013; Costanza et al., 2014). Analogously, rooted macrophytes can also play a significant role in improving ES delivered by aquaculture, in both freshwater and saltwater settings, through the phytoremediation of wasted dissolved nutrients and production of valuable biomass with several applications (Custódio et al., 2017; Goddek et al., 2015).

7.4.3. Filter-feeders and bottom feeders

Bivalves feed on suspended particulate organic matter in the water column, potentially enhancing regulation and maintenance ES by improving water quality, reducing eutrophication and providing habitat for microbenthic species. Recent models of shellfish production that integrate environmental interactions have been proven useful for EBM of coastal aquaculture and several studies have shown their capacity to mitigate the leaching of nutrients from coastal fish farms (Ferreira et al., 2012; Nobre et al., 2010).

Following a model by Saurel et al. (2014), individual Manila clams (*Venerupis philippinarum*) are capable of a net removal of 0.28 g N year^{-1}, with a follow-up modeling study estimating that 700,000 metric tons of bivalves could remove 46,800 tons N year^{-1} (Ferreira & Bricker, 2016). In the fjords of Denmark, farmed mussels significantly improved regulation services by filtering phytoplankton (Nielsen et al., 2016). The authors suggested that the filtration rate could be increased by 80–120% without affecting growth. Previous studies in the Chesapeake Bay (USA) have also demonstrated that oyster reefs and oyster farming enhance denitrification (Higgins et al., 2011; Kellogg et al., 2013). Nitrogen removal by shellfish is potentially more cost-effective than wastewater treatment plants (Rose et al., 2015). Nonetheless, it is important to analyze trade-offs between shellfish remediation and organic deposition below grow-out structures (e.g. cages and tables), as this affects benthic biodiversity and substrate chemistry (Quintino et al., 2012).

Bottom feeder organisms, such as polychaetes and sea cucumbers, have also been cultivated under aquaculture conditions due to their economic value and their integration in IMTA systems has been explored. Besides the valuable biomass, these organisms can deliver regulation and maintenance ES on a similar fashion as filter-feeders, through bioremediation of sediments and nutrient recycling (Marques et al., 2017; Purcell et al., 2016). Polychaeta species (e.g. *Hediste diversicolor*) and sea cucumbers (e.g. *Holothuria tubulosa*) can be integrated into aquaculture sand filters or placed below offshore fish-cages, feeding on the organic matter that is retained in the sediment (Marques et al., 2017; Neofitou et al., 2019). Moreover, they can incorporate the valuable nutraceutical compounds from wasted aquafeeds, such as EPA and DHA fatty acids, adding value to the production (Marques et al., 2018).

7.4.4. A framework for better decision-making

Transdisciplinary communication is at the core of every ES assessment for any given ecosystem, principally in those affected by intense human activity. Marine and coastal systems are particularly exposed to multiple anthropogenic stressors, mainly driven by human economic activities, which destabilize ecological homeostasis by pushing ecosystem properties away from equilibrium (Durrieu de Madron et al., 2011; Halpern et al., 2007;).

Southeastern Asian countries have experienced an intensification of shrimp farming, a highly profitable activity for investors, and a source of employment for local people. Yet, externalities emerging from aquaculture added to the loss of mangroves have proven disastrous in many fronts, with loss of biodiversity and ES, with consequent grave economic costs to local communities and society (Polidoro et al. 2010; Brander et al. 2012). The published studies discussed in this review consistently revealed substantially higher ES value for intact mangroves, advising decision-makers about which development scenarios to pursue (van Oudenhoven et al., 2015). Nonetheless, ideal trade-offs might be achieved at partial conversion states, without affecting the optimal flow of ES. Thus, evaluating integrative scenarios is key to promote constructive dialogue and improve relations among stakeholders.

As seen above, certain types of aquaculture can have a positive impact on the capacity of ecosystems to deliver ES. Seaweeds, rooted macrophytes, and bivalves, besides being important food providers and sources of compounds with many applications, are also important at remediating eutrophic water bodies and at promoting the increase in biodiversity. Thus, culturing such species can enhance provisioning services along with regulation & maintenance ES of marine and coastal ecosystems, which could be achieved through the adoption of, for example, IMTA-based solutions.

The ES framework approach exposes trade-offs associated with management alternatives using a common transdisciplinary language and valuation measures on which to base negotiations, ultimately improving communication among groups with competing interests and differing worldviews (Peterson et al., 2018). Several valuation methods exist, from direct monetary valuation techniques to assess direct use services, to deliberative approaches for less tangible services, to help provide a more complete picture of ES capacity, flow, and demand. The choice of valuation methods is paramount and will ultimately dictate the reliability of the assessment since some methods elicit better value estimates than others depending on the type of service being valued. For instance, use-values are usually elicited quantitatively using ‘revealed preference methods’ (e.g. market price, replacement cost, benefit-transfer) for consumptive products traded in markets and are the most used valuation methods (Himes-Cornell et al., 2018; Vo et al., 2012). But non-marketed use-values and nonuse values are more difficult to assess using those same market-based methods and ‘stated preference methods’ (e.g. contingent valuation), which rely on participatory processes (e.g. surveys, workshops), are more reliable and informative (Gómez-Baggethun et al., 2014).

Stakeholders' involvement through participatory processes is a central part of an ES assessment, especially in coastal areas, where many groups, institutions, and industries coexist and interact. In this context, stakeholders are usually fish farmers, fishermen, watershed recreational users, local community, research institutions, managers, maritime authorities, government representatives and NGOs, depending on the location and scale of the assessment.

The Integrated Coastal Zone Management (ICZM) is an important policy instrument that aims to coordinate the different strategies affecting the coastal zone and associated with activities such as fisheries, aquaculture, agriculture, renewable energy, shipping, tourism, conservation, and coastal protection infrastructures (European Commission, 2007). Its approach takes into consideration the state of natural resources and ecosystem boundaries to which the ES framework would be an important assessment tool. Due to the overlapping of human activities at the sea-land interface, EU recommendation on the implementation of ICZM (Recommendation 2002/413/EC) is to be implemented in coherence with existing EU Coastal and Marine Policy.

Relevant examples are the Maritime Spatial Planning (MSP), concerned with the sustainable use of the maritime space (Directive 2014/89/EU); the Marine Strategy Framework Directive (MSFD), which aims at Good Environmental Status in marine waters, following an ecosystem-based approach (Directive 2008/56/EC), the Water Framework Directive (WFD), addressing community actions in the field of water policy, including transitional and coastal waters (Directive 2000/60/EC), the Common Fisheries Policy (CFP), which aims to achieve sustainable use of fishery resources (Regulation EU 1380/2013). This implementation has the potential to improve planning and management in both the environmental and socio-economic dimensions such as, for instance, to minimize the effects of maritime infrastructures (e.g. coastlines protections, oil platforms) on coastal activities (e.g. aquaculture and fisheries) and protected areas. Most importantly, the principles of EAA should become fully operational in ICZM, MSP, and EU directives to preserve marine and coastal ES capacity and flow to meet human populations' demand (Ansong et al., 2017; Katsanevakis et al., 2011).

7.5. The step-forward: pluralistic valuation

Monetary valuation measures the contributions of nature to human well-being from a utilitarian perspective using monetary metrics and is suitable for assessing certain types of ES, mostly within the provisioning and regulation and maintenance sections. However, it often fails to capture the importance of nature beyond economic values (Jacobs et al., 2018). To elicit the diversity of values associated with nature, non-monetary approaches are essential methods to examine the relevance of preferences, values, and demands of people towards nature and provide a holistic assessment through integrated valuation (Chan et al., 2012; Jacobs et al., 2018; Norgaard, 1989). These approaches aim to demonstrate the pluralistic value of nature and its importance within the ES framework (Figure 7.3), where monetary value is only one type of value among others, including cultural, spiritual and symbolic values (Díaz et al., 2018; Garcia-Rodrigues et al., 2017; Pascual et al., 2017).

In that sense, ES should be considered under three value domains: economic, ecological, and sociocultural (Braat & de Groot 2012). Ecological value is obtained using ecological indicators (e.g. diversity and integrity) to assess the flow of services from the supply side, the ecosystem. Sociocultural value involves non-tangible services, such as cultural and spiritual identity, and is usually estimated through surveys and deliberative approaches (e.g. Q-methodology) which assess stakeholders' perceptions and preferences. Economic value is typically obtained using the Total Economic Value framework, through methods that allow for monetary-based assessments (Science for Environmental Policy, 2015). Developments on this domain have led to the creation of a novel environmental policy tool designated as Payment for Ecosystem Services (PES). It aims to internalize the positive externalities generated by ecosystems, producing incentives for landowner behavior that creates and ensures the delivery of ES that belong to the realm of public goods (Salzman et al., 2018). Nonetheless, PES captures only a fraction of the value, since existence and option values and other benefits are not usually captured by this mechanism.

Furthermore, modern information technology tools such as 'remote sensing' and 'geographic information systems' are being used to map ES and can be integrated with valuation data to better understand ES state and dynamics within EBM (de Araujo Barbosa et al., 2015; Schägner et al., 2013; Science for Environmental Policy, 2015). Integrated ES valuation should also feed on other knowledge systems, such as folk knowledge and traditional ecological knowledge, most importantly in locations where scientific data are still scarce or even inexistent (Díaz et al. 2018; Tengö et al. 2014). As an example, IPBES, through its 'nature's contribution to people' approach, already acknowledges such alternative worldviews, defined as 'local and indigenous knowledge', and incorporates them within its framework (Díaz et al., 2018).

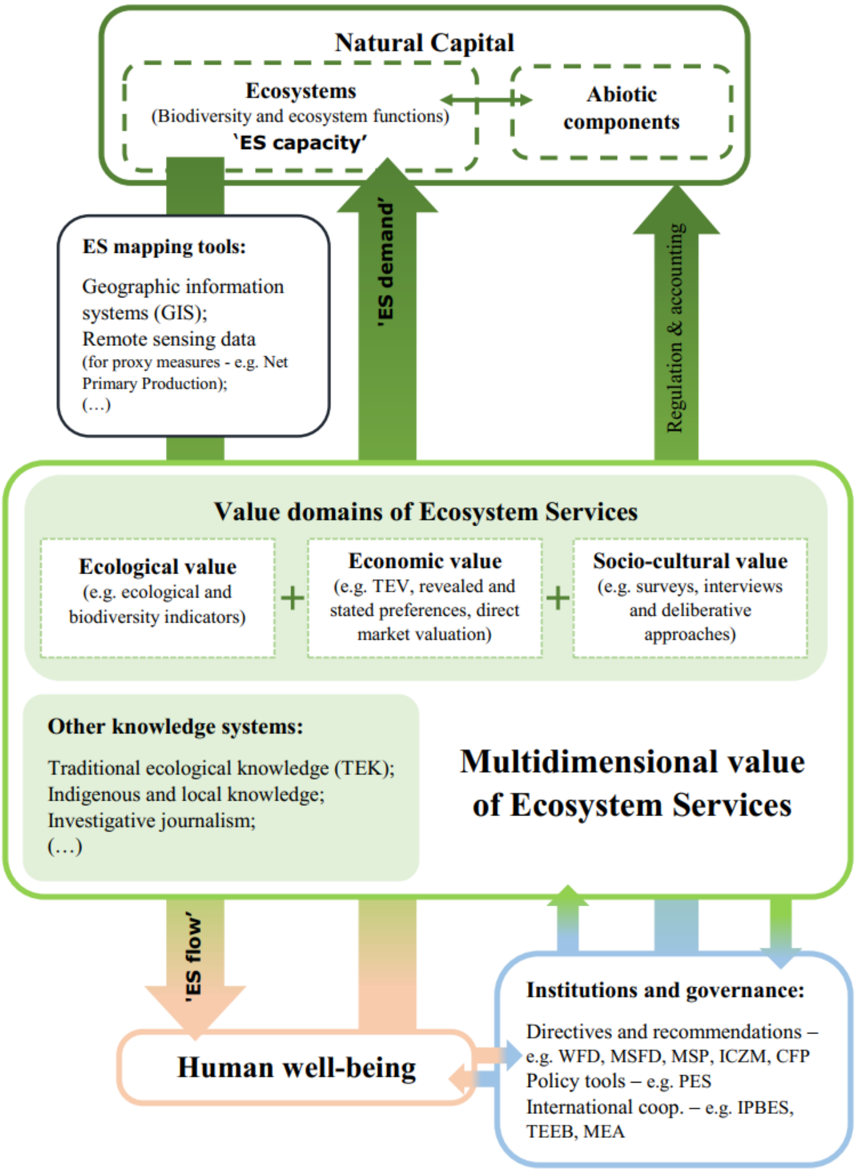

Figure 7.3: Conceptual framework for the pluralistic valuation of ES.

Undoubtedly, the most important next step in ES valuation is to operationalize an integrated valuation framework that endorses 'value pluralism' to better support global policy initiatives in EBM of marine and coastal ecosystems, where aquaculture is increasingly becoming an important driver of change. In this way, a greater portion of society will be involved in assessing the value of ES and both 'natural capital' and 'social capital' will be further integrated within national and global accounts of economic development (Drakou et al., 2018; Garcia-Rodrigues et al., 2018).

7.6. Conclusion

By identifying the ES delivered by marine and coastal ecosystems and aquaculture and by using pluralistic valuation approaches to reveal ES trade-offs between different scenarios, researchers can provide a more accurate forecast of the environmental and socio-economic impacts of aquaculture development. Aquaculture not only consumes but also provides ES beyond the provision of goods and the recognition of the positive effects of certain modes of aquaculture will enable more accurate accounting of economic, ecological, and social values. This approach can ultimately improve decision-making, improve the effective implementation of EBM options, and allow policymakers to use knowledge-based solutions that foster sustainable aquaculture development scenarios. From the present review, it became evident that many more valuation studies are necessary to assess ES trade-offs between aquaculture and the environment in which it occurs, to demonstrate the validity of ES conceptual frameworks to effectively support an EAA. The strengths and limitations of the different valuation methods must be pondered and a combination of them should make the valuation process more reliable. Practical reasons (e.g. available data and resources, expertise), stakeholder-oriented reasons (e.g. stakeholder participation, the inclusion of local knowledge, ease of communication) and decision-oriented reasons (e.g. purpose of the assessment, Ecosystem Services at stake) should be key considerations in selecting those methods.

Even though the literature on marine and coastal EBM is already diverse, its practical application has been generally impaired by the diversity of perspectives among management players on how to operationalize it. Moreover, outputs from previous marine and coastal ES assessments performed to inform decision-makers did not translate into the decision-making process. Thus, the application of the ES framework to foster sustainable development of aquaculture will depend on the research efforts carried out in the future, the valuation methodologies chosen to correctly elicit value, the successful communication of results to key players, and the actual application of conforming measures into decision-making. Additionally, government incentives towards the mapping of ES in marine and coastal areas most likely to be selected for and impacted by the development of aquaculture are also paramount. Only by shifting towards this approach will it be possible, in the future, to sort through different development scenarios and conscientiously support projects that sustain ES capacity and maintain or enhance ES flow to local communities and human societies.

Chapter 8

Conclusion

8. Conclusion

Aquaculture is rapidly becoming a major player of the Blue Bioeconomy, soon surpassing fisheries as the main provider of aquatic products to humanity. With increasing economic significance comes greater social and environmental responsibility and several efforts are being made by societal institutions and stakeholders to effectively integrate aquaculture within the objectives of Blue Growth. This initiative has been promoted by FAO and adopted by the EU as a strategy that aims to introduce responsible and sustainable approaches to reconcile economic growth in maritime sectors with the conservation of aquatic resources. Moreover, aquaculture development, if undertaken sustainably and equitably, can contribute significantly to several of the SDGs targets.

The IMTA production framework has been the underlying concept throughout this thesis, as it represents a real sustainable solution for the aquaculture industry. This production framework aims to mitigate the impacts of nutrients wastage by harnessing the extractive capacity of low-trophic level organisms and create value from their co-production. Halophytes, in particular, have been the main focus of this research, given their unique features in terms of withstanding saline conditions and the untapped potential they hold in the context of healthy diets and sustainable food systems. Therefore, this thesis set out to answer the question "*are halophytes suitable crops for sustainable coastal aquaculture?*" with a set of objectives that followed a transdisciplinary approach, supported by systematic reviews. Overall, the objectives were accomplished, and the following was concluded:

i. A growing body of scientific literature supports the integration of several edible halophytes in IMTA systems, following successful cultivation in saline (soil and hydroponics) conditions, efficient extraction of dissolved nutrients, and potential for economic valorization (e.g. food, source of raw materials and bioactive compounds).

ii. The sea purslane *H. portulacoides* can be successfully cultivated in controlled saline hydroponic conditions, efficiently extracting DIN and DIP from saline water, and produces harvestable edible biomass under a wide range of inorganic N and P concentrations with above-average productivity rates, compared with other halophytes.

iii. Planting density and hydraulic retention times must be managed, as a function of nutrients concentration in the effluent, to optimize nutrient extraction efficiency and maximize yields and achieve a balanced trade-off between remediation and productivity of extractive units.

iv. The edible biomass fraction of *H. portulacoides* presents an interesting nutritional profile that puts it in the categories of fresh vegetable products and can be used as a low-sodium salt alternative.

v. The polar lipidomic profile is maintained under a wide range of N and P concentrations and can be further investigated in terms of product development (functional foods) and applications for the nutraceutical industry.

vi. Halophyte can be accepted by consumers as fresh vegetables, as Portuguese consumers demonstrate willingness-to-pay for fresh-cut *Salicornia,* and certain consumer segments are more likely to incorporate halophytes in their diets.

Halophytes *are suitable crops for sustainable coastal aquaculture* that can deliver valuable 'provisioning services' of nutrition for humans and raw materials for other applications, as well as 'regulation and maintenance services', for instance, of mediation of nutrients wasted in effluents. The development of halophyte crops has important implications in light of the Blue Growth and the SDGs, as these plants can play an important role in elevating aquaculture production to more sustainable standards and contribute to food security, nutrition, poverty alleviation, resource use efficiency, waste reduction and the protection of marine and coastal ecosystems. Therefore, balanced integration of extractive halophyte units into IMTA can contribute to the eco-intensification of coastal aquaculture.

However, despite being encouraged by EU policies (including the Blue Growth strategy), socioeconomic, administrative, and regulatory bottlenecks still hinder the uptake of IMTA on a more industrial scale. From an operational point-of-view, there is still a long way to go until IMTA becomes a profitable, low-risk food production framework, as further research is needed concerning trophic connectivity between cultured organisms, to anticipate and balance nutrient transfer for better management. Additionally, there is a most important need to develop markets for trading halophyte-based products before major investments are made in halophytes integration and production on commercial-scale. While the efficient removal of N and P from aquaculture effluent waters is key to successfully integrate secondary species in IMTA, a major determinant of halophytes integration will be the existence (or lack thereof) of demand for its biomass, preferentially associated with high-end uses and products.

Future investigations must continue to select for the most promising halophyte species for IMTA, from an economic and environmental perspective, and the possibility of bringing halophytes production indoors for a completely controlled production environment must be explored. Other factors that may influence *H. portulacoides* vegetative growth should also be addressed, such as optimal irradiance/PAR, temperature, salinity, the supply of other macro- and micronutrients, along with the interaction of all these factors. Biochemical studies are also necessary for more extensive screening of *H. portulacoides* biomass for known and new bioactive compounds, including the determination of production conditions that favor the synthesis and accumulation of those compounds for higher functionality and added-value of halophyte-based products. Additionally, the valorization of non-edible biomass (roots and stems) through, for example, the production of bioenergy should also be investigated to promote the whole valorization of the biomass produced, as advocated by the principles of a circular economy. Concerns are expressed over food-safety issues of IMTA products related to the uptake, by extractive organisms, of potentially noxious substances present in aquaculture effluents (e.g. antibiotics, metals), halophytes produced under these conditions must also be surveyed to guarantee maximum standards of quality and safety for halophyte-based products. More socioeconomics and marketing studies are also necessary to better understand in which ways can halophytes be integrated into healthy diets and be accepted by consumers, not only in Portugal where policies already encourage their promotion but also across Europe and in the global context.

The successful introduction of halophytes to consumers and the full characterization of their nutritional, health, and socioeconomic benefits will create the space for halophytes to become increasingly attractive saline vegetable crops for human use, further expanding their role in the promotion of healthy diets from sustainable food systems.

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Annex

I. Tables

Table A2.3: Relevant peer-reviewed articles on halophytes for aquaculture effluent remediation

Reference	Title
Boxman et al. (2016)	Effect of support medium, hydraulic loading rate and plant density on water quality and growth of aquaponic systems halophytes in marine.
Chen & Wong (2016)	Integrated wetlands for food production.
de Lange & Paulissen (2016)	Efficiency of three halophyte species in removing nutrients from saline water: a pilot study.
Ben Nejma et al. (2015)	New septanoside and 20-hydroxyecdysone septanoside derivative from *Atriplex portulacoides* roots with preliminary biological activities.
Benzarti et al. (2015)	Involvement of nitrogen in salt resistance of *Atriplex portulacoides* is supported by split-root experiment data and exogenous application of N-rich compounds.
Buhmann et al. (2015)	Optimization of culturing conditions and selection of species for the use of halophytes as biofilter for nutrient-rich saline water.
Quintã et al. (2015a)	Growth and nitrogen uptake by *Salicornia europaea* and *Aster tripolium* in nutrient conditions typical of aquaculture wastewater.
Quintã et al. (2015b)	Uptake of an amino acid (alanine) and its peptide (trialanine) by the saltmarsh halophytes *Salicornia europaea* and *Aster tripolium* and its potential role in ecosystem N cycling and marine aquaculture wastewater treatment.
Waller et al. (2015)	Integrated multi-trophic aquaculture in a zero-exchange recirculation aquaculture system for marine fish and hydroponic halophyte production.
Rodrigues et al. (2014)	Maritime halophyte species from southern Portugal as sources of bio-active molecules.
Turcios & Papenbrock (2014)	Sustainable treatment of aquaculture effluents - What can we learn from the past for the future?
Vilela et al. (2014)	Screening of lipophilic and phenolic extractives from different mor-phological parts of *Halimione portulacoides.*
Andrades-Moreno et al. (2013)	Growth and survival of *Halimione portulacoides* stem cuttings in heavy metal contaminated soils.
Buhmann & Papenbrock (2013)	Biofiltering of aquaculture effluents by halophytic plants: basic prin-ciples, current uses and future perspectives
de Lange et al. (2013)	'Halophyte filters': the potential of constructed wetlands for applica-tion in saline aquaculture.
Lymbery et al. (2013)	The potential of a salt-tolerant plant (*Distichlis spicata* cv. NyPa For-age) to treat effluent from inland saline aquaculture and provide live-stock feed on salt-affected farmland.

Shpigel et al. (2013)	Constructed wetland with Salicornia as a biofilter for mariculture effluents.
Webb et al. (2013)	The effect of halophyte planting density on the efficiency of constructed wetlands for the treatment of wastewater from marine aquaculture.
Benzarti et al. (2012)	Photosynthetic activity and leaf antioxidative responses of *Atriplex portulacoides* subjected to extreme salinity.
Cambrollé et al. (2012a)	Tolerance and accumulation of copper in the salt-marsh shrub *Halimione portulacoides.*
Cambrollé et al. (2012b)	Zinc tolerance and accumulation in the salt-marsh shrub *Halimione portulacoides.*
Webb et al. (2012)	Halophyte filter beds for treatment of saline wastewater from aquaculture
Sousa et al. (2011)	Influence of multiple stressors on the auto-remediation processes occurring in salt marshes.
Sousa et al. (2010)	Productivity and nutrient cycling in salt marshes: contribution to ecosystem health.
Sousa et al. (2008)	Heavy metal accumulation in *Halimione portulacoides*: intra- and extra-cellular metal binding sites.
Válega et al. (2008a)	Mercury in salt marshes ecosystems: Halimione portulacoides as biomonitor.
Válega et al. (2008b)	Mercury mobility in a salt marsh colonized by *Halimione portulacoides.*
Neves et al. (2007)	Primary production and nutrient content in two salt marsh species, *Atriplex portulacoides* L. and *Limoniastrum monopetalum* L., in Southern Portugal.
Redondo-Gómez et al. (2007)	Growth and photosynthetic responses to salinity of the salt-marsh shrub *Atriplex portulacoides.*
Lymbery et al. (2006)	Efficacy of a subsurface-flow wetland using the estuarine sedge *Juncus kraussii* to treat effluent from inland saline aquaculture.
Lin et al. (2005)	Performance of a constructed wetland treating intensive shrimp aquaculture wastewater under high hydraulic loading rate.
Lin et al. (2003)	The potential use of constructed wetlands in a recirculating aquaculture system for shrimp culture.
Lin et al. (2002)	Nutrient removal from aquaculture wastewater using a constructed wetlands system.
Brown et al. (1999)	Halophytes for the treatment of saline aquaculture effluent.
Jensen (1985)	On the ecophysiology of *Halimione portulacoides.*

Table A3.4: Molecular (I) and elemental (II) composition of treatment solutions. Note: values do not account for the additional elements in the artificial saline water used as base-solution.

Molecular composition	Molar mass (g/mol)	$[N,P]_{low}$ (mM)	$[N,P]_{med}$ (mM)	$[N,P]_{high}$ (mM)	Control (mM)
KNO_3	101.11	0.40	1.32	1.50	1.50
$Ca(NO_3)_2.4H_2O$	236.15	-	-	-	1.00
$NH_4H_2PO_4$	115.03	0.03	0.10	0.20	0.50
$MgSO_4 . 7H_2O$	246.48	0.25	0.25	0.25	0.25
KCl	74.56	1.155	0.23	0.05	0.05
H_3BO_3	61.83	0.03	0.03	0.03	0.03
$MnSO_4 . H_2O$	169.01	2e-3	2e-3	2e-3	2e-3
$ZnSO_4 .7H_2O$	287.56	2e-3	2e-3	2e-3	2e-3
$CuSO_4 . 5H_2O$	249.68	5e-4	5e-4	5e-4	5e-4
$(NH_4)_6Mo_7O_{24} . 4H_2O$	1235.86	5e-4	5e-4	5e-4	5e-4
FeNaEDTA	367.05	0.02	0.02	0.02	0.02
NH_4NO_3	80.04	-	-	2.65	-
Elemental composition	**Atomic mass**	$[N,P]_{low}$ (ppm)	$[N,P]_{med}$ (ppm)	$[N,P]_{high}$ (ppm)	**Control** (ppm)
N	14.00	5.92	19.92	98.04	56.04
P	30.97	0.77	3.10	6.19	15.49
S*	32.06	8.16	8.16	8.16	8.16
K*	39.10	60.61	60.61	60.61	60.61
Ca*	40.08	-	-	-	40.08
Mg*	24.31	6.08	6.08	6.08	6.08
Fe	55.85	1.12	1.12	1.12	1.12
Cu	63.55	0.03	0.03	0.03	0.03
Zn	65.38	0.13	0.13	0.13	0.13
Mn	54.94	0.11	0.11	0.11	0.11
B*	10.81	0.27	0.27	0.27	0.27
Mo	95.95	0.34	0.34	0.34	0.34

* These elements are present in very high concentrations in the artificial saline water. Elemental variations between treatments (which only occurred in Ca) due to N and P adjustments are therefore considered negligible. At 20 ppt, Red Sea® saline water has, according to manufacturer's information: 235-248 mg L^{-1} Ca; 703-742 mg L^{-1} Mg and 213-226 mg L^{-1} K.

Table A3.2: Average water temperature, pH, and photosynthetically active radiation (PAR) at the end of each remediation week.

	Week									
	1	**2**	**3**	**4**	**5**	**6 †**	**7**	**8**	**9**	**10**
Temperature (ºC)	23.5 ± 0.1	23.1 ± 0.0	21.5 ± 0.1	22.5 ± 0.1	22.6 ± 0.0	25.0 ± 0.2	20.9 ± 0.3	21.3 ± 0.0	21.2 ± 0.0	21.5 ± 0.1
pH	7.7 ± 0.0	7.7 ± 0.0	7.7 ± 0.0	7.5 ± 0.0	7.7 ± 0.1	6.7 ± 0.1	7.1 ± 0.7	7.0 ± 0.6	6.9 ± 0.7	7.1 ± 0.6
PAR (μmol s^{-1} m^{-2})	332.0 ± 54.8	303.5 ± 44.8	321.3 ± 55.1	315.0 ± 42.0	331.3 ± 34.0	333.8 ± 47.5	356.3 ± 53.4	350.0 ± 66.3	303.8 ± 53.4	301.3 ± 61.4

† A drop in pH to values between 6.6 - 6.8 was registered in all treatments at the end of week 6. This occurrence coincided with an abrupt increase in air temperature in the facilities due to a failure in the ventilation system. Plants were exposed to this increase in room temperature for a maximum of 16 hours overnight. Treatment solutions were renewed the next day, coinciding with the end of a nutrient extraction period.

Table A5.5: Chemical composition of the hydroponic medium.

	Molar mass (g/mol)	Concentration (mM)
Molecules		
KNO_3	101.11	1.90
$Ca(NO_3)_2.4H_2O$	236.15	1.25
$NH_4H_2PO_4$	115.03	0.10
$MgSO_4 . 7H_2O$	246.48	0.25
KCl	74.56	0.05
H_3BO_3	61.83	0.03
$MnSO_4 . H_2O$	169.01	2e-3
$ZnSO_4 .7H_2O$	287.56	2e-3
$CuSO_4 . 5H_2O$	249.68	5e-4
$(NH_4)_6Mo_7O_{24} . 4H_2O$	1235.86	5e-4
FeNaEDTA	367.05	0.02
	Atomic mass	**Concentration (ppm)**
Chemical elements		
N	14.00	63.00
P	30.97	3.10
S	32.06	8.16
K*	39.10	76.25
Ca*	40.08	50.01
Mg*	24.31	6.08
Fe	55.85	1.12
Cu	63.55	0.03
Zn	65.38	0.13
Mn	54.94	0.11
B*	10.81	0.27
Mo	95.95	0.34

***** These elements are already present in high concentrations in the base saline solution, which are not accounted for in this table. At 20 ppt, Red Sea® saline water has, according to manufacturer's information: 235-248 mg L-1 Ca; 703-742 mg L-1 Mg and 213-226 mg L-1 K.

Table A7.6: Relevant peer-reviewed articles on aquaculture ecosystem services.

Reference	Title
With valuation	
Barbier *et al*. (2008)	Coastal ecosystem–based management with nonlinear ecological functions and values
Blayac *et al.* (2013)	Perceptions of the services provided by pond fish farming in Lorraine (France)
Farley *et al.* (2010)	Conserving mangrove ecosystems in the Philippines: transcending disciplinary and institutional borders
Liu *et al.* (2010)	Valuation of shrimp ecosystem services – a case study in Leizhou City, China
Malik *et al*. (2015)	Economic valuation of mangroves for comparison with commercial aquaculture in South Sulawesi, Indonesia
McDonough *et al*. (2014)	Wetland ecosystem service values and shrimp aquaculture relationships in Can Gio, Vietnam
Schmitt and Brugère (2013)	Capturing ecosystem services, stakeholders' preferences and trade-offs in coastal aquaculture decisions: a Bayesian belief network application
Thompson *et al.* (2014)	Locally assessing the economic viability of blue carbon: a case study from Panay Island, the Philippines
Zhang *et al.* (2012)	Analysis of stakeholder attitudes towards the coastal aquaculture environment – empirical study on willingness value estimation method
Without valuation	
Chopin *et al*. (2012)	Open-water integrated multi-trophic aquaculture: environmental biomitigation and economic diversification of fed aquaculture by extractive aquaculture
Deines *et al.* (2016)	Tradeoffs among ecosystem services associated with global tilapia introductions
Ferreira and Bricker (2016)	Goods and services of extensive aquaculture: shellfish culture and nutrient trading
Kim *et al*. (2017)	Seaweed aquaculture: cultivation technologies, challenges and its ecosystem services
Marcianò (2015)	Aquaculture in Lake Storsjön: an ecosystem services-based investigation - *BSc thesis*
Nielsen *et al.* (2016)	Magnitude, spatial scale and optimization of ecosystem services from a nutrient extraction mussel farm in the eutrophic Skive Fjord, Denmark
van Oudenhoven *et al*. (2014)	Effects of different management regimes on mangrove ecosystem services in Java, Indonesia
Saurel *et al.* (2014)	Ecosystem goods and services from Manila clam culture in Puget Sound: a modelling analysis
Walls (2017)	Ecosystem services and environmental impacts associated with commercial kelp aquaculture – *PhD thesis*
Walton *et al*. (2015)	A model for the future: Ecosystem services provided by the aquaculture activities of Veta la Palma, Southern Spain

II. Figures

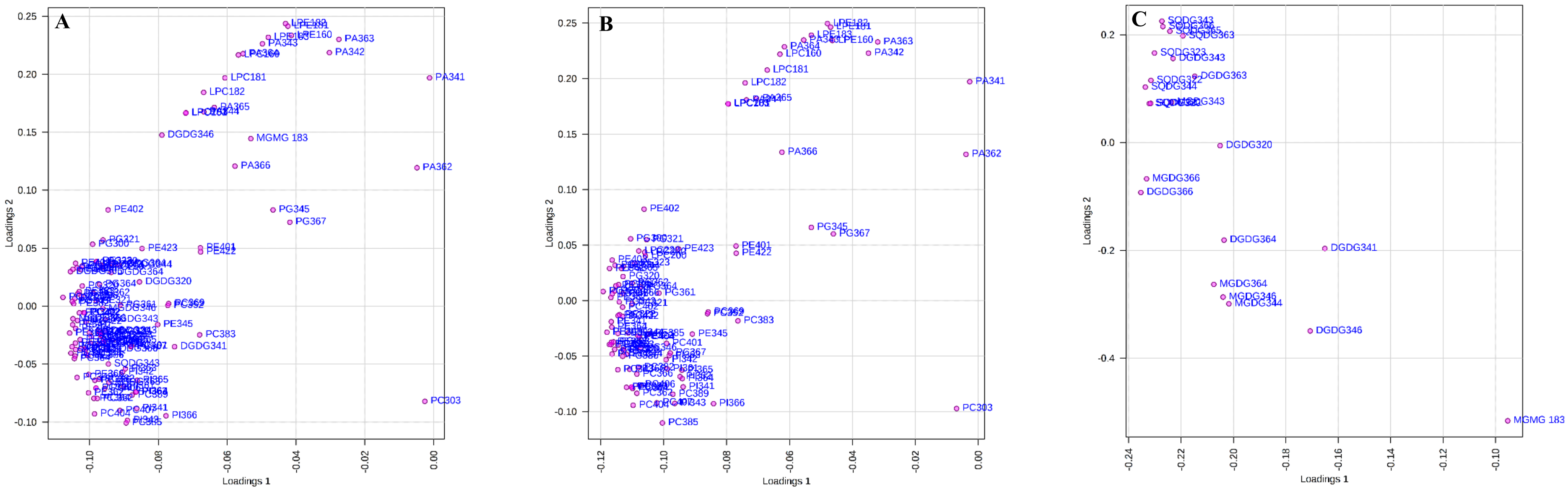


Figure A4.1: PCA loadings plot of total lipidome (A), phospholipids (B) and glycolipids (C).

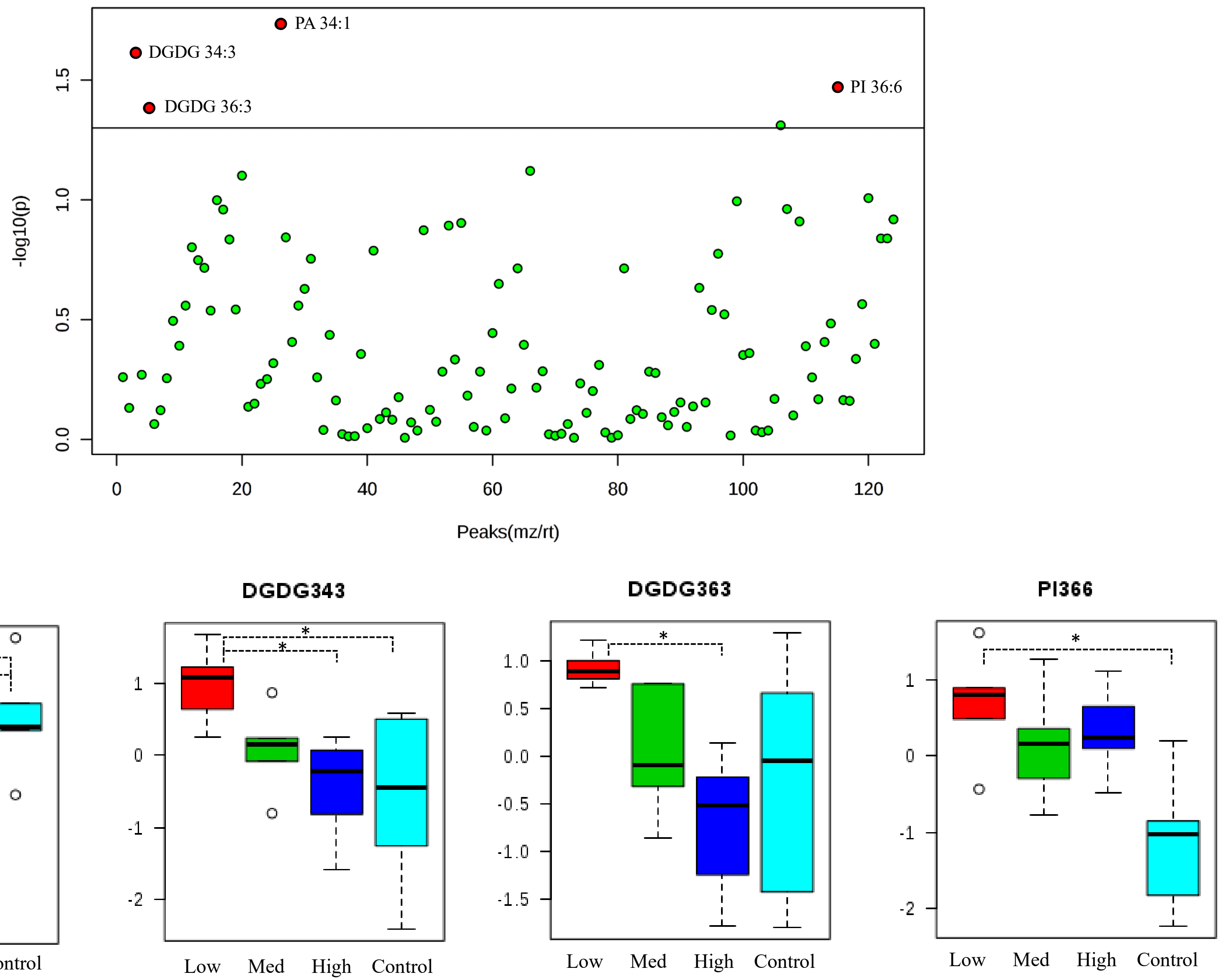
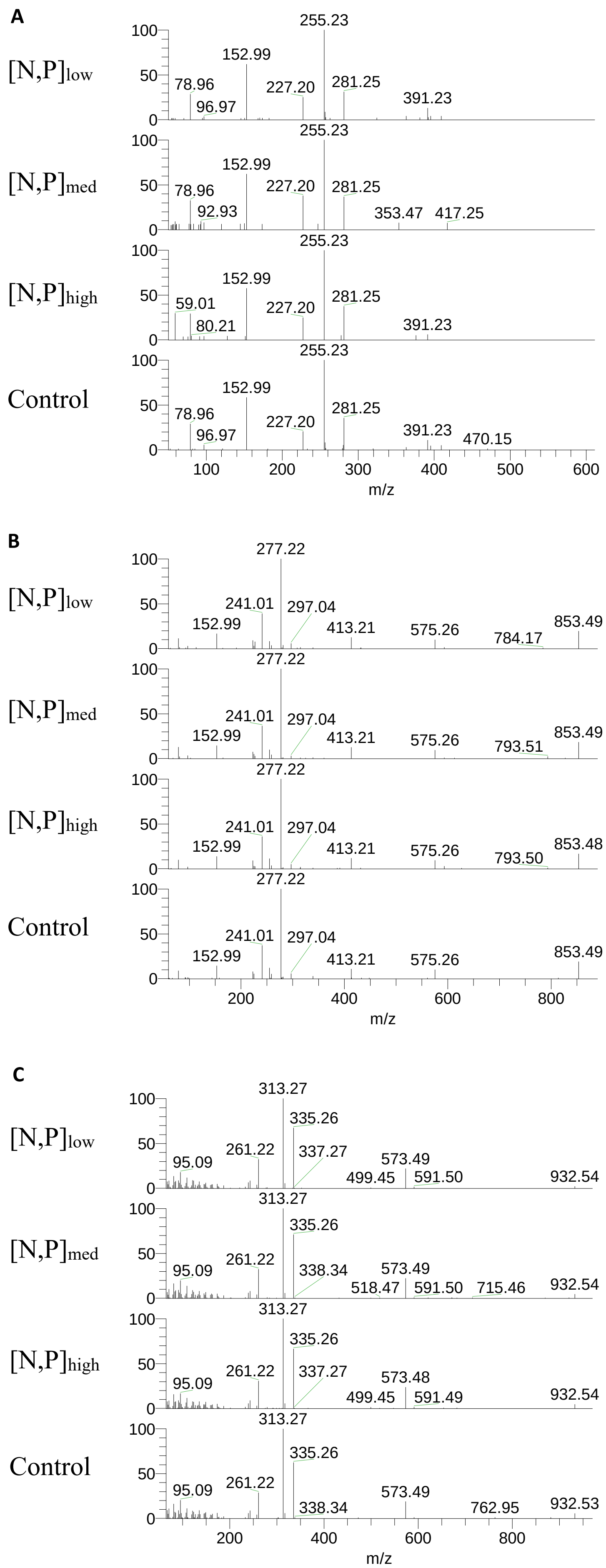
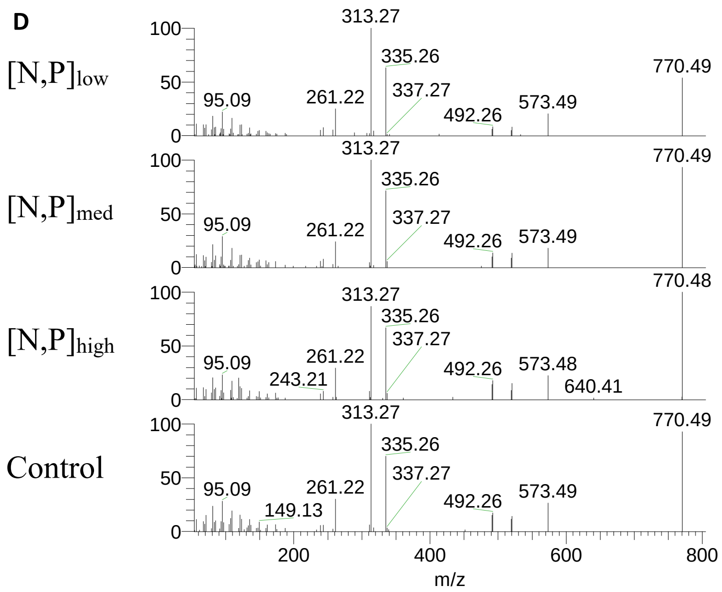


Figure A4.2: Univariate non-parametric Kruskal Wallis plot of peak-intensities (red dots - $p < 0.05$) and box-whiskers plots of significantly different species (* $p < 0.05$).


A
[N,P]low
255.23
152.99
78.96
96.97
227.20
281.25
391.23
[N,P]med
255.23
152.99
78.96
92.93
227.20
281.25
353.47
417.25
[N,P]high
255.23
152.99
59.01
80.21
227.20
281.25
391.23
Control
255.23
152.99
78.96
96.97
227.20
281.25
391.23
470.15
100
50
0
100 200 300 400 500 600
m/z
B
[N,P]low
277.22
241.01
152.99
297.04
413.21
575.26
784.17
853.49
[N,P]med
277.22
241.01
152.99
297.04
413.21
575.26
793.51
853.49
[N,P]high
277.22
241.01
152.99
297.04
413.21
575.26
793.50
853.48
Control
277.22
241.01
152.99
297.04
413.21
575.26
853.49
200 400 600 800
m/z
C
[N,P]low
313.27
335.26
261.22
95.09
337.27
573.49
499.45
591.50
932.54
[N,P]med
313.27
335.26
261.22
95.09
338.34
573.49
518.47
591.50
715.46
932.54
[N,P]high
313.27
335.26
261.22
95.09
337.27
573.48
499.45
591.49
932.54
Control
313.27
335.26
261.22
95.09
338.34
573.49
762.95
932.53
200 400 600 800
m/z

D



E

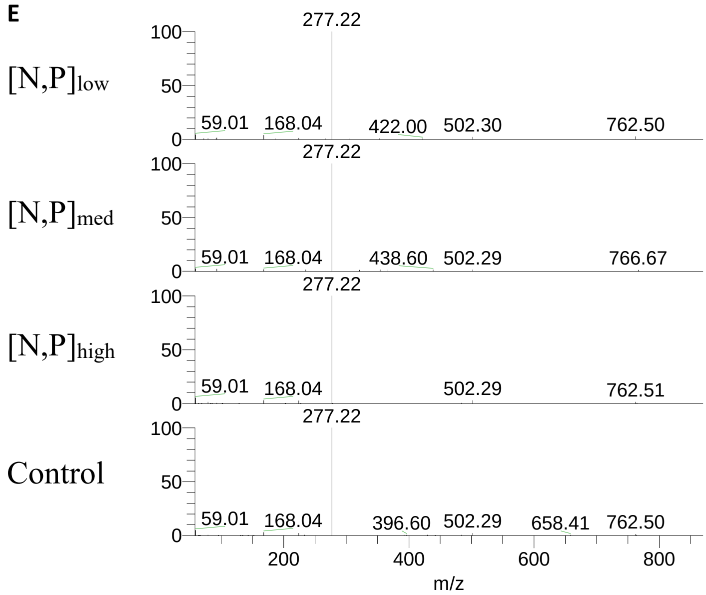
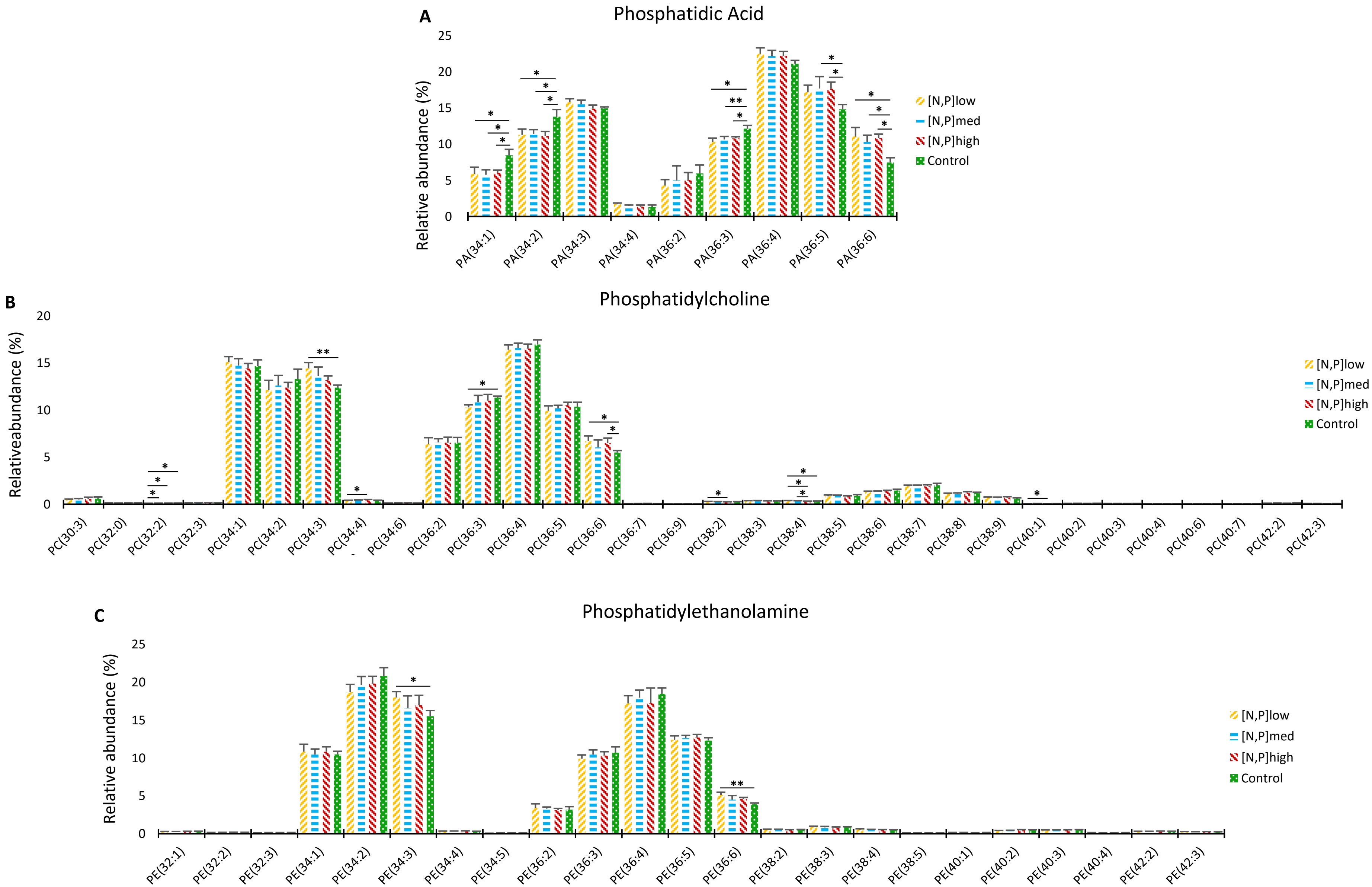


Figure A4.3: MS/MS spectra from all conditions of top VIP (Variable Importance in Projection) features in the 'total lipidome' PLS-DA projection: PA 34:1 (A), PI 36:6 (B), DGDG 34:3 (C), MGDG 34:3 (D), PC 36:6 (E).



A
Phosphatidic Acid
Relative abundance (%)
0
5
10
15
20
25
PA(34:1)
PA(34:2)
PA(34:3)
PA(34:4)
PA(36:2)
PA(36:3)
PA(36:4)
PA(36:5)
PA(36:6)
[N,P]low
[N,P]med
[N,P]high
Control
B
Phosphatidylcholine
Relativeabundance (%)
0
5
10
15
20
PC(30:3)
PC(32:0)
PC(32:2)
PC(32:3)
PC(34:1)
PC(34:2)
PC(34:3)
PC(34:4)
PC(34:6)
PC(36:2)
PC(36:3)
PC(36:4)
PC(36:5)
PC(36:6)
PC(36:7)
PC(36:9)
PC(38:2)
PC(38:3)
PC(38:4)
PC(38:5)
PC(38:6)
PC(38:7)
PC(38:8)
PC(38:9)
PC(40:1)
PC(40:2)
PC(40:3)
PC(40:4)
PC(40:6)
PC(40:7)
PC(42:2)
PC(42:3)
[N,P]low
[N,P]med
[N,P]high
Control
C
Phosphatidylethanolamine
Relative abundance (%)
0
5
10
15
20
25
PE(32:1)
PE(32:2)
PE(32:3)
PE(34:1)
PE(34:2)
PE(34:3)
PE(34:4)
PE(34:5)
PE(36:2)
PE(36:3)
PE(36:4)
PE(36:5)
PE(36:6)
PE(38:2)
PE(38:3)
PE(38:4)
PE(38:5)
PE(40:1)
PE(40:2)
PE(40:3)
PE(40:4)
PE(42:2)
PE(42:3)
[N,P]low
[N,P]med
[N,P]high
Control

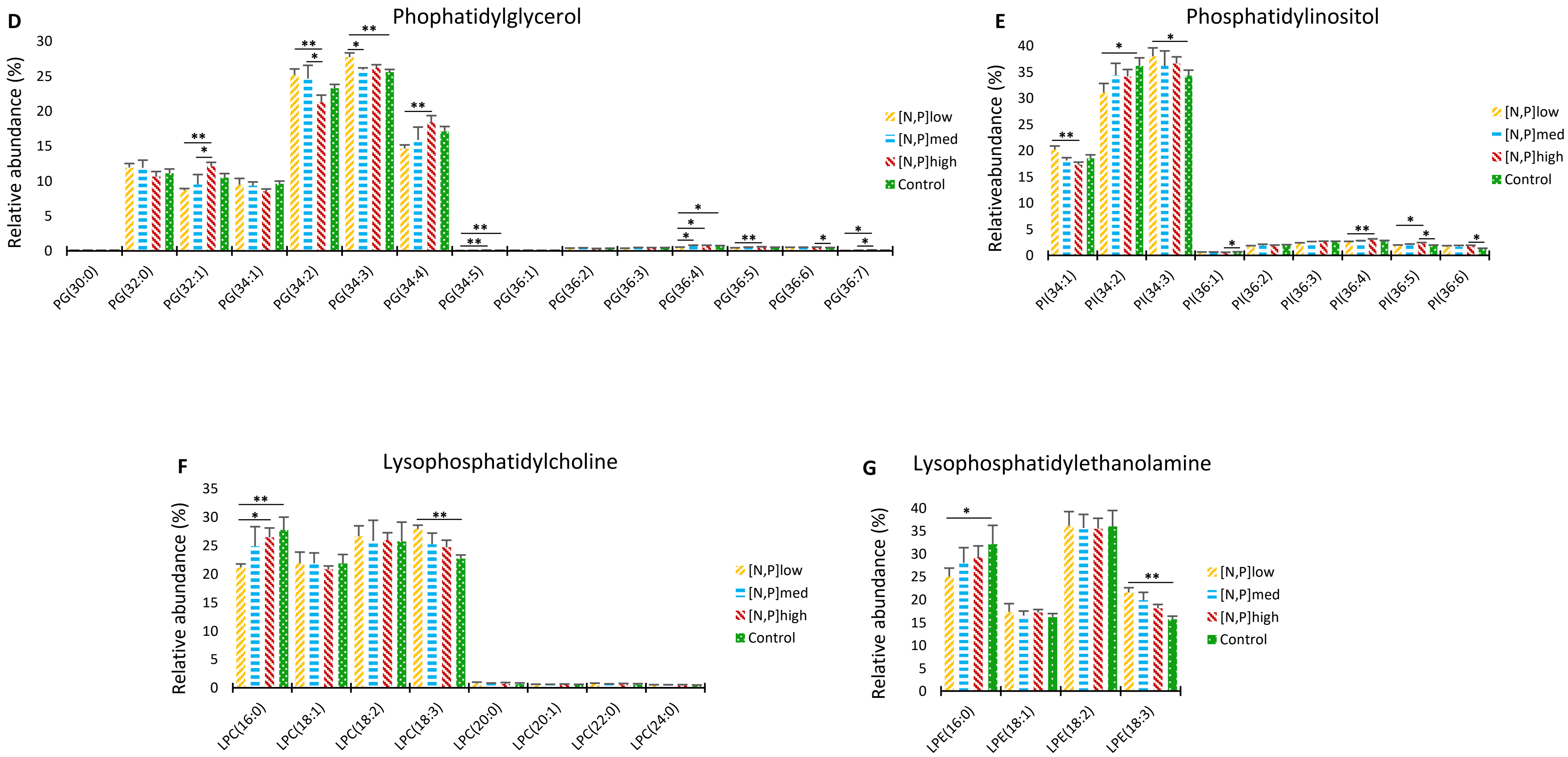


Figure A4.4: Relative abundance of phospholipid molecular species identified after LC-MS analysis. The results are expressed as a percentage, obtained by dividing the normalized peak areas of each molecular species by the sum of the total peak areas. Error bars represent standard deviations and horizontal lines represent significant differences: * p < 0,05; ** p < 0,01.

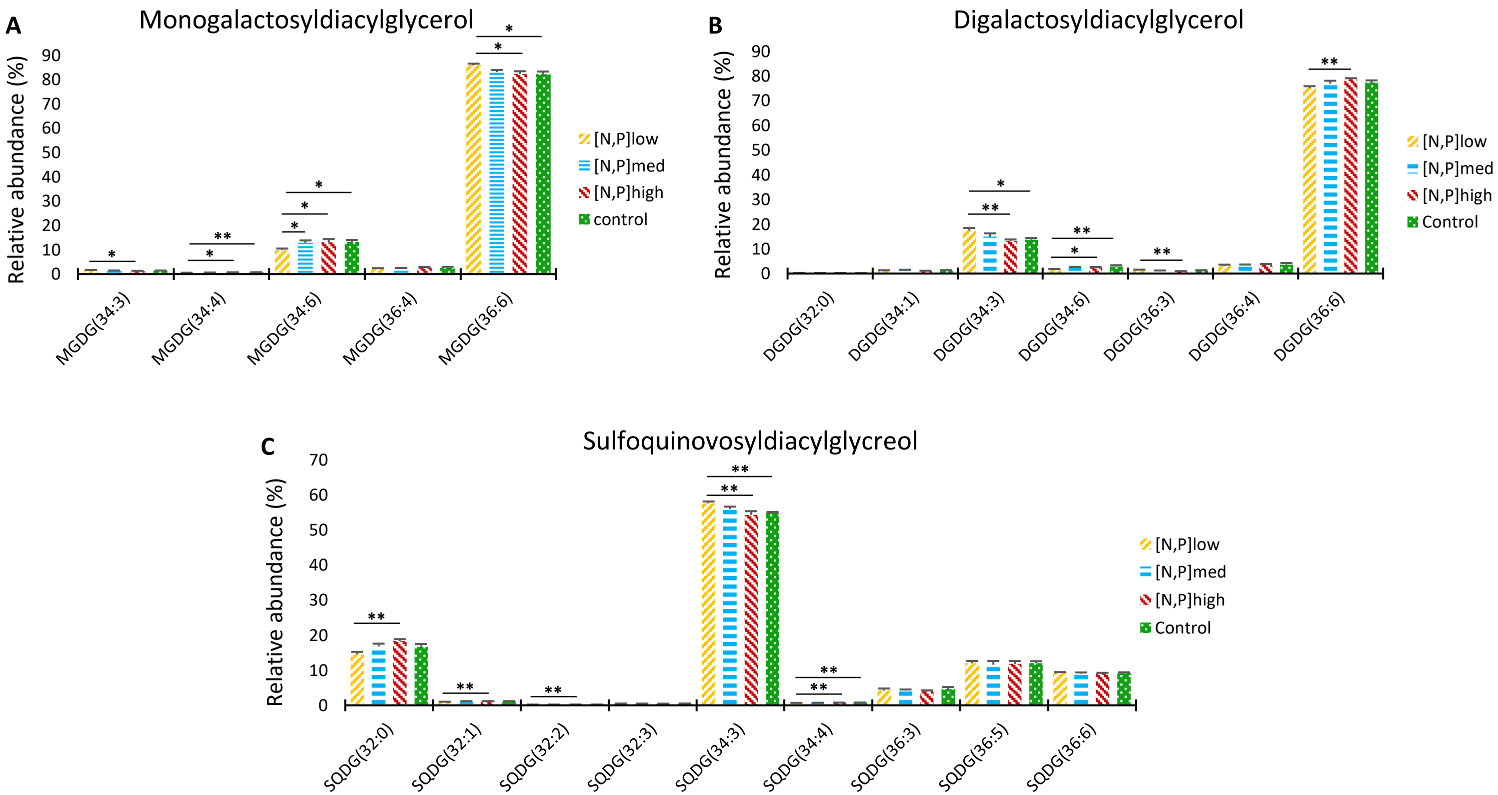


Figure A4.5: Relative abundance of glycolipid molecular species identified after LC-MS analysis. The results are expressed as a percentage, obtained by dividing the normalized peak areas of each molecular species by the sum of the total peak areas. Error bars represent standard deviations and horizontal lines represent significant differences: * p < 0,05; ** p < 0,01.

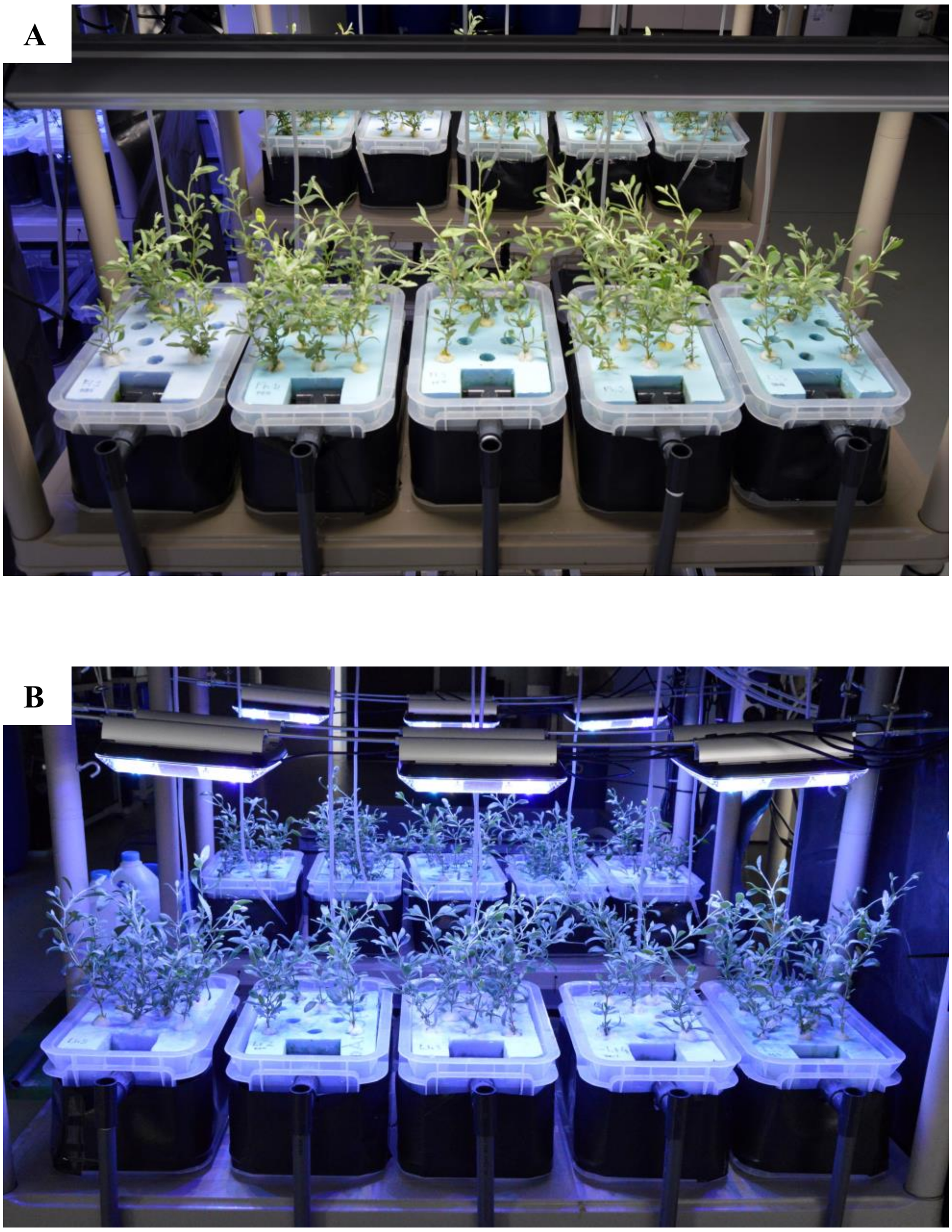


Figure A5.1: Experimental growth systems with fluorescent lights (A) and blue LEDs (B)

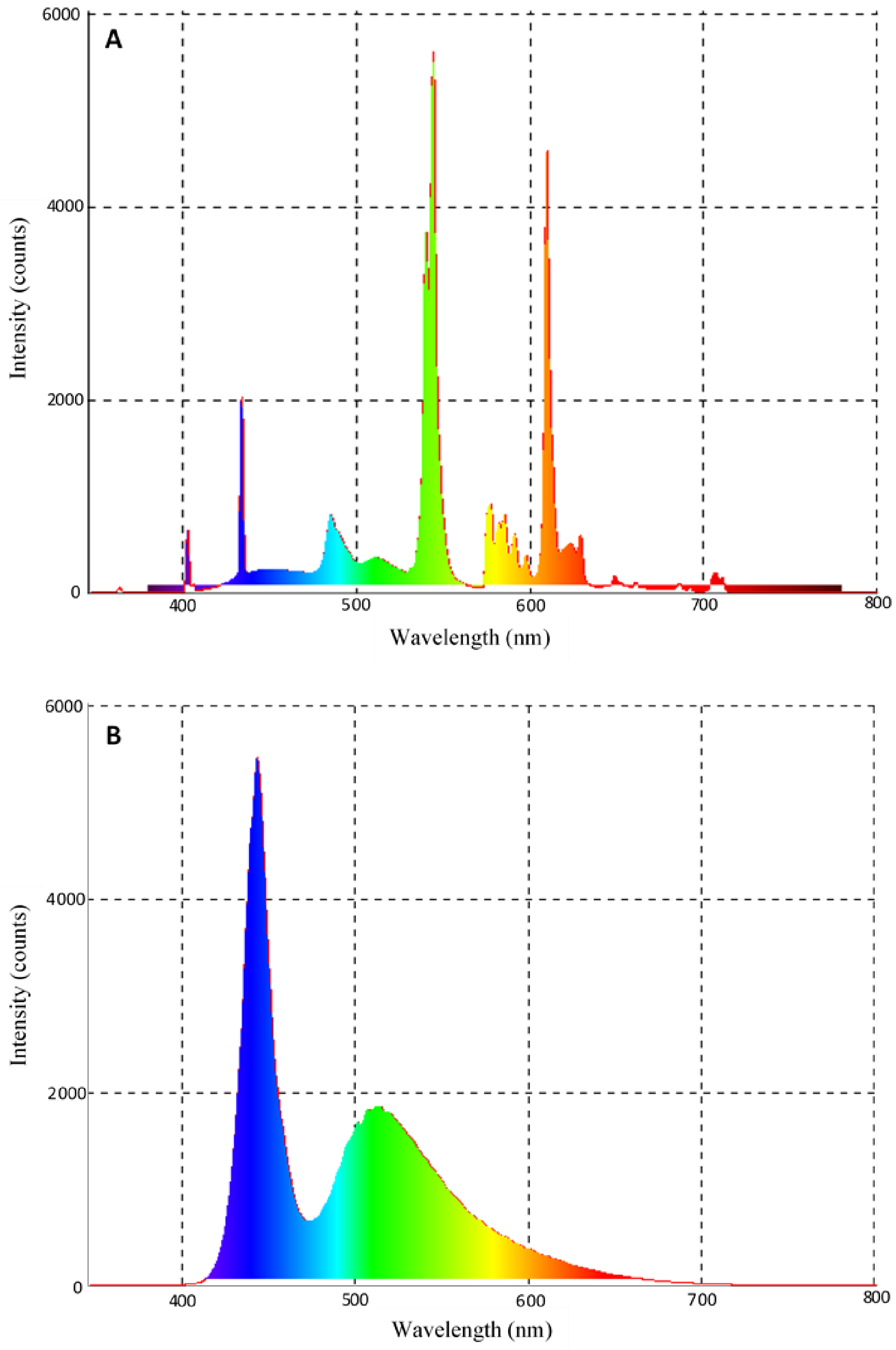


Figure A5.2: Light spectra of fluorescent lamps (A) and LED (B) measured with an Ocean Optics modular spectrometer, model FLAME-T-VIS-NIR (Ocean Optics Inc., Dunedin, FL, USA).

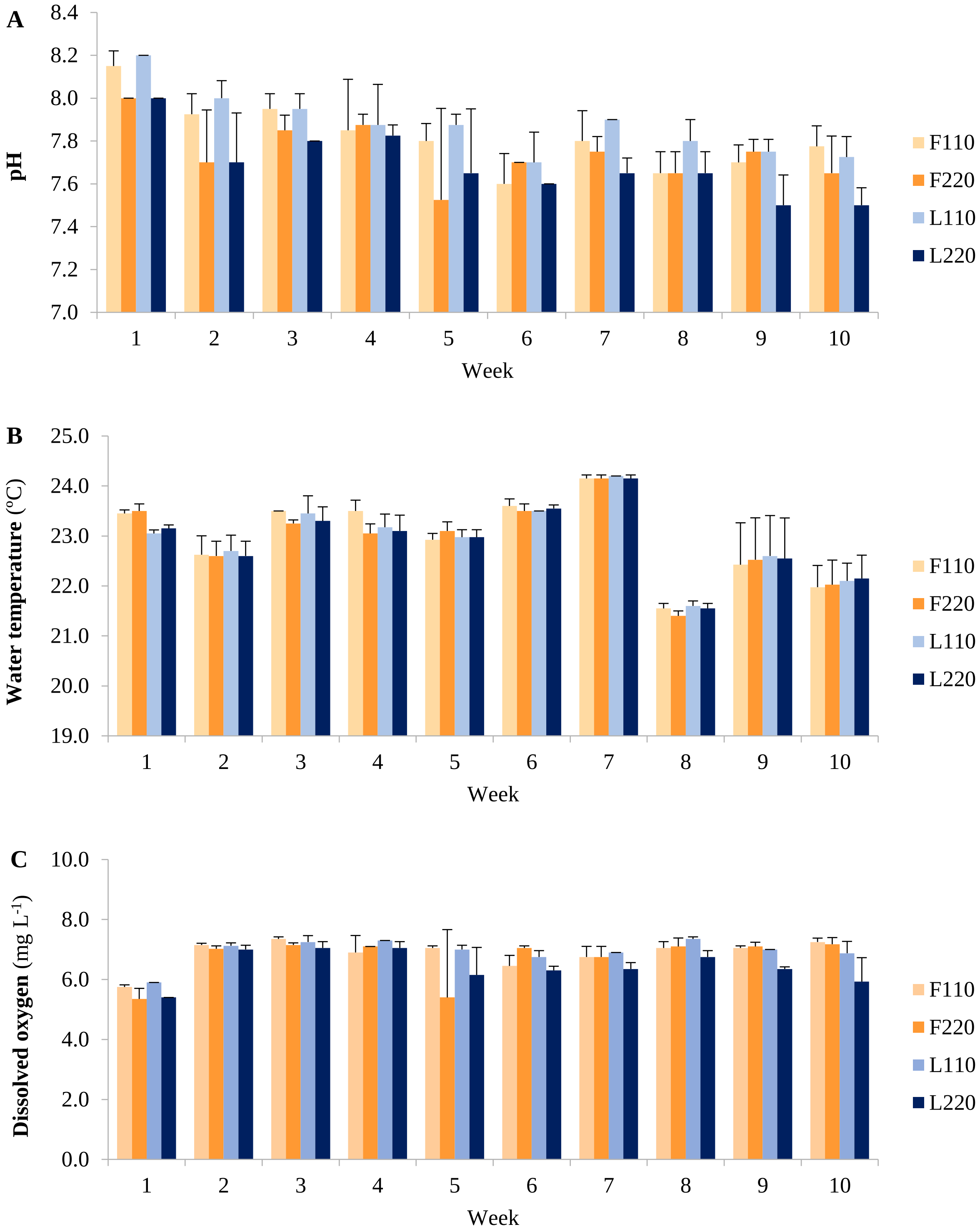


Figure A5.3: Average water pH (A), temperature (B) and dissolved oxygen (C).

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Date & location:

Part 1

1. What percentage of your day-to-day diet is composed of vegetable products?

0%--------------------25%--------------------50%-------------------75%--------------------100%

2. Do you diversify your vegetable intake in your day-to-day diet? □ Yes □ No

3. On a scale of 1 to 7, what is your level of agreement with the following sentences:

i. I try to choose food produced with minimal impact on the environment

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

Totally disagree	Disagree	Somewhat disagree	Neither agree nor disagree	Somewhat agree	Agree	Totally agree

ii. I love to try recipes from different countries

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

iii. Eating and food are important part of my social life

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

iv. I am concerned about the conditions under which the food I buy is produce

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

v. I like to try new foods that I have never tasted before

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

vi. Decisions on what to eat and drink are very important for me

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

vii. I try to choose food that is produced in a sustainable way

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

viii. I look for ways to prepare unusual meals

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

ix. Eating and drinking are a continuous source of joy for me

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

Figure A6.1: Questionnaire (1/3)

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Part 2

4. **Do you know what halophyte plants are?**
   - □ Yes
   - □ No

5. **Did you ever try or eat an halophyte (e.g. Salicornia)?**
   - □ Yes
   - □ No

6. **This is a package** [show] **with fresh Salicornia which can be eaten raw or prepared. Would you like to try this product?**
   - □ Yes
   - □ No

7. **On a scale from 1 to 7, how do you agree with the following sentence:**
   I liked the product

1-----------------2-----------------3-----------------4-----------------5-----------------6-----------------7

1	2	3	4	5	6	7
Totally disagree	Disagree	Somewhat disagree	Neither agree nor disagree	Somewhat agree	Agree	Totally agree

8. **Consider this package with 50 g of fresh Salicornia. What is the maximum price in € you would be willing to pay for this product?**

Willingness-to-pay:_______________€

Observations:

Figure A 6.1: Questionnaire (2/3)

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Part 3

9. **What is your gender?** □ Female □ Male

10. **How old are you?** □ 18-29 □ 30-39 □ 40-49 □ 50-59 □ > 60

11. **What is your level of education?**
    - □ Secondary school or less
    - □ University

12. **What is your employment status?**
    - □ Employee
    - □ Self-employed
    - □ Unemployed
    - □ Retired
    - □ Student
    - □ Other:____________________

13. **What is your monthly income (after taxes)?**
    - □ 0 – 599 €
    - □ 600 – 1000 €
    - □ 1001- 2000 €
    - □ 2001- 3000 €
    - □ > 3000€

14. **What is your household size?** ____________

Thank you for participating in this study!

Figure A 6.1: Questionnaire (3/3)

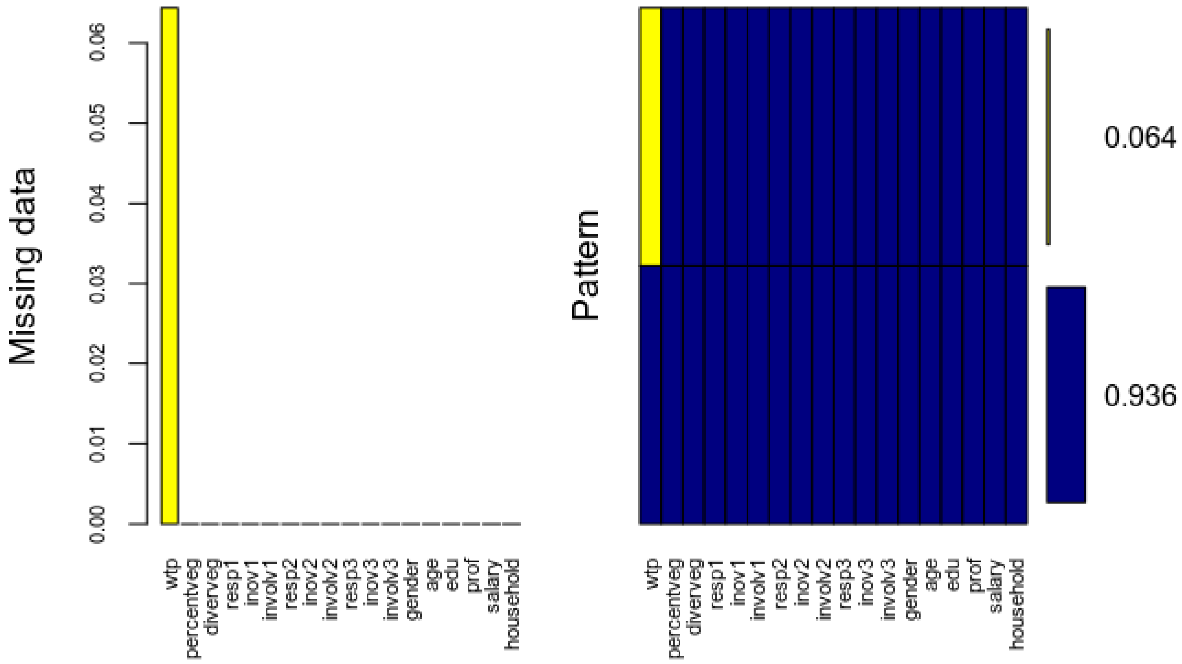

Figure A6.2: Missing data in the survey dataset.

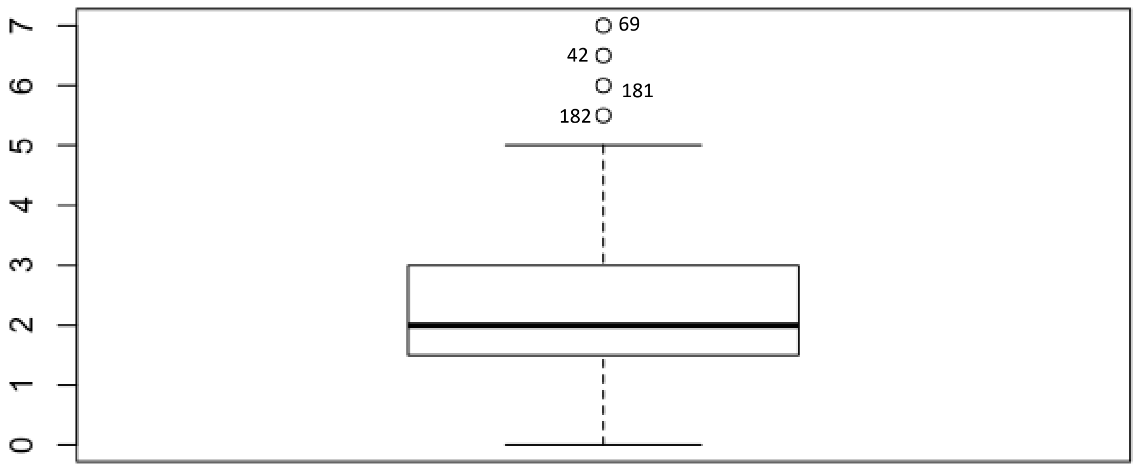

Figure A6.3: Boxplot of 'willingness-to-pay' responses and outliers.

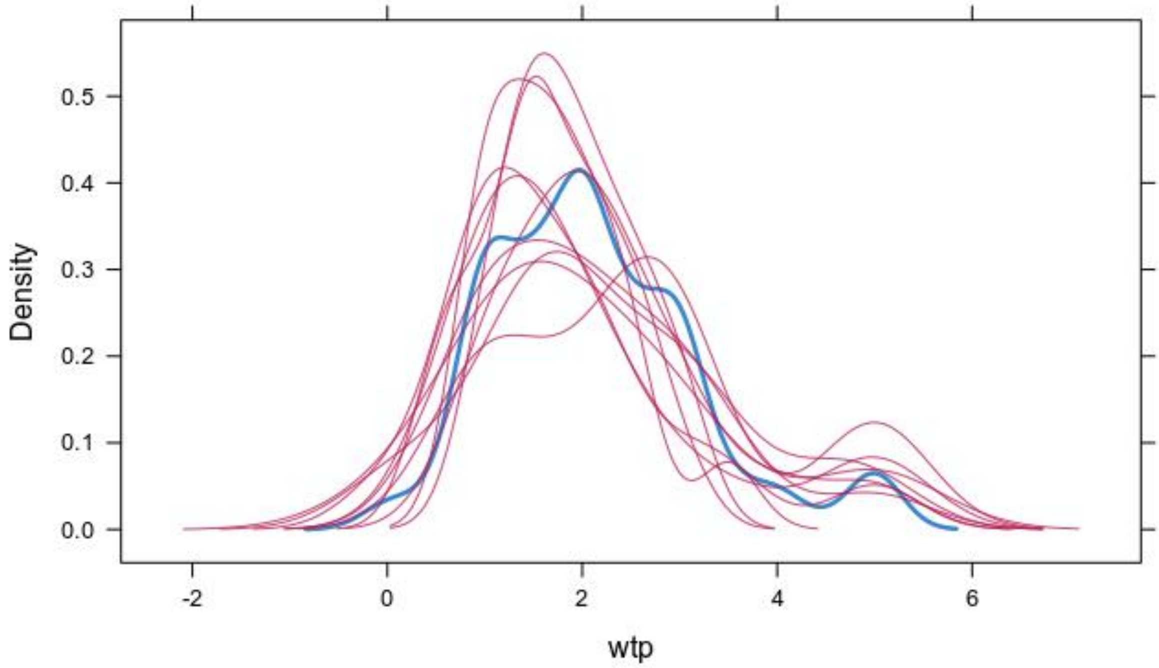

Figure A6.4: Missing data in 'willingness-to-pay' responses imputed using the MICE method. Density distribution of imputations (red curves) using "predictive mean matching" (number of multiple imputations: 10, number of iterations: 20). The blue curve represents the original 'willingness-to-pay' density distribution.

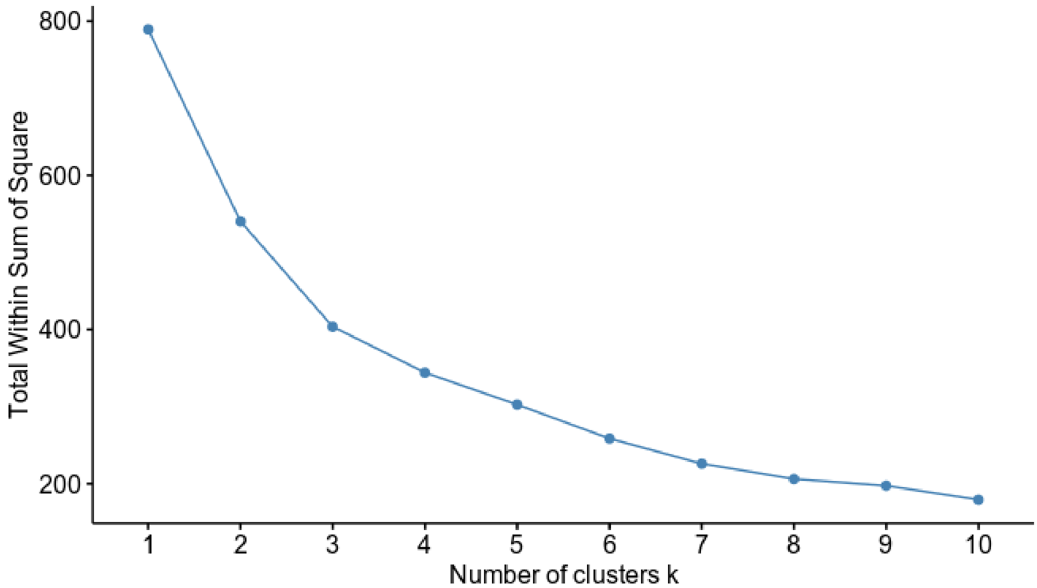

Figure A6.5: Within-Cluster Sum of Squares (WCSS).

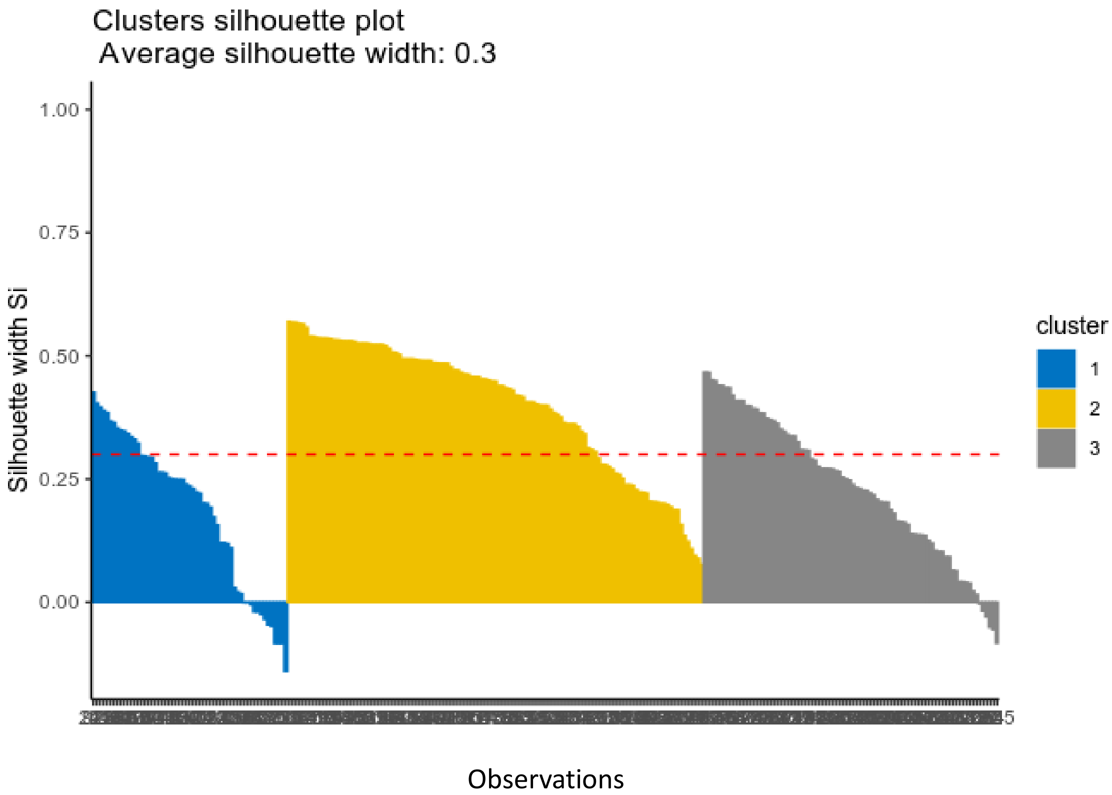

Figure A6.6: Silhouette plot (clustering validation).