



Floating wetland islands to promote water quality enhancement

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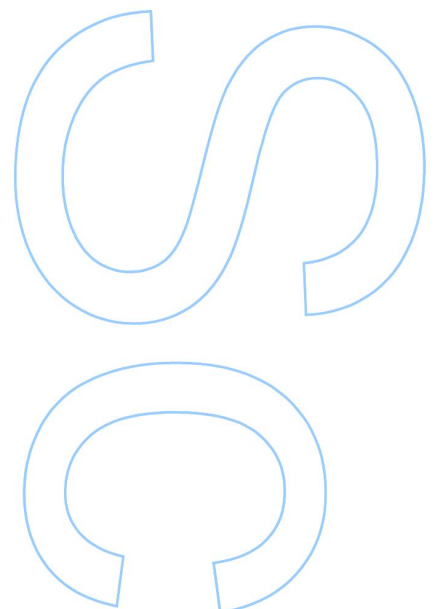
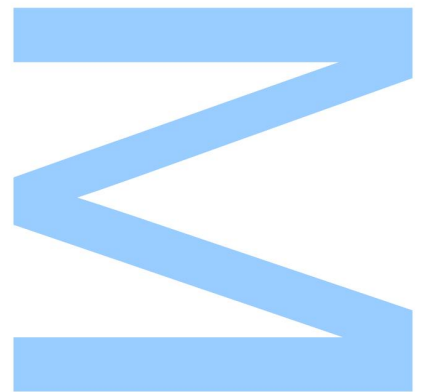
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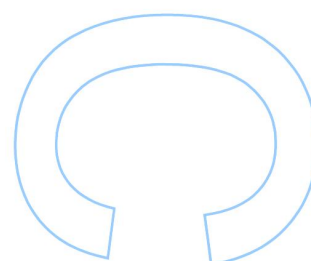
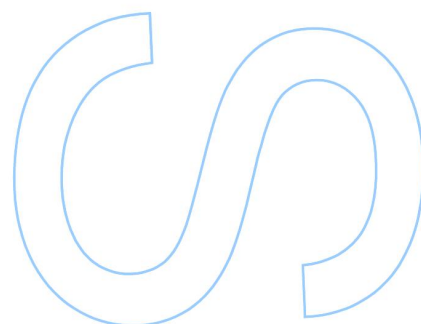
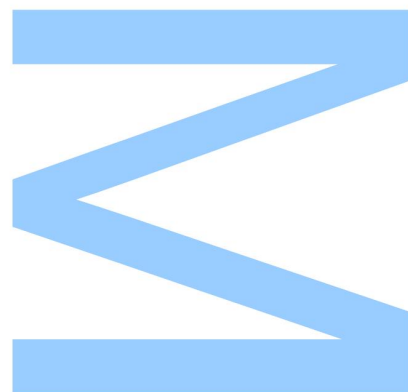




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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ABSTRACT

Floating wetland islands (FWIs) are considered nature-based solutions with great potential to promote biodiversity and water quality enhancement through phytoremediation processes. They have been used in many countries, but most studies have focused on their application to freshwater bodies. On the other hand, most research has explored nutrients removal rather than pollutants of another kind.

Taking these factors into account, to our knowledge, the present work is the first to scientifically document the *in situ* application of an FWI to a seawater port marina. In this sense, the work was divided into two phases. The first consisted of applying an FWI pilot system to the Marina of the Cruise Terminal of the Port of Leixões to evaluate the establishment of different plant species (*Sarcocornia perennis*, *Juncus maritimus*, *Phragmites australis*, *Halimione portulacoides*, *Spartina versicolor*, *Spartina maritima*, *Limonium vulgare*) to the imposed environmental conditions. During this period, the water in the study area was characterized physicochemically (with a focus on hydrocarbons measurement) and microbiologically. In parallel, a micro and macroscopic characterization of the diversity of organisms present in the FWI was made. The second phase aimed, at the laboratory level, to subject the plant species more successfully established in the FWI to marina water (doped with diesel from recreational boats and non-doped) to assess the hydrocarbons removal potential in this hypothetical spill scenario.

The main conclusions were: (1) total petroleum hydrocarbons (TPHs) were detected at the marina up to ca. 6 mg/L, although polycyclic aromatic hydrocarbons (PAHs) were not detected; the chemical oxygen demand values were, generally, considered high (135-630 mg/L); (2) FWI withstood to the 485 test days under the imposed environmental conditions; (3) *S. perennis* was the species with the highest survival rate (80-88%) and growth; (4) the structure of the microbial community showed clear differences between those established in the FWI and those in water; in macroscopic terms, *Mytilus* sp. was the predominant taxa, contributing considerably to the weight increase of the FWI; (5) in the laboratory tests, platforms with *S. perennis* obtained maximum nutrient removal rates for ammonium ion (initial concentration: 0.18 mg/L), nitrate ion (initial concentration: 1.67 mg/L), nitrite ion (initial concentration: 0.04 mg/L) and phosphate ion (initial concentration: 0.09 mg/L) of 95%, 95%, 92% and 73%, respectively; exposure to a concentration of 1550 mg/L of TPHs was lethal for this species and plants did not seem to influence the removal of hydrocarbons from the water.

Keywords: floating wetland islands, phytoremediation, *Sarcocornia perennis*, port marina, saltwater, hydrocarbons, water quality.

RESUMO

As ilhas flutuantes (IFs) são consideradas soluções baseadas na natureza com grande potencial para promover a biodiversidade e a melhoria da qualidade da água através de processos de fitorremediação. Estas têm vindo a ser utilizadas em muitos países, mas a maioria dos estudos tem-se focado na aplicação das mesmas em massas de água doce. Por outro lado, a maior parte da investigação realizada tem explorado a remoção de nutrientes ao invés de poluentes de outro tipo.

Tendo estes fatores em conta, para nosso conhecimento, o presente trabalho é o primeiro a documentar cientificamente a aplicação *in situ* de uma IF a uma marina portuária de água marinha. Neste sentido, o trabalho foi dividido em duas fases. A primeira consistiu em aplicar uma IF piloto na Marina do Terminal de Cruzeiros do Porto de Leixões para avaliar o estabelecimento de diferentes espécies de plantas (*Sarcocornia perennis*, *Juncus maritimus*, *Phragmites australis*, *Halimione portulacoides*, *Spartina versicolor*, *Spartina maritima*, *Limonium vulgare*) face às condições ambientais impostas. Durante este período, a água da zona de estudo foi caracterizada físico-quimicamente (com foco na medição de hidrocarbonetos) e microbiologicamente. Em paralelo, foi realizada uma caracterização micro e macroscópica da diversidade de organismos presentes na IF. A segunda fase pretendeu, a nível laboratorial, sujeitar as plantas estabelecidas com maior sucesso na IF à água da marina (dopada com diesel dos barcos de recreio e não dopada) para avaliar o potencial de remoção de hidrocarbonetos neste hipotético cenário de derrame.

As principais conclusões foram: (1) hidrocarbonetos totais de petróleo (HTPs) foram detetados na marina até cerca de 6 mg/L, embora não tenham sido detetados hidrocarbonetos aromáticos policíclicos (HAPs); os valores obtidos da carência química de oxigénio foram, em geral, considerados altos (135-630 mg/L); (2) a IF resistiu aos 485 dias de teste nas condições ambientais impostas; (3) *S. perennis* foi a espécie que registou maior taxa de sobrevivência (80-88%) e crescimento; (4) a estrutura da comunidade microbiana mostrou diferenças claras entre as estabelecidas na IF e as existentes na água; em termos macroscópicos, *Mytilus* sp. foi o taxa predominante, contribuindo consideravelmente para o aumento do peso da IF; (5) nos testes de laboratório, as plataformas com *S. perennis* obtiveram taxas máximas de remoção de nutrientes de ião amónio (concentração inicial: 0.18 mg/L), ião nitrato (concentração inicial: 1.67 mg/L), ião nitrito (concentração inicial: 0.04 mg/L) e ião fosfato (concentração inicial: 0.09 mg/L) de 95%, 95%, 92%, 73%, respetivamente; a exposição a uma concentração de 1550 mg/L de HTPs foi letal para esta espécie e as plantas parecem

não ter influenciado a remoção de hidrocarbonetos da água.

Palavras-chave: ilhas flutuantes, fitorremediação, *Sarcocornia perennis*, marina portuária, água salgada, hidrocarbonetos, qualidade da água.

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ABBREVIATIONS AND SYNCRONIMS

ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
COD	Chemical Oxygen Demand
DIY	Do It Yourself
DO	Dissolved Oxygen
eDNA	Environmental Deoxyribonucleic Acid
ETR_m	Maximum Electron Transport Rate
F_m	Maximal Fluorescence Yield
F_o	Basic Fluorescence Yield
FTIR	Fourier-Transform Infrared Spectroscopy
FWI	Floating Wetland Island
GC-MS	Gas Chromatography-Mass Spectrometry
HDPE	High Density Polyethylene
NaCl	Sodium Chloride
NGS	New Generation Sequencing
O&M	Operation and Maintenance
OHCB	Obligate Hydrocarbonoclastic Bacteria
ONG	Observed Number of Genera
PAHs	Polycyclic Aromatic Hydrocarbons
PAM	Pulse Amplitude Modulation
PCoA	Principal Coordinates Analysis
PVC	Polyvinyl Chloride
RLC	Rapid Light Curve
SD	Standard Deviation
SIMPER	Similarity Percentage Analysis
SPME	Solid-phase Microextraction
TPHs	Total Petroleum Hydrocarbons
USD	United States Dollar
VAT	Value-Added Tax
XPS	Extruded Polystyrene

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CHAPTER 1

INTRODUCTION

1.1 Floating wetland islands

1.1.1. Definition and terminology

Floating wetland islands (FWIs) are man-made systems, which intend to mimic the processes that occur in natural wetland systems with the major difference of plants being grown in hydroponic mode (no soil requirement) instead of being supported in a substrate [1]. Thus, FWIs consist of establishing emergent vegetation in a floating platform that is applied to the surface of a water body [1; 2]. In general, conventional FWIs have, as main components: floating platform, planting media, the emergent vegetation and anchoring system (Figure 1).

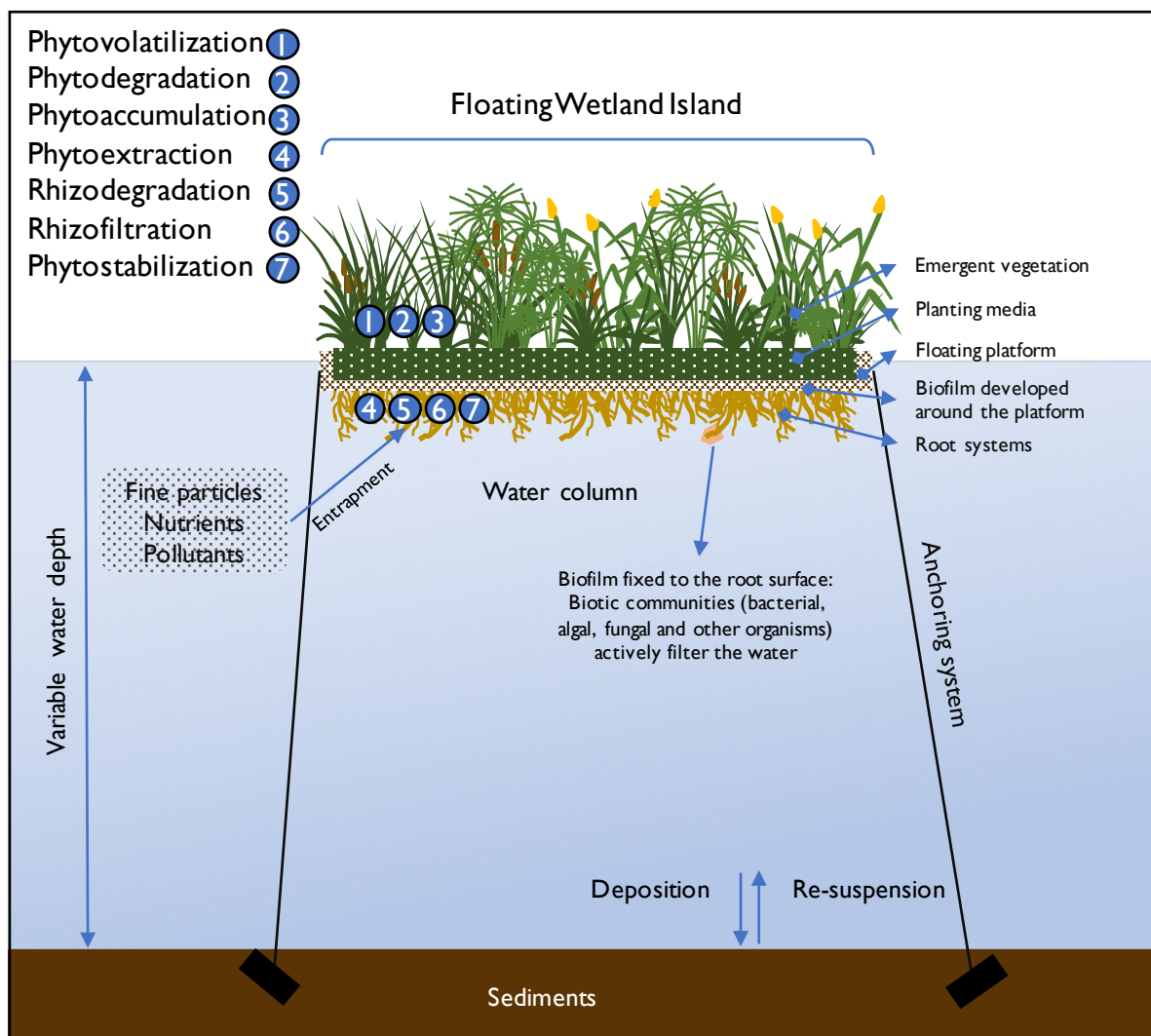


Figure 1. Representation of an FWI in a cross-sectional view, showing the physicochemical and biological associated phenomena.

FWIs are being used in several countries around the globe [2]. This relatively recent technology received especially interest since the 1980s when, in Germany, this knowledge was used to create rafts on rivers and lakes that they called *Schwimmkampen* (*schwimmen* means “to swim/float”; *kampe* means in its closest English translation “camp”) [3]. These FWIs had the shape of equilateral triangles vegetated or gravel-topped and could create habitat for birds among other uses [3]. Currently, there are several applications of FWIs as it is discussed ahead.

Regarding terminology, the term applied in describing this technology presents plenty of variations. There is no global unanimity in the designation and a vast terminology is being used worldwide [1; 2; 4-7]. Some of the keywords (synonyms) found, in the literature, are generally composed of two or more words such as “artificial floating islands” or “floating emergent macrophyte treatment wetlands” (this last term is following the systematic classification for constructed wetland treatments developed by Fonder and Headley [8]). The terms used in the literature were compiled and are present in Table 1.

Table 1. List of compiled terminology used to describe FWIs technology with example references.

Terminology used	Example references
Artificial floating bed/island/reed beds	[4; 9; 10]
Artificial vegetation island	[11]
Constructed floating wetland	[2; 7; 12]
Constructed macrophyte floating bed system	[13]
Ecological floating bed	[14; 15]
Ecologically engineered floating vegetation mat	[16]
Floating bed	[17]
Floating emergent macrophyte treatment wetland	[8]
Floating mat treatment system	[18]
Floating phyto-bed	[19]
Floating treatment wetland	[20; 21]
Floating wetland island	[22]
Hydroponic root mat	[5]
Planted floating bed system	[23]
Vegetated floating island	[24]

Therefore, with this compiled list, it was possible to reach more information about this technology approach, when applied to ScienceDirect® and Google Scholar search engines. For this present thesis, the term adopted was “floating wetland island”.

1.1.2. Construction design and materials

The floating platform can be homemade or commercially acquired [25]. Concerning the first alternative, there are a few “do it yourself” (DIY) options available online (e.g. [26]).

Several principles must be taken into account during the process of FWI construction such as functionality, durability, anchoring system, weight and buoyancy achievement [27]. More recently, Bi *et al.* [7] established a framework for the proper FWI construction design requirements, based on the combination of four aspects: the management purpose, the

target system (the type of water body), pollutants type and operation and maintenance (O&M) costs involved.

According to Headley *et al.* [1], there are three methods to reach the buoyancy on an FWI: (1) the use of a buoyant structure where is attached a planting media (most common approach) [1] or (2) a material that includes the buoyant property and simultaneously allows the growth of the vegetation [1] (e.g. [28]); (3) the last method consists of choosing the right plant species to create a natural auto-buoyant plant platform that sustains itself over the time, where, at a first phase, a small buoyant support for plants establishment and development is provided [1].

Several materials can be chosen for the floating platform and planting media [1; 2].

In terms of planting media, there are presented the following compilation of example materials that have been tested to date: coir fibre [10; 28; 29], soft foam [30], bamboo charcoal and soil [31], cotton [32], peat moss [33], natural fibres and cork granules [3], green zeolite, sepiolite, absorbent paper, ceramsite [14], burlap, mulch [34] and wood ash [28]. A few of these referred materials (for instance the zeolite) have pollutant sorption qualities and can be advantageous in water treatment approaches [14; 35]. Particular attention is necessary for the planting media selection process since some of them can add additional nutrients into the water column as reported by Chang *et al.* [33].

Concerning the materials used to handmade the floating platform, they were used, to date, mainly plastic derivate: polystyrene (PS) blocks and wooden frames [36], polyvinyl chloride (PVC) pipes and plastic mesh [29], polypropylene random copolymer with buoyant bottles [37], extruded polystyrene (XPS) foam [30] and high density polyethylene (HDPE) foam [13]; but natural materials have also been used such as bamboo [31], combination of raw materials (dredged sludge as main material, treated industrial slag and expanded perlite) [32] and as, last example, decomposing plants as base layer [28]. Interestingly, Seo *et al.* [11] studied different floating platform materials and used rubberized coconut fibre in their floating platforms, reporting its hydrophobic property as being useful for a faster and likely stronger bacterial adhesion, for nutrients absorption and showing low desorption, compared to the hydrophilic floating platforms. Section 1.1.6. provides more types of materials used for the case of commercially available floating platforms.

Regarding the anchoring system, it varies accordingly to the geography of the site, water physicochemical characteristics, water surface movements and the depth of the water body [1].

Plants are another important component in an FWI, contributing for instance to the aesthetical value (e.g. [38]), wildlife habitat support (e.g. [11]) and biodiversity promotion (e.g. [39]). Besides that, plants play a major role in water treatment [2; 4; 20] through phytoremediation processes that consist of an environmentally-friendly approach that uses living plants and associated organisms for clean-up contaminated sites [40]. Therefore, the plants placed in the FWI are responsible for several phytoremediation processes that can occur [41] (Figure 1).

The vegetation species used in FWIs, at a global scale, was recently reviewed by Pavlineri *et al.* [2] showing *Canna* genus as the most common species used. Concerning the plants used in FWIs, until the moment, the aquatic plants were the most studied in this type of technology, while the terrestrial ones were less explored [2].

In-field scale approaches have tested polycultures (use of more than one species) such as: *Calamus* sp., *Iris* sp., *Lythrum* sp., and *Hydrocotyle vulgaris* [42]; *Pontederia sagittata* and *Cyperus papyrus* [38]; *Pontederia cordata* L. and *Schoenoplectus tabernaemontani* [29]; *Juncus effusus* and *Pontederia cordata* [33]; *Iris p.*, *Lycopus europaeus*, *Myosotis palustris*, *Lythrum salicaria*, *Carex rostrata*, *Carex acutiformis*, *Carex gracilis*, *Mentha aquatica* and *Apium nodiflorum* [12]. In the case of monocultures (one single species used) plants such as: *Carex virgata* [43] and *Phragmites karka* [10] were tested. The polyculture can be interesting to understand the pollutant removal efficiency associated with the combination and synergy of different species, whereas the use of monocultures might be useful to attribute the performance pattern and efficiency to a specific plant species [4].

The underwater section of the FWI has significant importance to water treatment processes, because it is where the roots develop into the water column and act through several mechanisms for pollutant removal [2; 4] such as biosynthesis and rhizofiltration [2]. Thereby, the root growth generates a large matrix that operates by filtering and trapping particles [43; 44]. Besides that, plants are responsible for producing organic compounds released into the water, promoting the flocculation of suspended matter as well as improving pollutant settlement [4; 43-45]. Furthermore, in contact with water, a root-biofilm network is established, where microbial communities attach [46], influenced by a process called chemotaxis [47], and help to reduce pollutant loads [37]. On the other hand, microbial communities beneficiates from the oxygen and carbon released by the upper part of the plant to the roots [24]. Thus, in an aquatic system, this matrix of associated microorganisms (mainly bacteria members) and the relationships established with the plants are responsible for the mitigation of several pollutants present in the water [48].

1.1.3. Pollutants removal

The processes that are driven by plants and the synergy with the associated microorganisms, discussed above, play a major role in FWIs pollutant removal processes. In general, the FWIs are applied to water bodies for nutrients removal [10; 20; 21; 33; 49; 50]. Although less studied, other types of pollutants having been targeted such as heavy metals [23; 43], petroleum-based [51], faecal coliforms [38] and veterinary antibiotics [13]. Furthermore, the major contributions result from laboratory-scale or mesocosm studies, since it is important at the first phase of the investigation to obtain valuable data before full-scale application [52] and it is easier to control the experimental conditions in small-scale rather than on full-scale approaches [7]. Nonetheless, Bi *et al.* [7] described that the removal efficiencies based on small-scale were considerably higher when compared to in-field studies and overrated when extrapolation from small to large-scale was made. Additionally, Lucke *et al.* [52] claimed that the compared results are often not transferable.

In addition to conventional FWIs, more sophisticated systems have been explored, particularly in the last 10 years, aiming to enhance the efficiency of FWIs by using complementary technologies [7]. For instance, Li *et al.* [37] tested the use of bio-carriers and freshwater clams suspended in FWIs to enhance water treatment efficiency in outdoor mesocosm experiments; Dai *et al.* [53] also used bio-carriers but composed by elastic-plastic fillers located under the FWI in a greenhouse; similarly Wang *et al.* [42] suspended zeolite and sponge iron reporting water improvement in the reclaimed water tested; Chang *et al.* [39], in a lake, added solar power units to an FWI that generated energy for underwater aerators and, in the middle of the island, installed an underwater net cage containing submerged plants; Guo *et al.* [54] also designed an underwater cage with submerged plants to treat domestic wastewater while, recently, Shen *et al.* [55] tested aluminium-based drinking water treatment residual as substrate in cages beneath the FWI to improve a synthetic water quality. Lastly, the novel application by Kadam *et al.* [19] of an FWI for water treatment of textile wastewater and, simultaneous, electricity generation.

1.1.4. FWIs surface coverage in water bodies

FWIs can be applied to a variety of water bodies [4] such as rivers [10; 23], ponds [22; 29; 33; 38; 43], lakes [36], to different types of wastewater [13; 51; 56; 57] and to aquaculture systems [58]. Having into consideration the water body typology, the sizing calculation of a conventional FWI to define the surface coverage of a water body is a hard task, and multiple circumstances that interact with each other, as described ahead, must be considered.

Borne *et al.* [44], considers that one of those variables is the limitation of dissolved oxygen (DO) depletion and the shading effect caused by the FWI in the water column. The physical barrier formed through the FWI decreases the photosynthetic activity and the water aeration, which leads to DO reduction. Besides, the microbial community under the platform also potencies the consumption of DO [44]. Contrarily, Wang and Sample [59] reported a study where chlorophyll-a was not affected by the FWI shading effect, however, parameters such as the water temperature and thermal pollution were reduced by 61% of FWI coverage.

Concerning treatment efficiency, in-field studies in ponds demonstrated that 50% surface coverage can improve performance compared to the control [43] and testing 9% and 18% surface coverage, results also suggested that greater percentage coverage of FWIs had a better pollutant removal [50]. On the other hand, Garcia Chance and White [60] established mesocosm experiments, wherein addition to FWI surface coverage on the water body (50% and 100%) and with different plant densities applied to the FWI itself, also tested the tanks with and without aeration supply. The results showed that the lowest DO value was registered in non-aerated tanks with 100% density plants, which proves that uncovered areas are important to gas exchange [60]. Ultimately, a recent review by Lucke *et al.* [52], critically analysed different FWI experimental designs to better understand what could be improved. For each research study, they presented a schematic configuration of the study area and the FWI disposal, pointing possible gaps. Based on these observations, they observed that the design efficiency could be more effective than the percentage of surface coverage area, e.g. positioning of the FWIs in the water body accordingly to the water flow patterns [52].

1.1.5. Operation and maintenance

To achieve a long-term successful FWI application, a well-designed operation and management (O&M) strategy must be developed [7]. Many design recommendations to the construction and installation were discussed above (literature review sections 1.1.2. and 1.1.4.).

Borne *et al.* [44], based on their experience working with FWIs field-scale applied to ponds, established guidelines for O&M, including periodic tasks such as: monitoring the plant health and evolution (especially at the beginning of installation); trimming upper plant parts; replantation of free vegetation areas; pulling out not wanted invasive plants; removing detritus or filtering gross solids and checking punctually the anchoring system.

Some researchers point plant harvesting as also a maintenance task to avoid phyto-uptake nutrients (nitrogen and phosphorus) return into the water column when plant senescence occurs, since, in their studies, they noticed a correlation between the water nutrients concentration and the plant decay [29; 61].

1.1.6. FWIs available on the market

This section aimed to provide a brief market review, where, in the first phase, it is presented the FWIs solutions available from the main national (Portuguese) commercializing companies (Table 2) (the names of the companies were occulted due to confidentiality issues). Currently, there are several options for modular prefabricated FWIs available on the market, that essentially differ, in the dimension and type of materials used (Tables 2 and 3).

Table 2. Examples of floating wetland island solutions available in the national (Portuguese) market.

Main materials	Modules dimension (l x w x h)
Cork agglomerate	1 x 0.5 x 0.04/0.10 m
Coconut fibre blanket and plastic-covered wire netting as buoyant structure	Not available
Matrix of coconut fibres treated with natural latex and buoyant structure	2 x 1 x 0.10 m
Matrix of coconut fibres and HDPE, polypropylene and polyethylene foam as buoyant structure	5/10 x 1/2 x 0.05/0.075 m

In the second phase, there were summarized international FWIs commercialized systems. Hence, in Table 3 there are shown, for example, five floating platforms used in scientific studies with example references.

Table 3. Floating platforms available in the international market tested in scientific studies.

Company	Product designation	Main materials	Modules dimension	Example references
BeeMats, LLC. (USA)	BeeMats	Buyoant closed-cell foam	Customized: (e.g. around 2.97 m ²)	[20; 21; 62]
Bestmann Green Systems (Germany)	AquaGreen® Repotex® I2	Plastic-covered wire netting	n.a.	[12]
BioHaven Environmental Solutions, LLC. (USA)	Biohaven®	Recycled polyethylene terephthalate and polyurethane	Starting at around 0.46 m ²	[21; 22; 33]
De Rebus Plantarum (Italy)	Tech-ia®	Ethylene vinyl acetate	0.5 m ²	[63]
Maryland Aquatic Nurseries, Inc. (USA)	n.a.	Polypropylene and fibrous coir materials	n.a.	[57]

n.a.: information not available in the company website at the time of the present thesis preparation

1.1.7. Economical aspects

For this economical aspects section was only considered, for evaluation, the conventional FWIs. One of the cost-saving benefits is the fact that installation is, in general, made onto an existing water body, not requiring additional terrain areas [59; 64].

Only few studies report the real costs involved in FWIs: Wang and Sample [59] described a cost of 19 USD/m² (for the floating platform) and approximately 52 USD/m² (for planting media) and Billore *et al.* [10] reported a total cost (manufacturing and installation) of 60 USD/m². On the other hand, research conducted by Lynch *et al.* [21], testing two commercially available floating platforms, reported the individual cost (without the price of planting media and vases) of 38 USD/m² for BeeMats and 377 USD/m² in the case of Biohaven[®].

In three villages of Bangladesh, Irfanullah *et al.* [65] presented an almost inexpensive FWIs that were constructed with raw materials to cultivate vegetables and other crops in this developing country. The estimated cost for a so-called “floating garden” (7 m²) was 4.30-10.90 USD (counting labour, seeds, and materials expenses). Here, the produced vegetables were consumed or sold out, obtaining economic benefits.

Ultimately, in the case of Portugal, based on a market survey (4 companies inquired) conducted within the framework of the present thesis, for an FWI system (floating platform and planting media, no plants or labour costs included) costs range between 72.00 to 185.70 €/m² (plus value-added tax - VAT).

In sum, the costs associated with FWIs are related to the materials used in either manufacturing or commercial acquisition and the labour associated with the installation and maintenance operations.

1.1.8. FWIs ecosystem services and applications

Ecosystem services present the known freely benefits (direct and indirect) provided by the ecosystem to human well-being, which includes provisioning, regulating, cultural and supporting services [66]. In this sense, Bi *et al.* [7] mention the necessity of further field studies, involving FWIs, to estimate the related ecosystem service values.

FWIs are used in a large range of cases depending, essentially, on the main function for which they are planned to be used. Consequently, to demonstrate the current relative value associated with FWI systems, Table 4 shows the ecosystem services categories

and the corresponding FWI services resulting from real applications where this technology has been tested to date (in alignment with Millennium Ecosystem Assessment [66]).

Table 4. Ecosystem services and example of FWI applications.

Ecosystem services	Function/Application	Example references
Provisioning		
Food	Crop production	[17; 28; 65]
Fuel	Electric energy production	[19; 67]
	Biogas production	[17]
	Bioenergy feedstock	[68]
Regulating		
Ornamental resources	Harvested plants for commercial purposes	[62]
Water purification and waste treatment	Water quality improvement	[2; 4; 20]
Cultural		
Educational values	Knowledge and awareness increase	[65]
Aesthetic values	Landscape improvement	[38]
Social relations	Human social dynamics	[65]
Recreation and tourism	Recreational area	[38]
Supporting		
Nutrient cycling	Nutrient uptake and removal	[58]
Biodiversity	Biodiversity enhancement and wildlife habitat support	[11; 34; 36; 39; 69]

Although there are reports from commercializing companies mentioning the FWIs application to wave attenuation and coastal erosion reduction [70], no scientific detailed studies were found in the search engines referred previously and, therefore, not included in the table.

Most studies and applications of FWIs are focused on freshwater systems while the potential of FWIs to be applied to saline waters, which is of interest to the present thesis, were less addressed. In general, FWIs have been adopted in marine aquaculture approaches [18; 30; 58] and Sanicola *et al.* [64] have addressed the performance of five plant species to identify the most appropriate ones for FWI application in saline environments, testing them for different salt concentrations in an FWI mesocosm.

To the best of our knowledge, no detailed scientific research has been carried out to investigate the potential of FWIs technology when applied to seawater marina ports. However, the following two cases mention the application of FWIs to harsh environments in areas associated with navigation activity: (1) two FWIs installed in the Elfin Cove area in Southeast Alaska, USA and (2) Baltimore's Inner Harbor at Maryland, USA. The first report showed that the FWIs resist the harsh environment that they were exposed to, in which the vegetation (variety of plant species, including vegetables) bloomed in the Spring and the floating platforms were colonized by marine organisms [71]. The second case is a project that aims to reinvent the area surrounding the main waterfront campus of "The National Aquarium" and, in 2010, it was implemented the first small-scale FWIs in the "Inner Harbor", showing promising results concerning their establishment [72; 73].

1.2. Environmental issues related to seawater marinas

According to Dafforn *et al.* [74], coastal zones are suffering such an urban expansion that in areas of Europe, Asia, Australia, and USA, at least 50% of the shoreline is transformed into highly visible man-made structures. Hence, the creation of new structures to support maritime activities such as the creation of marinas comes up. In these sites, there is a group of anthropogenic activities that stands out, such as diesel spills during vessel refuelling; runoffs from boat cleaning and reparation operations (as painting), and maritime traffic with the associated diesel combustion [75; 76]. Additionally, the enclosed shapes of these areas generate inadequate pollutant input dispersal due to the low hydrodynamics and tend to accumulate these pollutants in high levels [77].

The mentioned issues influence the water quality, being found pollutants such as heavy metals [78], biocides [79], and hydrocarbons [78; 80]. Additionally, the presence of pollutants and the low hydrodynamics within marinas affect and shape the microorganism communities' composition in water and sediments [75] and, as well, the relative abundance of certain macroorganisms is known to be affected by the pollutant existence in harbour sediments [81]. In these marine environments, the accumulation of organisms on the underwater structures (e.g. pier) receives the designation of biofouling [82].

Moreover, among these pollutants, hydrocarbons were the ones of interest since were the organic pollutants studied in the present thesis. These compounds are presented as a mix of saturated (or aliphatic) and aromatic hydrocarbons among the different mixtures of other compounds present in crude oil, fuel oil, diesel and gasoline [83].

Saturated hydrocarbons are more easily and rapidly degradable [84]. Total petroleum hydrocarbons (TPHs) measurements provide an estimation of the amount of the saturated hydrocarbons (e.g. [85]). On the other hand, aromatic hydrocarbons can take longer to be degraded [84]. Polycyclic aromatic hydrocarbons (PAHs) are the most commonly monitored and being 16 of them considered a priority by the United States Environmental Protection Agency [86].

Thus, reports referring to these pollutants related to marinas, ports and coastal waters were searched and are summarized in Table 5. Various studies show results concerning the presence of hydrocarbons mainly in the sediments than in the water column and the levels of contamination can vary from non-contaminated to highly contaminated.

Table 5. Aliphatic hydrocarbons (measured through total petroleum hydrocarbons - TPHs) and polycyclic aromatic hydrocarbons (PAHs) values from water column and sediment at global-scale.

Sampling site (country)	Type of location	Matrix [#]	TPHs	ΣPAH ₁₆	Ref.
Alagoa Bay (South Africa)	Coastal	SSW	0.045-0.307 mg/L	12,780-78,940 ng/L	[87; 88]
		SD	0.72-27.03mg/Kg dw	1.17-10.47 mg/Kg dw	
Barcelona Harbour (Spain)	Port	SD	n.d.	2-4 mg/Kg dw	[81]
Port Fouad and Port Said (Egypt)	Ports	SD	1.86-32.25 mg/Kg dw*	n.d.	[89]
Khark Island (Iran)	Coastal	SSW	<d.l.-0.331 mg/L	79.48-16,657.9 ng/L	[90]
		SD	80-618 mg/Kg dw	0.003-0.253 mg/Kg dw	
Mar del Plata Port (Argentina)	Port	SD	n.d.	0.180-17.094 mg/Kg dw	[91]
Marina of Portoroz (Slovenia)	Marina	SD	n.d.	1,173 mg/Kg dw	[80]
Marinas of the Apulia region (Italy)	4 Marinas	SD	n.d.	0.1-21.5 mg/Kg dw	[78]
Northern Portuguese Coast (Portugal)	Coastal	SD	<d.l.	0.0021-0.0053 mg/Kg dw	[92]
Porto Atlantic Coast (Portugal)	Coastal	SSW	n.d.	48.5-60.3 ng/L	[93]
		SD	n.d.	25.2-106.1 mg/Kg dw	
Port of Koper (Slovenia)	Port	SD	n.d.	0.496 mg/Kg dw	[80]
Sichang Island - Sriracha (Thailand)	Coastal	SSW	0.00001-0.013 mg/L*	n.d.	[94]
		SD	1.1-153.4 mg/Kg dw*	n.d.	
Sultanate of Oman (Western Asia)	Harbours, marinas, terminals and ports	SD	<d.l.-21 mg/Kg dw	n.d.	[95]
Tema Harbour (Ghana)	Port	SD	n.d.	2.75-5.13 mg/Kg dw	[96]
Tianjin Bohai Bay (China)	Coastal	SSW	0.024-0.508 mg/L	n.d.	[97]
Ushuaia Bay (Argentina)	Various types of ports	SD	n.d.	<d.l.-0.36 mg/Kg dw	[98]

[#]SD: Sediment; SSW: Surface seawater; d.l.: detection limit; n.d.: not determined; dw: dry weight; *include other hydrocarbons than aliphatic

1.3. Aim and outline of this thesis

The present project aimed to investigate the application of a FWI to promote water quality enhancement in a high salinity environment, a seawater marina near a commercial seaport, as well as to assess the biotic communities that could establish in the FWI to support a strategy for biodiversity promotion.

The development of these types of solutions is aligned with the objectives of the United Nations' Agenda of 2030, goal nº 14 – “conserve and sustainably use the oceans, seas and marine resources for sustainable development”.

The specific aims of this thesis were the following:

- (1) Characterize the water body of the seawater port marina under study in terms of physicochemical parameters, such as nutrients and hydrocarbons contents, and water microbiome;
- (2) Evaluate the performance of an *in situ* FWI applied to a seawater port marina in terms of resistance to the environmental conditions, vegetation development and biotic (micro and macro) communities' establishment;

(3) Test, at lab-scale, the potential of the more FWI resistant plants for the removal of water hydrocarbons.

This thesis is structured in 4 chapters: a general introduction (Chapter 1) presenting the FWI technology and discussing the environmental issues related to seawater marina environments; materials and methods (Chapter 2) describing the FWI implementation and the lab-scale experiment setup as well as the analytical methodologies; obtained results and their discussion (Chapter 3); and, finally, the main conclusions and future directions (Chapter 4).

CHAPTER 2

MATERIALS AND METHODS

2.1. *In situ* FWI applied to a seawater port marina

2.1.1. Study area

The FWI was implemented on February 22, 2018 and the experiment had a duration of 16 months (485 days), ending on June 22, 2019. Based on the record with an OH503 logger (Greutor), at each monthly sampling campaign, the air temperatures and relative humidity ranged from 13.1 to 20.7 °C and 26 to 71%, respectively. The FWI was set up *in situ*, and was located next to the pier of the Marina of the Porto Cruise Terminal in Matosinhos (Portugal) (41°10'41.13" N; 8°42'13.99" W). This recent marina is situated in the south jetty, between the Porto Cruise Terminal and the outer pontoon of the Port of Leixões (Fig.2 A) and has a surface water area of 2 ha (Fig.2 B). As it is possible to see on the map (Fig.2 A), the marina is positioned at the port entrance, being influenced by the traffic of cargo ships, fishing boats and cruise ships that pass by to the respective section in the port. This marina is working in complementarity with an existing one in the north jetty, in Leça da Palmeira and it has been mainly frequented by recreational boats during the experiment (Fig.2 C). Besides that, there is also the influence of tides and the river mouth (Leça river) that flows through the Port and sometimes brings detritus as seen in Fig.2 D.

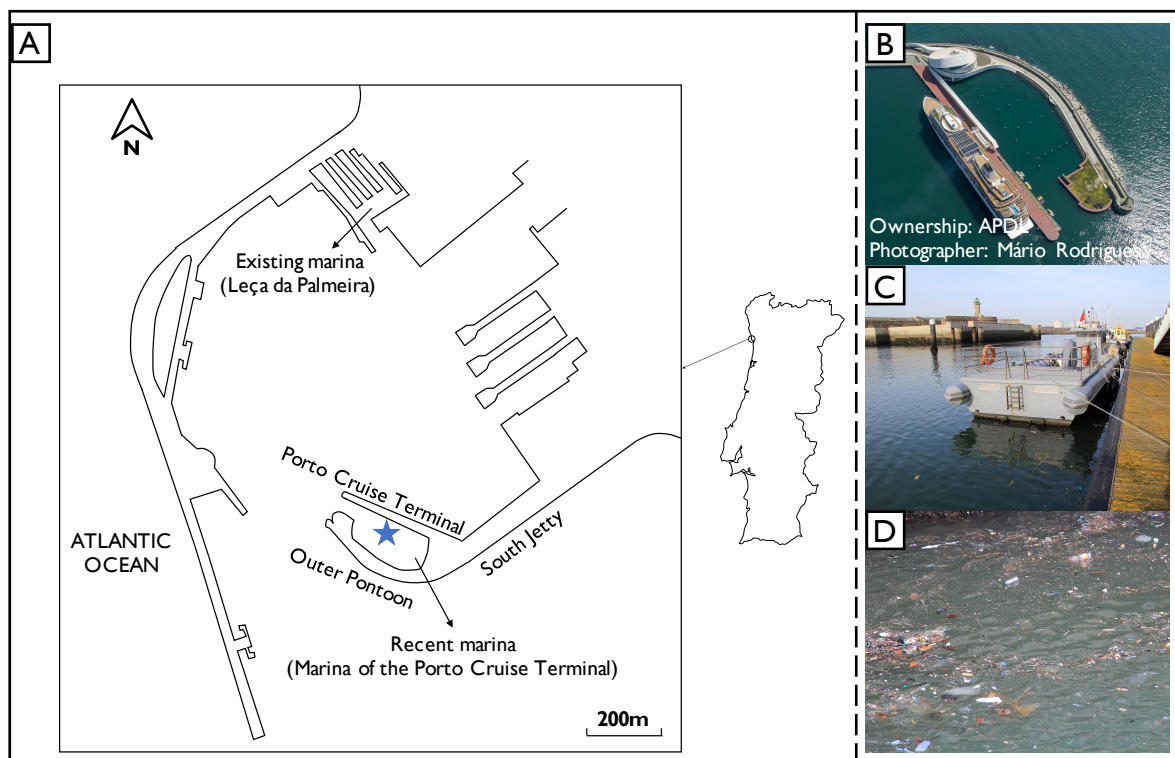


Figure 2. Study area: **A** – Map of Port of Leixões (the blue star represents the FWI); **B** – Aerial photo of the Marina of the Porto Cruise Terminal; **C** – Recreational boats present in the marina under study; **D** – Visually polluted water body in the marina.

During the experimental time, 91 cruise ships arrived at the Porto Cruise Terminal (according to APDL - Port Authority of Douro, Leixões and Viana do Castelo).

2.1.2. FWI system design and maintenance

The FWI system was constituted by a set of 3 modules which were interconnected (Fig.3 A), with 39 pre-cut holes, being 37 available for plants. Each module (Cork Floating Island[®], Bluemater, S.A., Porto, Portugal) meet the following technical characteristics: made of cork agglomerate (density of 0.2); with frustoconical holes of two different sizes (8 cm and 16 cm diameter for the small and the larger types, respectively); a size of 100 x 50 x 4-10 cm (*l* x *w* x *h*); capacity to support until 16 Kg/m² of plants and up to 24 plants/m², according to the manufacturer [99].

The FWI had two individual cables (Fig.3 A), serving as an anchoring system to moor the floating platform in the same place during the experiment time. Each cable had a weight attached at the end, passed through a hole located in a corner of each side of the floating platform and tied to a metal structure on the top of the wall (allowing the FWI to accompany the tidal level fluctuations). Adjustable plastic clamps were used to fortify the joint between the modules and between the holes and the vases (Fig.3 B).

The plant species were collected from selected sites (as described in section 2.1.3.) and were placed in a planting media of coconut fibre vases, fixed with rock wool (Fig.3 C) and watered with seawater during 24 hours. The planted vases were inserted into the holes of the floating platform with a completely random distribution of the plant species. In the implementation, each hole held the same planting density (eight seedlings of the same size for each species tested). Later, three more plantations were made.

The holes available for planting were enumerated from positions 1 to 37, as demonstrated in Figure 3. D, to help in the plant monitoring over time.

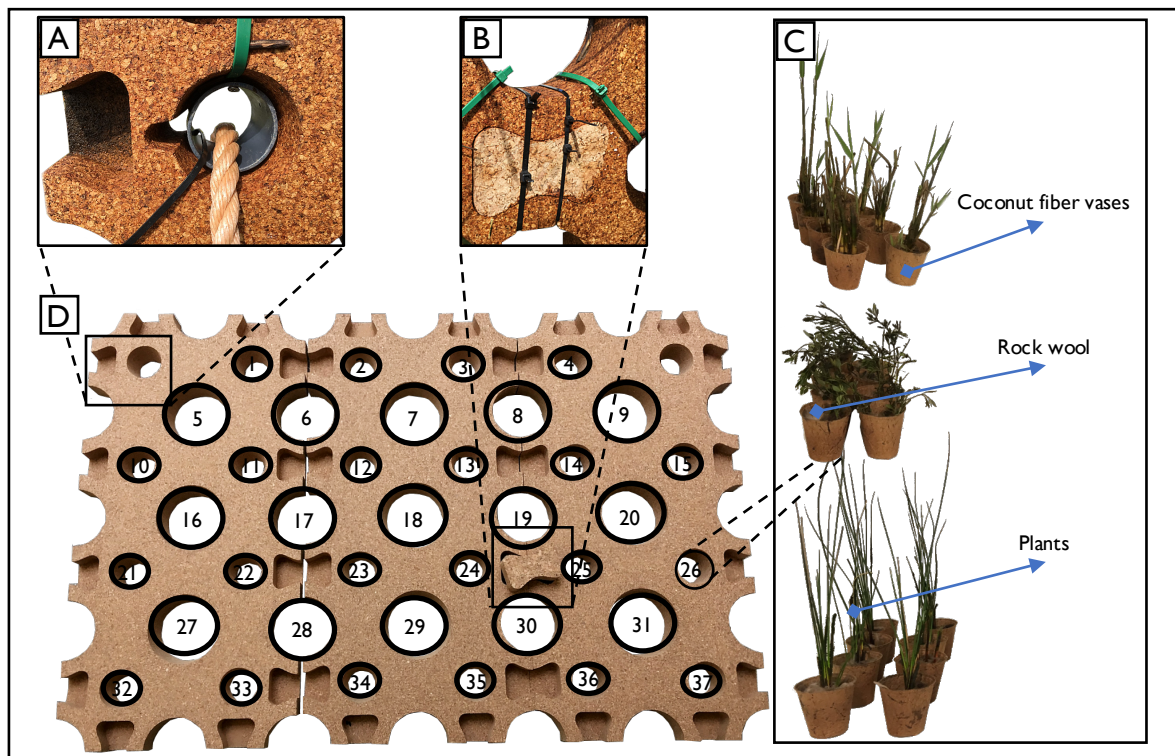


Figure 3. FWI system design: **A** – Cable responsible for the anchoring system; **B** – Joints between modules; **C** – Plants and materials used; **D** – Floating platform modules enumerated from 1 to 37 available holes.

In terms of maintenance, the intention was to minimize human intervention, causing minimum interference. Therefore, it was made a small cleaning of the detritus, mainly plastics, accumulated over the platform and small adjustments when it was extremely necessary.

2.1.3. Plant selection

For the process of plant selection, the following criteria, adapted from a previous approach described in Wang and Sample [59] were used (ordered by decreased relevance): (1) native to Portugal; (2) potential to survive in hydroponic mode with salinity tolerance levels; (3) preferentially perennial species, (4) favourably with aerenchyma and (5) abundance in the nature. Furthermore, the intention was to test different plant families to maximize the potential of having, in the end, a polyculture FWI system.

Overall, 7 halophyte plants were tested. Halophytes are plants that have adaptations to support salty environments of around 200 mM NaCl concentrations or more [100]. These plants were collected in the natural environment and placed in the FWI after 24 hours, as referred in section 2.1.2. Table 6 shows when plants were placed in the FWI, the species used and their collection location.

Table 6. Origin and diversity of plant species used in this study with the respective plantation month.

Plantation month	Plant species	Origin
February, 2018	<i>Juncus maritimus</i>	Cávado river estuary (Esposende)
	<i>Halimione portulacoides</i>	
	<i>Phragmites australis</i>	Mouth of Onda river (Matosinhos)
March, 2018	<i>Sarcocornia perennis</i>	Ria de Aveiro (Aveiro)
September, 2018	<i>Sarcocornia perennis</i>	
	<i>Spartina versicolor</i>	
	<i>Limonium vulgare</i>	
November, 2018	<i>Spartina maritima</i>	
	<i>Sarcocornia perennis</i>	

2.1.4. Sampling procedure

Sampling survey occurred monthly, in the last days of each month, starting in April and excluding October, November and December 2018 months. In the latter mentioned months, two new plantations were done and, for that, no sampling was undertaken since it was considered as an acclimation period for the plants.

Physicochemical parameters of the surface seawater (temperature, salinity, pH, and conductivity) were measured in the field, at a location of 2-3 m distance from the FWI, using a portable multi-parameter (MU 6100 L pHenomenal[®], VWR). Air temperature and relative humidity were recorded with an OH503 logger (Greutor), and observed weather conditions registered.

Surface seawater samples (considered for this case the first 20 cm below the water surface) were collected to one 250 mL pre-cleaned amber glass Schott for the laboratory measurement of TPHs and PAHs or to falcon tubes for nutrients (nitrogen and phosphorus) as well for chemical oxygen demand (COD) determination. Samples were preserved in a thermic box with ice, during the sampling procedure.

Samples from different matrices were collected to access the microbial communities present in each type of sample: (1) biofilm from the planting media; (2) marine biofouling from the floating platform and (3) surface seawater.

Biofilm from the planting media (mainly rock wool), around plant rhizosphere, was collected with sterilized forceps from each plant species. Marine biofouling developed in the floating platform (non-planted holes and under surface) was collected by scratching the surface with a sterilized bistoury blade. Each sample type was composed by a heterogeneous mix of four subsamples (e.g. for the representation of non-planted holes, one sample was composed of the same amount of biofouling retrieved from four different non-planted holes). A surface seawater sample was additionally collected aseptically for a 5 L plastic bottle. Figure 4 shows, schematically, the sampling procedure.

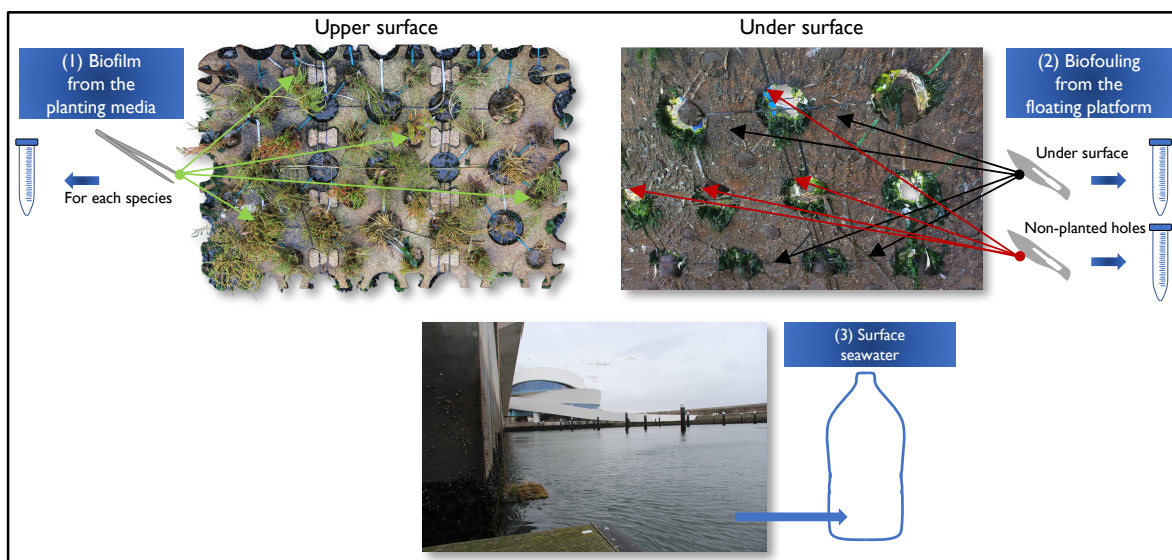


Figure 4. Sampling procedure of biofilm, biofouling and water for posterior molecular biology analyses.

Once in the laboratory, samples of biofilm and biofouling were preserved at $-80\text{ }^{\circ}\text{C}$ for further molecular biology analysis. The 5 L of collected seawater were filtered through a SterivexTM filter with a $0.22\text{ }\mu\text{m}$ pore size (SVG010RS, Merck Millipore) with the assistance of a peristaltic pump and two replicate filters were sealed and stored at $-80\text{ }^{\circ}\text{C}$ for later DNA extraction. Table 7 summarizes the type of samples collected for molecular biology approaches and the given code assigned to each sample.

Table 7. Characterization of the 13 samples collected in this study.

Type of sample	Sampling points	Season*	Code
Marine biofouling from the floating platform	Under surface	Summer	U-S18
		Summer	H-S18
	Non-planted holes	Winter	H-W19
		Spring	H-SP19
Biofilm from the planting media around plant rhizosphere	<i>Sarcocornia perennis</i>	Summer	SA-S18
		Winter	SA-W19
		Spring	SA-SP19
	<i>Phragmites australis</i>	Summer	P-S18
	<i>Spartina maritima</i>	Winter	SP-W19
		Spring	SP-SP19
Seawater	Surface seawater	Summer	W-S18
		Winter	W-W19
		Spring	W-SP19

*Summer = June of 2018; Winter = January of 2019; Spring = April of 2019

Samples for later TPHs, PAHs, and COD analyses were preserved at $-20\text{ }^{\circ}\text{C}$. For nutrient determinations samples were filtrated through a WhatmanTM glass microfiber filter with a $1.2\text{ }\mu\text{m}$ pore size (GE Healthcare Life Sciences) and stored also at $-20\text{ }^{\circ}\text{C}$ until further analysis.

To access the FWI, initially, the entire system was pulled up from the water to the pier but, after 11 months from the implementation, it was accessed directly with the system placed in the water due to the increased weight acquired that enabled this procedure.

During the sampling campaign, evaluation of the apparent health status of plants was performed by visual observation and posterior registration of mortality or survival of each plant species per hole. The survival rate at each plantation period (by plant species) was calculated as:

$$\text{Survival rate (\%)} = \frac{\text{N}^\circ \text{ of holes with survival plants}}{\text{N}^\circ \text{ of total planted holes}} \times 100$$

It was considered that a plant died when the whole aerial parts turned brown or the whole plant started to slowly decompose after two months of persistent status.

Additionally, visual observations looking for macro-biota present in the FWI system were also performed and registered. Photos were taken in each month to register detailed and general aspects.

2.2. Evaluation of hydrocarbon degrading potential by *Sarcocornia perennis*

2.2.1. Microcosm setup

A microcosm experiment was carried out to evaluate the hydrocarbon degrading potential of the plant species that better adapted in the pilot FWI implemented in the seawater port marina, i.e. *Sarcocornia perennis*. The experiment was set up to simulate an acute water contamination due to a boat diesel spill, i.e. a “worst-case scenario”, with a high concentration of diesel. This experiment was set up in a greenhouse during four weeks at the Portuguese Institute for Sea and Atmosphere (IPMA) facilities, in the following conditions: temperature was kept between 16 and 24 °C (mean 21 ± 2 °C) and the mean intensity (lux) recorded was 2456 ± 270 lux with a HOBO® Pendant® temperature/light data logger (MX2202, Onset Computer Corporation). A Solux® metal halide lamp (400 W, 35000 lumens, 4500 K) was installed to ensure adequate intensity light during the experience (to not being a limiting factor for the plants), coupled to a light reflector and a programmed timer (under a 16 h light / 8 h dark photoperiod).

Eight polypropylene plastic tanks (19 x 26 x 15 cm, *l* x *w* x *h*) were placed in a clear plastic greenhouse. Six equally spaced holes with diameters of 3 cm were drilled on extruded polystyrene floating platforms (18 x 25 x 3 cm, *l* x *w* x *h*). Plants were collected in the natural environment, in a salt marsh (Ria de Aveiro, Aveiro, Portugal). In the vegetated floating platforms, six seedlings with similar size were fitted into the holes. Before placing

the plants, roots were washed with tap water to remove soil matter.

Doped and non-doped seawater treatments were carried as shown in Figure 5 and Table 8. Four treatments were considered (each in triplicate, except the non-doped one) and followed a completely randomized distribution of the tanks. The doped treatment was composed of a solution of seawater with 2.5% of diesel. This percentage was based on previous work described in Ribeiro *et al.* [101]. To later measure the initial TPHs concentration, a sample of this solution was collected.

The diesel used in the tanks was a coloured diesel used for recreational boats and has the same properties of a normal or road diesel, being the main difference in the green colour (hence the colour designation). This diesel has a tracer additive to detect its use even if it has been discoloured.

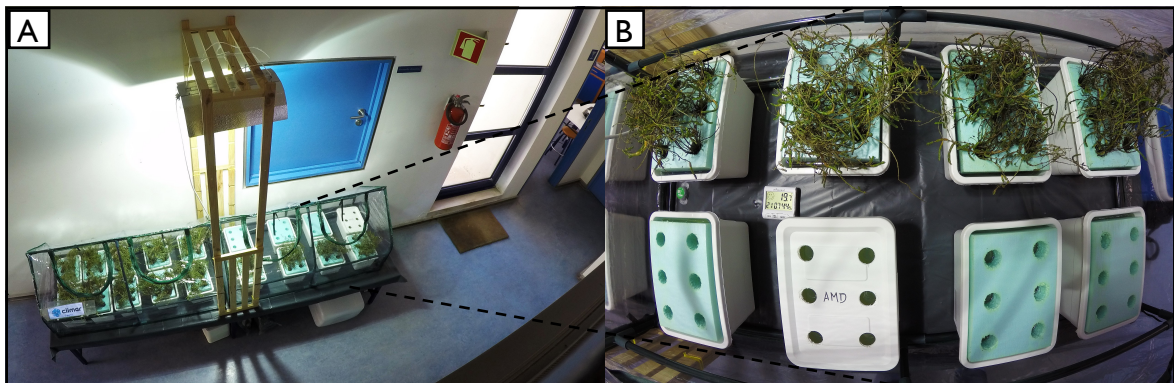


Figure 5. Microcosm setup: **A** – General view of the experiment installation; **B** – Close-up of the four treatments arranged inside the greenhouse.

Tanks were fulfilled with seawater collected at the same point of section 2.1.1. (Marina of the Porto Cruise Terminal). In the case of the diesel doped treatments, 3.9 L of seawater was mixed with 100 mL of coloured diesel fuel (2.5% diesel). For the other non-diesel doped treatments, a total of 4.0 L of seawater was used.

Table 8. Summary of the four treatments used.

Treatment	Description	Replicates (tanks)
DP	Floating platform + Diesel doped seawater marina + Plants	3
DWP	Floating platform + Diesel doped seawater marina	3
HD	Cover with same hole configuration as in the floating platforms + Diesel doped seawater marina	1
SP	Floating platform + Non-diesel doped seawater marina + Plants	1

Every two days, tanks were disposed of in a different random design to secure the same abiotic conditions for each tank during the experiment. To compensate water evaporation, deionized water was added up to a pre-defined mark, when necessary. Two batches were considered: for each batch: *w0* – initial time, *w1* – first week, *w2* - second week. For that,

the water solutions were exchanged one time at the end of the first two weeks (*w2*), with new seawater, diesel doped or non-diesel doped, being added to the tanks.

2.2.2. Sampling procedure

Sampling occurred weekly at 10:00 a.m. In each week, water from *SP* treatment was collected for nutrients and COD analyses. Physicochemical parameters (temperature, salinity, pH, and conductivity) were also measured in this tank with a portable multi-parameter (MU 6100 L pHenomenal[®], VWR). Then, water from the other treatment tanks (*HD*; *DP* and *DWP*) was collected (after homogenisation with a glass pipette through circular movements) from the middle of each tank at the same depth of the water column (having the scale of the pipette as reference) for further TPHs analysis. Samples were preserved in a thermic box with ice, during the sampling procedure.

2.3. Analytical techniques

2.3.1. Chemical analyses

2.3.1.1. Chemical oxygen demand

Chemical oxygen demand was determined after a previous dilution of the samples in deionized water (1:15) and using reagent vials (0 – 150 mg/L range, HI 93754A-25, Hanna Instruments). The vials were heated for 2 h at 150 °C in a COD reactor (HI 839800, Hanna Instruments) accordingly to the manufacturer's instructions. After complete digestion and posterior cooling, samples were measured in a multiparameter bench photometer for wastewater treatment application (HI 83214, Hanna Instruments).

2.3.1.2. Nutrients

For the quantification of nutrients, four dissolved inorganic compounds were measured in the water samples: phosphate ion (PO_4^{3-}) and ammonium ion (NH_4^+) using the Koroleff method [102]; nitrate ion (NO_3^-) using the method described by Jones [103] and adapted by Joye & Chambers [104]; and nitrite ion (NO_2^-) using the method described in Grasshoff *et al.* [102].

Briefly, for phosphate ion, it was used the molybdenum blue method. The blue complex measured results from the presence of ascorbic acid that induces the reaction of

orthophosphate with the excess of ammonium molybdate.

In terms of ammonium ion, the method consists in quantifying the intense blue colour of the complex resulting from the reaction of the monochloramine compound with phenol, catalyst nitroprusside and the excess of hypochlorite (after an incubation of about 6 hours).

For nitrate ion, the method consists of reducing the nitrate ion to nitrite ion and then using the same method for measuring nitrite ion. The reduction results from the constant stirring of the sample with a solution of ammonium chloride and an amount of spongy cadmium.

Lastly, for nitrite ion, the method quantifies the intensity of the final pink complex (that is proportional to the amount of nitrite ion in the sample). This complex is formed by the reaction of the sulfanilamide with the nitrite ion and the product formed reacts with the N-(1-naphthyl)ethylenediamine.

Analyses were performed in triplicates for each water sample and using a V-1200 spectrophotometer (VWR) to measure the absorbance related to the colour of each solution.

2.3.1.3. Total petroleum hydrocarbons

For the measurement of TPHs in water, a previously optimized method was used, as described in Almeida *et al.* [85]. This method provides an estimation of the aliphatic hydrocarbons amount.

Briefly, frozen samples were left in the fridge to gradually unfreeze, overnight. The day after, three replicates were prepared per water sample. Each replicate consisted of mixing 5 or 10 mL of the sample with 10 mL of tetrachloroethylene (>99%, Sigma), followed by extraction of 15 min in an ultrasonic bath (Transsonic 460/H, Elma). After 10 min rest, the organic phase was pipetted to a new glass vial containing 1 g of sodium sulphate (to retain any remaining water from the organic extract) and 0.3 g of activated silica 2% (m/m) (to remove any lipids that might interfere with the analysis). In the case of the samples from the microcosm experience, for each replicate, 1 mL of sample was used.

The mixture was then agitated for 10 min with a mechanical shaker (P-Selecta, Unitronic OR). After that, samples were filtrated through a 1 mL pipette tip filled with 0.15 g glass wool to newly cleaned glass vials.

For the samples from the microcosm experience, a dilution (1:10) of the extracts in

tetrachloroethylene (>99%, Sigma) was made.

Lastly, the quantification of TPHs in sample extracts was performed by Fourier-transform infrared spectroscopy (FT/IR, Jasco FT/IR-460 Plus) using a 1 cm quartz cell. The external calibration was prepared with standard solutions as described in Almeida *et al.* [85].

2.3.1.4. Polycyclic aromatic hydrocarbons

For the measurement of PAHs in water, a previously optimized method was used as described in Gonçalves *et al.* [105]. This measurement considers 10 (headspace methodology) or 16 (immersion methodology) of the PAHs considered a priority by the United States Environmental Protection Agency: Naphthalene (Nap), Acenaphthylene (Acy), Acenaphthene (Ace), Fluorene (Flu), Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (Fla), Pyrene (Pyr), Benz[a]anthracene (BaA), Chrysene (Chr), Benz[b]fluoranthene (BbF), Benz[k]fluoranthene (BkF), Benz[a]pyrene (BaP), Benz[g,h,i]perylene (BghiP), Indeno[1.2.3-cd]pyrene (InP) and Dibenzo[a,h]anthracene (DahA).

For the samples of 2018, it was used the immersion methodology, while for the samples of 2019, it was performed the headspace methodology due to operational issues that did not permit to perform all the measurements in immersion methodology.

Briefly, the frozen samples were left in the fridge to gradually unfreeze overnight. The day after, triplicates were prepared per sample, consisting in 10 mL of each sample transferred to amber glass vials and mixed with an internal standard (20 µL of 2 mg/L methanolic solution) for headspace method or 18 mL of each sample mixed with 20 µL of the same internal standard when using the immersion method. Ultimately, to evaluate the efficiency of the method, control samples were doped with PAH standard solutions to assess the recoveries of the spiked samples.

Then, water samples were pre-concentrated by solid-phase microextraction (SPME) followed by gas chromatography-mass spectrometry (GC–MS) analysis: Varian Saturn 2000 MS coupled to a Varian 3900 GC with a CP-Sil 8 CB-MS column (50 m length x 0.32 mm diameter, 0.40 µm film thickness, Agilent); autosampler SPME CombiPal model (CTC Analytics). Hydrocarbons were quantified by external calibration with aqueous PAHs standard solutions, corrected with internal PAHs standards signals as described in Gonçalves *et al.* [105].

2.3.2. Molecular biology analyses

2.3.2.1. DNA extraction, quantification and sequencing

Environmental DNA (eDNA) was extracted from the FWI samples (biofilm from the planting media and biofouling from the floating platform) using the DNeasy Powersoil Kit (Qiagen) according to the manufacturer's instructions. Then, the final amount of two replicates for each sample was combined, followed by a concentration step in a Concentrator plus (Eppendorf) to enhance the extracted DNA quantity.

The eDNA extraction from water samples was performed using the DNeasy PowerWater Sterivex Kit (Qiagen) according to the manufacturer's instructions.

Later, extracted DNA was quantified in QubitTM 3 Fluorometer (ThermoFisher Scientific) using the QubitTM dsDNA HS Assay Kit (Invitrogen) following the manufacturer's instructions.

The eDNA obtained after extraction was used for 16S rRNA gene sequencing and metabarcoding analysis to target prokaryotic communities. The sequencing method includes the amplification of the 16S rRNA gene fragment (hypervariable V4-V5 region; ≈412 bp) using the forward primer 515YF (5'- GTGYCAGCMGCCGCGGTAA-3') and reverse primer Y926R (5'- CCGYCAATTYMTTTRAGTTT-3') [106] and the paired-end sequencing was carried out by an Illumina MiSeq[®] platform at Genoinseq facilities (Cantanhede, Portugal) for analysis.

The first quality steps were performed by the Genoinseq company: raw reads were extracted from Illumina MiSeq[®] System in fastq format and quality-filtered with PRINSEQ version 0.20.4 [107] to remove sequencing adapters, reads with less 100 bases for the 16S gene region and trim bases an average quality lower than Q25 in a window of 5 bases. The forward and reverse reads were merged by overlapping paired-end reads with AdapterRemoval version 2.1.5 [108] using default parameters. Merged reads in fastq format were converted into fasta format by Mothur software (version 1.35.1; <https://www.mothur.org/>) and then uploaded to the SILVAngs analysis pipeline (<https://ngs.arb-silva.de/silvangs/>).

2.3.2.2. Bioinformatics analysis

All sequence reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) [109]. Each read was aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)) [110] against the SILVA SSU rRNA SEED and quality controlled [109]. Reads shorter than 50 aligned nucleotides and reads with more than 2% of homopolymers, or 2% of ambiguities, respectively, were excluded from additional processing. Artefacts, suspected contaminations and, reads with a low alignment quality (40 alignment score reported by SINA, 50 alignment identity), were excluded from downstream analysis after their identification.

After these initial steps of quality control, identical reads were identified in a dereplication phase, the unique reads were clustered (OTUs), on a per sample basis, and the reference read of each OTU was classified. Dereplication and clustering were performed using cd-hit-est (version 3.1.2; <http://www.bioinformatics.org/cd-hit>) [111] running in accurate mode, ignoring overhangs, and with identity criteria of 1.00 and 0.98, respectively. The classification was executed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 132; <http://www.arb-silva.de>) using blastn (version 2.2.30+; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with standard settings [112].

The classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU. Reads without any BLAST reads or hits with weak BLAST hits, where the function “($\% \text{ sequence identity} + \% \text{ alignment coverage}$)/2” did not exceed the value of 93, remain unclassified. The meta group “No Relative” in the SILVAngs fingerprint was formed by these reads.

2.3.2.3. Molecular biology downstream analysis

Abundance tables at different taxonomic levels: higher (phylum) and lower level (genus) have been produced to show the relative abundance of each taxonomic group within each sample (the total number of sequences assigned to each taxonomic path). These tables were used as input to further molecular downstream analyses.

Alpha-diversity (within-sample diversity) and beta-diversity (between-sample diversity) analyses have been also performed, using PAST software (version 3.24; <https://folk.uio.no/ohammer/past/>). Bray–Curtis dissimilarity [113] was calculated with a transformed taxonomic table at the lower level (z-score transformation to reduce the

discrepancy of sequencing depth across samples). The resulting matrix was used to calculate the Shannon diversity index [114] and observed number of genera (ONG). This matrix was also visualized by principal coordinates analysis (PCoA) method to analyse the community structure. Furthermore, a one-way analysis of similarities (ANOSIM) was calculated based on the Bray-Curtis distance measure to determine the statistical different sample groups. Then, based on these results the similarity percentage analysis (SIMPER) was performed to identify the taxa (at the lower level) that most contributed to those sample groups' differences.

2.3.3. Chlorophyll fluorescence measurement

To assess the health status of plants, an *in situ* and no-destructive method was used to measure the chlorophyll fluorescence. After plants were kept in dark conditions, for 25 minutes (covered by an opaque black cloth), a pulse-amplitude modulated (PAM) chlorophyll fluorometry (JUNIOR-PAM, Walz), fitted with a 1.5 mm light guide plastic fibre and a blue led (445 nm) light source, was applied to the plant. The measurements were performed following the manufacturer's instructions, using the standard default settings for rapid light curve (RLC) determination. The basic fluorescence yield (F_o) value was recorded after applying a modulated light that was sufficiently low ($< 0.1 \mu\text{mol}/\text{m}^2\cdot\text{s}$) and the maximal fluorescence yield (F_m) was measured by applying a strong light pulse of $10,000 \mu\text{mol}/\text{m}^2\cdot\text{s}$ for 0.8 s. The parameters attained were automatically calculated by the WinControl-3 software (version 3.29; Walz) to quantify the characteristic parameters: maximum electron transport rate (ETR_m) that reflects the photosynthetic capacity at light saturation conditions [115]; quantum efficiency of photosynthesis (α) that estimates the efficiency of light-harvesting complexes [116] and the F_v/F_m ratio that is defined as the maximum photochemical quantum yield of photosystem II if all the available reaction centres were open (lower values are associated with fewer open reaction centres available) [117].

In the FWI, PAM measurements were performed in 4 different plants of *Sarcocornia perennis* (3 records for the same predefined plant) during July and September of 2018 and February and April of 2019 survey. In the microcosms experiment (section 2.2.1.), 2 PAM measurements in each *DP* treatment replicate and 6 PAM measurements for the *SP* treatment were carried out at different times ($w1$ and $w2$ for each batch). Due to the deteriorated state of the aerial plant tissues, PAM measures became inaccurate and the records were discarded for the final measurements ($w2$ of the second batch). All the measurements were achieved by applying the fibre guide to the bright green parts of the plant.

2.3.4. Statistical analyses

The statistical analysis was performed using PAST software (version 3.24; <https://folk.uio.no/ohammer/past/>) to analyse data at a 95% confidence level and significance between treatments was considered as statistically significant if the *p-value* < 0.05. All the data subjected to statistical analyses were examined with Shapiro–Wilk test to confirm the existence of normal distribution and an evaluation of the homogeneity of variance assumption was performed by applying Levene's test. When data presented normal distribution, a one-way analysis of variance (ANOVA) was performed and when statistically significant differences for ANOVA were found, a Tukey post-hoc test was executed. All data were expressed as mean $\pm$ standard deviation (SD). The alphabet letters labelled over the values represent the statistically significant/non-significant differences between or within treatments.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. *In situ* FWI applied to a seawater port marina

3.1.1. Water characterization

To have an overview characterization of the water body under study (seawater port marina), the physicochemical parameters considered are presented in Table 9. Considering the obtained values for salinity and conductivity, the water of the marina can be classified, as expected, as very highly saline [118]. COD values ranged between 135 and 630 mg/L in the marina water. Although no water quality standards for this type of water body exist, taking into consideration the Portuguese legislation for wastewater discharge (*Decreto-Lei n 236/98 – 1 August*), the COD values were, in general, higher than those referenced (150 mg/L). Nutrient values for the four inorganic compounds measured were higher than the ones found in the coastal areas along the northwest Portuguese coast [92] and lowest than those reported by a study from coastal areas of the same region and relatively close to the present study area [93]. The coastal waters have a high hydrodynamic, therefore, water renewal can lead to lower nutrient levels, unless there is a significant source such as rivers discharge. In the case of the marina, the waters are more confined, with lower hydrodynamic [77], but with the direct influence of the Leça river, because the marina stands on the river mouth. These facts can explain the intermedium nutrient values and high COD values observed in the present study.

Regarding water hydrocarbon levels, TPHs concentrations were often below the detection limit but were also detected at a level of ca. 6 mg/L. No reports of values for TPHs in waters in Europe were found, but the obtained values were much higher (up to 6 mg/L) than the ones found in coastal areas influenced by shipping activities or near oil explorations of South Africa [87], Iran [90], Thailand [94] and China [97]. In these cases, levels detected were up to 0.51 mg/L. These values (although only detected in just three months of 2019) in this marina water were not unexpected since these areas are known to be subject to potential sources of fuel leaks. On the other hand, PAHs were below the detection limit in all the water samples analysed (Table 10). Rocha *et al.* [93] detected PAHs in the water near to the present study area between 48.5 and 60.3 ng/L, indicating their sources were both pyrogenic and petrogenic [93]. A possible explanation for the detection of TPHs and no PAHs, in the present study, can be related to the tendency of the latest for deposition and accumulation on sediment bed [119; 120]. Since marinas are relatively confined areas, as discussed above, they can accumulate more fine sediments with more organic matter which can adsorb these organic pollutants [119]. Finally, it must be considered that this marina has been working at a low intensity. Hence, with more recreational boats navigating, higher hydrocarbons levels can be expected in the future.

Table 9. Water parameters in the surface seawater of the Marina of the Porto Cruise Terminal during the experiment.

Date	Water parameters									
	Temp. (°C)	Cond. (mS/cm)	pH	Sal. (ppt)	PO ₄ ³⁻ (mg/L)	NH ₄ ⁺ (mg/L)	NO ₂ ⁻ (mg/L)	NO ₃ ⁻ (mg/L)	TPHs (mg/L)	COD (mg/L)
2018	Apr.	15.4	47.4	8.03	30.7	0.09	0.14	1.35	<d.l.	135
	May	14.0	49.3	7.86	32.1	0.13	0.19	2.20	<d.l.	210
	June	15.3	47.3	7.93	30.4	0.11	0.07	1.00	<d.l.	270
	July	19.8	44.9	8.25	28.8	0.07	0.06	0.83	<d.l.	150
	Aug.	18.5	46.6	8.21	30.0	0.10	0.05	0.38	<d.l.	210
2019	Sept.	17.2	48.8	7.89	31.6	0.43	0.34	1.27	<d.l.	375
	Jan.	13.8	51.8	8.37	33.5	0.12	<d.l.	1.31	6	630
	Feb.	13.1	51.7	8.33	33.3	0.11	0.16	0.93	5	225
	Mar.	13.5	54.2	8.09	35.1	0.13	0.04	1.36	<d.l.	435
	Apr.	14.1	44.3	8.10	28.1	0.17	0.59	3.91	<d.l.	360
	May	15.2	49.7	7.97	32.0	0.31	0.68	3.03	<d.l.	330
	June	16.3	53.2	8.05	34.6	0.15	0.30	1.13	4	375

d.l. detection limit; NH₄⁺ = 0.01 mg/L; TPHs = 2 mg/L

Table 10. Polycyclic aromatic hydrocarbons (PAHs) in the surface seawater of the Marina of the Porto Cruise Terminal during the experiment.

Date		PAH ₁₆ (ng/L)															
		Low molecular weight PAHs (LPAHs)								High molecular weight PAHs (HPAHs)							
		Nap	Acy	Ace	Flu	Phe	Ant	Fla	Pyr	BaA	Chr	BbF	BkF	BaP	BghiP	InP	DahA
2018 ^a	May	<5.1**	<8.6*	<4.4*	<5.0*	<4.3*	<3.4*	<10.0*	<10.0*	<10.0*	<7.5*	<49.7**	<49.7**	<15.1*	<32.9*	<50.6*	<43.8*
	June	<5.1**	<8.6*	<4.4*	<5.0*	<4.3*	<3.4*	<10.0*	<10.0*	<10.0*	<7.5*	<49.7**	<49.7**	<15.1*	<32.9*	<50.6*	<43.8*
	July	<5.1**	<8.6*	<4.4*	<5.0*	<4.3*	<3.4*	<10.0*	<10.0*	<10.0*	<7.5*	<49.7**	<49.7**	<15.1*	<32.9*	<50.6*	<43.8*
	Aug.	<5.1**	<8.6*	<4.4*	<5.0*	<4.3*	<3.4*	<10.0*	<10.0*	<10.0*	<7.5*	<49.7**	<49.7**	<15.1*	<32.9*	<50.6*	<43.8*
	Sept.	<5.1**	<8.6*	<4.4*	<5.0*	<4.3*	<3.4*	<10.0*	<10.0*	<10.0*	<7.5*	<49.7**	<49.7**	<15.1*	<32.9*	<50.6*	<43.8*
2019 ^b	Jan.	<1.6*	<30.0*	<10.0*	<7.6*	<6.0*	<7.5*	<15.1*	<10.0*	Not determined							
	Feb.	<1.6*	<30.0*	<10.0*	<7.6*	<6.0*	<7.5*	<15.1*	<10.0*								
	Mar.	<1.6*	<30.0*	<10.0*	<7.6*	<6.0*	<7.5*	<15.1*	<10.0*								
	Apr.	<1.6*	<30.0*	<10.0*	<7.6*	<6.0*	<7.5*	<15.1*	<10.0*								
	May	<1.6*	<30.0*	<10.0*	<7.6*	<6.0*	<7.5*	<15.1*	<10.0*								
	June	<1.6*	<30.0*	<10.0*	<7.6*	<6.0*	<7.5*	<15.1*	<10.0*								

Nap: Naphthalene; Acy: Acenaphthylene; Ace: Acenaphthene; Flu: Fluorene; Phe: Phenanthrene; Ant: Anthracene; Fla: Fluoranthene; Pyr: Pyrene; BaA: Benz[a]anthracene; Chr: Chrysene; BbF: Benz[b]fluoranthene; BkF: Benz[k]fluoranthene; BaP: Benz[a]pyrene; BghiP: Benz[ghi]perylene; InP: Indeno[1,2,3-cd]pyrene; DahA: Dibenzo[a,h]anthracene

*detection limit; **quantification limit; ^aMeasured by immersion method; ^bMeasured by headspace method

3.1.2. Evaluation of the FWI performance

In terms of structural integrity, facing the dynamic and challenging environment of the seawater port marina, the system resisted various storms, tides, currents and other environmental deteriorating characteristics such as the salinity. In February of 2019, during the manipulation of the cables, one of the corners broken accidentally and, in general, due to the impact of the floating platform against the marina pier, the other corners slowly suffered a notable impact. Over time, the macro-biota, which is further discussed in section 3.1.4., was fixing to the floating platform (sides and under surface). Consequently, this constituted a problem related to the accumulated weight that might eventually compromise the island's buoyancy, in the future. In a similar application, the same issue related to biofouling in an FWI was manifested by Mccarty *et al.* [73]. Likewise, the under surface of the floating platform, at a certain point, was full-covered (including holes) making it impossible to roots pass through. However, plant development did not seem to be affected since the planting media remained wet and they continued to develop.

3.1.3. Evaluation of plant establishment and development

One of the preliminary objectives was to evaluate which plant species would survive and if they would show visual growth under the tested conditions. Appendix I shows a compilation of monthly photos seen from above of the FWI, showing visually how the plants covered the floating platform over time.

From the tested plants, only two species survived: *Spartina maritima* and *Sarcocornia perennis* (Figure 6).

In this study, *Spartina maritima* exhibited resilience (67% of survival), especially when Spring months arrived and the plants started to recover, displaying emerging green shoots. Although, the optimal growth for this species seems to occur at 20% of seawater [121].

Overall, *Sarcocornia perennis* demonstrated the greatest potential among the other six plant species tested, presenting survival rates of 80-88%, depending on the plantation date. This marsh plant evidenced visual growth in such a way that it began to spread to other holes of the FWI, particularly in the last month of the experience (June of 2019) as it is possible to confirm in Appendix I photos. This propagation tendency is characteristic from this perennial succulent species, in which the subshrubs tend to merge, forming

agglomerates and forming mats of at least 1 m diameter [122]. Additionally, showing accordance with the obtained results, Sanicola *et al.* [64] reported a plant from the same genus (*Sarcocornia quinqueflora*) as the responsible for the better root biomass growth in saltwater (rather on freshwater) among the other four wetland plant species tested in their FWI mesocosm studies. Moreover, Calheiros *et al.* [123] used *Sarcocornia fruticosa* for highly saline wastewater treatment and the species also demonstrated high resilience.

Other plant species decayed rapidly, remaining no specimens alive after November of 2018. Thus, the two resistant and promising plant species to this environment, *Spartina maritima* and *Sarcocornia perennis*, seem to be suitable species for future FWI applications in highly saline water bodies, obtaining a polyculture.

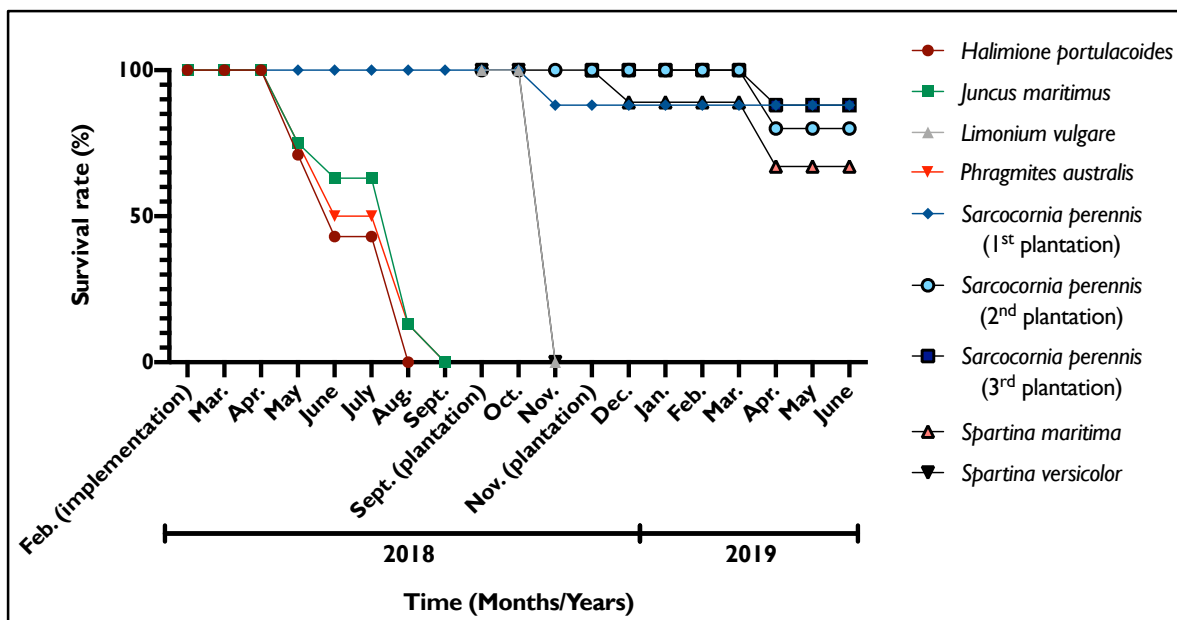


Figure 6. Survival rate (%) for each of the 7 plant species tested in the FWI during the experimental time (16 months).

Furthermore, PAM measurements were performed in *Sarcocornia perennis* (Figure 7). Results showed the highest and significantly different ETRm value in April of 2019 (presenting high values for the other two parameters registered as well). On the other hand, February of 2019 registered the lowest values for all the three parameters tested, including significantly different Fv/Fm ratio and quantum efficiency of photosynthesis. These results suggested a better photosynthetic performance in the months related to the higher temperature and a higher daily number of light hours (Summer and Spring), considering that in the first PAM measurement (5 months after the FWI implementation), plants had already adapted to the environmental conditions. Thus, these results indicated the absence of major concerns, considering the photosynthetic performance. Nevertheless, no measurements were found, in the literature, for similar environmental conditions, to establish a direct comparison.

For the other plant species, PAM measurements were not performed because early *S. perennis* showed the most promising results (survival rate) and so it was the focus.

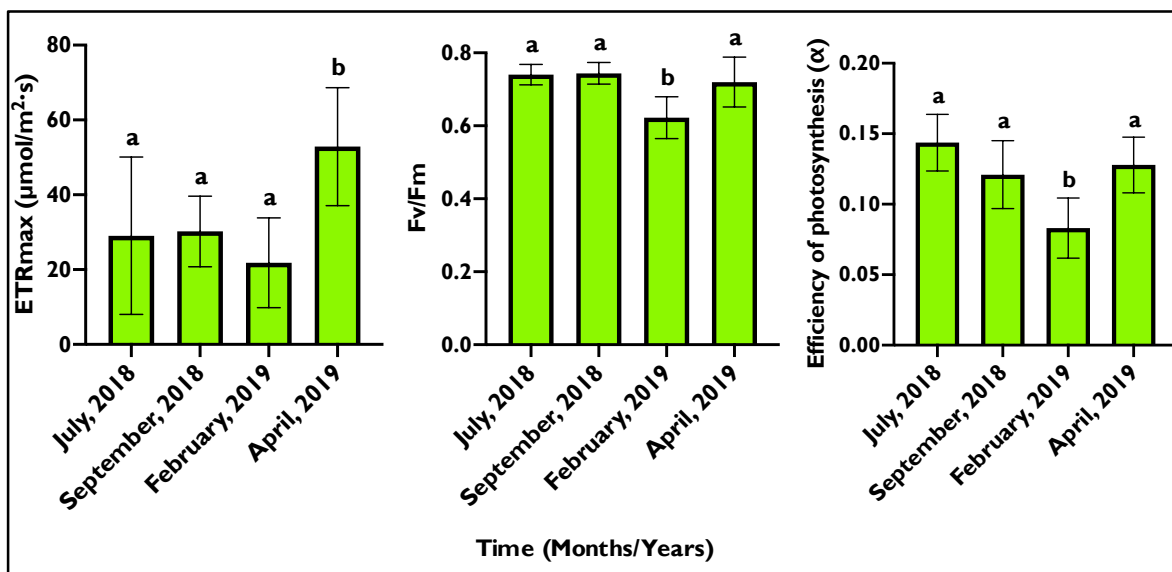


Figure 7. Maximum electron transport rate (ETR_m); the Fv/Fm ratio and the quantum efficiency of photosynthesis (α) registered for *Sarcocornia perennis* (mean $\pm$ SD, $n = 4$) for 4 different months of 2018 and 2019. The different letters placed on the bars show the statistically significant differences ($p < 0.05$) between months.

3.1.4. Assessment of microbial diversity in the FWI and water

A total of 764,759 of 16S rRNA gene sequences were obtained by Illumina MiSeq sequencing for all the samples processed by SILVAngs analysis pipeline, which decreased to a total of 606,549 (79.31%) after quality filtering. From those, 593,753 (77.64%) were classified and 12,796 (1.67%) were not classified. Rarefaction curves (Appendix II) indicated that microbial diversity was not fully covered from the sequencing depth in the samples since a plateau phase was not achieved.

The analysis of alpha-diversity indices across all the samples (Fig.8 A), detected a pattern within each type of samples in relation with the observed number of genera (ONG), with an increase from the Summer of 2018 to the Winter of 2019, followed by a decrease in the Spring of the same year but with values still higher than the Summer of the previous year, showing a possible seasonality. The lower value of ONG in Summer can be related to more intense maritime traffic detected in the present study area. A previous study by Nogales *et al.* [124] also mentioned shifts in the bacterial composition related to the impact caused by the activities of recreational coastal areas. However, other factors must be considered such as the natural colonization process, and the possible seasonality referred.

The Shannon diversity index reflects the diversity within each sample, considering the

observed number of taxa and their abundance. This index remained stable over time for each type of sample. However, the highest values were detected for the biofilm from the planting media of *Sarcocornia perennis*, presenting slightly differences in comparison to the other plant species. Oliveira *et al.* [125] also reported a higher Shannon index in bacterial communities of *Sarcocornia perennis* (subsp. *perennis*) rhizosphere comparing to *Halimione portucaloides* rhizosphere in sediments of their natural environment (Ria de Aveiro, Portugal) affected by hydrocarbons. In the present study, a hypothesis for explaining the higher values recorded for *S. perennis* can be related to the best resilience shown (section 3.1.3.) that could influence microbial diversity.

When the same type of samples (biofouling from the floating platform, biofilm from the planting media and water) was grouped (Fig.8 B) and compared, no significant ONG differences were detected. But Shannon diversity varied significantly for the samples of biofilm from the planting media compared to the water samples and, although not significantly different, this index was also higher compared to the samples of biofouling from the floating platform.

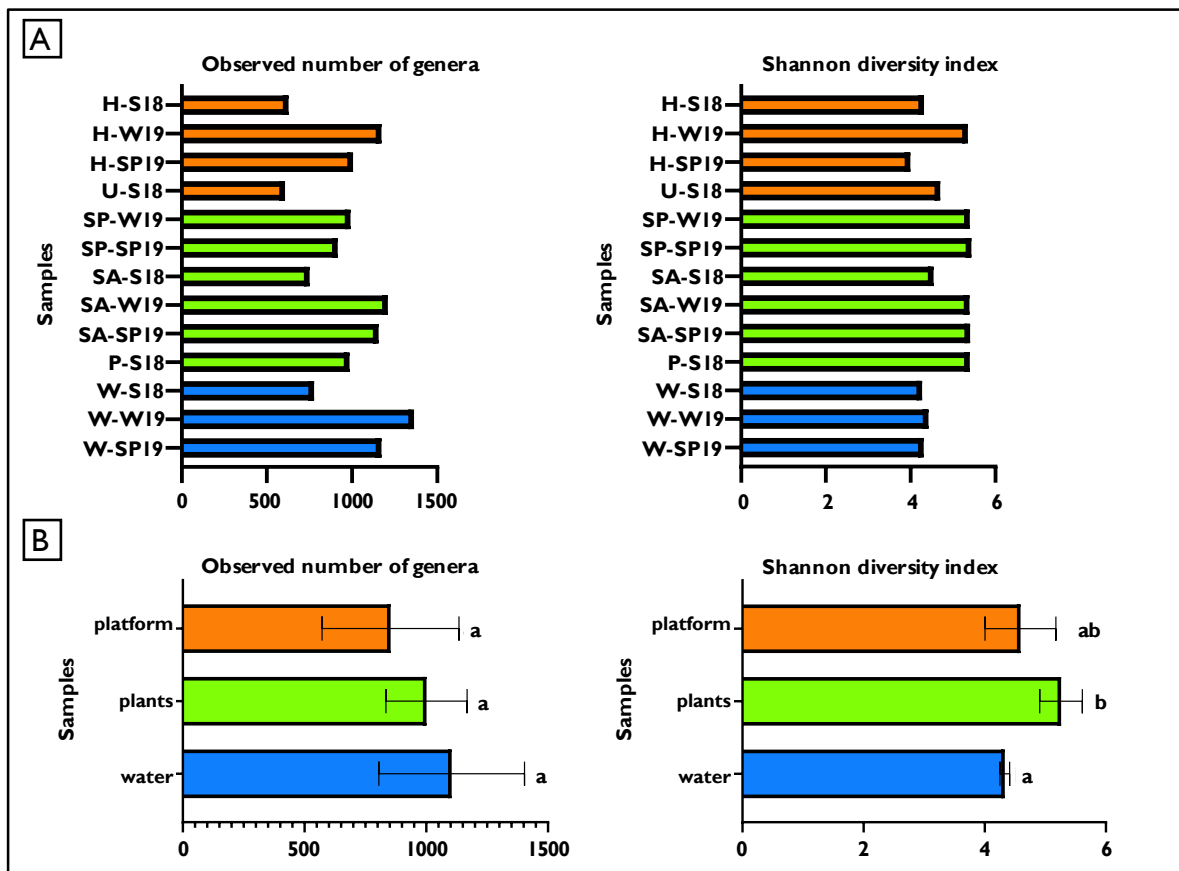


Figure 8. Alpha-diversity metrics (Shannon diversity index and Richness - Observed number of genera): **A** – Across all the individual samples; **B** – Comparison between type of samples. Orange bars represent the biofouling samples from the floating platform (*H* = non-planted holes and *U* = Under surface), green bars represent the biofilm samples from the planting media (*SP* = *Spartina maritima*; *SA* = *Sarcocornia perennis* and *P* = *Phragmites australis*) and blue bars represent the water samples (*W*); -*S18* = Summer of 2018; -*W19* = Winter of 2019 and -*SP19* = Spring of 2019. The different letters placed on the bars show the statistically significant differences ($p < 0.05$) between the type of samples.

At a higher level, Figure 9 displays the top 10 phyla found in the analysed samples. A total of 74 microbial phyla were detected across all the samples, being Bacteria the principal domain.

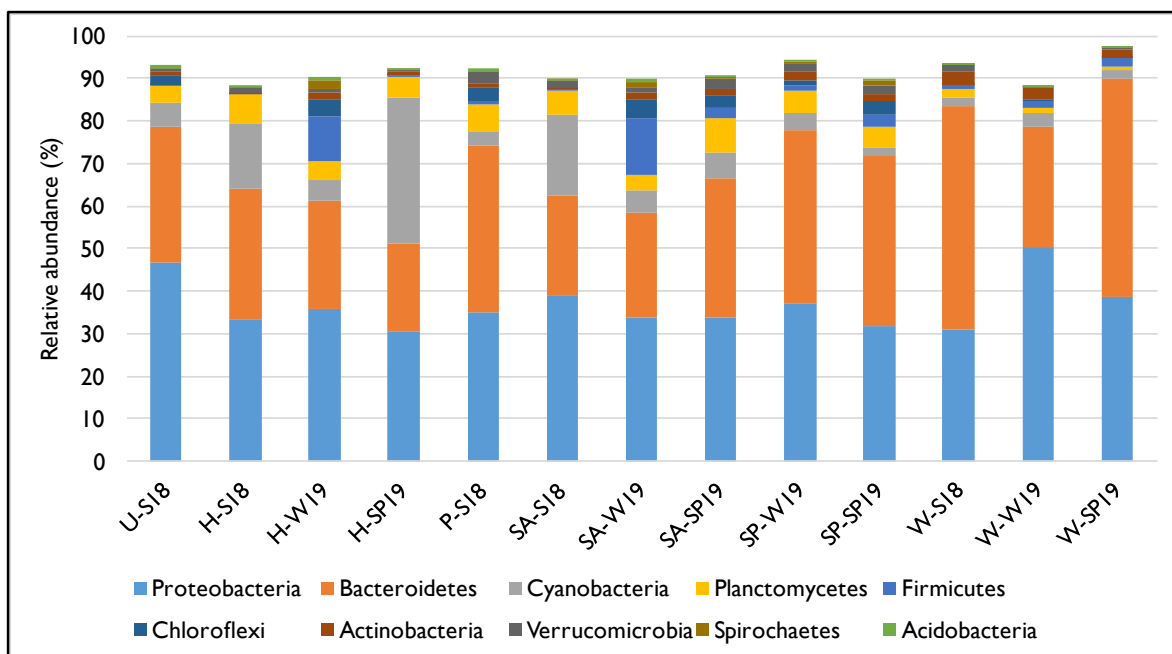


Figure 9. Taxonomic profile of top 10 prokaryotic taxa at higher level (phylum) across all samples. Top 10 represents 91% of the total sequences dataset. Codes: biofouling samples from the floating platform (*H* = non-planted holes and *U* = Under surface); biofilm samples from the planting media (*SP* = *Spartina maritima*; *SA* = *Sarcocornia perennis* and *P* = *Phragmites australis*) and water samples (*W*); -*S18* = Summer of 2018; -*W19* = Winter of 2019 and -*SP19* = Spring of 2019.

Regarding the average relative abundance of the main phyla for each type of samples (biofouling from the floating platform, biofilm from the planting media and water), it is displayed in Figure 10.

Through the interpretation of Fig. 10, it was observed, in general, a predominance of Proteobacteria and Bacteroidetes (especially in the water samples that accounted for around 40-44% of relative abundance, respectively). The phyla Actinobacteria and Acidobacteria were also found in higher relative abundance in the water samples (≈ 2.7 - 0.1% , respectively).

Similar percentages of the most abundant phyla detected in the present marina's water have been reported also in different studies focused on microbial diversity in harbour waters [75].

On the other hand, the biofouling samples from the floating platform and the biofilm samples from the planting media showed more variability in their taxonomic composition compared with water samples. Overall, they presented a higher relative abundance of the

phyla Cyanobacteria, Planctomycetes, Firmicutes, Choloroflexi, Verrumicrobia (particularly in the biofilm from the planting media) and Spirochaetes.

Furthermore, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Planctomycetes, Proteobacteria, and Verrucomicrobia are described as minor phyla in the initial stages of marine biofilm formation [126]. In the present study, there were analysed the microbial communities in an advanced stage of marine biofilm development. Thus, these phyla were contrarily within the top 10 of the more abundant phyla in the marine biofouling from the floating platform.

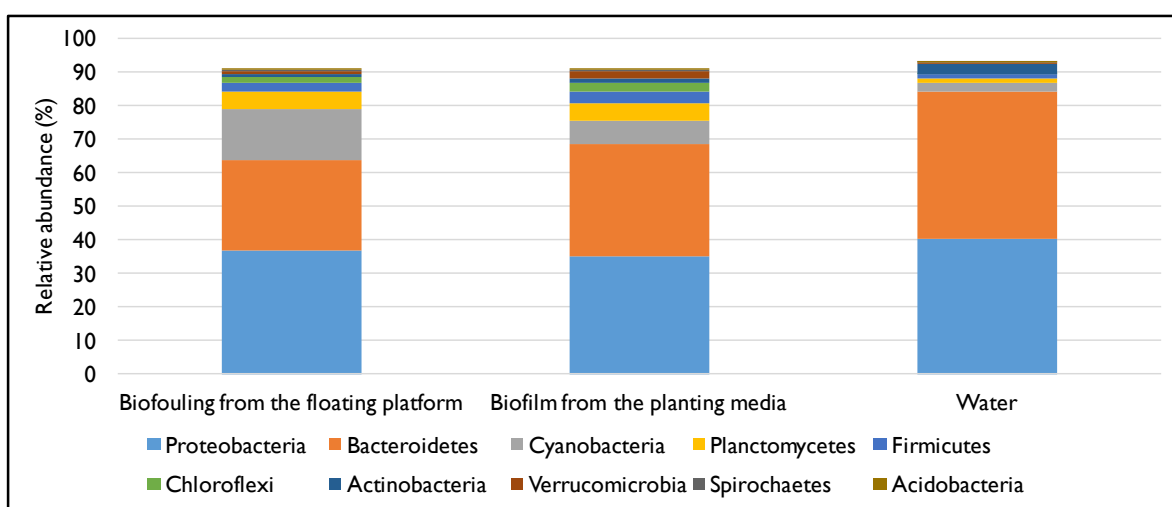


Figure 10. Taxonomic profile of top 10 prokaryotic taxa at higher level (phylum) in each type of samples (biofouling from the floating platform, biofilm from the planting media and water). Top 10 represents 91% of the total sequences dataset.

At a lower level, Figure 11 shows the top 20 taxa across the dataset, which includes genera from seven different families: Flavobacteriaceae (*Aurantivirga* sp., uncultured representative, *Maribacter* sp., *Winogradskyella* sp., *Psychroserpens* sp., *Maritimimonas* sp.), Rhodobacteraceae (uncultured representative, *Loktanella* sp., *Sulfitobacter* sp., *Amylibacter* sp., *Lentibacter* sp.), Saprospiraceae (uncultured representative, *Lewinella* sp.) Alteromonadaceae (*Glaciecola* sp.), Halieaceae (*Halioglobus* sp.), Cryomorphaceae (uncultured representative), Gottschalkiaceae (*Gottschalkia* sp.). This top 20 also includes one taxon from the Flavobacteriales order and one from the Gammaproteobacteria class and, lastly, a chloroplast organelle. This organelle was associated with Cyanobacteria phylum (namely Oxyphotobacteria class) and was not removed from the dataset because it compromised a great relative abundance across most of the samples and so could be important for the present discussion.

The latter three groups (Favobacteriales order, Gammaproteobacteria class and chloroplast organelle) did not classify until the genus level because they did not have enough similarity with any reference in the SILVA database to be classified at a lower

taxonomic level.

The taxa found in the present study were compared with other ones in the literature that manifested the potential to degrade hydrocarbons in similar environments. However, Nogales and Bosch [75] report the limitation of comparing environmental sequences obtained, at the genus level, with known hydrocarbon degrading bacteria to deduce the presence of putative hydrocarbon degraders. Indeed, it is known that within the same genus some species do not degrade hydrocarbons [75; 127] or within the same species, different strains have different degradation potential [75; 128]. Among the taxa known to have degradation potential, there is a group called obligate hydrocarbonoclastic bacteria (OHCB) that is well-known due to their specialization to use almost exclusively hydrocarbons as the unique source of nutrition [127; 129]. Specifically, the genera *Alcanivorax*, *Cycloclasticus*, *Neptunomonas*, *Oleibacter*, *Oleiphilus*, *Oleispira*, and *Thalassolituus* [127], all found in the present study.

However, the relative abundance of OHCB genera, within each type of sample (biofouling from the floating platform, biofilm from the planting media and water) detected in the present results was quite low: *Alcanivorax* sp. (≈ 0.08 ; $\approx 0.08\%$; $\approx 0.01\%$), *Cycloclasticus* sp. ($\approx 0.002\%$; not present; $\approx 0.001\%$), *Neptunomonas* sp. ($\approx 0.007\%$; $\approx 0.02\%$; $\approx 0.002\%$) *Oleibacter* sp. ($\approx 0.003\%$; $\approx 0.0004\%$; $\approx 0.006\%$), *Oleiphilus* sp. ($\approx 0.05\%$; $\approx 0.2\%$; $\approx 0.01\%$), *Oleispira* sp. ($\approx 0.02\%$; $\approx 0.02\%$; $\approx 0.02\%$), *Thalassolituus* sp. ($\approx 0.001\%$; not present; $\approx 0.003\%$). This fact was not surprising because, in general, no significant concentration of hydrocarbons was detected in the marina waters (section 3.1.1.).

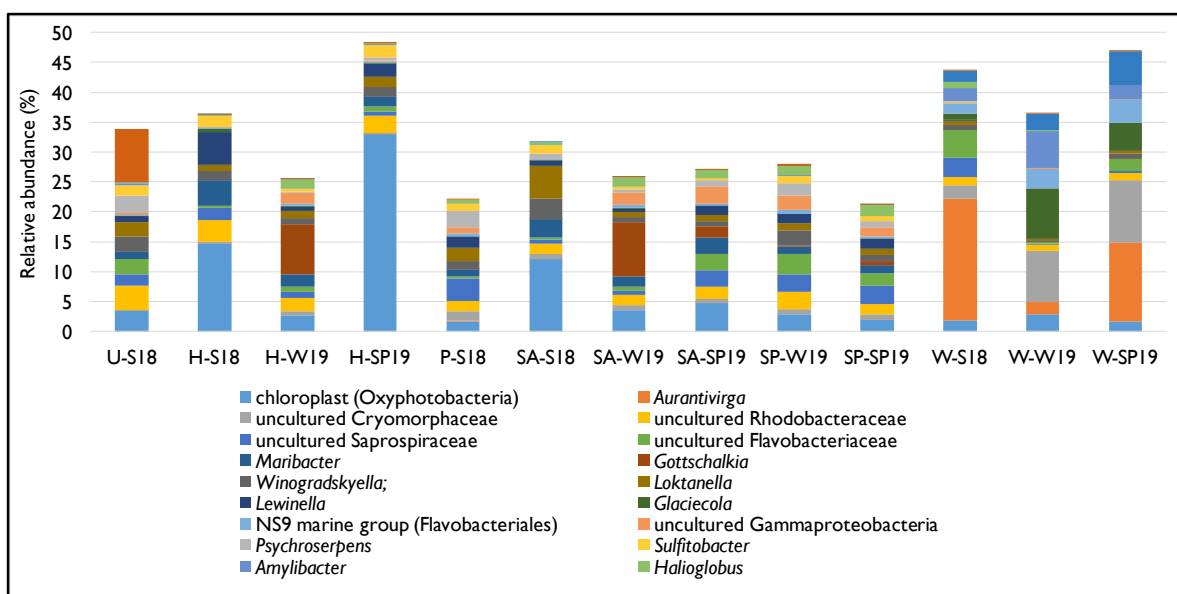


Figure 11. Taxonomic profile of top 20 prokaryotic taxa at lower level (genus) across all samples. Top 20 represents 33% of the total sequences dataset. Codes: biofouling samples from the floating platform (*H* = non-planted holes and *U* = Under surface); biofilm samples from the planting media (*SP* = *Spartina maritima*; *SA* = *Sarcocornia perennis* and *P* = *Phragmites australis*) and water samples (*W*); -*S18* = Summer of 2018; -*W19* = Winter of 2019 and -*SP19* = Spring of 2019.

The beta-diversity analysed through the principal coordinates analysis (PCoA) (Figure 12) showed that the principal coordinate 1 (PC1) explains 38% variance between the water samples and the other two types of samples. These results showed a clear distinct communities' composition developed in the water compared with the other two communities. The distinctness between water and marine biofilm communities was also, previously, reported by other authors [130; 131].

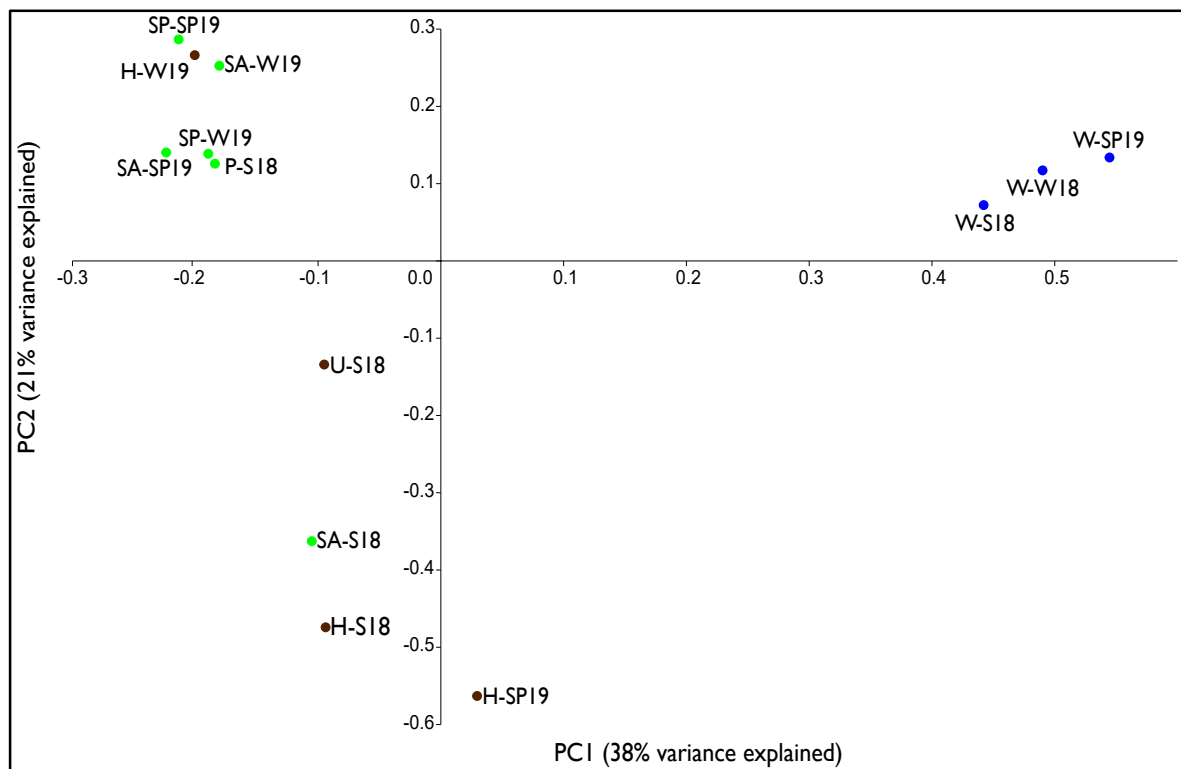


Figure 12. Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity of all the samples analysed: brown points represent biofouling samples collected from the floating platform; green points represent the biofilm samples from the planting media and blue points represent samples from the water. Codes: biofouling samples from the surface of the floating platform (*H* = non-planted holes and *U* = Under surface); biofilm samples from the planting media (*SP* = *Spartina maritima*; *SA* = *Sarcocornia perennis* and *P* = *Phragmites australis*) and water samples (*W*); -*S18* = Summer of 2018; -*W19* = Winter of 2019 and -*SP19* = Spring of 2019.

Based on these results, ANOSIM confirmed, statistically, a significant difference ($R^2 = 1$, $p = 0.012$) between the water samples and the biofilm samples from the planting media as well between the water samples and the biofouling samples from the floating platform ($R^2 = 1$, $p = 0.027$). Whereas, ANOSIM between the biofouling samples from the floating platform and the biofilm samples from the planting media showed no significant difference ($R^2 = 0.28$, $p = 0.073$).

Additionally, SIMPER analysis was performed to identify the taxa (at genus level) that most contributed to the differences between the two types of samples in comparison to the water samples (Table 11 and 12).

Table 11. SIMPER analysis results displaying top 10 genera responsible for the dissimilarity and the contribution percentage for that dissimilarity between water samples and biofilm samples from the planting media.

Taxa (genera)	Mean Abund. Plants (%)	Mean Abund. Water (%)	Contrib. (%)
<i>Aurantivirga</i>	0.04	28.90	4.79
Chloroplast (Oxyphotobacteria)	19.4	5.69	2.44
Uncultured Cryomorphaceae	4.85	19.50	2.42
<i>Glaciecola</i>	0.42	13.00	1.98
Uncultured Saprospiraceae	12.70	2.76	1.64
<i>Amylibacter</i>	0.14	9.82	1.53
Gottschalkia	8.95	0.01	1.49
Maribacter	8.87	0.02	1.44
<i>Lentibacter</i>	0.09	8.85	1.41
Uncultured Gammaproteobacteria	8.65	0.15	1.30

Obs.: Taxa more abundant in the biofilm from the planting media rather on water are shown in bold

Table 12. SIMPER analysis results displaying top 10 genera responsible for the dissimilarity and the contribution percentage for that dissimilarity between water samples and biofouling samples from the floating platform.

Taxa (genera)	Mean Abund. Platform (%)	Mean Abund. Water (%)	Contrib. (%)
<i>Aurantivirga</i>	0.03	28.90	6.45
Chloroplast (Oxyphotobacteria)	29.40	5.69	5.87
Uncultured Cryomorphaceae	0.99	19.50	3.94
<i>Glaciecola</i>	0.53	13.00	2.60
<i>Amylibacter</i>	0.35	9.82	1.99
<i>Lentibacter</i>	0.13	8.85	1.88
Maritimimonas	8.57	0.01	1.61
Gottschalkia	9.42	0.01	1.59
NS9 marine group (Flavobacteriales)	0.82	8.00	1.55
Maribacter	7.02	0.02	1.44

Obs.: Taxa more abundant in the biofouling from the floating platform rather on water are shown in bold

This analysis showed a more relative abundance of *Maritimimonas* sp., *Gottschalkia* sp., *Maribacter* sp. and of Cyanobacteria phylum members. More particularly, results showed also a more relative abundance of uncultured Saprospiraceae and Gammaproteobacteria classes in the case of the biofilm samples from the planting media.

Previous studies reported the presence of the *Maritimimonas* genus, in great percentage, in biofilms developed on biocidal coatings [132] and plastic surfaces at harbour environments [133], suggesting a great resistance capacity. In the present study, it was found, mainly, in the under surface of the floating platform (Figure 11). The *Gottschalkia* genus is characterized by the ability to degrade purines [134]. Interestingly, *Gottschalkia* genus members are classified as obligatory anaerobic [134], although, this genus was found in the floating platform (presenting aerobic conditions). Members affiliated to the *Maribacter* genus are, usually, found in association with macroalgae *Ulva* sp. [135; 136]. This may be correlated with the visual observations reported in the next thesis section (3.1.5.), in which *Ulva* sp. greatly contributed as one of the macroorganisms colonizing the FWI. Concerning the Oxyphotobacteria class, members are known for its importance as primary producers, capable of beneficiate other microorganisms trough secondary metabolites and oxygen, for instance [137]. For this reason, can be advantageous been found in such abundance in the FWI.

With relation to the Saprospiraceae family, members are mostly isolated from marine environments (but also freshwater) and activated sludge, with the capacity of breakdown

complex organic compounds [138]. For this reason, it can be considered another group of important organisms for bioremediation processes, been, mainly, present in the biofilm from the planting media in the present study. Finally, the Gammaproteobacteria class that contributed to the dissimilarity between water and the biofilm from the planting media, in this study, was previously found in other studies, in a high relative abundance associated with the rhizosphere of salt marsh plants [139], as well.

3.1.5. Assessment of macro-diversity in the FWI

Over time, successive colonization was observed on the floating platform by marine biota. Table 13 summarizes the macroorganisms found on or surrounding the FWI and their localization (especially at the submerged surfaces). At the initial phase, the first organisms found to be colonizing the FWI (under surface) were members from the Polychaeta class and *Chthamalus* genus. At the end of this study, the floating platform was covered mainly by *Mytilus* sp. and by a variety of macroalgae. The upper surface was in general dry (with exception of periodic waves from the tides), receiving total light exposure, which may explain the less colonization. However, over time, with the FWI sinking partially (due to the overall weight of the platform), this part received more water and so organisms such as *Ectocarpus* sp. and *Ulva* sp. installed in a major proportion in that area. Considering mobile organisms, *Palaemon* sp. was observed actively interacting with the detritus present in the non-planted holes and *Mugil* sp. was observed surrounding the FWI, pecking the under surface, possibly for feeding interest.

Table 13. Macro-biota found on or surrounding the FWI during the experimental time (16 months).

Macro-biota	Location	
	Genera	
Macroalgae	<i>Ceramium</i>	S
	<i>Chthamalus</i>	S; Un
	<i>Ectocarpus</i>	H; Up
	<i>Fucus</i>	H; S; Un
	<i>Gelidium</i>	S
	<i>Laminaria</i>	S; Un
	<i>Ulva</i>	H; S; Un; Up
Macroinvertebrate	<i>Mytilus</i>	S; Un
	<i>Palaemon</i>	H; Un
	<i>Patella</i>	S; Up
	unidentified Polychaeta	S; Un
Fish	<i>Mugil</i>	Su

H: Non-planted holes; S: Sides; Un: Under surface; Up: Upper surface; Su: Surrounding the FWI

Appendix III shows photos of the biodiversity found on or surrounding the FWI mentioned in Table 13. Other authors also recorded several organisms present in FWI but in freshwater ecosystems [29; 34; 39]. This biodiversity increment observed in the present study might be advantageous since some of these organisms, such as *Ulva* genus members, might have bioaccumulation capacity [140], retaining pollutants from the water

(acting synergistically with the plants and with the microbial communities present in the FWI) and contributing for aesthetical purposes as well. However, the uncontrolled proliferation might compromise the buoyancy of the FWI as discussed above.

3.2. Evaluation of hydrocarbon degradation potential by *Sarcocornia perennis*

Due to the previous results (section 3.1.3.), *Sarcocornia perennis* was selected for the lab-scale experiments. This species has been already studied as a biotool restoration for degraded salt marshes, showing favourable results since transplanted plants improved the metal extraction from the contaminated soil [141].

3.2.1. Water characterization

The physiochemical parameter results from the non-diesel doped treatment are presented in Table 14. The similar decrease in pH values over time for both batches can be attributed in part to plant exudation [61]. Whereas, the water temperature increased 3 to 7 °C due to the indoor conditions. On the other hand, COD results showed a general decreasing tendency, except for the first week (w1) of the first batch, probably due to oxygen consumption by plants and microbial communities in the plant roots and water.

Table 14. Water parameters from the non-diesel doped treatment during the experiment (4 weeks).

Time	Water parameters					
		Temp. (°C)	Cond. (mS/cm)	pH	Salinity (ppt)	COD (mg/L)
1 st Batch (2 weeks)	w0	14.2	52.7	8.22	34.0	270
	w1	17.8	52.9	6.71	34.5	435
	w2	20.4	51.5	6.72	33.6	360
2 nd Batch (2 weeks)	w0	13.5	54.2	8.09	35.1	435
	w1	20.7	53.5	6.82	35.1	360
	w2	17.8	53.6	6.82	35.0	315

Regarding nutrients, the plants not exposed to the diesel, showed, in general, high removal efficiencies (Figure 13), with nutrient levels decreasing notably.

Values of phosphate ion (PO_4^{3-}) removal from the initial concentration (0.09 and 0.14 mg/L), varied between 23-73% for the first batch and 43-59% for the second batch. Values of ammonium ion (NH_4^+) removal from the initial concentration (0.18 and 0.14 mg/L), ranged between 19-95% for the first batch and 72-94% for the second batch. The values of nitrate ion (NO_3^-) removal from the initial concentration (1.67 and 1.36 mg/L), ranged between 69-95% for the first batch and presented 92% for the second batch. Values of nitrite ion (NO_2^-) removal from the initial concentration (0.03 and 0.04 mg/L),

presented 92% for the first batch and presented 93% for the second batch. Overall, the second batch presented a higher percentage of nitrogen ions removal, related to a possible adaptation of the plants to the experimental conditions. On the other hand, PO_4^{3-} removal percentage was, in general, lower, probably due to system saturation as this ion is removed by plant accumulation or water precipitation [142].

There is a lack of similar studies using *Sarcocornia perennis*. However, in a horizontal subsurface flow constructed wetland (which consists of water percolating a planted bed) with *Sarcocornia fruticosa* for the treatment of highly saline wastewater [143], showed the removal of total phosphorus of 40-92% (from a mean inlet: 0.71 mg/L), removal of NH_4^+ of 31-89% (from a mean inlet: 3.9 mg/L) and of NO_3^- around 96% (from mean inlet: 23.1 mg/L) [143], similar to the present study (although no total phosphorus was measured). Furthermore, Sanicola *et al.* [64] referred to *Sarcocornia quinqueflora* (applied to saltwater FWIs) by having an impressive amount of nutrient uptake. Results showed a substantial nitrogen shoot mass increase (966% from an initial 0.022 g) and a less notable phosphorus increase (68% from an initial 0.022 g) [64].

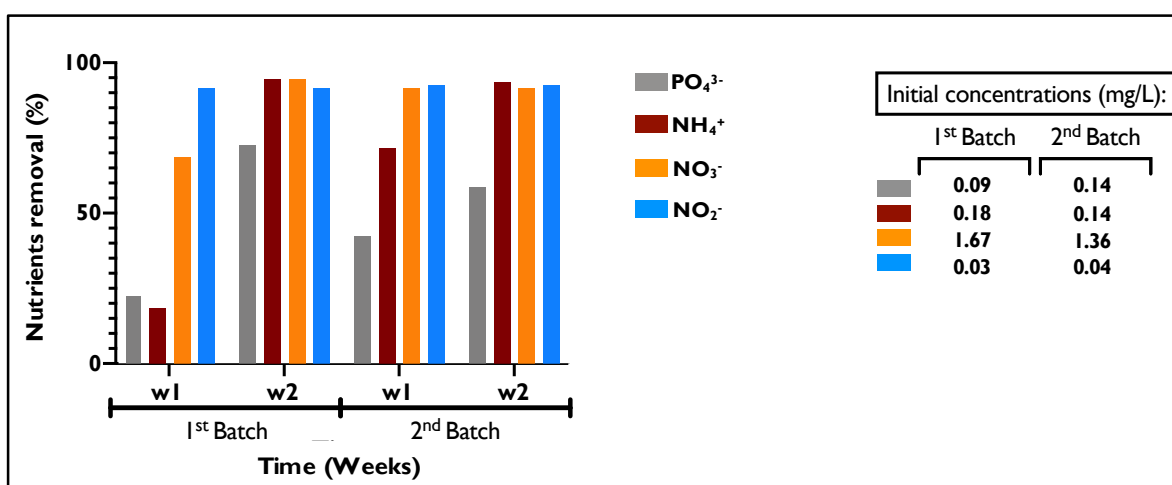


Figure 13. Nutrients removal (%) in non-diesel doped seawater during the experimental time (4 weeks) and the initial concentrations (mg/L) for each batch.

These removal efficiencies of nutrients from the non-diesel doped medium demonstrated the potential of *Sarcocornia perennis* to enhance seawater quality when applied in FWIs.

Concerning water hydrocarbons, in the present microcosm experiment, initial TPHs concentration measured in the water column was 1555 mg/L. Regarding TPHs removal rate (Figure 14), no statistically significant differences were observed among treatments, with very variable results along the experimental time. This great variability could be related to the difficulty to well homogenise the solution in the tanks and collection of properly representative samples.

The tested plants (*Sarcocornia perennis*) did not seem to influence the removal of hydrocarbons from the water. Furthermore, observed TPHs removal rates were probably due to abiotic factors namely the sorption of hydrocarbons to the floating platform, photodegradation, and natural volatilization. These last two abiotic factors have been described in other studies without plants [144; 145]. In the present study, plants may also have decreased the effect of these abiotic factors, due to the “shading effect” and the reduction of open spaces in the surface of the tanks which might have reduced the photodegradation and the volatilization of hydrocarbons.

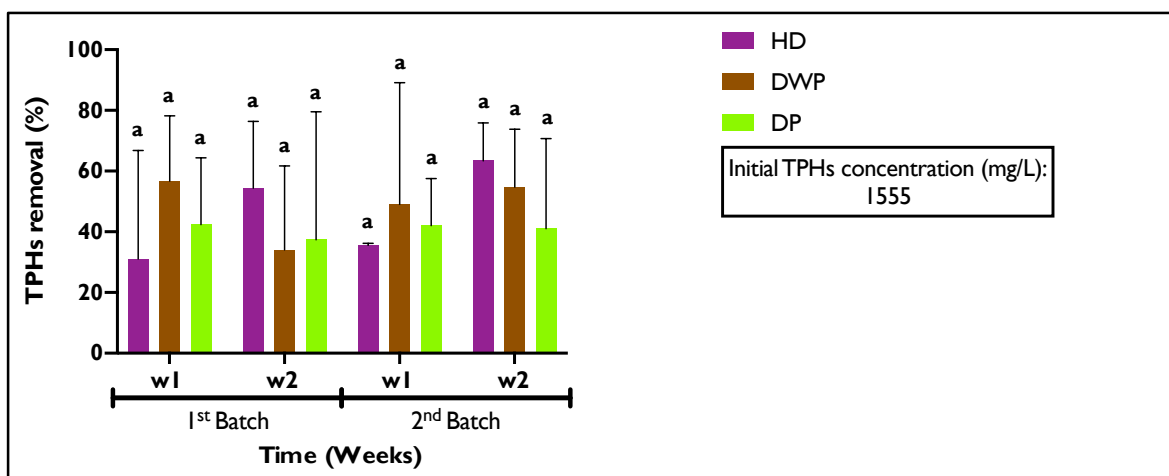


Figure 14. Total Petroleum Hydrocarbons (TPHs) removal (%) analysed from the three diesel doped treatments (*HD* - Cover with same hole configuration as in the floating platforms; *DWP* - Floating platform without plants; *DP* - Floating platform with plants) during the experimental time (4 weeks) and the initial TPHs concentration (mg/L) (mean $\pm$ SD, $n = 3$). The different letters placed on the bars show the statistically significant differences ($p < 0.05$) between treatments and time.

Contrasting the results from this study, some investigations have used FWIs to treat water contaminated with hydrocarbons [51; 146-148] as well as in similar technologies (e.g. horizontal subsurface flow systems) [145], but all for freshwater. All studies showed promising results as following described.

Rehman *et al.* [148] used 20 L tanks with plants (*Brachiaria mutica* and *P. australis*) and with bacterial enrichment, revealing that, after 42 days of experience, the maximum crude oil removal was observed (reduced by 97% from the original oil concentration of 325 mg/L). Effendi *et al.* [147] tested *Vetiveria zizanioides* L. Nash for the removal of crude oil in 25 L tanks with 1% of oil in water, observing reductions of 91.39% after 4 weeks. Li *et al.* [51] filled 75 L tanks with refinery wastewater, starting with 1720 mg/L of TPHs and, over 35 days, observed decreases of 40-55%, depending on the plant species tested (*Geophila herbacea* O. Kuntze, *Lolium perenne* CV. Caddieshack, *Lolium perenne* Topone and *Lolium perenne* L.). In a recent study conducted by Afzal *et al.* [146], an FWI of 3058 m² was applied in-field to a water stabilization pit contaminated with crude oil, using plants (*P. australis*, *Typha domingensis*, *Leptochloa fusca* and *Brachiaria mutica*)

and a consortium of hydrocarbon-degrading bacteria. Results showed a reduction of 99.1% in the hydrocarbon content (319 mg/L) within 18 months [146].

Finally, Al-Baldawi *et al.* [145] also evaluated the treatment of water contaminated with diesel (like the one used in the present study) but in a horizontal subsurface flow constructed wetland, observing removal of 91.5% from the initial TPHs concentration of 17,400 mg/L, after 72 days, using *Scirpus grossus*.

3.2.2. Plant physiological response

The plants subjected to the diesel doped water did not resist at the end of the experiment, manifesting aerial tissue senescence. On the other hand, the plants subjected to the non-diesel doped water, resisted, showing only some symptoms of damage in a few aerial parts, possibly related to the low nutrient concentrations in the water.

In terms of PAM measurements, results indicated an increasing trend over time in the values of ETR_m for the plants in non-diesel doped water (Figure 15). This trend suggested an adaptation on this group of plants to the high salinity of the water and the transfer from the soil to the hydroponic mode. Other studies have also used PAM measurements to efficiently study factors of stress in *S. perennis*, i.e. different tidal exposures such as submersion periods [149].

In the present study, however, accurate PAM measurements were not always possible due to the damaged aerial tissues and the difficulty in maintaining the static contact between the plastic fibre guide and the plant tissue. Nevertheless, these measurements still suggested that the plants in diesel doped seawater presented more constraints due to the extra-stress factor (hydrocarbons presence).

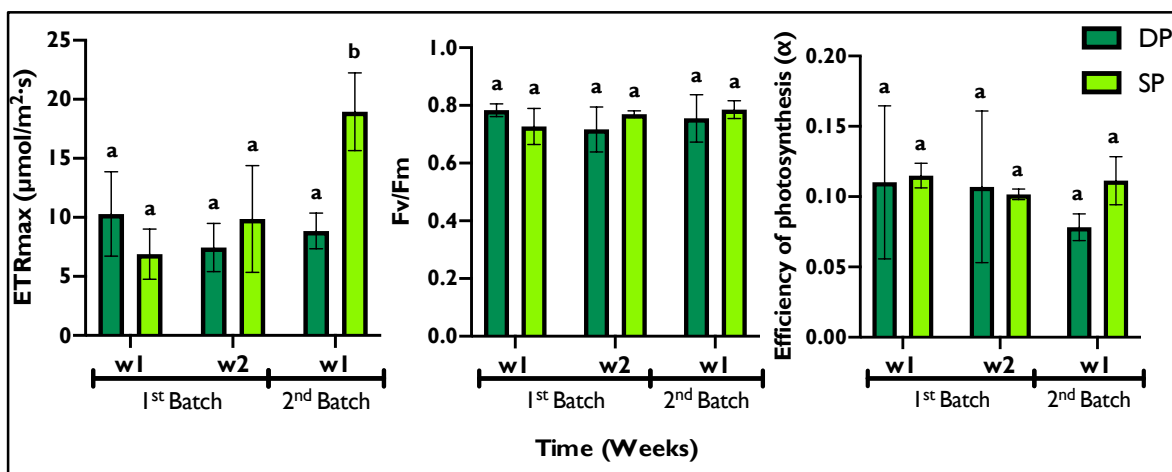


Figure 15. Maximum electron transport rate (ETR_m); the Fv/Fm ratio and the quantum efficiency of photosynthesis (α) registered for *Sarcocornia perennis* in diesel doped (DP) and non-diesel doped (SP) treatments (mean ± SD, *n* = 3) during 3 experimental weeks. The different letters placed on the bars show the statistically significant differences (*p* < 0.05) between treatments and time.

As mentioned, in the present study, plants did not survive to diesel exposure. Contrarily, wetland plants (*Vetiveria zizanioides* L. Nash), showed higher growth (upper plant biomass and higher roots length) in crude oil-contaminated water [147]. Furthermore, authors suggested that plants tended to display this response because, in the presence of crude oil, plants used oil as a source of nutrients while the decomposition of this pollutant was occurring in the water [147]. Afzal *et al.* [146] in their FWI in-field approach also reported plant survival and growth (*P. australis*, *T. domingensis*, *L. fusca* and *B. mutica*) in hydrocarbon contaminated water. In that study, the plants were enhanced by inoculation of 10 hydrocarbon-degrading bacteria that are suggested as plants' growth promoters [146]. On the other hand, Rehman *et al.* [148] reported that their tested plants (*B. mutica* and *P. australis*) had approximately 50% growth inhibition in the contaminated water compared to the control (tap water). Lastly, Li *et al.* [51] reported the survival of five different wetland grasses species, although displaying different biomass increment and so suggesting different species tolerance to the water contaminated with petroleum hydrocarbons.

These examples indicate that wetland plants in hydroponic mode (applied to FWI) can tolerate and grow in water contaminated with hydrocarbons. Despite *S. perennis* survival in the imposed environmental conditions of the marina and the treatment with non-diesel doped water, it may not be sufficiently tolerant to hydrocarbons (at least under this concentration).

CHAPTER 4

CONCLUSION
AND FUTURE DIRECTIONS

The use of FWIs to promote water quality enhancement of highly saline water bodies demonstrated interesting results, adding knowledge in this type of application.

The water analyses of the Marina of the Porto Cruise Terminal indicated the presence of TPHs, although, PAHs were not detected in the water surface. Further on, high COD values were registered. These results constitute an important dataset for future comparison with other harbours/marinas in Europe and at a global level since the existent lack of data.

Regarding the tested floating platform, made of cork agglomerate, showed resistance to the environmental conditions of the seawater port marina. However, some adjustments for robustness improvement could beneficiate the system. For instance, a higher thickness could better support the weight caused by marine biofouling.

This study showed that the halophyte plants *Sarcocornia perennis* and *Spartina maritima* can be used in future FWIs seawater applications. Particularly, *S. perennis* proved to be promising in phytoremediation approaches, reducing COD and inorganic nutrients. However, for water hydrocarbons removal, further investigation might be conducted using *S. perennis* under different diesel concentrations to better understand the specific tolerance of this species.

The use of the new generation sequencing (NGS) proved to be an interesting tool to assess the composition and diversity of the microbial communities in three compartments, raising knowledge about the water microbiome present in the Marina of Terminal Cruise of the Port of Leixões. Moreover, it pointed out the main differences established between those microbial indigenous communities and the ones developed in the biofouling of the floating platform, as well, in the biofilm from the planting media of the plants. Overall, the knowledge gained about the micro and macroorganisms diversity developed on the FWI will be important, in future applications, from the perspective of the use of FWIs for water quality improvement.

Finally, the in-field FWI provided important ecosystem services such as biodiversity promotion and contributing to the aesthetic improvement of the seawater port marina landscape. With this work, further investigation using FWIs to improve highly saline water quality is expected, having this knowledge raised, as a starting point.

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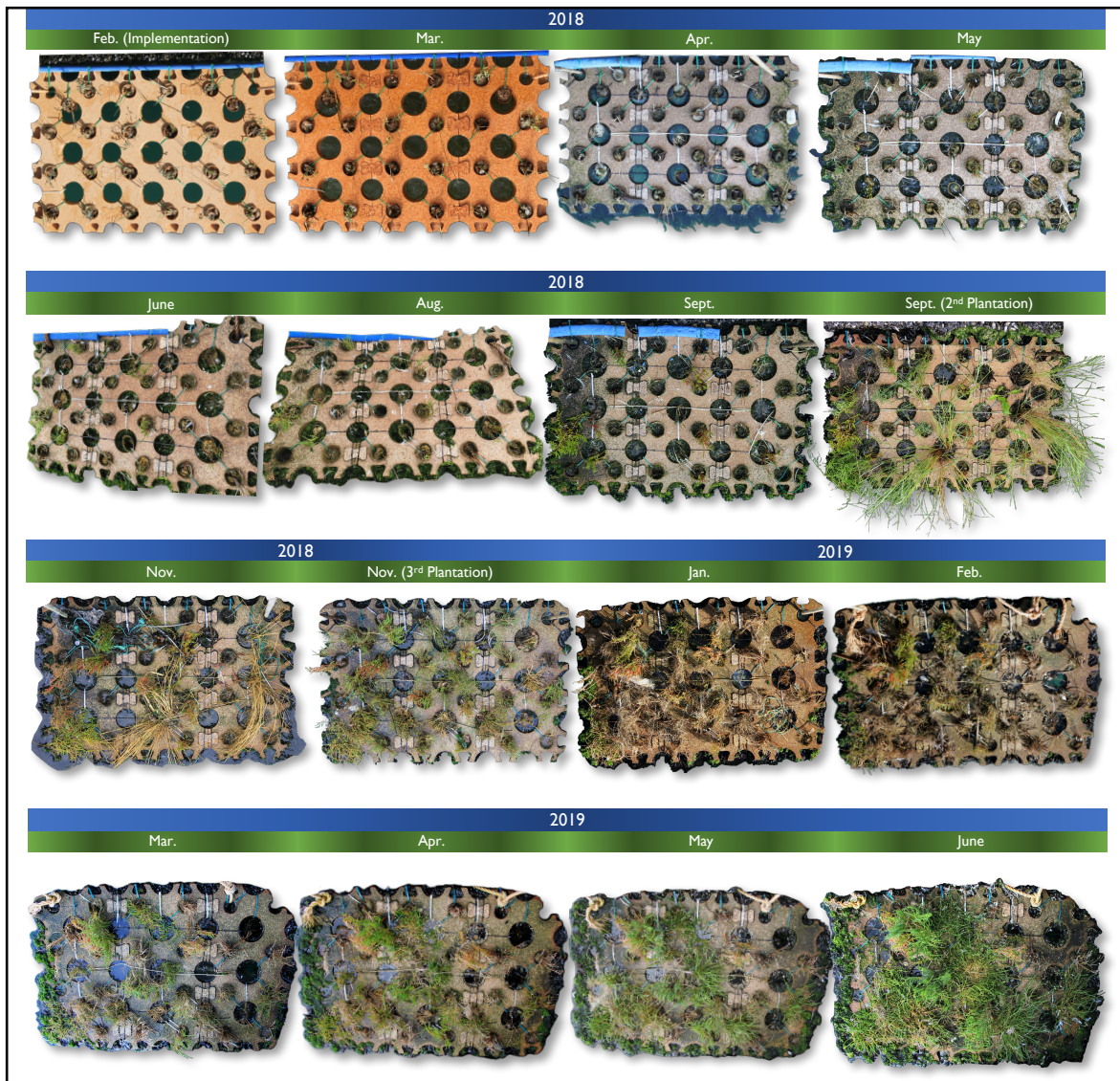
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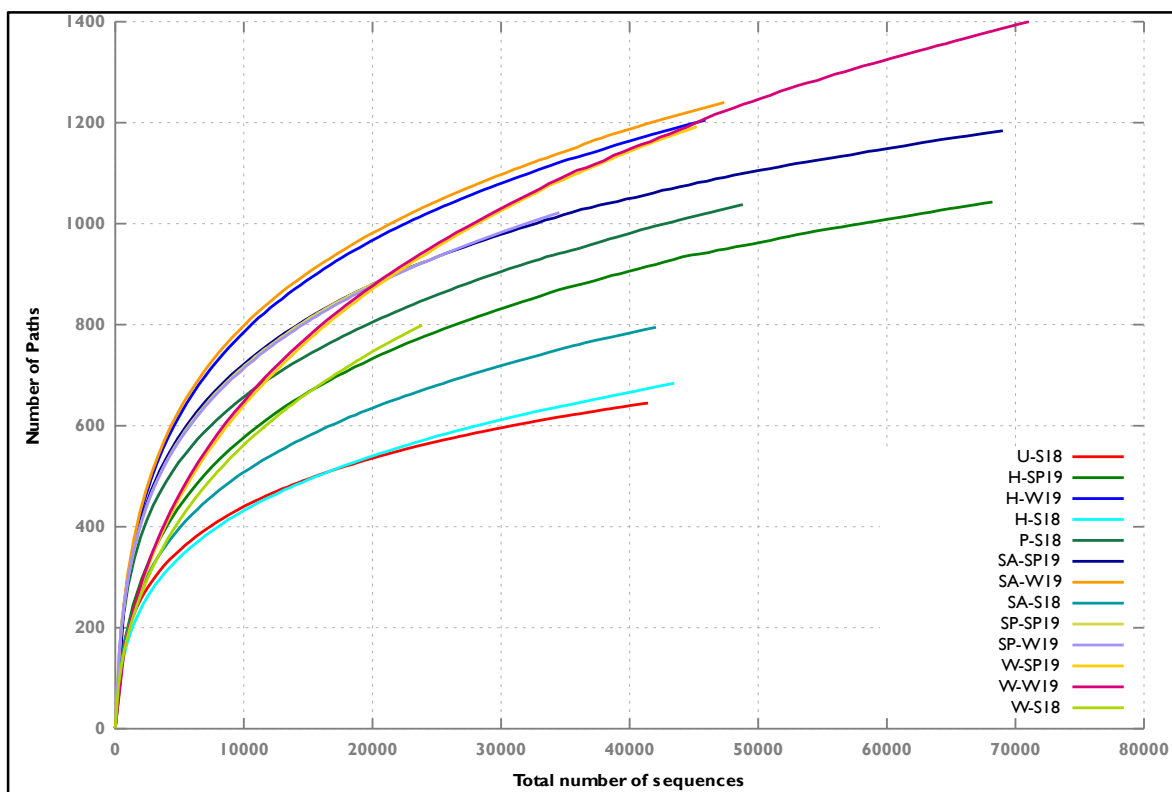
APPENDIX

Appendix I. Plant establishment and development



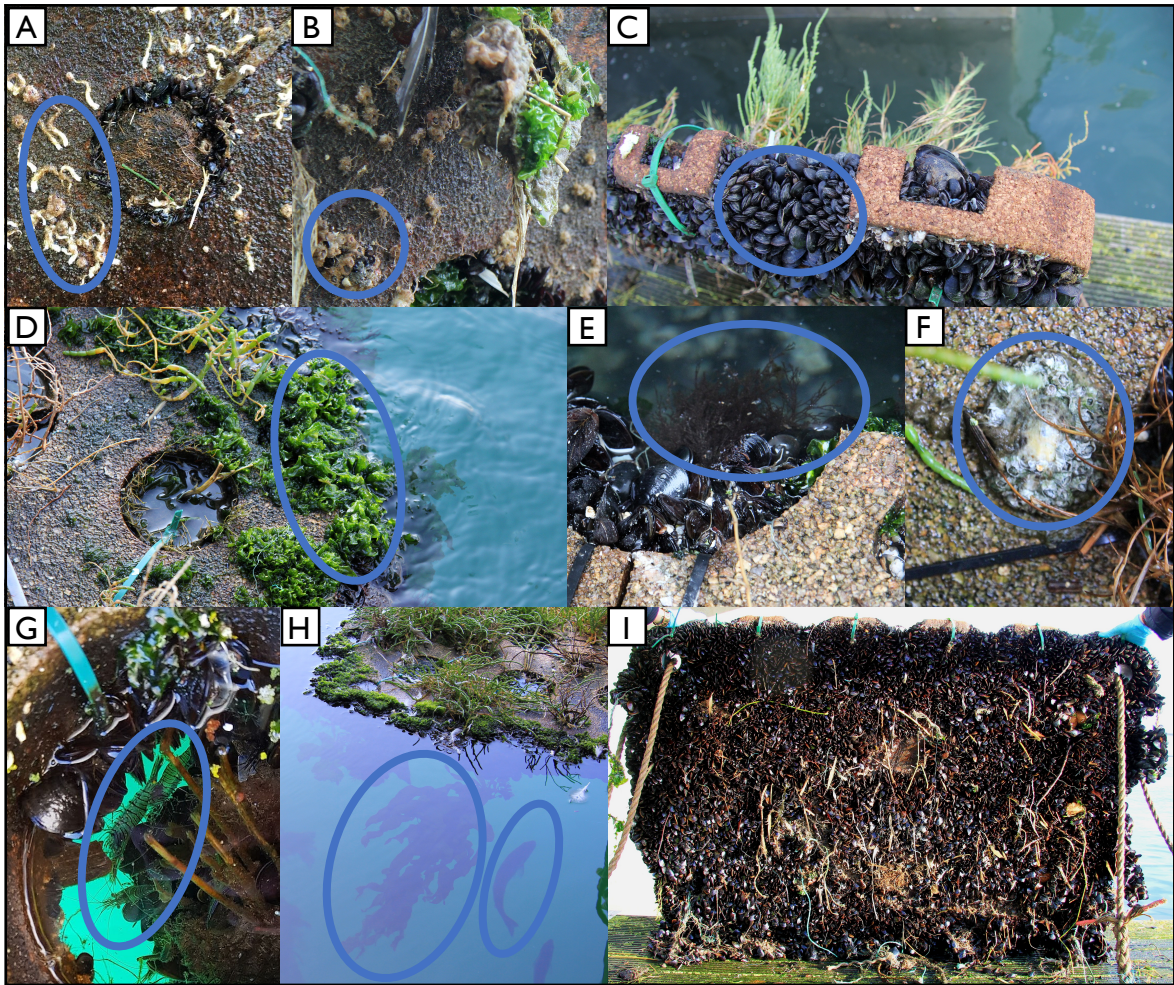
Supplementary figure 1. Top view photos showing the general aspect of the FWI for each month of the experiment (February of 2018 until June of 2019).

Appendix II. Rarefaction curves from 16S rRNA gene sequencing



Supplementary figure 1. Rarefaction curves across all the sequenced samples in this study. Codes: biofouling samples from the floating platform (H = non-planted holes and U = Under surface); biofilm samples from the planting media (SP = *Spartina maritima*; SA = *Sarcocornia perennis* and P = *Phragmites australis*) and water samples (W); -S18 = Summer of 2018; -W19 = Winter of 2019 and -SP19 = Spring of 2019.

Appendix III. Macro-diversity in the FWI



Supplementary figure 1. Macroorganisms found on or surrounding the FWI: **A** – Polychaeta; **B** – *Chthamalus* sp.; **C** – *Mytilus* sp.; **D** – *Ulva* sp.; **E** – *Ceramium* sp.; **F** – *Patella* sp.; **G** – *Palaemon* sp.; **H** – *Laminaria* sp. (left elypte) and *Mugil* sp. (right elypte); **I** – *Mytilus* sp..

COMMUNICATIONS

BASED ON THE WORK
DESCRIBED IN THIS THESIS

COMMUNICATIONS IN NATIONAL SCIENTIFIC SYMPOSIUMS

Carecho, J., Favas, R., Tomasino, M. P., Azevedo, J. P., Almeida, C. M., Mucha, A. P., Calheiros, C. S. C. 2019. Floating wetland islands to promote water quality enhancement. (ref. 15273) In: Book of Abstracts of the 12th Meeting of Young Researchers at U. Porto (IJUP012). 13-15/02/2019, Porto, Portugal. ISBN: 978-989-746-203-0 (*Poster communication*)

Favas, R., Carecho, J., Azevedo, J., Almeida, C. M., Mucha, A. P., Calheiros, C. S. C. 2019. Biocenosis associated to floating wetland islands. 7th AEICBAS Biomedical Congress. 14-17/03/2019, Porto, Portugal (*Poster communication*)

COMMUNICATIONS IN INTERNATIONAL SCIENTIFIC SYMPOSIUMS

Carecho, J., Favas, R., Tomasino, M.P., Azevedo, J., Almeida, C.M., Mucha, A.P., Calheiros, C. S. C. 2019. Assessment of floating wetland island implemented in a port marina. (pp. 382) In: Book of Abstracts of the 8th International Symposium on Wetland Pollutant Dynamics and Control (WETPOL2019). 17-21/06/2019, Aarhus University, Denmark. ISBN: 978-87-91486-1-7 (*Poster communication*)

Carecho, J. Favas, R., Tomasino, M.P., Azevedo, J., Silva, R., Pinto, G., Arenas, F., Almeida, C.M., Mucha, A.P., Calheiros, C.S.C. 2019. The potential of *Sarcocornia perennis* applied to a floating wetland island in port marina environment. 2nd International Meeting on New Strategies in Bioremediation Processes (BIOREMID2019). 24-25/10/2019, Porto, Portugal (*Poster communication*)

Carecho J., Lopes M., Calheiros C.S.C. 2019. Educational resources to raise environmental awareness in school. 18th Encontro Nacional de Educação em Ciências (XVIII ENEC) | 3rd International Seminar of Science Education (III ISSE) (ENEC/ISSE2019). 5-7/09/2019, Porto, Portugal (*Oral communication*)