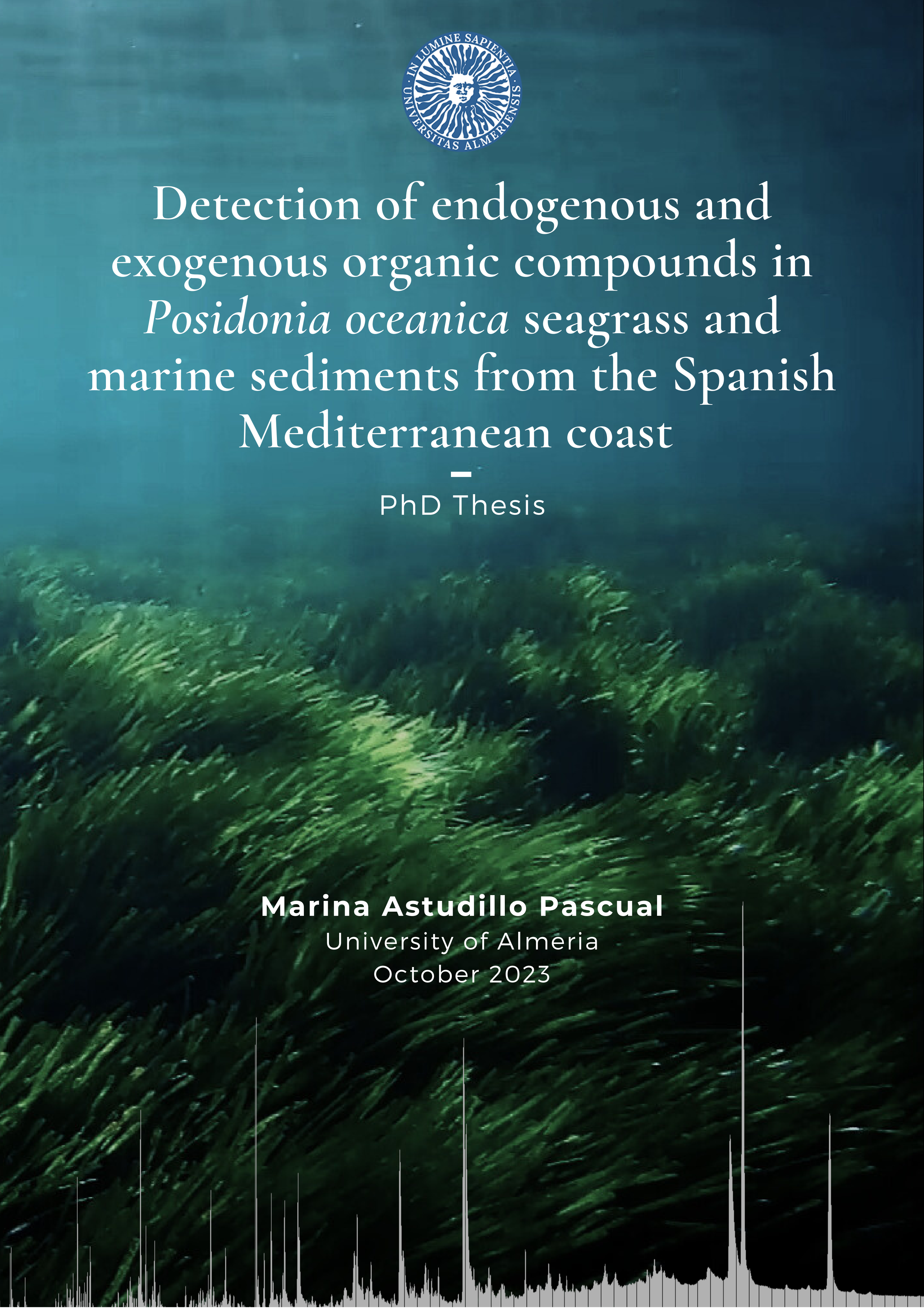

Detection of endogenous and exogenous organic compounds in *Posidonia oceanica* seagrass and marine sediments from the Spanish Mediterranean coast

PhD Thesis

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University of Almeria
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UNIVERSIDAD
DE ALMERÍA

Department of Biology and Geology &
Department of Chemistry and Physics

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Detección de compuestos endógenos y exógenos en *Posidonia oceanica* y sedimentos marinos de la costa mediterránea española

Marina Astudillo Pascual
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Memoria presentada por Marina Astudillo Pascual para optar al grado de Doctora por la Universidad de Almería con Mención Internacional por el Programa de Doctorado en Ciencias Aplicadas al Medio Ambiente.

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Summary

Posidonia oceanica (*P. oceanica*) meadows are widely recognized as a unique ecosystem in the Mediterranean Sea, providing key services to human beings. However, this relevant and protected seagrass is currently in recession. Considering its low resilience, the need to investigate potential early warnings or biomarkers, such as phenolic compounds (PhCs), increases. PhCs are naturally produced compounds which concentrations might vary when undergoing stress. Additionally, marine pollution might be affecting its recovery, yet the organic pollutant (OP) composition in this seagrass has not been explored in depth. Therefore, this Thesis is seeking to present an investigation on the *P. oceanica* chemical composition in the form of PhCs (endogenous compounds), as well as the OPs signature, hereafter exogenous compounds. In this last case, the investigation is broadened to marine sediments. This research aims to provide the phenolic fingerprint of each *P. oceanica* tissue (leaves, rhizomes, and roots), enabling future studies to test the PhCs' stress response and their potential as early warnings. Additionally, this research is expected to contribute to our understanding of how land-borne pollution (current and legacy alike) is accumulated and transformed in coastal ecosystems.

The sampling areas were selected based on the OP-generating activities taking place, such as agriculture, industrial production, and tourism, potentially forming sedimentary reservoirs and causing stress to *P. oceanica*. Samples were analyzed by liquid and gas chromatography (LC and GC, respectively) coupled to high resolution mass spectrometry (HRMS) using the Orbitrap analyzer. This makes this work the first investigation applying the potentials of the HRMS analyses to *P. oceanica* studies, as well as the search for simple, reliable, and precise methods.

The consideration of several Green and White Chemistry Principles in this work is of special interest considering the protection state of *P. oceanica* and the conventional, non-environmentally friendly methods for extracting OPs from marine sediments.

The conducted investigation has been divided into two sections, depending on the type of targeted organic compound (endogenous or exogenous). Firstly, in Section I, different extraction techniques for PhCs extraction and analytical methods were optimized. The feasibility of the resulting method was verified by applying it to collected *P. oceanica* samples.

In more detail:

- For an exhaustive phenolic characterization, different tissues of *P. oceanica* were taken by scuba diving from 5 different meadows belonging to the Almeria region. Sampling points with different characteristics in terms of depth and pressure acting on the meadows were selected. This variability between sampling points is meant to broaden the types of PhCs detected.
- The performance of different extraction procedures was evaluated.
- Similarly, the analytical method for the determination of PhCs in *P. oceanica* was also optimized, namely the mobile phases, chromatographic conditions, and mass spectrometric parameters.
- The feasibility of the different methods was verified by applying them to the different tissues (leaves, rhizomes, and roots) of five *P. oceanica* samples, obtaining tissue-specific extraction methods.
- Determination of PhC compartmentation among *P. oceanica* tissues.

As a result, the most complete phenolic fingerprint for *P. oceanica* was obtained to date. From this array of PhCs, several of them were reported for the first time, opening new possibilities for future biomarker research.

Secondly, in Section II, a variety of simple and affordable extraction methods were optimized and validated for the study of OPs in *P. oceanica* and marine sediments. These methods, which were used for the first time in *P. oceanica* tissues, facilitated the detection of a multiclass group of OPs: polychlorinated biphenyls (PCBs), pesticides, and polycyclic aromatic hydrocarbons (PAHs). The following work was conducted in Section II:

- Numerous samples of *P. oceanica* (21 leaves, 20 rhizomes) and 43 sediment samples were collected by scuba diving from three singular regions: Almeria, Murcia, and Alicante. Sampling points were situated where OPs might be transferred from their source into the sea (water courses, pipelines, ports…).
- Development of analytical methods for the monitoring of OPs in *P. oceanica* (leaves and rhizomes) and marine sediments, based on miniaturized, eco-friendly (minimal sample size and solvents) extractions. Methods validation and analysis of the marine samples were performed using GC coupled to HRMS (GC-Q-Orbitrap MS), and a 3-way analytic approach (target, suspect, and unknown).

- Study of the *P. oceanica* tissues and sediments compartmentation in terms of encountered OPs.
- Detection frequency of the detected OPs in the environmental samples.

Consequently, the most complete OPs signature in *P. oceanica* was obtained through a method able to preserve its ecological integrity. Certainly, this brings, for the first time, the possibility of conducting sustainable monitoring programs for the detection of OPs in *P. oceanica*.

Resumen

Las praderas de *Posidonia oceanica* (*P. oceanica*), ampliamente reconocidas como un ecosistema único en el mar Mediterráneo, brindan servicios claves a los seres humanos. Sin embargo, esta planta marina relevante y protegida, se encuentra actualmente en recesión. Considerando su baja resiliencia, la necesidad de investigar biomarcadores potenciales o "alertas tempranas", tales como los compuestos fenólicos (PhCs), aumenta. Estos PhCs son producidos de manera natural y sus concentraciones pueden verse afectadas bajo situaciones de estrés. A esto hay que sumarle la contaminación marina, que podría estar afectando a la recuperación de *P. oceanica*. Sin embargo, el contenido de los diversos contaminantes orgánicos (OP) en dicha planta marina no ha sido explorado aún en profundidad. Por lo tanto, la presente Tesis busca investigar la composición química de *P. oceanica* en forma de PhCs (o compuestos endógenos), así como de OPs (compuestos exógenos). En este último caso, la investigación también se amplía a los sedimentos marinos.

Esta investigación tiene por objetivo proporcionar la huella fenólica de cada tejido de *P. oceanica* (hojas, rizomas y raíces), permitiendo que estudios futuros prueben la respuesta de los PhCs al estrés y su potencial como "señales tempranas". Así mismo, se espera que esta investigación contribuya a nuestra comprensión de cómo la contaminación terrestre (tanto la actual como la histórica) se acumula y transforma en los ecosistemas costeros.

Las áreas de muestreo se seleccionaron en función de las presentes actividades generadoras de OPs, como la agricultura, la producción industrial y el turismo, las cuales pueden llegar a formar reservorios sedimentarios en la costa y causar estrés en *P. oceanica*. Las muestras fueron analizadas mediante cromatografía de líquidos y de gases (LC y GC, respectivamente) acopladas a espectrometría de masas de alta resolución (HRMS) utilizando el analizador Orbitrap. Esto convierte a este trabajo en la primera investigación que aplica las potencialidades de los análisis de HRMS a los estudios de *P. oceanica*, así como el primero en buscar métodos simples, confiables y precisos.

Es de especial interés, la consideración en este trabajo de varios de los principios de la Química Verde y Química Blanca, considerando el estado de protección de *P. oceanica* y los existentes métodos convencionales no respetuosos con el medio ambiente para extraer OPs de los sedimentos marinos.

Resumen

La investigación que se ha llevado a cabo se ha dividido en dos secciones, en función del tipo de compuesto orgánico (endógeno o exógeno). En primer lugar, en la Sección I, se optimizaron diferentes técnicas de extracción de PhCs y métodos analíticos. La viabilidad del método resultante se verificó aplicándolo a muestras de *P. oceanica*.

En mayor detalle:

- Toma de muestras mediante buceo de diferentes tejidos de *P.* oceanica en cinco praderas diferentes de la región de Almería para una caracterización fenólica exhaustiva. Se seleccionaron puntos de muestreo con diferentes características en términos de profundidad y presión. Esta variabilidad entre los puntos de muestreo tenía como objetivo ampliar los tipos de PhCs detectados.
- Evaluación de la eficacia de diferentes procedimientos de extracción.
- Optimización del método analítico para la determinación de PhCs en *P. oceanica*, incluyendo la fase móvil, condiciones cromatográficas y otros parámetros espectrométricos.
- Evaluación de la viabilidad de los diferentes métodos mediante su aplicación a los diferentes tejidos (hojas, rizomas y raíces) de cinco muestras de *P. oceanica*, obteniendo métodos de extracción específicos para cada tejido.
- Determinación de la distribución de los PhCs entre los tejidos de *P. oceanica*.

Como resultado, se obtuvo la huella fenólica más completa de *P. oceanica* existente hasta la fecha. De este conjunto de PhCs detectados, varios de ellos fueron observados por primera vez, abriendo nuevas posibilidades para futuras investigaciones de biomarcadores.

En segundo lugar, en la Sección II, se optimizaron y validaron una variedad de métodos de extracción simples y asequibles para el estudio de OPs en *P. oceanica* y sedimentos marinos. Estos métodos, los cuales se utilizaron por primera vez en tejidos de *P. oceanica*, facilitaron la detección de varias clases de OPs: bifenilos policlorados (PCB), plaguicidas e hidrocarburos aromáticos policíclicos (PAH). El trabajo realizado en la Sección II incluye lo siguiente:

- Toma de numerosas muestras de *P. oceanica* (21 hojas, 20 rizomas) y 43 muestras de sedimentos mediante buceo en tres regiones: Almería, Murcia y Alicante. Los

puntos de muestreo se situaron en zonas donde los OPs podrían estar siendo transferidos desde su fuente al mar (cursos de agua, tuberías, puertos, etc.).

- Desarrollo de métodos analíticos para el monitoreo de OPs en *P. oceanica* (hojas y rizomas) y sedimentos marinos, basados en extracciones miniaturizadas y respetuosas con el medio ambiente (manteniendo un tamaño mínimo de muestra y disolventes). La validación de los métodos y el análisis de las muestras marinas se realizaron utilizando GC acoplada a HRMS (GC-Q-Orbitrap MS) y un enfoque analítico triple (target, suspect y unknown).
- Estudio sobre la distribución de los OPs en los tejidos de *P. oceanica* y sedimentos.
- Estimación de la frecuencia de detección de los OPs en las muestras ambientales.

Como resultado, se obtuvo la señal de OPs más completa en *P. oceanica* hasta el momento, usando un método capaz de preservar la integridad ecológica. Esto brinda, por primera vez, la posibilidad de llevar a cabo programas de monitoreo sostenibles para la detección de OPs en *P. oceanica*.

Objectives

This work aims to investigate firstly the most complete chemical composition *P. oceanica* seagrass, in the form of PhCs. For this purpose, all *P. oceanica* tissues (leaves, rhizome, and roots) were studied. Additionally, this Thesis discusses the compartmentation of the PhCs along the three different tissues. Secondly, this research aims to examine the OPs present in the leaves and rhizomes of *P. oceanica*, as well as in certain marine sediments from the coastal environment of the Mediterranean Spanish coast.

To fulfill these purposes, different matrix-specific methods were developed, optimized, and applied to environmental samples for the extraction and analysis of PhCs and OPs. Among these OPs, three different classes of substances were investigated: PCBs, pesticides, and PAHs. Subsequently, the multiclass method was validated for a total of 54 OPs in each matrix included in the study. Furthermore, this work has presented a novel approach to the field of marine ecology by incorporating advances in analytical chemistry. GC and LC coupled to HRMS have been employed providing the maximum information to date.

The resulting scientific information has been separated into two distinct parts, one per compound type. The first one focuses on PhCs in *P. oceanica* (leaves, rhizome, and root), which are also named endogenous compounds in this Thesis, using LC-HRMS. The second part studies instead the OPs signature, hereafter exogenous compounds, by GC-HRMS. The following objectives were achieved:

- Optimization of a miniaturized extraction procedure of PhCs from *P. oceanica* tissues in which sample amount, extraction solvent, and time have been reduced in comparison to other studies and reconsidered to improve extraction efficiency. Moreover, the chromatographic conditions were also optimized to obtain an improved signal resolution, assessing diverse chromatographic columns and mobile phases. An additional novelty was the usage of HRMS, allowing for the exhaustive processing (target and non-target analyses) of the environmental samples. All together help satisfactorily identify 42 phenolic compounds (**Article I**).
- Development, optimization, and validation of miniaturized, multiclass methods for the analysis of priority OPs and other compounds of concern in *P. oceanica*

seagrass and marine sediments. The targeted OPs were, in the first instance, PCBs and priority pesticides (**Article II**), later adding to the list several priority PAHs (**Article III**). These methods were applied in environmental samples, additionally running non-target analysis, such as suspected and unknown, thus broadening the range of OPs detected (**Articles II** and **III**)

Scientific Contributions included in this Thesis

The list of articles related to this Thesis is shown below:

I. **New Phenolic Compounds in *Posidonia oceanica* Seagrass: A Comprehensive Array Using High Resolution Mass Spectrometry**
Astudillo-Pascual, M., Domínguez, I., Aguilera, P.A., Garrido Frenich, A., 2021. *Plants*, 10, 864. https://doi.org/10.3390/plants10050864

II. **Target and Suspect Analysis with High-Resolution Mass Spectrometry for the Exhaustive Monitoring of PCBs and Pesticides in *Posidonia oceanica* Meadows and Sediments**
Astudillo-Pascual, M., Aguilera, P.A., Garrido Frenich, A., Domínguez, I., 2022. *Chemosensors*, 10, 531. https://doi.org/10.3390/chemosensors10120531

III. **Determination of PAHs, PAH-Derivatives and Other Concerning Substances in Posidonia oceanica Seagrass and Marine Sediments by High Resolution Mass Spectrometry**
Astudillo-Pascual, M., Tudor, R., Domínguez, I., Aguilera, P.A., Frenich, A.G., 2023. J. Mar. Sci. Eng. 11, 36. https://doi.org/10.3390/jmse11020369

Author's contribution in each publication

Article I, II, and III: Marina Astudillo Pascual participated in the literature review, sampling and experimental design, sample retrieval, execution of the experimental part, data collection, implementation of the employed software, data analysis and writing of the manuscript.

Abbreviations and Acronyms

1-MOF	1-Methoxymethylfluorene
2,3-DMAQ	2,3-Dimethylanthraquinone
2,4-DTBP	2,4-Di-tert-butylphenol
2-BrN	2-Bromonaphthalene
7,9-DTBO	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
Ac	Acenaphthene
Ace	Acetone
Acy	Acenaphthylene
ADR	Adriatic Sea
AEG	Aegean Sea
ALB	Alboran Sea
Ald	Aldrin
Ant	Anthracene
Atr	Atrazine
BaA	Benz[a]anthracene
BAC	Background Assessment Concentration
BaP	Benz[a]pyrene
BbF	Benzo[b]fluoranthene
BghiP	Benzo[ghi]perylene
BkF	Benzo[k]fluoranthene
C18	Octadecylsilane
CCD	Charge-Coupled Device
CEN	Cretan Sea
Chlp	Chlorpyrifos
Chry	Chrysene
cycloHx	CycloHexane
d.w.	Dry Weight
DAD	Diode Array Detector
DahA	Dibenz[a,h]anthracene
DAIP	Diallyl Isophthalate
DBAL	Dimethyl Benzaldehyde
DBF	Dibenzofuran
DCHP	Dicyclohexyl Phthalate
DCM	Dichloromethane
Die	Dieldrin
DMMN	1,6-Dimethyl-4-(1-methylethyl)naphthalene
DMNs	Dimethylnaphthalenes
Dodem	Dodemorph
EAC	Assessment Criteria Limit
ECHA	European Chemicals Agency
EM	Electron Multiplier
End	Endrin

EtAc	Ethyl Acetate
FD	Fluorescence Detector
FID	Flame Ionization Detector
Flt	Fluoranthene
Flu	Fluorene
GC	Gas Chromatography
GIS	Geographical Information System
HCD	Higher-energy Collision-induced Dissociation
HESI	Heated Electrospray Ionization
HMW	High Molecular Weight
HP	High Performance
HRMS	High Resolution Mass Spectrometry
Hx	Hexane
HxCl	Hexachlorobenzene
IEO	Spanish Institute of Oceanography
InP	Indene[1.2.3-cd]pyrene
ION	Ionian Sea
Irg 168	Irgafos 168
Isd	Isodrin
Isop	Isoprocarb
LAB	Linear Alkylbenzene
LC	Liquid Chromatography
LMW	Low Molecular Weight
LOD	Limit of Detection
log k_{ow}	n-Octanol/water Partition Coefficient
LOQ	Limit of Quantification
LP	Low Performance
LRMS	Low Resolution Mass Spectrometry
MCP	Microchannel Plate
MPA	Marine Protected Area
MS	Mass Spectrometry or Mass Spectrometer
MSD	Mass Selective Detector
n.d.	Not described
Naph	Naphthalene
NEW	Northwestern Sea
NLE	North Levantine Sea
NMR	Nuclear Magnetic Resonance
OP	Organic Pollutant
OSPAR	Convention for the Protection of the Marine Environment in the North-East Atlantic
P. oceanica	*Posidonia oceanica*
PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated Biphenyl
PDA	Photodiode Array
PE	Polystyrene
PEC	Predicted Environmental Concentration

Pest	Pesticide
PhC	Phenolic Compound
Phe	Phenanthrene
PLE	Pressurized Liquid Extraction
PMT	Photomultiplier Tube
PNEC	Predicted No-Effect Concentration
POP	Persistent Organic Pollutant
PSA	Primary Secondary Amine
PtCl	Pentachlorobenzene
Pyr	Pyrene
Q	Quadrupole
QQQ	Triple Quadrupole
QuEChERS	Quick, Easy, Cheap, Effective, Rugged & Safe extraction method
R^2	Determination Coefficient
REDD+	Reducing Emissions from Deforestation and Forest Degradation
REDIAM	Andalusian Environmental Information Network
Ret	Retene
rs	Revolutions per Second
RSD	Relative Standard Deviation
RT	Retention Time
Simz	Simazine
SLE	South Levantine
SWE	Southwestern Sea
TBP	Tributyl Phosphate
TDC	Time-to-Digital Converter
TDN	1,1,6-Trimethyl-1,2-dihydronaphthalene
TIC	Total Ion Chromatogram
TOF	Time-of-Flight
TP	Transformation Product
Trif	Trifluralin
TRY	Terryhenian Sea
UAE	Ultrasound Assisted Extraction
UHP	Ultra High Performance
UNECE	United Nations Economic Commission for Europe
UP	Ultra Performance
UV	Ultraviolet
VL	Validation Level
w.w.	Wet Weight
WFD	Water Framework Directive

Chapter I

Introduction

1. Marine angiosperms

Approximately 100 million years ago various land flowering plants evolved and returned to the marine environment (Les et al., 1997). These marine sessile flowering plants are known as marine angiosperms or seagrasses. The adaptation to the new environment was possible in part by developing different ecophysiological traits, such as the clonal growth.

Seagrasses differ in several points from marine algae. For example, unlike algae, seagrass are macrophytes with differentiated and specialized tissues. Such tissues can be broadly categorized into three parts: leaves, which main activities are photosynthesis and nutrients uptake from the water column (Khan and Belik, 1995); rhizomes, which are above- and below-ground stems, key for the attachment of the specimen and storage of nutrients and carbohydrates (Alcoverro et al., 2001; Romero, 2004); and roots, which are in charge of the nutrients uptake from the sediment pore water (Lepoint et al., 2004, 2002).

Currently, there are merely 72 recognized seagrass species, grouped into four main families or types: *Cymodoceaceae*, *Hydrocharitaceae*, *Posidoniaceae,* and *Zosteraceae* (Pérez et al., 2015). As can be seen in Figure 1.1, seagrasses are spread around the globe, mostly in coastal ecosystems. However, their specific distribution is not yet clear and is based on estimations that vary greatly from study to study. A recent research study has claimed that seagrasses probably occupy a total of 160,387 km^2, but also possibly 266,562 km^2 with lower confidence (McKenzie et al., 2020). On the other hand, in a previous publication, the area obtained was 1,646,788 km^2, reflecting the lack of consensus (Figure 1.1; Jayathilake and Costello, 2018).

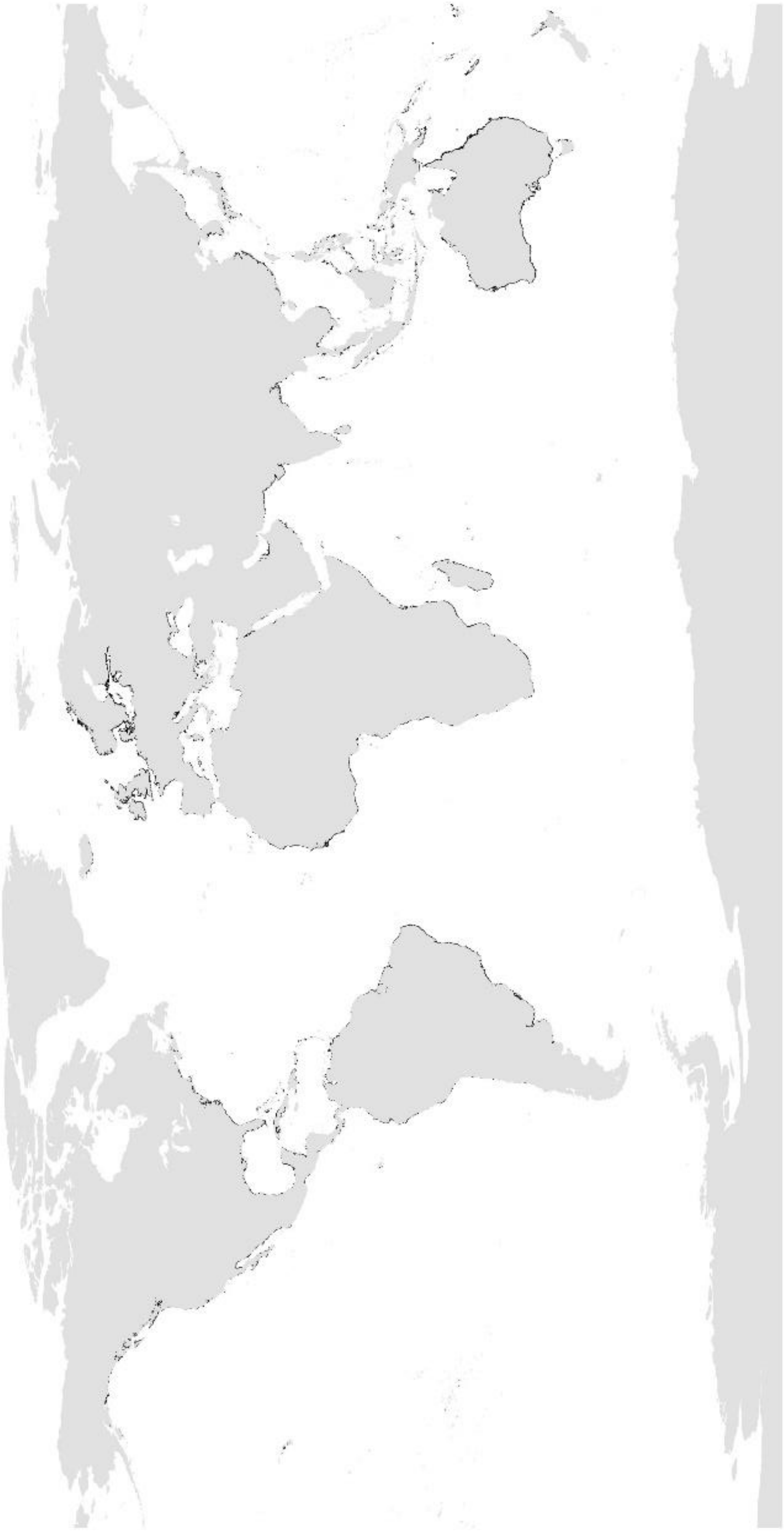

Figure 1.1. Seagrass distribution map according to Jayathilake and Costello (2018).

In addition to the scientific uncertainty, there is a generalized lack of awareness regarding seagrasses. These marine plants are frequently overlooked at several levels, by ordinary people, stakeholders, civil servants, and policy makers. Therefore, the multiple benefits provided and the consequent improvement in human well-being might be ignored according to some public perception studies (Barañano et al., 2022; Losciale et al., 2022). In some cases, seagrasses are even related to negative connotations, such as unpleasant or dirty, among others (Daby, 2003; De Falco et al., 2008; Ruiz-Frau et al., 2018).

Contrary to popular belief, seagrass meadows represent valuable marine ecosystem service providers with a major role in coastal protection, being also shelter and nursery for a myriad of species, thus fostering fisheries, food security and local economy (Unsworth et al., 2022). Additionally, their oxygen production rates and carbon sequestration capacity (blue carbon) are greater than many other ecosystems (Duarte et al., 2008). Lately organizations are seeing seagrasses blue carbon as natural-based solution to help mitigate the climate change impacts (IUCN, 2021; Miles et al., 2021). Seagrasses are also considered a promotor of water quality by reducing the effects of the water acidification, fostering sedimentation, as well as accumulating chemicals in their tissues acting as potential phytoremediators (Bergstrom et al., 2019; Gerstenbacher et al., 2022; Lewis and Devereux, 2009).

1.1 Global decline

Since conservation and restoration efforts are being taken, the loss of the seagrass in certain areas is decelerating (de los Santos et al., 2019). However, the worldwide meadows still face a net state of decline (Dunic et al., 2021). Their lost or change in distribution is correlated to environmental changes, such as pH, temperature, and water quality, among others, which makes them suitable sentinel species (Bonanno and Di Martino, 2017). The main factors to understand why these efforts do not reach an actual mitigation or reversion of seagrass ecosystem destruction have been pointed out. Firstly, research and conservation efforts on seagrasses are limited to certain parts of the globe, which is especially problematic when considering their almost worldwide distribution (Hind-Ozan and Jones, 2018). As shown in Figure 1.2-A, the efforts and the attention received by seagrasses pale in comparison to other coastal ecosystems, such as corals (Unsworth et al., 2022, 2019). This partly explains the lack of fundamental knowledge in their distribution, biology and the biophysiochemical traits needed to provide the ecosystem services (Nordlund et al., 2016). Rather than being a past problem, this imbalance in research effort seems to be widening as shown in Figure 1.2-B*.

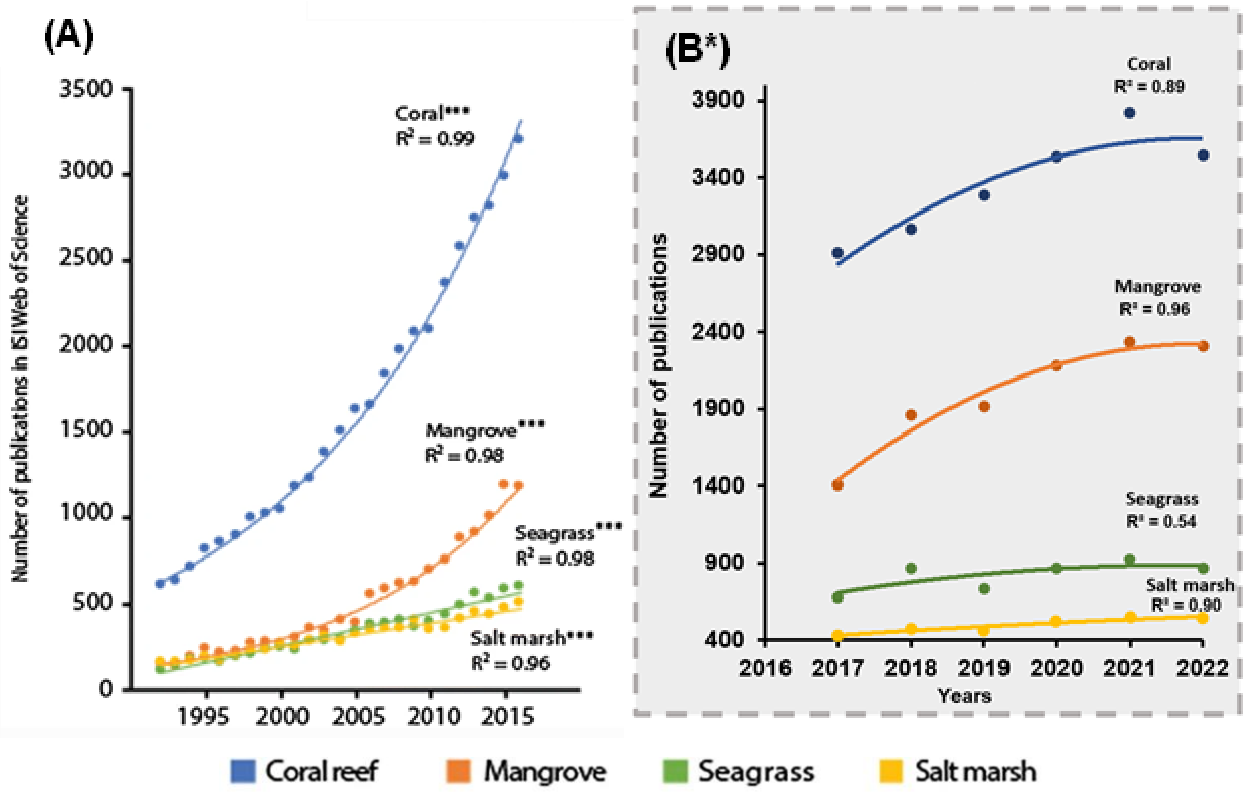

Figure 1.2. Imbalance in: (A) research effort between 1992-2016 (number of publications per year) allocated to each of the four ecosystems, and (B*) research effort from 2017 to 2022 calculated in this Thesis. Original figure extract retrieved from Unsworth et al. (2019) and edited by the author.

Secondly, the lack of allure or charisma of these ecosystems hamper the probability to fund seagrass-related research, as confirmed by several authors. For instance, Duarte et al. (2008) pointed out that "*seagrass research and conservation is underfunded, conducted by fewer people, and grows at an increasingly slower rate, than that on coral reefs (and to a lesser extent mangrove)*". Additionally, Unsworth et al. (2019) stated that "*the need for novel science and the drive for researchers to publish unique findings can skew our understanding of seagrass ecosystems, e.g. by limiting local or regional study replication*".

1.2 *Posidonia oceanica* meadows

1.2.1 Structure, functioning and distribution

Among Mediterranean seagrasses, one of them is endemic, i.e., native and restricted to the basin: *P. oceanica*. These meadows of special interest are thought to be foundation species namely habitat-forming organisms (Ellison, 2019). Their growth rate is considerably low, especially when compared to other fast growing Mediterranean seagrasses, such as *Cymodocea nodosa, Zostera noltii, Zostera marina, Halophila stipulacea, and Ruppia sp.* (Ruíz et al., 2009). According to Marbà and Duarte (1998) *P. oceanica* horizontal elongation rate estimated in 1-6 cm/yr. Despite this, *P. oceanica* is the dominant seagrass in the oligotrophic Mediterranean basin. This is part due to its prevailing clonal reproduction over the sexual propagation (Diaz-Almela et al., 2007), which has helped this seagrass cover extensive surfaces. Contrary to theoretical beliefs and certain empirical data, the clonal reproduction, and the resulting reduced genetic diversity, seems to favor *P. oceanica* survival. An exception to this finding occurs when extreme disturbance events take place (Arnaud-Haond et al., 2010). In fact, the *P. oceanica* clone, or genet, might occupy a meadows area that goes from meters up to hundreds of kilometers. This seagrass is also considered one of the most long-living beings (Arnaud-Haond et al., 2012; Mateo et al., 1997; Mínguez et al., 2018; Rovere et al., 2010). Currently, the largest genet is found in Formentera (Spain), dating from 80,000 to 200,000 yrs, meaning that it might be the eldest organism yet found in the planet (Arnaud-Haond et al., 2012). When focusing on individual shoots or plants, and not clones, the age varies. In this case, leaves live approximately 1 yr being short-lived tissues, while rhizomes live roughly 30-40 yrs (Boudouresque, 2012; Duarte, 2004; Pergent et al., 1989).

Even though *P. oceanica* is acknowledged as one of the most valuable ecosystems of the Mediterranean Sea, the meadows merely occupies ~ 2% of the entire basin (Sanchez-Lizaso, 2004). The settlement area comprises depths from 0 to 45 m (Duarte et al., 2007). In these infralittoral areas *P. oceanica* can thrive either in soft-sandy bottoms or rocky floors, depending on the average intensity of the hydrodynamics (Badalamenti et al., 2015; Sanchez-Lizaso, 2004). To anchor, the rhizomes develop roots which have been seen to reach up to 70 cm length below the sediment surface (Boudouresque, 2012). However, other studies have observed that normally roots grow up to 5–7 cm on rocky bottoms and 13 cm in soft-sandy bottoms (Balestri et al., 2015). Their direct root-sediment contact, and with the pore water facilitates not only the nutrients uptake, but also the intake of other hazardous substances present in the sediment (Gerstenbacher et al., 2022; Lewis and Devereux, 2009).

1.2.2 Bioindicator

P. oceanica is a common plant species of the infralittoral Mediterranean, which appearance and development seems to be correlated with the ecological succession of other precursors, such as *Cymodocea nodosa* (Moliner and Picard, 1952). The ecosystems are dynamics and natural disturbances are needed to ensure species composition and diversity (Mori, 2011). On the other hand, human disturbances seem to be gaining spatial distribution and temporal frequency, potentially having an elevated intensity and severity (define as the effect on organisms and ecosystems). This magnification of the disturbances in the sea has been recently called as The Blue Acceleration (Jouffray et al., 2020). Additionally, these human disturbances do not commonly occur isolated, but in synergisms (e.g., ocean acidification, marine heatwaves driven by climate change or plastic and chemical pollution). Thus, instead of fostering biodiversity in the ecosystem, human disturbances impact the structure and function of ecosystems, being these damages difficult to revert. Due to the sensitivity of *P.* oceanica to the aforementioned changes, this seagrass has been long considered a biological quality element in the context of the Water Framework Directive (WFD), and biomarker of the ecological status of the coastal waterbodies (Directive, 2000). To assess the status, their presence/absence and certain morphological parameters are measured, such as shoot density, leaves lengths, necrosis or epiphyte load (Boissery and Pergent-Martini, 2012; Ferrat et al., 2003; Lewis and Devereux, 2009; Montefalcone, 2009; Romero et al., 2007).

1.2.3 Ecosystem services

The diverse benefits provided by ecosystems to humans are named ecosystem services and are classified into three main categories (Reid et al., 2005). The first one, applied to the case of *P. oceanica*¸ is the Provision Service which includes food supply, pharmaceutical uses, construction, usage as fertilizers (Cornara et al., 2018; Maciá et al., 2016; Photiou and Vyrides, 2021) or mattresses, animal bedding, roof thermic insulation, compost, curative properties, among others (Diviacco et al., 2012; Renzi et al., 2022). Although nowadays some of these usages have gone out of favor, *P. oceanica* still provides plenty of benefits to society. Secondly, *P. oceanica* offers Regulatory Services, which play a crucial role in climate stability with actions such as CO_2 sequestration (or blue carbon), coastal protection and improvement in water quality (Kennedy et al., 2022). Thirdly, Cultural Services, as ecotourism, sense of belonging and scientific knowledge. Due to the prehistorical presence of *P. oceanica*, there is a millennia-old relationship with the coastal Mediterranean societies and culture.

Research on the economic valuation of the *P. oceanica* meadows ecosystem services were done, proving its significantly contribute to the human welfare. Results showed that *P. oceanica* ecosystems services are valued from 57 to 184 thousands €/ha/yr (Paoli et al., 2018; Rigo et al., 2021). Interestingly, *P. oceanica* contribution escapes the local scale, being its significance already recognized in a global perspective. This is mostly due to their efficiency in absorbing and storing CO_2 (Pergent et al., 2014). This has led to the inclusion of *P. oceanica* in official mitigation strategies like REDD+ (Reducing Emissions from Deforestation and Forest Degradation; Duarte et al., 2013).

1.2.4 Facing pressures

This emblematic seagrass, with scientifically recognized crucial services with influence in the human welfare, has been granted certain protection at different levels. For instance, the European Union's Habitat Directive (Council Directive, 1992) has classified *P. oceanica* as priority habitats (Type 112) due to their endemic character. Likewise, the Barcelona Convention has devoted an Action Plan merely for these meadows (UNEP, 2005a). Regardless of the current jurisdiction, there are still pressures exerting an undeniable impact on *P. oceanica* meadows. Examples are climate change driven problematics (such as marine heat waves), physical changes (e.g., coastal development, shipping, illegal or destructive fishing anchoring) or chemical alterations (brine and/or sewage discharges, runoff of contaminants and fertilizers, atmospheric deposition from industrial emissions, and so forth (Capó et al., 2020; Gerstenbacher et al., 2022; Guerrero-Meseguer et al., 2017; Lewis and Devereux, 2009; Sánchez-Lizaso et al., 2015). The activities lead to chemical alterations, transport exogenous compounds as organic micropollutants, trace metals or microplastics into the sea, deteriorating the water quality. According to a recent article, a strong correlation was observed between the fast decline in seagrass meadows (i.e., >25% loss within 2000 and 2010) and destructive demersal fishing as well as poor water quality (Turschwell et al., 2021). As for the poor water quality, this pressure is related to several contaminants and, among them, organic chemicals. Considering the few studies conducted in organic micropollutants in *P. oceanica* and the consequent lack of knowledge, this pressure should require special consideration when studying this Mediterranean seagrass.

Additionally, the vulnerability of *P. oceanica* meadows also resides in its low resilience and low recovery capacity from decline. This is particularly due to its slow growth and spread rate in comparison to the current declining rate, which is hundred-fold greater (Marbà et al., 2005; Sintes et al., 2006; Waycott et al., 2009). This indicates that once meadows have retrieved, their recovery is hardly possible. Some studies have observed that even long after the pressure (e.g., bottom-trawling fisheries) had ceased, the revival of the former meadows did not take place (Martínez-Daranas et al., 2021). Thus, effort must be put on earlier stages such as loss prevention, preservation, and conservation, instead of restoration. The prevention section should include the determination and monitoring of early warnings or biomarkers of great sensibility, which react to low concentrations of compounds that deteriorate the water quality, such as the organic micropollutants.

2. Endogenous compounds: Phenolic compounds in *P. oceanica*

Seagrasses, alike their terrestrial relatives, rely on two metabolic pathways for their functioning (Bernards, 2010). Under normal circumstances, the primary metabolism, where essential metabolites involved in the seagrass growth and development are produced, prevails. Notwithstanding, when exposed to severe or long-lasting pressures, either nature-driven (temperature, turbidity, marine currents, salinity, grazing) or human-driven, the secondary metabolism is fostered and outweighs the primary (Iriti and Faoro, 2009; Subhashini et al., 2013). This is the so-called metabolism shift, varying from an optimum state (or normal metabolism) to mild stress (stress metabolism) as shown in Figure 1.3 (Phase A and B). When the stress surpasses the tolerance threshold, the organism faces a state of metabolic distress. This compromises the secondary metabolism and irreversible damages occur (Phase C). The metabolism shift favors the production of chemical defenses (phytochemicals) to counteract the effects of the stress, over the production of metabolites required for other relevant activities, such as growth, photosynthesis, or nutrients uptake (Singh, 2009). This changes the chemical composition of the plant (Phase B). More in detail, the stress leads to an increased generation of damaging free radicals known as oxidative stress. These free radicals cause biochemical and physiological harm, and ultimately pathologies which jeopardize the survival of the organism. Likewise, the rise of free radicals promotes the synthesis of secondary metabolites (endogenous bioactive compounds) with a broad-spectrum antioxidant capacity and radical scavenging activity (Ammar et al., 2021; Cornara et al., 2018; Halliwell et al., 1995).

PhCs are an example of endogenous bioactive compounds. In the case of the seagrasses, PhCs are primarily generated via the secondary metabolism of the shikimic acid pathway (Subhashini et al., 2013).

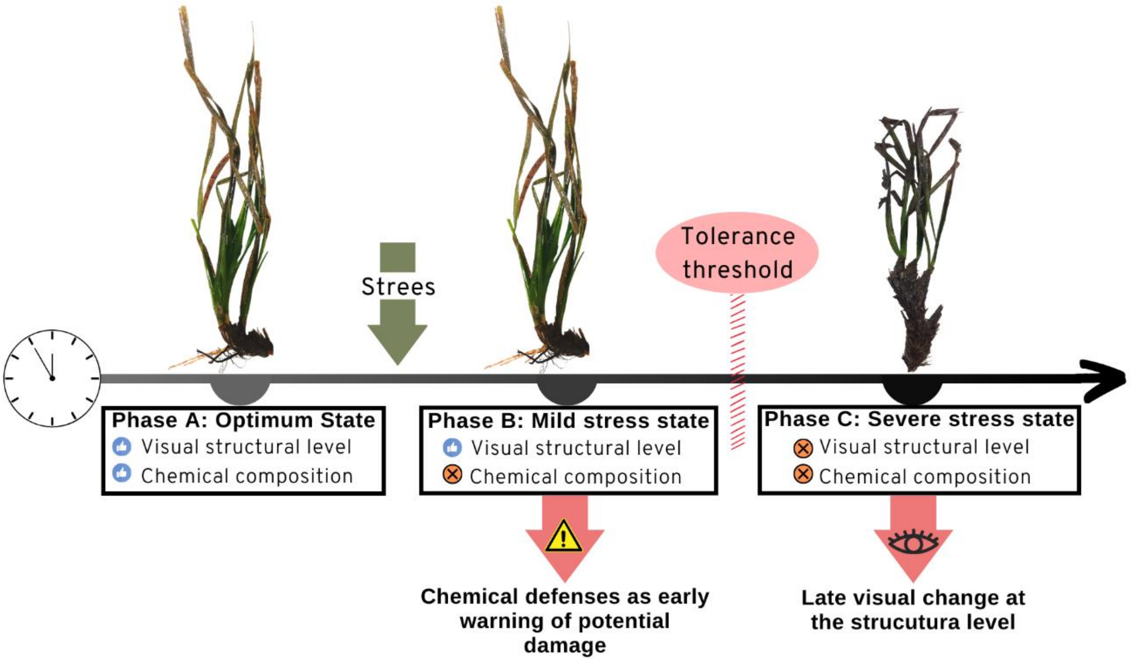

Figure 1.3. Temporal line in *P. oceanica* before and after being subject to stress, and their subsequent alterations in the morphology (visual structural level) and the chemical composition or defense system (such as the PhC content). Figure made by the author.

As mentioned before, PhCs hold relevant properties for the protection of the organisms. One of the most relevant properties is the high antioxidant activity to trap or intercept free radicals, neutralizing or reducing the severity of the damage. Due to their attributes these compounds have interesting applications, and therefore gather a growing scientific attention. For instance, PhCs are used in the health sector as antitumoral drugs, anti-inflammatories, and immunostimulants (Haznedaroglu and Zeybek, 2007). They are also employed as constituents in personal care products and cosmetics, or in the agri-food industry as nutritional medicine, dietary antioxidant supplementation, and antioxidant micronutrients used to develop functional food (Cornara et al., 2018; Lordan et al., 2011).

As for the PhCs structure, these comprise a wide range of chemical shapes, being the phenol the common basic structure. This molecule is made up of a hydroxyl group (OH) bound to an aromatic ring (Peñarrieta et al., 2014). From this basic molecule other ones more complex take form. To date more than 8000 structures have been identified (de la Rosa et al., 2019). There are a great number of PhCs. These can be broadly grouped into flavonoids and nonflavonoids. The flavonoids aggrupation is made up of 6 groups or families, such as the anthocyanidins or anthocyanins, flavanols (or flavan-3-ols), flavanones, flavones, flavonols, and isoflavones (Dias et al., 2021). The nonflavonoids are generally smaller and simpler than the flavonoids. These are the phenolic acids (also divided into hydroxybenzoic acids, hydroxycinnamic acids, and other hydroxyphenyl acids), the simple phenols and the lignans (de la Rosa et al., 2019).

2.1. Biomarkers of *P. oceanica*

Due to their rapid stress-specific response, PhCs lure also attention due to the potential usage as biomarkers or early warnings. In fact, published studies stated that PhCs, as well as other biochemical descriptors, seem to more accurately reflect, and earlier, environmental alterations (Migliore et al., 2007; Rotini et al., 2013). Despite these findings, current monitoring programs still assess the health status and trends of *P. oceanica* meadows using primarily traditional methods. These traditional methods are morphological or physiological alterations, such as meadows fragmentation and extent, cover of thriving and dead *P. oceanica*, shoot density, shoot length, lower limit depth, and so forth (Moreno et al., 2001; Rotini et al., 2013). Although traditional descriptors have been proved to satisfactorily depict the meadow's sate, they show a clear downside: when damage is visible and measurable, the seagrass capability to recover is already low (Figure 1.3, Phase C; Marbà et al., 2005). Contrary, the shift in the chemical composition precedes the morphological change, potentially warning and giving time to react, as depicted in Figure 1.3, Phase B. In an experiment testing eutrophication and burial effects in *P. oceanica* it was observed that the total phenolic content was associated with the meadows' survival acting as a useful stress predictor. Although, this variable did not alert of the meadow's imminent collapse, being therefore poorly trustworthy (Ceccherelli et al., 2018). Nonetheless, this study tracked the behavior of the total phenolic signal, not delving into individual PhCs which may provide a more reliable and precise warning.

Different PhCs, such as ferulic acid, caffeic acid, chicoric acid, caftaric acid, and cinnamic acid have been detectetd in *P. ocenica* leaves. However, rhizomes were explored merely in terms of total phenolic content (Kaal et al., 2018; Migliore et al., 2007; Rotini et al., 2013) through the Folin-Ciocalteu assay (Singleton and Rossi, 1965). Roots were similarly disregarded, being used solely in 2 studies (Cariello et al., 1979; Kaal et al., 2018). The main phenolic apportioning registered in *P. oceanica,* according to these articles, were hydroxycinnamic acids, belonging to the subclass phenolic acid and the nonflavonoids class. *P. oceanica* is believed to be a highly interesting matrix in terms of phenolic content, due to its *promising reservoir of potent and cell safe molecules* with capability to act against the oxidative stress and illnesses (Cornara et al., 2018; Haznedaroglu and Zeybek, 2007; Leri et al., 2018; Vasarri et al., 2021). A summary of the conducted studies focusing on individual PhCs in *P. oceanica* tissues is shown in Table 1.1, together with the followed extraction and analytical techniques.

3. Exogenous compounds: Organic pollutants in *P. oceanica*

Due to water pollution, other substances that can be found in *P. oceanica* tissues are chemical pollutants (i.e., exogenous compounds). These compounds trigger a natural removal strategy, or biodegradation process, by the host. The efficiency of this process relies on the physicochemical characteristics of such exogenous compounds. For instance, the most lipophilic or heavy tent to be more resistant to breakdown, thus more difficult to eliminate. When exogenous compound residues persist in the host, and bioaccumulate, detrimental impacts on the functioning of the hosting organism occur, even conditioning its survival capacity (Robinson et al., 2018). Examples are trace metals and OPs. In fact, and as mentioned before, researchers identified poor water quality (organic chemical pollution mainly) as the primary cause of the rapid seagrasses decline worldwide, together with destructive fishing (Turschwell et al., 2021).

Table 1.1. Compilation of studies that delved into the identification of individual PhCs present in *P. oceanica* leaves (◊), rhizomes (■) and roots (•) and their different extraction and analytical methods.

Source	Tissue	N° PhC	Sample (g d.w.)	Solvent (ml)	Cycles	Extraction Method	Total Extraction time	Analytical Technique
Sogin et al. (2022)	■ •	2	18.2	Methanol/water (3)	2	n.d.	48 h	GC-Q-MS
Hernán et al. (2022)	◊ ■	5	0.2	Methanol (5)	3	Shaking UAE	n.d.	UPLC-PDA-Q-TOF
Messina et al. (2021)	◊	9	5 w.w.; 1 d.w.	Methanol/ethanol (10)	1	Polytron	n.d.	HPLC-DAD
Ammar et al. (2021)	■	86	2000	Methanol (3500)	3	n.d.	n.d.	UPLC-Q-Orbitrap and HPLC-DAD
Astudillo-Pascual et al. (2021)	◊ ■ •	42	0.15	Methanol/water (3)	1	UAE	15 min	UHPLC-Orbitrap-MS
Cornara et al. (2018)	◊	7	100	Ethanol/water (1000)	2	Agitation	8 h	HPLC-MSD
Barletta et al. (2015)	◊	6	1	Ethanol/water (10)	1	Agitation	~15 h	UPLC-PDA
Grignon-Dubois and Rezzonico (2015)	◊	3	10	Ethanol/water (120)	2	UAE	48 h	HPLC-DAD-MS, NMR, and UV
Haznedaroglu and Zeybek (2007)	◊	7	5	Ethanol/water (200)	1	Heated bath	>3 h	HPLC-UV
Dumay et al. (2004)	◊	5	13	Ethanol/water (200)	1	Heated bath	>3 h	GC-FID-MS
Agostini et al. (1998)	◊	23	n.d.	Ethanol/water (200)	1	Heated bath	>3 h	HPLC-UV
Cuny et al. (1995)	◊	22	n.d.	Ethanol/water (200)	1	Heated bath	>3 h	HPLC*
Cariello et al. (1979)	◊ •	3	n.d.	Acetone (n.d.)	3	n.d.	9 days	HPLC*

DAD: Diode Array Detector; d.w.: dry weight; ESI: Electrospray Ionization; FID: Flame Ionization Detector; GC: Gas Chromatography; HPLC: High Performance Liquid Chromatography;; n.d.: not described; MS: Mass Spectrometry, MSD: Mass Selective Detector; NMR: Nuclear Magnetic Resonance; PDA: Photodiode Array Detector; Q: Quadrupole; TOF: Time-of-Flight; UAE: Ultrasound Assisted Extraction; UHPLC: Ultra High Performance Liquid Chromatography; UV: Ultraviolet; and w.w.: wet weight.

* Detector and/or analyzer not described.

3.2 Organic pollutants

The OPs are carbon-based chemicals with elevated toxicity. They are also known as organic micropollutants since even trace concentrations (from ng to µg L^{-1}) in the environment develop adverse effects in living organism and humans. These OPs are commonly classified into three groups: persistent, priority and emergent.

The first group, persistent OPs (hereafter POPs) show a long half-life and bond to the lipid tissues with ease, which promotes their persistency in the marine environment, bioaccumulation in the organisms and the consequent entrance into the food chain (Dromard et al., 2018). From the OPs, those regulated due to their remarkable threat that they supposed to the quality of surface waters (high toxic, hydrophobicity and widespread use and detection) are classified as priority OPs (the second group) by the WFD (2000/60/EC; Directive, 2000). Currently the list includes 45 priority substances or groups in the Annex X, although several more potential candidates are under consideration after being proposed by the Member States (Article 16; EC, 2019, 2000). The selection is based on an assessment of the risk and exposure, being commonly employed the ratios "predicted environmental concentration" (PEC) and "predicted no-effect concentration" (PNEC). The first ratio is obtained from exposure models while the second one employs ecotoxicology studies in water organisms. Overall, OPs which surpass a PEC/PNEC > 1 are considered a risk to the aquatic environment. Regarding the third group, the emerging OPs are compounds not considered in the routine monitoring programs of the marine environment and are only partially regulated (Pintado-Herrera et al., 2016). Unlike the other two groups, the emerging OPs are being recently detected thanks to the improvement of the analytical techniques, although they are not necessarily new substances. Examples of the last group are pharmaceuticals and personal care products (Arpin-Pont et al., 2016).

Three chemical classes of POPs are highlighted in this Thesis due to their potential detrimental impact onto the environment and human health: PAHs and pesticides listed as priority substances (Directive, 2013, 2000), and PCBs. Regarding PCBs, these are one of the original twelve classes POPs covered by the Stockholm Convention, also known as "The dirty Doze". The three classes here selected are considered xenobiotics, i.e., compounds not naturally present in living organisms. A list of the target POPs is included in Table 1.2.

Concerning PAHs, these represent a group of complex chemicals composed of multiple aromatic rings. Their physicochemical characteristics are critical to understand their accumulation in the organisms, such as the nonpolar nature, weight and log k_{ow} (n-octanol/water partition coefficient), which affect solubility (Patel et al., 2020). Regardless of the low or high molecular weight of the PAHs (LMW and HMW), both have been observed to be damage precursors, ranging from significant acute toxicity for LMWs to carcinogenic potential in presence of HMWs. The PAH class is particularly ubiquitous, considering their dual source, natural and anthropogenic combustion of organic material (from volcanos, forest fires, cosmic dust, or crude oil combustion and petrol derivatives). Overall, the vast majority of PAHs come from the decay or combustion of organic matter in environments with reduced oxygen and extreme temperatures. Nonetheless, they can also be found as manufactured by-products (impurities) of industrial processes, as pesticide co-formulants, and as the result of migration from plastics and rubbers (European Commission, 2018; Zeinali et al., 2011). To ensure environmental public safety, several EU legislations regulate their presence in food, water and ambient air (Commission Regulation, 2018; Directive, 2004). Note that further information regarding legislation involving the studied POPs is later described in the section *Current legislation on OPs*.

Table 1.2. Classes of POPs considered in this Thesis and their subsequent identification CAS number.

PAHs	CAS	PCBs	CAS	PCBs	CAS	Pest	CAS
Ac	83-32-9	PCB 18	37680-65-2	PCB 153	35065-27-1	Ald	309-00-2
Acy	208-96-8	PCB 28	7012-37-5	PCB 156	38380-08-4	Atr	1912-24-9
Ant	120-12-7	PCB 31	16606-02-3	PCB 157	69782-90-7	Chlp	2921-88-2
BaA	56-55-3	PCB 44	41464-39-5	PCB 167	52663-72-6	Die	60-57-1
BaP	50-32-8	PCB 52	35693-99-3	PCB 169	32774-16-6	End	72-20-8
BbF	205-99-2	PCB 66	32598-10-0	PCB 170	35065-30-6	HxCl	118-74-1
BkF	207-08-9	PCB 77	32598-13-3	PCB 180	35065-29-3	Isd	465-73-6
BghiP	191-24-2	PCB 81	70362-50-4	PCB 189	39635-31-9	o.p'-DDT	789-02-6
Chry	218-01-9	PCB 101	37680-73-2	PCB 194	35694-08-7	p.p'-DDD	72-54-8
DahA	53-70-3	PCB 105	32598-14-4	PCB 206	40186-72-9	p.p'-DDT	50-29-3
Flt	206-44-0	PCB 114	74472-37-0			PntCl	608-93-5
Flu	86-73-7	PCB 118	31508-00-6			Simz	122-34-9
InP	193-39-5	PCB 123	65510-44-3			Trif	1582-09-8
Naph	91-20-3	PCB 126	57465-28-8				
Phe	85-01-8	PCB 128	11096-82-5				
Pyr	129-00-0	PCB 138	35065-28-2				

Ac: Acenaphthene; Acy: Acenaphthylene; Ald: Aldrin; Ant: Anthracene; Atr: Atrazine; BaA: Benz[a]anthracene; BaP: Benz[a]pyrene; BbF: Benzo[b]fluoranthene; BghiP: Benzo[ghi]perylene; BkF: Benzo[k]fluoranthene; Chlp: Chlorpyrifos; Chry: Chrysene; DahA: Dibenz[a.h]anthracene; Die: Dieldrin; End: Endrin; Flt: Fluoranthene; Flu: Fluorene; HxCl: Hexachlorobenzene; InP: Indene[1.2.3-cd]pyrene; Isd: Isodrin; Naph: Naphthalene; Phe: Phenanthrene; PtCl: Pentachlorobenzene; Pyr: Pyrene; Simz: Simazine; and Trif: Trifluralin.

On the other hand, PCBs consist of a group of synthetic organic chemicals, composed by carbons, hydrogen and chlorine atoms (OSPAR, 2004; USEPA). There are 209 PCB congeners, i.e., clearly define chemical species inside the PCB category. The chlorine atoms are of special interest since the number and position in the molecule determines many of their physicochemical properties. For instance, these compounds show great stability, being able to resist both extreme temperatures and pressures. Due to their properties, PCBs had applications as electrical insulators, heat conductor or lubricants in hydraulic equipment, pigments, and dyes, but also in paints as plasticizers or in plastics/rubber products, among others. Because of their detrimental impacts their commercial production came to an end in the 80s in most countries (EFSA, 2022). However, due to their persistence it is still common to find certain PCBs in different environmental compartments (Domínguez et al., 2018; Merhaby et al., 2015).

Alike the PAHs, PCBs are fat-soluble, being easily absorbed by the fatty tissues embedded in the organisms. Once in the tissues, PCBs are usually not properly removed, which fosters their bioaccumulation. PCB congeners show a wide range of toxicity. In marine animals, PCBs have been seen to develop cancer in a relative short period, whilst in humans, long exposure causes alterations at a nervous, reproductive, and immune system levels. Because of their detrimental impacts their commercial production came to an end in the 80s in most countries (EFSA, 2022).

As for the pesticides, these are chemical compounds utilized to prevent or eradicate pest or disease vectors such mosquitos, but also are plant regulators (USEPA, 2023; WHO, 2020). Each pesticide is manufactured for a determined plague/s or pests such as acaricide, bactericide, fungicide, herbicide, insecticide, miticide, and so forth. This means that their components and their nature differ from one to another, encountering carbamates, organochlorines, organophosphates, pyrethroids pesticides, among others. Therefore, each pesticide shows individual properties and toxicological effects. Several pesticides can also be classified as persistent, remaining in the environment for long periods and enhancing their hazard. In fact, an ample fraction of the current “most unwanted POPs” are pesticides (Annex A; UNEP, 2019).

The application of pesticides dates to the early stages of the agriculture, when Romans and Greeks used “chemical methods” (EU Food Safety, n.d.). When these chemicals are utilized on crops, a noteworthy percentage does not reach their target and contaminate soil and water (Carriger et al., 2006; Parween et al., 2016). Nowadays there is still a dependence on (synthetic and natural) pesticides to guarantee food security and meet the market demand. Rather than reverse this trend, and considering that projections indicate that by 2050 it a ~ 60 percent boost in food production between 2005/07 and 2050 would be required (Alexandratos and Bruinsma, 2012), the application of pesticides will increase (EU Pesticides Directive 91/414/EC and the Pesticide Action Network; PAN, 2023).

Further descriptions of the compounds of study (PAH, PCBs, and pesticides), their hazards and current legal state can be found in the European Chemicals Agency (ECHA; https://echa.europa.eu/), searching by name o CAS number (Table 1.2).

Summarizing, PAHs, PCBs, and pesticides are still found in the environment and biota, being relatively ubiquitous (Jin et al., 2020; Joksić et al., 2022). Once in the organism, these complex substances are difficult to eliminate, potentially leading to severe and irreversible health impacts such as cancer, damage in the immune system, malfuctioning reproductive systems, birth defects, greater vulnerability to disease, and others (UNEP, 2019). Moreover, in the case of the mammals, these compounds can be transferred to the offspring via the placenta and breast milk (Antignac et al., 2016; Aznar-Alemany and Eljarrat, 2020). Their ubiquity in the environment have led to the development of baselines o background concentrations that are considered in the monitoring programs. Such Background Assessment Concentrations (BACs) stress the limit above which baseline (natural or assumed concentration of contaminants established with historical and current data) levels are surpassed (OSPAR, 2008).

3.3 Current legislation

The legislation regulating OPs is dynamic, constantly updating the number of prohibited compounds. However, despite the dangerous traits of OPs, several economic sectors still rely on the usage of such substances. It seems that, current society still cannot afford to stop using them. For these cases, certain international and national organisms and legislations have set maximum amounts that can be traded or released into the environment, as described in Table 1.3. These organisms and legislations work together to define lists of subtances of concern, trying to reduce their usage and promote frequent monitoring programme. While somes do not refer directly to a specific environmental compartment (such as the Roterdam and the Stockholm Convention), other does, such as the UNECE Convention, OSPAR Convention, the WFD, among others.

Table 1.3. Operating legislation on OPs sorted from global, to European and national levels, together with a brief description of their contributions and requirements.

Legislation and Compartment	Title	Brief description
Rotterdam Convention (UNEP and FAO, 1998)	Convention on Prior Informed Consent (PIC) procedure for certain hazarous substances and pesticides in international trade	Banned or severely restricted chemicals or pesticides are listed in Annex III and are subject to the PIC procedure. A Decision Guidance Document must be prepared, giving basic information in the chemicals, hazards and alternatives. The PIC procedure collect and share the decisions made by importing Parties on their interest in receiving shipments of chemicals listed in Annex III and to ensure compliance by exporting Parties.
UNECE Convention (UNECE, 1998, 1979) - Air	Convention on Long-range Transboundary air pollution-POPs and Heavy Metals protocols	This convention gave rise to the Protocol on Persistent Organic Pollutants. Each Party must cease production and use of the substances listed in Annex I and deal with the waste as indicated. Likewise, Parties shall only use the substances listed in Annex II for the purposes described therein. Additionally, it is encouraged to reduce the total annual air emissions of each of the substances listed in Annex III. Referent limit values are seen in in Annex IV.
Stockholm POPs Convention (UNEP, 2019)	22 May 2001, demanding to actively take *meassurements to eliminate or reduce the release of POPs into the environment*	Each Party is encouraged to prohibit and/or take the legal and administrative actions to cease the fabrication, use and trade of the chemicals listed in Annex A. Additionally, each Party is intend to restrict the production and use of the chemicals listed in Annex B.
OSPAR Convention (OSPAR, 1992) - Water, sediment and biota.	Convention for the Protection of the Marine Environment in the North-East Atlantic	Parties are meant to prevent and eliminate pollution from land-based sources (Annex I), dumping or incineration (Annex II), offshore sources (Annex III) or other sources, in order to protect the maritime area. Likewise, Parties shall conserve the marine ecosystems and restore those affected. Furthermore, Parties shall regularly evaluate the quality status of the marine areas (Annex IV) and report to the Commission.
EU Water Framework Directive (WFD); (Directive, 2000) - Water	Directive 2000/60/EC, and the Daughter Directive 2008/105/EC	Requires Parties to protect and restore water bodies to achive a "good status", chemically and ecologically (Annex V). Additionally, Parties are intended to periodically monitor a list of priority substances in surface waters (Annex X).
EU Directive 2013/39/EU, Decision 2018/840; (Directive, 2013; EU Decision, 2018) - Water and biota	Regarding priority substances in the field of water policy, and amending the Directives 2000/60/EC and 2008/105/EC	The Annex X of the Directive 2000/60/EC is replaced by Annex I of this Directive 2013/39/EU. Moreover, the Environemntal Quality Standards (EQS) are implemented (Part A of Annex I). In Decision 2018 a new list of subtances to be monitored is rleased.
Royal Decree 817/2015 (BOE, 2015) - Water	Trasposting the EU Directive 2013/39/EU, Decision 2018/840	Establishing the Environmental Quality Normative of priority subtances in Spain. It also publishes several steps to assess the chemical, physicochemical, and biological status of a water body. The aim is to ensure the "good status" of a water body. For this purpose, a technical description of the analysis are described (Annex III). An additional list of priority substances and other pollutants is given (Annex IV and V).

3.4 Organic pollutants in the marine ecosystem

At the same time, numerous studies focused their effort on investigating manners to replace the OPs (e.g., using greener compounds or safeners) or stopping their entrance to the marine environment. Examples are the usage of incinerators, solvent extractions in wastewater plants or other techniques, such as filters in industries or bio-remediation (Björklund and Li, 2017; Gaur et al., 2018; Moško et al., 2021). However, these methods are insufficient to eradicate the problem due to their cost, or simply because the obtained transformation products (TPs) might be of higher toxicity than the parent OP (Chibwe et al., 2015; Kang et al., 2019). Hence, OPs might still scape the protective barriers, reaching the marine ecosystems (McKnight et al., 2015). In the case of the organic marine pollution, the key sources are atmospheric deposition, runoff, and discharge from urban (e.g., wastewater, sewage effluents), as already depicted by Tielens (2008) in Figure 1.4.

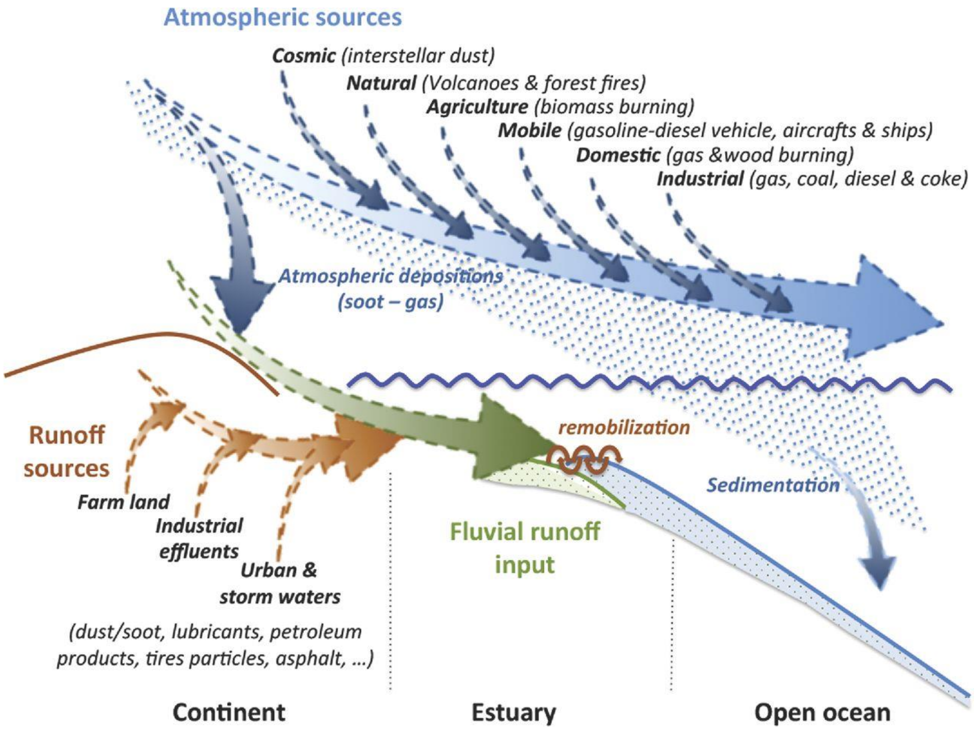

Figure 1.4. Main sources of OPs entering the marine environment. Source: Tielens (2008).

Once in the marine environment, these chemicals follow a highly complex cycling controlled by numerous variables at different scales (regional and global), even interacting with the carbon cycling. In a very reduced and simplified manner, these mostly hydrophobic substances can follow several pathways as described in Figure 1.5. Briefly, some suspended OPs might be directly filtered or uptake by biota, such as mussels and seagrasses. If these remain in the water column, they usually get attach or bond to floating particles (such as organic matter, sediment, plastic fibers, or phytoplankton). Two paths can be followed for those OPs linked to the suspended particles: (i) incorporation to the food web, feeding zooplankton, accumulating in higher trophic levels (biomagnification), and growing in toxicity; or (ii) the colloidal suspension sinks onto the sea bottom, where they tightly adhere to sediments. This late process usually occurs with those HMW OPs, transforming the sediment into an OP reservoir with potential to feed the water column by resuspension due to the marine dynamics or OPs remineralization (Farrington and Hideshige, 2014; Tielens, 2008). Additionally, this sedimentary stock of OPs, can be also taken up by the seagrass' roots or filtered by sediment-related fauna such as sea cucumbers or fish (e.g., red mullet), forming a new entrance into the food chain (Farrington and Hideshige, 2014).

Considering the different OP routes and accumulation points, the Barcelona Convention proposed the surficial sediments as the abiotic environmental compartments to be assessed in the monitoring programs (UNEP/RAMOGE, 1999; UNEP, 2005b). Nowadays, the Marine Strategy Framework Directive (Directive 2008/56/CE), conduct periodical analyses of a wide range pollutants, including OPs, in surficial sediments as well as sediment-related fauna, such as the mussels *Mytilus galloprovincialis* and the red mullet fish (*Mullus barbatus*). The ultimate objective of this Marine Strategy is to achieve good environmental status in the European marine systems (Law et al., 2010) by acknowledging the current pollution magnitude in a first stage.

The exposure of other Mediterranean seagrasses to organic xenobiotics has been seen to trigger defensive responses stressing the organisms, but also jeopardizing other relevant activities and causing the meadow's recession (Espel et al., 2019; Malea et al., 2022, 2020). Moreover, the microbiota associated with seagrasses, required for the N_2 fixation, is also affected by the pollution, affecting therefore the plant's survival capacity (Brackup and Capone, 1985). Note that there is no information regarding the specific toxicity response in *P. oceanica*, perhaps due to the so far impossibility to cultivate them in the laboratory. Solely the seeds and the early stages of the plants have thrive under laboratory conditions, but not the mature plants (REE, 2018).

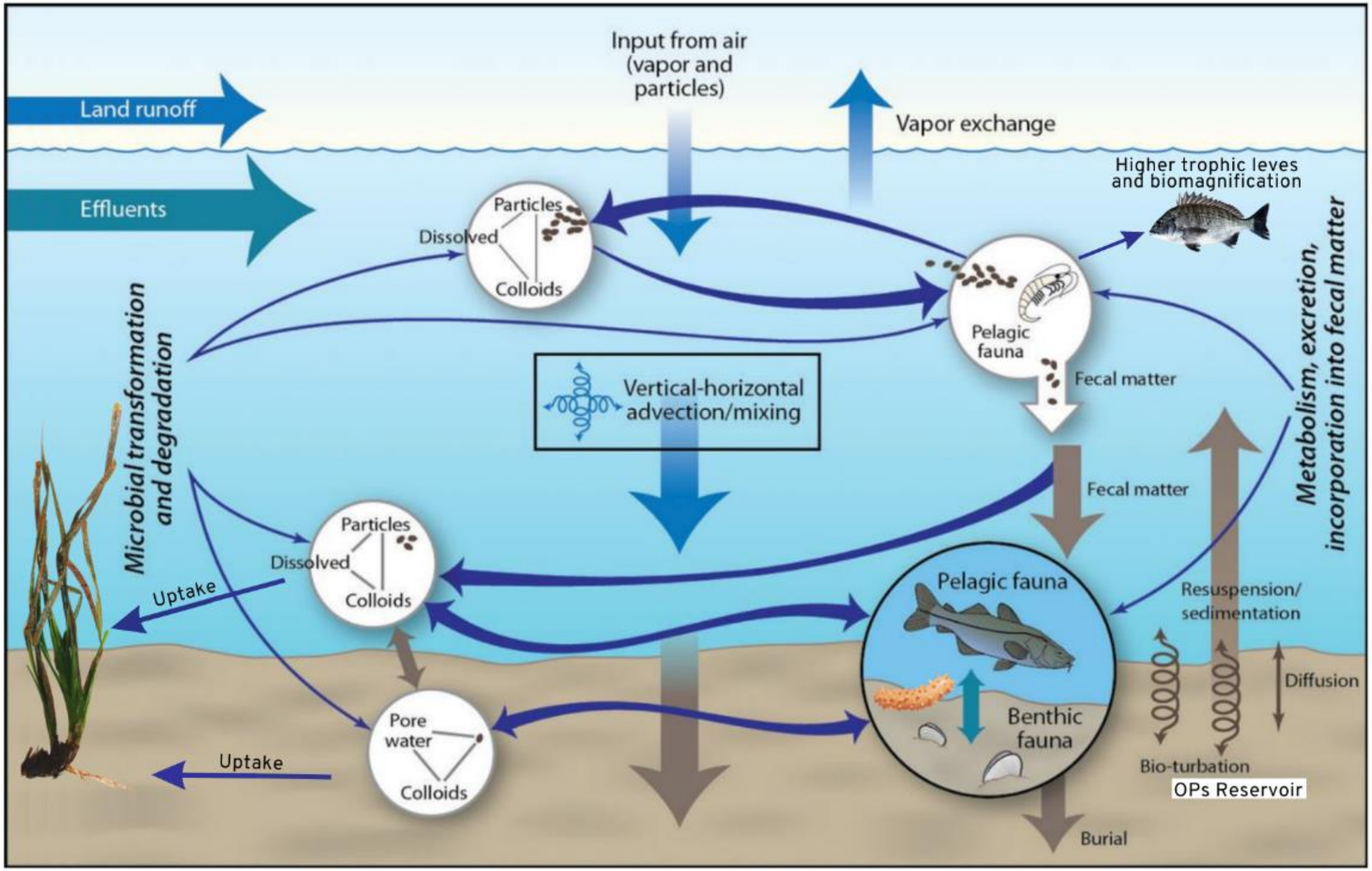

Figure 1.5. Diagram of the common pathways taken by the OPs in the marine environment and acquisition routes of exogenous compounds into *P. oceanica*. Images retrieved from Farrington and Hideshige (2014) and adapted for this Thesis.

3.5 Organic pollutants in *P. oceanica* and sediments

The environmental OPs can be: (i) accumulated in fatty tissues of organisms; (ii) abiotically transformed (e.g., via photodegradation) forming TPs; and (iii) biotically metabolized to metabolites. Compared to their parent form, the TPs and metabolites show distinct environmental and ecotoxicological traits and are usually of greater toxicity, requiring special attention (Anagnostopoulou et al., 2022; Bandowe and Meusel, 2017; Chibwe et al., 2015; Clergé et al., 2019; Kang et al., 2019; Virág et al., 2007). The key to their toxicity character lays on their higher solubility, which promotes the mobility and bioavailability. In fact, the not detection of parent-OPs dog not necessarily correspond to unpolluted environmental samples, as it is likely that TPs and/or metabolites are present. Despite this, OP TPs and metabolites, which have been long neglected, should be considered for an accurate assessment of marine OPs. This is also the case of the seagrass *P. oceanica* and marine coastal sediments in the Mediterranean. *P. oceanica* has been frequently used as a bioindicator of coastal contamination by trace metals, but barely considered for OPs (Bonanno and Di Martino, 2017; Bravo et al., 2016). The unbalance research effort between trace metals and OPs studies in *P. oceanica* can be up to certain extend ascribed to the difficulty to extract OPs and cultivate mature *P. oceanica* shoots under laboratory conditions. As a result, only a few studies have been published regarding OPs in *P. oceanica*. Table 1.4 compiles these different studies with reference to the extraction and analytical methods used for the determination of the compounds. Likewise, the *P. oceanica* specific biochemical response to OPs has not been investigated. This might be again also due to the so far impossibility to cultivate this seagrass in the laboratory or aquarium, hindering ecotoxicological tests. Nonetheless, from studies carried out with similar seagrasses, is possible to speculate analogous outcomes. In this sense, certain seagrasses seem to have the capability to counter act the OP-derived oxidative stress by producing PhCs and other enzymatic mechanisms (Malea et al., 2022). Nonetheless, detrimental effects are likely to occur such as growth inhibition, damage in nutrients uptake systems and a lowering in the resilience (Brackup and Capone, 1985; Espel et al., 2019; Malea et al., 2020).

Before this Thesis merely six articles were published regarding POPs in *P. oceanica,* as can be observed in Table 1.4. Out of these 6 articles, three were published between 2011 and 2014, and within 2021-2022 another 3, showing an apparent recent increasing interest for this matter. Originally, scientific interest seemed to focus on PAHs, shifting towards wider studies including PCBs and pesticides in the recent years. However, none of them considered TPs or metabolites.

Table 1.4. Brief description of the known studies regarding POPs in *P. oceanica* tissues, such as leaves (◊), rhizomes (■) and roots (•).

Source	**Tissue**	**POPs**	**Sample (g d.w.)**	**v/v Solvent ml**	**Extraction Method**	**Total Time**	**Analytical Technique**
Pergent et al. (2011)	◊	PAHs	0.5	n.d.	PLE	n.d.	HPLC-FD-UV
Mauro et al. (2013)	◊	PAHs	n.d.	1:1 Hx/DCM (n.d.)	PLE	n.d.	HPLC-FD
Apostolopoulou et al. (2014)	◊ ■ •	PAHs	0.5	3:1 Hx/Ace (n.d.)	Soxhlet	24 h	GC-Q-MS
Balcioğlu (2021)	◊	PAHs	n.d.	n.d. CycloHx (n.d.)	Saponification-evaporation	n.d.	HPLC-DAD
Jebara et al. (2021)	◊	PAHs PCBs Pest	5.0	9:1 Hx/EtAc (10)	QuEChERS-evaporation	n.d.	HPLC-QQQ-MS/MS
Renzi et al. (2022)	◊	PAHs PCBs Pest	n.d.	n.d.	PLE	n.d.	GC-Q or GC-Ion trap
Astudillo et al (2022 and 2023)	◊ ■ •	PAHs PCBs Pest	0.15	9:1 Hx/EtAc (3)	Polytron	~12 min	GC-Q-Orbitrap-MS

Ace: Acetone; EtAc: Ethyl acetate; CycloHx: Cyclo hexane; DCM: Dichloromethane; d.w.: dry weight; FD: Fluorescence detector; GC: Gas Chromatography; HPLC: High Performance Liquid Chromatography;; Hx: hexane; MS: Mass Spectrometry; PAHs: Polycyclic Aromatic Hydrocarbons; PCBs: Polychlorinated Biphenyls; Pest: pesticides; PLE: Pressurized Liquid Extraction; Q: Quadrupole; QQQ: Triple Quadrupole; QuEChERS: Quick, Easy, Cheap, Effective, Rugged & Safe extraction method; and UV: Ultraviolet Detector.
n.d.: not described.

On the other hand, between 2005 and 2019, a greater number of scientific articles were published, compared to *P. oceanica*, referring to OPs in sediments from the Mediterranean Sea. Likewise, the number of studies varied considerably among sub-basins. In this sense, a remarkable unbalanced in terms of research effort could be appreciated, highlighting the Alboran Sea (ALB) as the sub-basin less monitored (Figure 1.6-A; see Annex section for the list of papers considered in this analysis). Additionally, PAH studies seem to gather the bulk of the scientific attention (Figure 1.6-B). Interestingly, and as occurred with *P. oceanica*, no monitoring programs included TPs or metabolites.

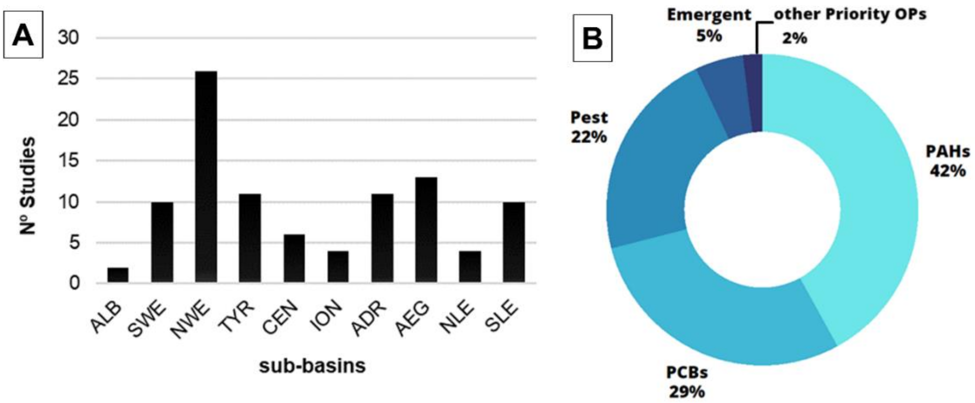

Figure 1.6. (A) Number of studies in sediments per sub-basin in the Mediterranean Sea from 2005 to 2019 (ALB: Alboran; SWE: Southwestern; NEW: Northwestern; TRY: Terryhenian; CEN: Cretan; ION: Ionian; ADR: Adriatic; AEG: Aegean; NLE: North Levantine; and SLE: South Levantine). (B) Percentage of studies researching different OPs in marine sediments from the Mediterranean Sea during between 2005 and 2019 (OPs: Organic Pollutants; PAHs: Polycyclic Aromatic Hydrocarbons; PCBs: Polychlorinated Biphenyls; and Pest: Pesticides)

4. Handicaps in phenolic compound and organic pollutant studies conducted in *P. oceanica*

Skimming the state-of-the-art before 2021 for PhCs and exogenous OPs in *P. oceanica*, there is no doubt that the previous investigations brought relevant unknown information to the scientific community. However, the need for a more complete investigation is clear since there are still well-defined information gaps in both areas of study and therefore room for improvement. These handicaps can be summarized in 6 main points:

1. *P. oceanica* has been explored incompletely, mostly narrowing the study to the temporal foliar tissues. Usually interesting tissues, such as roots and rhizomes, are not considered. For instance, the phenolic signal here occurring is usually neglected. Additionally, seeing the connection to the sediment and the longevity of these tissues, these might hold relevant data of the sedimentary OP stock and historical pollution information. The record of the past chemical events would be of special interest in coastal areas were no background pollution information is available.
2. The Green Chemistry Principles are not followed, neither are the White Analytical Chemistry principles (Gałuszka et al., 2013; López-Lorente et al., 2022; Nowak et al., 2021). There is a general disuse of miniaturized methods, lacking an optimization aiming to reduce the amount of sample material required and extraction solvent. Therefore, the extractions were not eco-friendly (i.e., respectful with the matrix of study and environment). In fact, we

can presume to an extend that rhizomes and roots have been poorly explored due to the need of an elevated amount of sample, likely causing the death of this relevant seagrass which collection is restricted.

3. The described experiments were complex and time consuming. Taking from hours to days in some cases (Table 1.1 and 1.4). This is translated into a higher economical cost and a delay in the presentation of the results. At the same time, simple and affordable methods long used in other fields of study, such as in the agri-food analytical labs, have not been tested. Meanwhile, simple gestures, such as adopting good resulting extraction methods from other areas, might simplify the experiment and make it available for a wider range of researchers.
4. Regarding PhCs, the majority of the studies provided information about total phenols content (through Folin–Ciocalteu assay) or the lignin content (Dumay et al., 2004; Kaal et al., 2018; Migliore et al., 2007; Rotini et al., 2013) instead of individual compounds.
5. Concerning OPs, several areas from the Mediterranean basin have been long disregarded, as well as the study of TPs and metabolites (Figure 1.6).
6. The analytical techniques employed were LC and GC coupled to traditional detectors using UV-Vis and fluorescence detection, and low resolution mass analyzers such as quadrupole (Q) and triple quadrupole (QQQ). Therefore, no studies have used the current improvements in mass spectrometry (MS) associated with the implementation of high-resolution analyzers, such as to broaden the search mode to non-target analysis (i.e., suspected and unknown). For a better comprehension of this point, a detailed description of MS is given in the following section.

Note that several of the recently published articles (from 2021 onwards) have considered some of the aforementioned lacks (such as the study of individual secondary metabolites instead of total phenols, and the usage of more actual analytical techniques; Table 1.I). Nonetheless, other advantages are still disregarded, such as for instance the incorporating the screening for suspected and unknown compounds.

5. Mass spectrometry approaches for the monitoring of phenolic compounds and organic contaminants in environmental samples

To understand how MS could contribute to the analysis of *P. oceanica* and other ecological studies, is necessary to comprehend certain analytical concepts. LC and/or GC is commonly coupled to MS since the late 1950s and early 1970s. In terms of utility, what makes a study choose between LC or GC is the average behavior of the compounds of study. For more polar chemicals (less volatile), such as the PhCs, is recommended to used LC, whereas GC is suitable for less polar substances (e.g., the vast majority of the POPs). Regardless of using LC or GC, here is where the separation of the different molecules occurs, more precisely in the analytical columns. Afterwards, these molecules are subject to a fragmentation into smaller ions inside a collision cell (Figure I.7-A). These ions are then trap in the C-trap and sent to the mass analyzer, such as the Exactive Orbitrap or Q-Orbitrap employed in this Thesis. In general terms, these molecules in the mass spectrometer are: (1) ionized by the ion source and fragmented; (2) then reorganized and separated regarding their exact mass and charge (*m*/*z*), and (3) detected together with their relative abundances (Figure I.7-B). Although mass analyzer is considered the core of the mass spectrometer, the detector also plays a key role by converting the ions into detectable (electrical) signals. The precision and resolution of MS, has increased along time, offering a wide array of possibilities. Commonly used analyzers, such as those employed in PhCs and OPs studies (Table I.1 and I.4), are classified into LRMS and HRMS in Table I.5. In this sense, an increase in resolution means the capability of providing higher precision of *m*/*z* measurement, giving 4 to 5 decimal digits and therefore greater capability to identify compounds. LRMS is less sensitive, providing 2 decimal digits instead, thus unable to distinguish two substances with similar masses.

Once the analysis finalizes, a raw data file per sample is obtained, containing the spectral data and a “Total Ion Chromatogram” (TIC). The TIC gathers information from multiple compounds present in the sample, including other matrix components. To screen all the information and to elucidate which compounds are present in the sample, the raw data file must be thoroughly processed.

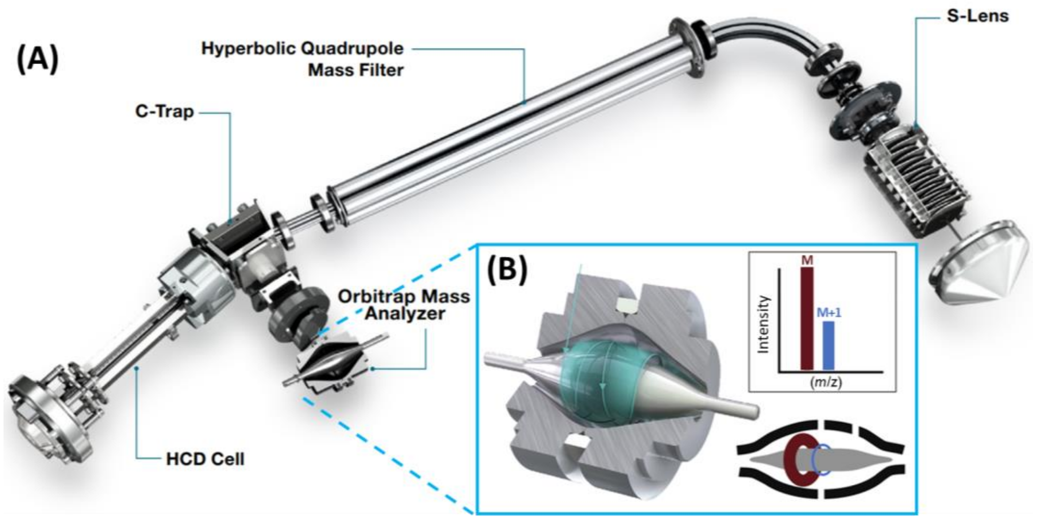

Figure 1.7. (A) Chart of a Q-Orbitrap MS ion path, and (B) Orbitrap Mass Analyzer diagram with ion trajectories and their corresponding mass spectrum. Images retrieved from Bromirski (2018) and Hofmann et al. (2020) and adapted for this Thesis. HCD Cell: Higher-energy Collision-induced Dissociation Cell.

Table 1.5. Several types of mass analyzers sorted out by resolution.

Resolution	Mass Analyzers
LRMS	Quadrupole (Q)
	Time-of-flight (TOF) without reflectron
	Ion Trap (3D or linear)
HRMS	TOF with reflectron
	Orbitrap
	Fourier Transform Ion Cyclotron Resonance
	Magnetic Sector[1]
	Hybrid analyzers[2]

[1] With high-resolution sector magnets
[2] Combining high-resolution techniques (e.g., Q-TOF and Q-Orbitrap)

5.2 New opportunities brough by high resolution mass spectrometry

Therefore, MS is classified as HRMS or LRMS depending on the used analyzer. As briefly introduced before, a beneficial improvement generally observed in HRMS, compared to the LRMS, is the increase in sensitivity and selectivity. The capability to measure up to 5 decimal digits, among other things, helps identify different compounds, even when these show the same *m*/*z*, which used to bring to confusion. This also allows for a better differentiation between genuine signals and artifacts (such as other components derived from the matrix, stationary or mobile phases, or lab-contamination; Belarbi et al., 2021). This sensitive analysis also gives the possibility to employ a small amount of sample, which is crucial in studies handling protected organisms, such as the case of *P. oceanica* seagrass.

The different screenings that can be conducted are also defined by MS resolution. Briefly, the high sensitivity of HRMS analyzers, concretely Orbitrap, allows for retrospective studies. This means that a greater amount of information can be obtained while saving time and resources, using the same raw data file (i.e., no need for extraction or injection repetition). Note that the resulting raw data file can be saved in a memory disk, transported as well as been re-processed at any time. Summarizing, in HRMS three different searching modes can be utilized to withdraw information (Du et al., 2022). When all modes are employed (hereafter 3-way analysis), a more accurate and comprehensive picture of the samples' characterization is attained. The 3-way analysis includes:

1. Target screening, which is the so far most employed analysis. In this case only compounds of known characteristics (through the injection of their corresponding reference standards) are searched.
2. Suspect screening, a list of compounds that are presumed to be in the sample are searched. In this case, due to the uncertainty, no reference standards are utilized, and the necessary information (such as the molecular formula, *m*/*z*, confirmation ions, and so forth) for the tentative identification information is obtained from *in-house* databases, open databases, specialized software, and scientific literature.
3. Unknown screening, use to (tentatively) identify additional compounds present in the samples that have not being yet considered in the target or suspect screening. This screening is conducted through computerized data processing, by comparison of the sample information to open database and online spectral reference libraries (such as the NIST, PubChem, ChemSpider, and Human Metabolomics, CheBI, KEGG, MolBank, Nature Chemistry, Sigma Aldrich or Phenol Explorers, among others).

The last two screenings are called non-target analysis (or retrospective studies). As mentioned before, non-target screenings cannot be successfully carried out when using LRMS, overlooking a multitude of compounds, such as TPs and emerging pollutants (Suman et al., 2022).

5.3 Compounds identification and confirmation

Through the analysis complex molecules (parent ion, precursor ion or quantification ion) have been fragmented into different ions (confirmation or characteristic ions). The presence of these characteristic fragments enlightens the researchers about the potential original molecule. The fragments of an elaborate molecule are being re-assembled to unravel the original compound. In this sense, it is mandatory to assure that the observed ions correspond to the same molecule, meaning that they should share virtually the same retention time (RT) in which they were detected by the equipment.

Despite finding the precursor ion and their corresponding characteristic ions at the same RT, the structure is not yet unequivocally identified or confirmed until reference standards of the potential compounds are also analyzed in the same conditions. When the reference standard and the potential compound match in terms of ions and RTs, confirmation is reached (Schymanski et al., 2014). Nonetheless, thanks to HRMS the number of detected chemical compounds has dramatically increase, being virtually impossible in most of the cases to be in possession of all the required reference standards. In these specific cases, the compound is compared to theoretical compounds rather than to reference standards. When the potential compound or candidate meets the following criteria, then a tentative identification has been reached:

(1) The exact mass of the candidate and the exact mass of the theoretical compound should not present a difference in mass error greater than ±5 ppm.
(2) Several confirmation or characteristic fragments are present.
(3) The isotopic pattern observed in the spectrum corresponds to molecular formula of the candidate.

Despite their markable benefits, HRMS usage was initially narrowed to the proteomic or metabolomics field, slowly starting to be implemented in other fields such as the food safety studies. Fortunately, recently HRMS is being gradually considered in the environmental area (Gil-Solsona et al., 2021; Goto et al., 2020; Menger et al., 2022; Sanganyado et al., 2021). These analytical incorporations into environmental research studies have led to a significant expansion of the current knowledge (broadening the search to suspected and unknown compounds). The aim of this study is to apply for the first time the potential of HRMS, broadening the search to suspected and unknown compounds, in the marine environment, specifically in the analysis of P. *oceanica* and related sediments. This is the only way to obtain a more accurate characterization of the real composition of the samples, and therefore of the environment.

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Chapter II

Endogenous bioactive compounds

1. Introduction

Land and marine plants are capable of neutralizing or reducing the adverse effects of xenobiotics (Malea et al., 2022). In this process of detoxification, the xenobiotics present inside the organism (mostly the most mobile) can be transformed into other compounds and eliminated by excretion. These biologically driven transformations are divided into four primary phases (Molina and Segura, 2021; van der Oost et al., 2003; Zhang and Yang, 2021):

- Phase I: Metabolization of OPs by plant enzymes, which catalyze the process and transform the original compounds.
- Phase II: Metabolization by conjugation, obtaining a more polar and easily transportable substance.
- Phase III: Translocation, where the conjugated substances are allocated by certain proteins that act as membrane pumps. This is also called compartmentalization (Zhang et al., 2018).
- Phase IV: Other regulatory processes. Here, different metabolic pathways (for example, salicylic acid and jasmonic acid production) are induced (Subhashini et al., 2013). This process alters the concentration of endogenous substances, such as the PhCs (Tandey et al., 2020).

Under the premise of phase IV, individual PhCs could act as stressor-specific biomarkers of the deterioration of *P. oceanica* by pollutants, and indirectly of poor coastal water quality (one of the major threads to seagrass survival; Turschwell et al., 2021). To delve into this question, a preliminary step is required: to unveil the phenolic fingerprint in *P. oceanica*. Although *P. oceanica* is a notable reservoir of bioactive compounds, so far only a few studies have focused on this issue, and those published still leave room for improvement (Barletta et al., 2015; Cariello et al., 1979; Cornara et al., 2018; Cuny et al., 1995; Dumay et al., 2004; Grignon-Dubois and Rezzonico, 2015; Haznedaroglu and Zeybek, 2007).

This chapter, therefore, aims to investigate in depth the phenolic fingerprints of *P. oceanica*, by implementing several improvements such as: (1) the usage of the complete array of tissues in *P. oceanica* (leaves, rhizomes, and roots); (2) inclusion of a previous optimization steps of both extraction and analytical methods; (3) the analysis by means of LC-HRMS (Exactive Orbitrap) allowing the performance of target and non-target studies to achieve exhaustive phenolic monitoring. The yielded results will set the baseline information for further studies regarding *P. oceanica* stress reactions and the potential usage of individual PhCs as specific biomarkers.

In this chapter is comprised the Scientific article I: New Phenolic Compounds in *Posidonia oceanica* Seagrass: A Comprehensive Array Using High Resolution Mass Spectrometry.

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Scientific Contribution 1

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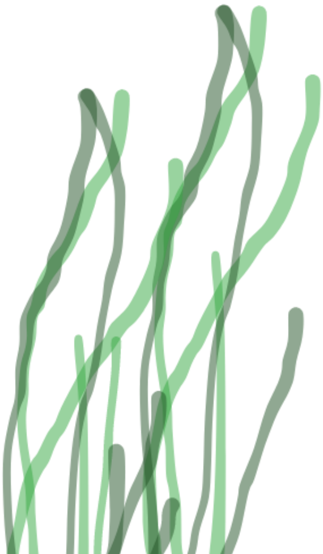

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New Phenolic Compounds in *Posidonia oceanica* Seagrass: A Comprehensive Array Using High Resolution Mass Spectrometry

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Abstract: The studies on the *Posidonia oceanica* Delile (*P. oceanica*) phenolic composition have been focused on the foliar tissues and have often neglected the phenolic compounds in rhizomes or roots alike. With the current improvements in high resolution mass spectrometry (HRMS) analyzers, such as the Orbitrap MS, there is a new opportunity to more deeply study *P. oceanica*. One of the benefits is the possibility of conducting an exhaustive phenolic monitoring, which is crucial in the search for new stressor-specific biomarkers of coastal deterioration. For this purpose, the different tissues (leaf, rhizome, and root) of *P. oceanica* seagrass from several marine sampling areas were analyzed through target, suspected, and non-target screenings. This paper brings a fast and tissues-specific extraction, as well as a detection method of phenolic compounds applying for the first time the potential of HRMS (Exactive Orbitrap) in *P. oceanica* samples. As a result, 42 phenolic compounds were satisfactorily detected, of which, to our knowledge, 24 were not previously reported in *P. oceanica*, such as naringenin, naringenin chalcone and pinocembrin, among others. Information here reported could be used for the evaluation of new stressor-specific biomarkers of coastal deterioration in the Mediterranean waters. Furthermore, the followed extraction and analytical method could be considered as a reference protocol in other studies on marine seagrasses due to the exhaustive search and satisfactory results.

Keywords: *Posidonia oceanica*; leaf; rhizome; root; UHPLC-Orbitrap MS; phenolic compounds

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1. Introduction

Posidonia oceanica Delile (*P. oceanica*) is a long-living, slow-growing marine angiosperm (seagrass) endemic to the Mediterranean Sea. The *P. oceanica* meadows have been identified as priority habitats by European conservation legislation [1]. These meadows supply essential ecosystem services for human well-being [2]. However, *P. oceanica* has been observed to be especially susceptible to anthropogenic disturbances [3–5]. Studies have detected the presence and bioaccumulation of pollutants in this seagrass, such as heavy metals. Ref. [6] brought insight into their application as contamination bioindicators. It has also been reported that *P. oceanica* responds to contamination-driven stress as well as to natural-driven stress (grazing, competition, microbial settlement, and eutrophication among others) by altering the amount of phenolic compounds [7,8]. Due to their specific and rapid response when changes in the environment occur, phenolic compounds have been proved to be suitable biomarkers or early warnings [9–12]. These compounds are specialized metabolites that benefit the bearing organism through several biological properties (e.g., antioxidant capacity) [13]. Interest in this field has been growing, given the potential medical contributions of such phenolic compounds to human health and personal care: immunostimulant and antitumoral drugs or products for skin aging [14,15].

P. oceanica phenolic content has been previously reported [14,16]. Of the researched compounds, ferulic acid, caffeic acid, chicoric acid, caftaric acid, and cinnamic acid have been identified as major phenolic compounds [17,18]. Studies commonly focused on the determination of phenolic compounds in foliar tissues (temporal structures marked by seasonal variations), and little attention has been paid to the tissues with a longer lifespan less marked by seasonal variations, i.e., rhizomes and roots. To the best of our knowledge, only one study reported the phenolic fingerprint in *P. oceanica* roots [19], and available studies based on rhizomes have only reported total phenols (following mainly the Folin–Ciocalteu assay) or lignin content [18,20–22]. However, several studies on terrestrial plants have observed specific phenols in these tissues reacting to specific changes in the environment or taking part in relevant activities, such as the reduction of contaminants in soil or their role in plant resistance to deficit conditions [23–25].

Additionally, studies based their identification/detection of the phenolic compounds on liquid (LC), gas chromatography (GC), or pyrolysis GC (Py-GC) coupled to low resolution mass spectrometry (LRMS) [15,18,26]. Nowadays, the improvements in high resolution mass spectrometry (HRMS) and its ability to detect a higher number of compounds, bring a new opportunity to obtain a comprehensive array of phenolic compounds in *P. oceanica*. The implementation of HRMS analyzers could also lead to (1) a better differentiation between genuine signals and artifacts driven signals, (2) accurate detection of target and non-target compounds, and (3) retrospective analysis of samples.

Hence, the objective of this study is double fold. Firstly, the determination of phenolic compounds in *P. oceanica* for the first time, to our knowledge, by ultra-high performance liquid chromatography combined with HRMS, concretely with an Orbitrap analyzer (UHPLC-Orbitrap MS), using target, suspected, and non-target analysis. Secondly, the evaluation of the phenolic content in the different *P. oceanica* tissues (leaf, rhizome, and root) from different sampling points. The findings of this study provide new reference information in the field of marine seagrass chemistry that could be useful in future investigations of *P. oceanica* phenolic compounds' suitability as stress-specific biomarkers.

2. Results

2.1. Extraction Procedure

Two different procedures were compared in the tissues of one of the collected *P. oceanica* samples (FAN7 leaves, rhizome, and roots) for extractant composition evaluation [17,27]. Procedures varied between a mixture of methanol/water 8:2 *v*/*v* and 5:5 *v*/*v* (pH 4 in both cases). After ca. 15 min extraction, samples were subject to a target screening using an in-house database. This database used 93 commercially available standards of the most common phenolic compounds detected in agri-food products [28,29]. In this preliminary screening, up to 20 compounds were identified and considered for the extractant composition evaluation. The results showed that the optimal composition was tissue-dependent (Table S1). For instance, for rhizomes and roots, an extractant mixture of 8:2 *v*/*v* methanol/water was proved to be the most adequate in terms of phenolic profile sensitivity in all cases, i.e., number of compounds, higher peak intensities (described as NL, Normalization Level), better peak shape, or a greater presence of confirmation fragments. In contrast, leaves showed overall relatively better results when using the 5:5 *v*/*v* mixture (~72% of the cases). An example of how the intensity varied according to the employed extractant composition and the studied tissue is shown in Figure 1. Observed differences in extraction efficiencies could be attributed to the type of plant material (e.g., ligneous and rigid in the case of the rhizome and roots) and the compounds affinity to the different tissues.

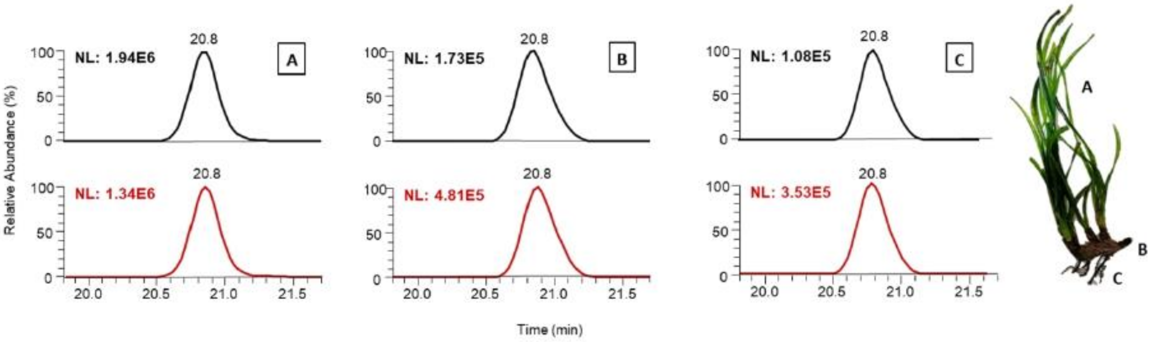

Figure 1. Extracted ion chromatogram for isorhamnetin-3-O-glucoside at 479.11840 *m/z* in full MS, with R_T 20.8 min, for FAN7 tissues: (**A**) leaves, (**B**) rhizomes, and (**C**) roots showing differences in base peak intensities (NL). Black chromatographic peaks were obtained using 5:5 *v/v* extraction mixture and brown peaks correspond to 8:2 *v/v* extraction mixture.

The results obtained for leaves confirmed that the extraction followed in previous studies (5:5 *v/v* mixture) yield relatively better results than the 8:2 *v/v* mixture [15–17,30]. Nonetheless, the scarcity of data on roots and rhizomes hindered the comparison between our results and other published procedures. No available information on rhizomes was found and, after a thorough search of the relevant literature, only one article was found regarding the extraction of the roots, in which non-diluted acetone was used in a three day extraction procedure [19]. In all observed articles, extraction times ranged from several hours to days or consumed elevated solvent-to-plant. In contrast, the extraction method employed in this study proved to be less time consuming and employed a reduced solvent volume, following the green chemistry approach.

Therefore, based on the assessment of the results, the extractant composition selected was a mixture of methanol/water 5:5 *v/v* for leaves, and 8:2 *v/v* in the case of rhizome and roots which allowed for the detection of a greater number of compounds (up to four) for each tissue.

2.2. Chromatographic Conditions

Two sub-2-µm columns, widely employed in the phenolic compound's studies, were also tested in the analysis of FAN7 leaves. A target screening was performed using the in-house database to evaluate the chromatographic separation performance. Such columns were Acquity C18 column (2.1 mm × 100 mm, 1.7 µm particle size; Waters, Milford, MA, USA) and Hypersil GOLD^{TM} (2.1 mm × 100 mm, 1.9 µm particle size; Thermo Fisher, San Jose, CA, USA).

Besides, two common aqueous mobile phases were also evaluated, 30 mM ammonium acetate aqueous solution pH 5 [27] and 4 mM formic acid aqueous solution pH 3 [17]. Methanol was employed as the organic eluent along the process.

Regardless of the employed columns, compound separation remained the same. On the other hand, elution times differed, showing an increase in R_T when using the Acquity C18 column, which could be attributed to the more reduced particle size. However, overall, relatively higher intensities or NL were reached when using Hypersil GOLD^{TM}, as shown in Figure S1.

As for the mobile phases and their respective pH, they have been seen to affect the peaks' intensity and the presence or absence of compounds and their confirmation fragments. In general, intensities were lower when ammonium acetate was employed, which in some cases hindered the apparition of confirmation fragments. Average better signals were observed in the formic acid mobile phase with pH 3, confirming effective binding of the phenolic compounds to the stationary phase. Up to six compounds more were observed when using formic acid. Examples are caffeic acid, p-coumaric acid or ferulic acid, phenolic compounds that were only confirmed when using formic acid. As an example, the case of ferulic acid is shown in Figure S2, which was absent when using ammonium. Such absence might be due to the charge of ferulic acid in increased pH, hampering the binding to the column.

As a result, the selected conditions for this analysis were: Hypersil GOLD^{TM} column, and formic acid as the aqueous mobile phase.

2.3. Phenolic Identification

After evaluating the extraction conditions, target, suspected, and non-target screenings of all tissues (leaves, rhizome, and roots) of five *P. oceanica* seagrasses were conducted as indicated in Figure S3. During the target screening, compound peaks of each molecule were located using information from the in-house database, such as the parent theoretical mass and confirmation/characteristic fragments, and performing pseudo MS/MS experiments. The risk of false-positive was also reduced by monitoring each parent's peak mass spectrum and comparing it to the theoretical molecule spectrum (or simulation) to confirm the ion ratios for the isotopic pattern [28].

Results of the target screening revealed 22 phenolic compounds from which 13 were detected for the first time to our knowledge in *P. oceanica* (Table 1). These newly reported specialized metabolites consisted of flavones (apigenin, baicalein, and luteolin), flavonols (galangin), isoflavones (biochanin A, genistein, and glycitein), flavonons/flavanones (eriodictyol, naringenin, pinocembrin, and sakuranetin/isosakuranetin), chalcones (naringenin chalcone), and kaempferol-3-O-glucoside/luteolin-4'-O-glucoside (IUPAC names can be found in Table S2). These phenolic compounds have already been proved to play essential roles inhibiting cancer or offering antioxidant properties [15,31,32]. More biological properties are summarized in Table S3, Supplementary Materials.

Table 1. Retention time (R_T) and *m/z* ions for the identification and confirmation of the detected target compounds in *P. oceanica* roots (●), rhizomes (■), and/or leaf (◊). Mass error corresponds to the smallest average mass error found among the three tissues.

R_T (min)	Compound Name	Elemental Composition	Polarity	Theoretical Mass (*m/z*)	Mass Error (ppm)	Fragment 1 (*m/z*)	Fragment 2 (*m/z*)	Fragment 3 (*m/z*)	Tissue
13.3	Catechin (+) [31]	$C_{15}H_{14}O_6$	ESI+	291.08631	0.080	139.03895	123.04502		● ■ ◊
14.9	Caffeic acid [17,19]	$C_9H_8O_4$	ESI−	179.03498	−0.127	135.04429	134.03628	89.03847	● ■ ◊
16.1	Epicatechin (−) [31]	$C_{15}H_{14}O_6$	ESI+	291.08631	0.122	139.03895	123.04502		● ■ ◊
16.3	Genistein *	$C_{15}H_{10}O_5$	ESI+	271.06010	0.189	153.01779	215.06962	243.06434	● ■ ◊
16.3	Baicalein *	$C_{15}H_{10}O_5$	ESI+	271.06010	0.056	253.04950	243.06520		● ■ ◊
16.9	Eriodictyol *	$C_{15}H_{12}O_6$	ESI−	287.05611	1.633	151.00235	107.01253		● ■ ◊
17.6	p-Coumaric acid [8,15]	$C_9H_8O_3$	ESI−	163.04007	−0.362	119.04881	93.03316	163.03950	● ■ ◊
18.1	Ferulic Acid [16,31]	$C_{10}H_{10}O_4$	ESI−	193.05063	−1.482	134.03643	149.06100	178.02640	● ■ ◊
19.6	Quercetin-3-O-glucoside [14]	$C_{21}H_{20}O_{12}$	ESI−	463.08710	0.821	300.02700	302.03696	301.03455	● ■ ◊
20.7	Kaempferol-3-O-glucoside * + Luteolin-4'-O-glucoside *	$C_{21}H_{20}O_{11}$	ESI−	447.09328	1.939	284.03200	255.02924	285.03995	● ■ ◊
20.8	Isorhamnetin-3-O-glucoside [14]	$C_{22}H_{22}O_{12}$	ESI+	479.11840	0.646	317.06550			● ■ ◊
22.4	Quercetin [7]	$C_{15}H_{10}O_7$	ESI+	303.04993	0.001	201.05453	153.01834	165.01837	● ■
23.2	Naringenin *	$C_{15}H_{12}O_5$	ESI−	271.06012	0.271	119.04879	151.00226	107.01350	● ■ ◊
23.4	Luteolin *	$C_{15}H_{10}O_6$	ESI−	285.04046	0.269	133.02834	151.00260	175.03898	● ■ ◊
26.3	Isorhamnetin [7]	$C_{16}H_{12}O_7$	ESI−	315.05103	2.013	300.02685	151.00245		● ■ ◊
26.5	Apigenin *	$C_{15}H_{10}O_5$	ESI+	271.06010	−0.037	153.01779	119.04943		◊
27.4	Naringenin Chalcone *	$C_{15}H_{12}O_5$	ESI−	271.06012	0.281	119.04879	151.00226	107.01350	● ■ ◊
30.6	Pinocembrin *	$C_{15}H_{12}O_4$	ESI−	255.06628	0.241	151.00241	213.05467		● ■ ◊
30.7	Biochanin A *	$C_{16}H_{12}O_5$	ESI−	283.06120	1.142	268.03634			● ■
30.7	Glycitein *	$C_{16}H_{12}O_5$	ESI+	285.07575	−0.667	270.05097	242.05613		● ■
33.3	Galangin *	$C_{15}H_{10}O_5$	ESI−	269.04555	0.068	213.05450			● ◊
34.4	Sakuranetin * + Isosakuranetin *	$C_{16}H_{14}O_5$	ESI−	285.07685	1.844	119.04883	221.15330	165.01802	● ■ ◊

* Compounds not detected before in *P. oceanica*.

Note that some of the detected phenolic compounds were isomers, with the same exact mass, R_T, and fragments. Since they could not be separated by chromatographic or mass resolution and the presence of both isomers could not be confirmed, these were recorded and counted as only one phenolic compound [28]. These phytochemicals were: luteolin-4′-O-glucoside and kaempferol-3-O-glucoside (*m/z* 447.09328; R_T: 20.7–20.8 min), and isosakuranetin and sakuranetin (*m/z* 285.07685, R_T: 34.4 min).

For the suspected screening, a second list with 38 compounds was developed by gathering published information on the *P. oceanica* phenolic fingerprint and other Mediterranean seagrasses (namely *Cymodocea nodosa*, *Zostera marina*, and *Zostera noltii*). Out of the 30 considered suspected compounds previously detected in *P. oceanica*, nine were tentatively identified in the samples and included in Table 2. On the other hand, the not detected suspected compounds are shown in Table S4. Note that the tentative identification of a compound was reached when (1) the difference between the exact mass of the candidate (calculated from the elemental formula) and the exact mass of the target compound felt within ±5 ppm of mass error, (2) confirmation fragments were present and, (3) spectrum matched among experimental and theoretical peaks in terms of ion ratios for the isotopic pattern (Table 2). Figure 2 shows a chromatogram and mass spectrum of isorhamnetin-3-O-malonylglucoside as an example. Suspected compounds identified in other Mediterranean seagrasses, such as apigenin-7-O-glucoside and rosmarinic acid or the sulfated flavonoids apigenin-7-sulfate, diosmetin-7-sulfate, and luteolin 7-sulfate, were not detected in this study in agreement with the literature [32–34].

Table 2. Retention time (R_T) and *m/z* ions of the detected suspected compounds in the Orbitrap system for *P. oceanica* and other Mediterranean seagrasses (@): *Cymodocea nodosa* and *Zostera marina* and *Zostera noltii*. Compounds detected in *P. oceanica* roots (•) rhizomes (■), and/or leaves (◊).

R_T (min)	Compound Name	Elemental Composition	Polarity	Theoretical Mass (*m/z*)	Mass Error (ppm)	Fragment 1 (*m/z*)	Fragment 2 (*m/z*)	Fragment 3 (*m/z*)	Tissue	Reference
7.6	Protocatechualdehyde	$C_7H_6O_3$	ESI−	137.02442	−4.850	136.01660	108.02050	109.03050	• ■ ◊	[16]
13.1	Zosteric acid@	$C_9H_8O_6S$	ESI−	242.99688	0.371	163.04010	145.02950	117.03460	• ■ ◊	[19,35]
16.1	p-Anisic acid	$C_8H_8O_3$	ESI−	151.04007	−1.210	133.02861	123.04398		• ■ ◊	[16]
16.3	Caftaric Acid	$C_{13}H_{12}O_9$	ESI−	311.04086	1.437	130.99800	161.02390	267.05050	• ■ ◊	[17]
16.3	Chicoric acid	$C_{22}H_{18}O_{12}$	ESI−	473.07255	1.672	311.04071	293.02844	149.00810	• ■ ◊	[14,19]
17.7	Fertaric acid	$C_{14}H_{14}O_9$	ESI−	325.05651	1.658	193.05010	130.99800	87.00820	■ ◊	[17]
18.3	Cinnamic Acid	$C_9H_8O_2$	ESI−	147.04515	−0.713	119.04916	117.03351	101.03851	• ■	[15]
19.9	Quercetin-3-O-malonylglucoside	$C_{24}H_{22}O_{15}$	ESI−	549.08859	1.007	505.10006	300.02737	301.03183	• ■ ◊	[14]
21.3	Isorhamnetin-3-O-malonylglucoside	$C_{25}H_{24}O_{15}$	ESI−	563.10424	2.148	459.09270	315.05050	299.01920	• ■ ◊	[14]

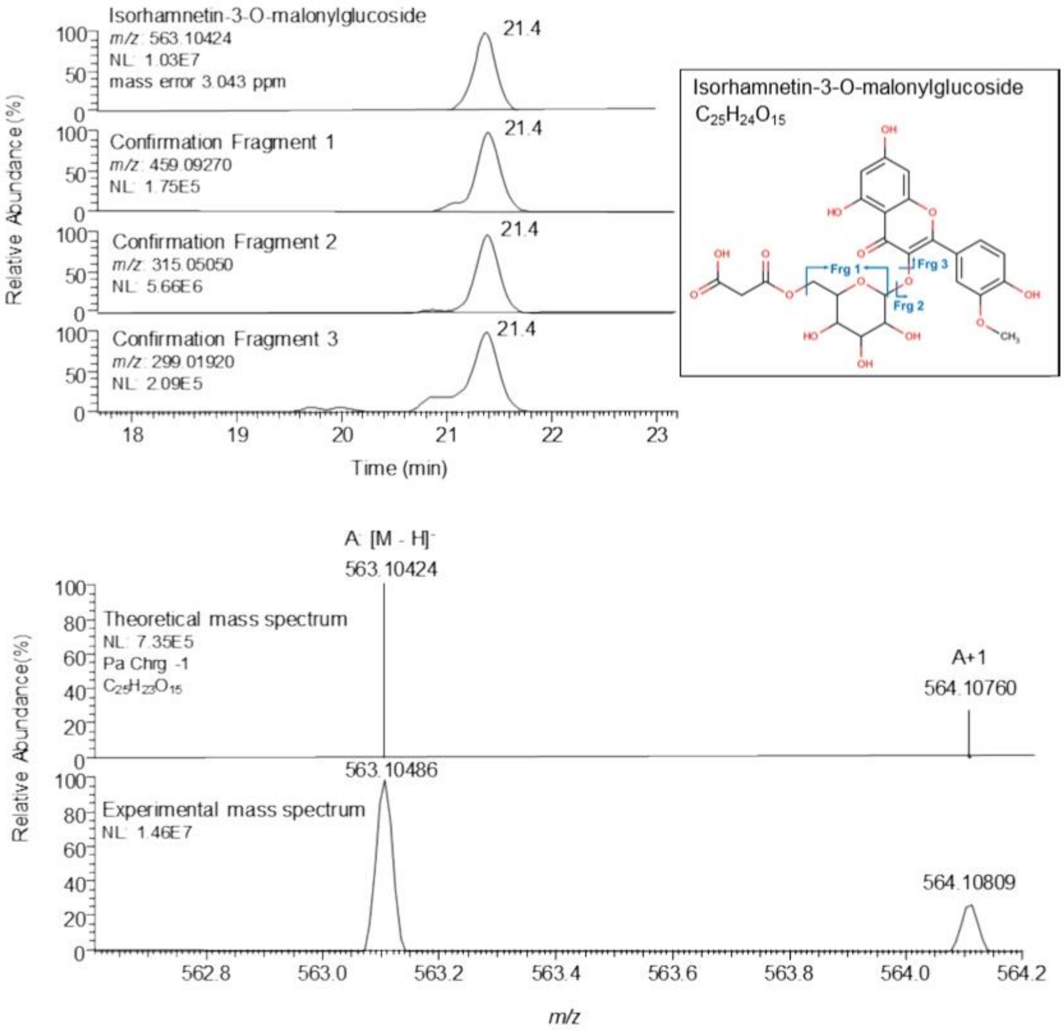

Figure 2. From top to bottom: Extracted ion chromatogram and mass spectrum of the suspected bioactive compound isorhamnetin-3-O-malonylglucoside (*m/z*: 563.10424) in foliar tissues from the sampling point AL2x. NL: intensity.

During the non-target screening, the Compound Discoverer Software (Thermo Fisher Scientific, Les Ulis, France) was employed to identify potential candidates. In this search, the software subtracts all exact masses and the respective molecular formulas. Finally, these formulas were compared to information on phenolic compounds gathered in open databases, searching for matches. Only those highlighted as a full match and meeting the criteria settled for a tentative identification were considered. The non-target analysis revealed 11 compounds not previously identified in *P. oceanica*, such as sophoraflavanone B and two curcuminoids (Table 3 and Table S2). In addition, through the non-target screening several compounds detected during the target and suspected analysis were further confirmed: chicoric acid, p-coumaric acid, ferulic acid, fertaric acid, bioachin A, genistein, naringenin, isorhamnetin, isorhamnetin-3-glucoside, and quercetin-3-O-glucoside. The fact that certain compounds were only observed in the non-target screening whilst others were only detectable during the target and suspected mode highlights the need to combine the three searching modes and the benefits of the retrospective analyses for environmental samples, as recently suggested [36].

Table 3. Average retention times (R_T) and *m/z* ions of the non-target compounds in the Orbitrap system for *P. oceanica*. Compounds detected in *P. oceanica* roots (•) rhizomes (■), and/or leaves (◊).

R_T (min)	Compound Name	Elemental Composition	Polarity	Theoretical Mass (*m/z*)	Mass Error (ppm)	Fragment 1 (*m/z*)	Fragment 2 (*m/z*)	Fragment 3 (*m/z*)	Fragment 4 (*m/z*)	Tissue
13.3	Gambiriin A1	$C_{30}H_{28}O_{12}$	ESI–	579.15080	2.881	125.02390	289.07120	151.03950	139.03950	• ■
18.1	Mascaroside	$C_{26} H_{36} O_{11}$	ESI–	523.21849	1.447	361.16510	331.15450			• ■ ◊
18.3	Astilbin	$C_{21}H_{22}O_{11}$	ESI–	449.10893	1.575	151.00322	150.03022	303.05050	285.03990	• ■ ◊
18.9	Tracheloside	$C_{27} H_{34} O_{12}$	ESI–	549.19775	1.640	387.14440	357.13380			• ■
21.0	Quercetin 3-O-sulfate	$C_{15}H_{10}O_{10}S$	ESI–	380.99219	0.372	301.03480	80.96460			• ■ ◊
33.7	Glabridin	$C_{20}H_{20}O_4$	ESI–	323.12888	1.317	187.07590	267.06570			◊
34.5	Piceatannol	$C_{14}H_{12}O_4$	ESI+	245.08084	0.002	135.04460	215.07080	227.07080		• ■ ◊
39.1	Sophoraflavanone B	$C_{20}H_{20}O_5$	ESI–	339.12380	1.093	219.06418	119.04871			• ■ ◊
40.7	Tetrahydrocurcumin	$C_{21}H_{24}O_6$	ESI–	372.15790	0.637	177.05520	193.08650	219.06570		• ■ ◊
41.8	Demethoxycurcumin	$C_{20} H_{18} O_5$	ESI–	337.10815	1.836	119.04970	161.06030	175.03950	217.05010	• ■ ◊
45.3	Xanthohumol	$C_{21}H_{22}O_5$	ESI–	353.13945	1.155	207.10210	119.04900	145.02900		• ■ ◊

Interestingly, among the first-time detected compounds, biochanin A, glycitein, gambiriin A1, and tracheloside were only observed in the underground tissues (Figure 3). To our knowledge it is also the first time that these compounds have been reported in any marine seagrass, it being more common to find them in other samples such as legumes and algae for biochanin A and glycitein, respectively [37,38].

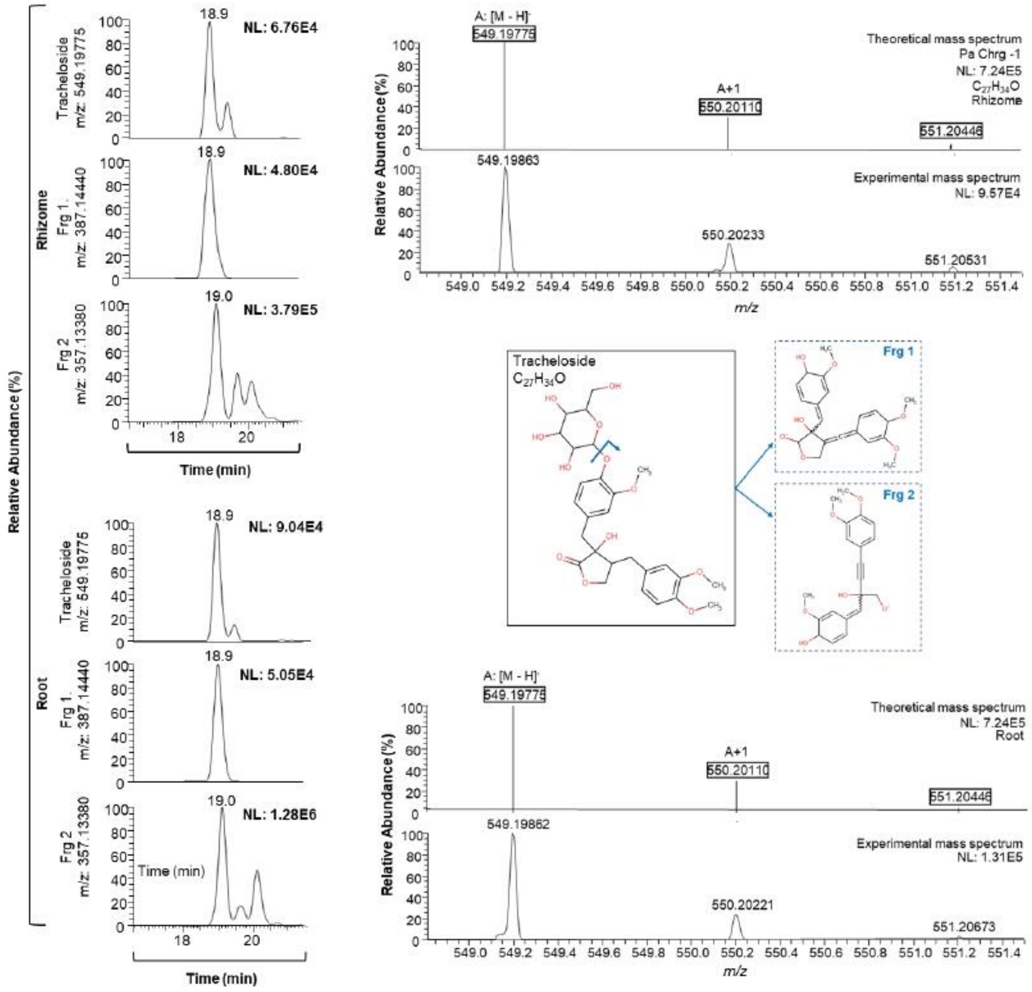

Figure 3. Extracted ion chromatogram of the first time detected and non-target bioactive compound tracheloside (*m/z*: 549.19775), in *P. oceanica* rhizome and root (sampling point CG4) and the corresponding mass spectrum. NL: intensity, Frg.: confirmation fragments.

Considering all *P. oceanica* samples and tissues, a total of 42 phenolic compounds were detected (Table S5). Among them, flavonoids and phenolic acids represented the main groups, as shown in Table 4. These two families were further investigated, revealing the cinnamic acid subclass responsible for the uneven distribution among tissues. Information on the average number of compounds per tissue is shown in Table S6. The rest of the phenolic subclasses show a relatively similar number of phenolic compounds in leaf, rhizome, and root. This difference in *P. oceanica* leaves could be ascribed to a greater exposition to UV radiation, current motion, shifts in water temperature, epiphytes load, or turbidity, among others [5,39]. For instance, marine water is relatively more unstable than sediment in terms of environmental conditions.

Table 4. Phenolic families detected in *P. oceanica* tissues and the maximum number observed in the plant.

		Flavonoids	Phenolic Acids	Other Polyphenols	Total Number
EE3	Leaf	20	9	3	32
	Rhizome	22	5	3	30
	Root	22	5	3	30
FAN7	Leaf	19	9	3	31
	Rhizome	24	5	3	32
	Root	20	4	3	27
AL2x	Leaf	21	9	3	33
	Rhizome	21	5	3	29
	Root	21	5	3	29
AL3	Leaf	22	9	2	33
	Rhizome	21	5	2	28
	Root	19	5	2	26
CG4	Leaf	23	8	3	34
	Rhizome	23	5	3	31
	Root	24	5	3	32
	Maximum	24	9	3	34

3. Material and Methods

3.1. Sampling

In this study, five samples of *P. oceanica* (EE3, AL2x, AL3, FAN7, and CG4) were taken from different sites along the coast of Almeria (Alboran Sea, Spain), as can be observed in Figure 4. Samples were collected manually by scuba divers in July, October, November, December 2019, and January 2020. The sampling sites were distributed at different water depths, from 1.5 m at CG4 to 9 m at EE3. More relevant information concerning the characteristics of the sampling points can be observed in Table 5.

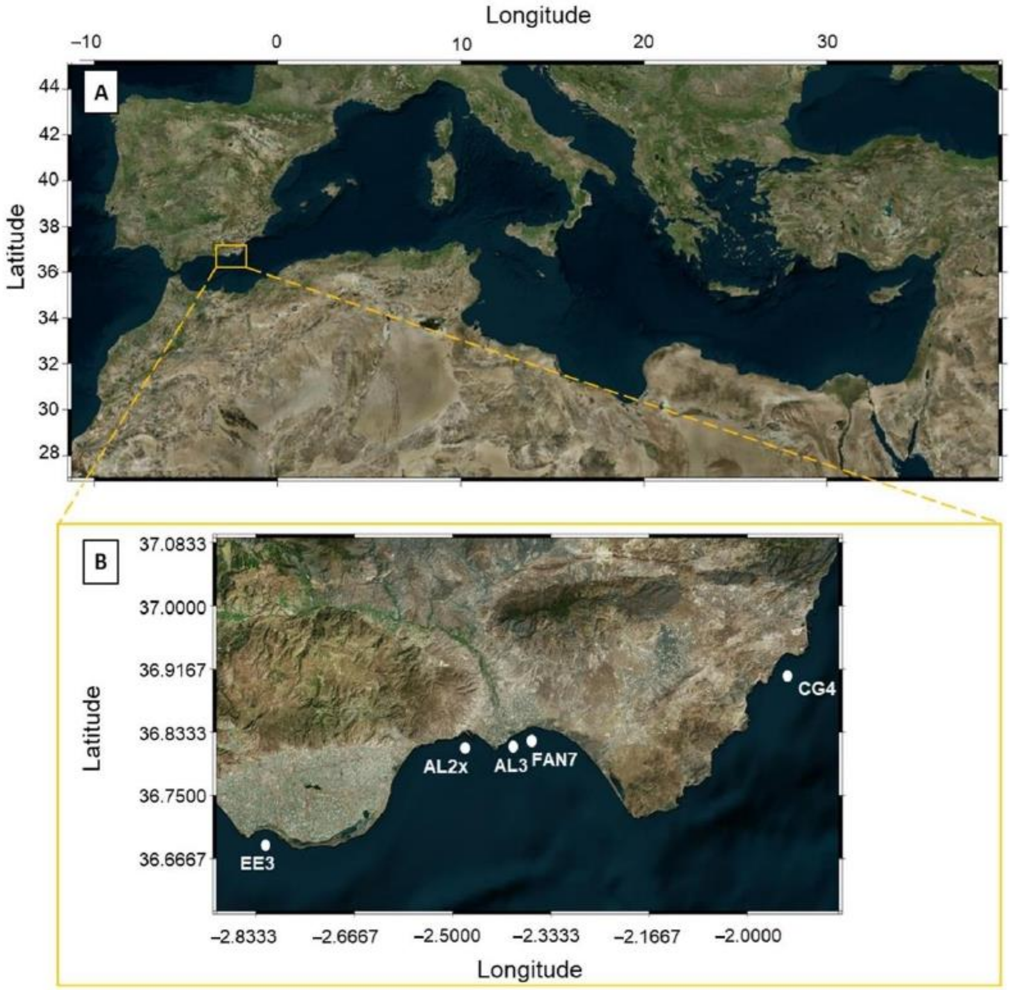

Figure 4. Study Area: (**A**) Map of the Mediterranean Sea Basin; the yellow square highlights Almeria coast, our study area. (**B**) Map of the coast of Almeria indicating the locations of the sampling stations. From west to east: EE3, AL2x, AL3, FAN7, and CG4. Coordinate System: WGS84, UTM.

Table 5. Characteristics of the considered sampling points in Almeria (Spain).

Site	Water Depth (m)	Location (WGS84_UTM)	Distance from Coast (m)	Area Description	Sampling Date
EE3	10.3	36.682721, −2.781700	670	Limit between a harbor and a nature spot. Influenced by watershed with intensive agriculture (greenhouses)	31 Oct 2019
AL2x	7.8	36.824655, −2.452103	80	Touristic city, harbor	18 Dec 2019
AL3	7.8	36.828547, −2.385920	540	Sewage, airport, and watercourse (seasonal)	11 Nov 2019
FAN7	-	36.835713, −2.352617	-	Submarine natural gas pipeline (MEDGAZ) and watercourse (seasonal)	2 Jul 2019
CG4	1.5	36.862794, −2.003661	5	Marine Protected Area	5 Jan 2020

3.2. Sample Pre-Treatment

After collection, samples were immediately stored in portable fridges at a low temperature until arrival at the laboratory. Subsequently, sand and salt were removed by rinsing with distilled water and samples were divided into three parts: leaf, rhizome, and root. All parts were kept in petri plates and weighted. Note that young leaves and basal sheathes were not considered in this study.

Afterward, samples were stored at −20 °C (48 h) and freeze-dried at −50 °C (48 h) in a Thermo Electron Corporation Heto PowerDry LL3000 freeze-dryer (Thermo Fisher Scientific, Bremen, Germany). In the case of the leaves, this part was conducted in two separate steps of 24 h. In between steps, leaves were gently cleaned from epiphytes (dried crust) using a brush. Subsequently, all samples were homogenized by powdering in a Mixer Mill MM 200 (2 min at 25 r/s) and stored in desiccators until extraction.

3.3. Chemical and Reagents

LC/MS-grade water and methanol were purchased from Merck KGaA (Darmstadt, DE) and Riedel-de-Haën™ (Seelze, Germany), respectively. Formic acid was purchased from Fisher Scientific (Waltham, MA, USA). Galangin standard was obtained from Extrasynthese (Genay, France). P-coumaric acid, kaempferol-3-O-glucoside, quercetin standards and ammonium formate were purchased from Sigma-Aldrich (St Louis, MA, USA). All reagents were of analytical grade. Employed standards had a purity of >99%. Individual standard solutions of 300 mg l^{-1} were prepared in methanol. Resulting stock standards solutions were kept in amber bottles. Subsequently, a multi-compound working solution (50 mg l^{-1}) was prepared by diluting each stock solution aliquot with methanol. All solutions were stored at 4 °C in an amber bottle until analysis.

3.4. Extraction Procedure

Specific extractions were developed regarding the investigated tissues from previous procedures with minor modifications [17,27]. Briefly, 3 mL of methanol/water 5:5 *v*/*v* solution (pH 4, acidified with formic acid) were added to 15 mL falcon tubes with 150 mg dry weight of leaf powder, whereas 3 mL of methanol/water 8:2 *v*/*v* solution was used in the case of rhizome and roots. All different mixtures were then sonicated for 4 min at room temperature (~25 °C) and centrifuged (5000 rpm, 10 min). One single extraction cycle was carried out since preliminary studies proved it to be adequate to monitor the phenolic compounds [27]. After ca. 15 min extraction the resulting supernatant was filtered (45 µm, Fisher Scientific, Madrid, Spain) into 2 mL LC vials and analyzed.

3.5. Chromatographic Conditions

Chromatographic analyses of *P. oceanica* tissues were conducted on a Thermo Scientific Transcend™ 600 liquid chromatography (Thermo Fisher Scientific, San Jose, CA, USA). The employed column was Hypersil GOLD™. The chromatographic separation was performed using a mobile phase that comprises water (1% formic acid and 4 mM ammonium formate) as eluent A and methanol as eluent B.

Elution from the UHPLC column gradient was carried out as follows: from 0 to 8 min, 5–30% B; from 8 to 13 min, 30–50% B; from 13 to 18 min, 50% B; from 18 to 23 min, 50–60% B; from 23 to 28 min, 60–70% B; from 28 to 33 min, 70–80% B; from 33 to 47 min, 80–100%; from 47 to 49 min, 100%; from 49 to 53.5 min, 100–10% and from 53.5 to 58 min, 10%.

The column temperature during analysis was maintained at room temperature (25 °C), the flow rate was settled at 0.2 mL min^{-1} and the injection volume at 10 µL along the process.

3.6. Orbitrap-MS Analysis

In this study, a single Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany) was used for MS analyses. The mass spectra were acquired employing four alternating acquisition functions: full MS, without fragmentation (higher collisional dissociation, HCD, collision cell was switched off), mass resolving power 25,000 FWHM (full width at half maximum); scan time 0.25 s, ESI+, and ESI−; all-ion fragmentation (AIF), with fragmentation (HCD on, collision energy 30 eV), mass resolving power 10,000 FWHM, ESI+, and ESI−. Mass range in full scan mode was set at *m/z* 100–1000, whereas, for MS/MS monitoring, it was set at *m/z* 70–700.

Data acquisition and processing were carried out using Trace Finder Version^{TM} 4.0 and Xcalibur^{TM} Version 2.2.1 (Thermo Fisher Scientific, Les Ulis, France) in Qual browser mode. Software searching criteria was set on 5 ppm mass tolerance error.

3.7. Phenolic Identification

As mentioned above, the identification of compounds was performed in three steps. Firstly, a target screening was carried out, submitting an in-house database containing 93 phenolic compounds to the software to elucidate the presence or absence of listed compounds. Further important information for the compound identification, such as molecular formula, retention time (R_T), exact theoretical mass of the molecular ion and characteristic fragments, and ionization mode, were also included in the in-house database.

Secondly, a suspected screening was conducted. In this case, previously detected phenolic compounds in *P. oceanica,* as well as in other Mediterranean seagrasses, were considered. This list was developed based on available data (literature and open databases such as PubChem, ChemSpider, and Human Metabolomics), adding to the study 30 suspected compounds detected in *P. oceanica* and eight only detected in the other Mediterranean seagrasses (Table 3 and Table S4). The exact mass of these suspected compounds was calculated using their chemical formula and the corresponding ionization mode (ESI−/ESI+). Confirmation fragments were also retrieved from the available literature and open databases. Note that several of the found suspected compounds were already present in our in-house database and were therefore treated as target compounds.

In third place, a non-target analysis was performed using Compound Discoverer Software (Thermo Fisher Scientific, Les Ulis, France). For that purpose, the raw data obtained from the sample and blank injections were submitted to the software. The software carried out a structural analysis, performing a metabolite profiling base on several selected databases (CheBI, KEGG, MolBank, Nature Chemistry, Sigma Aldrich, and Phenol Explorers) and the possible adducts ($[M{+}CH_2O_2{-}H]^{-1}$, $[M{+}H]^{+1}$, and $[M{-}H]^{-1}$). Subsequently, resulting potential precursor ions and characteristic fragments were confirmed using XcaliburTM Version 2.2.1 (Thermo Fisher Scientific, Les Ulis, France) in Qual browser mode.

4. Conclusions

The analysis of the three *P. oceanica* tissues using, for the first time, UHPLC-Orbitrap MS in target analysis mode in combination with retrospective analyses (suspected and non-target modes) contributed to the detection of 42 phenolic compounds. Out of these, 24 have not been previously reported in this marine angiosperm. The vast majority of the detected compounds belong to the flavonoid family, highlighting the presence of flavonols and flavanones, although some phenolic acids such as cinnamic and benzoic acids were also found.

Additionally, the distribution, in terms of number of total detected phenolic compounds, was relatively higher in leaves followed by rhizome and in last place roots. It seems that the cinnamic acids subclass is responsible for such a difference (caffeic acid, caftaric acid, chicoric acid, p-coumaric acid, fertaric acid, ferulic acid, and zosteric acid). However, only through the detailed research of the three tissues the complete *P. oceanica* phenolic fingerprint was unveiled, since several compounds were merely observed in underground tissues (e.g., bioachin A, glycitein, gambrin A1, and tacharoside).

This study brings new reference information in the field of bioactive compounds in marine seagrasses, namely a comprehensive array of phenolic compounds in *P. oceanica* tissues. In addition, the number of flavonoids, compounds widely employed as biomarkers, present in *P. oceanica* has been increased. Perhaps these first-time observed flavonoids could bring lacking information in the field of seagrasses chemical reactions.

The 42 detected compounds that make up the phenolic fingerprint of *P. oceanica* should be considered in future research to observe their behavior under different conditions and further evaluate their potential as specific-biochemical markers.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10050864/s1, Table S1: Compounds and their corresponding average intensities detected during the extractant composition evaluation (methanol/water, 5:5 v/v and 8:2 v/v) using *P. oceanica* tissues from FAN7 sampling point; Table S2: Traditional and IUPAC names of the first-time detected compounds in *P. oceanica* samples; Table S3: Biological properties of some bioactive compounds found in *P. oceanica* tissues; Table S4: List of suspected compounds retrieved from available literature for *P. oceanica* and other seagrasses (such as *Cymodocea nodosa*, *Zostera marina*, and *Zostera noltii*) that were not detected in this study. Theoretical mass in ESI- and ESI+ mode is provided for those compounds that were not present in our in-house database; Table S5: List of the phenolic compounds detected in *P. oceanica*, their precursor ions (Prc. Ion) and confirmation fragments (Frg.) in each tissue and sampling point; Table S6: Total phenolic compounds detected in *P. oceanica* tissues (target, suspected, and non-target) and the maximum number observed in the plant (grey). Subclasses from left to right: cinnamic acids (CA), benzoic acids (BA), flavones (FL), flavonols (FLL), isoflavones (i-FL), flavonons/flavanones (FLN), chalcones (CHL), catechins (CT), prenylated isoflavonoids (pr-IsF), dihydroflavonols (d-FLL), curcuminoids (CU), and stilbenes (ST); Figure S1: Extracted ion chromatogram for naringenin and naringenin chalcone (271.06012 *m/z*) in full MS of FAN7 leaves using Acquity C18 column and Hypersil GOLD column. NL: intensity; Figure S2: Extracted ion chromatogram for ferulic acid (full MS, *m/z*: 193.05063) and its confirmation fragment (MS/MS, *m/z*: 134.03643) in: (A) ammonium acetate and (B) formic acid as mobile phase. R_T: 17.9 min. NL: intensity; and Figure S3: Workflow indicating the conducted steps for the complete phenolic compound screening in the marine seagrass *P. oceanica*. Roots (●), rhizomes (■), and leaf (◊).

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Chapter III

Exogenous compounds

1. Introduction

Nowadays, it is common to detect current-use OPs, with potential detrimental impact, in the environment, even in remote areas far from their original sources (Cabrerizo et al., 2018). Furthermore, prohibited OPs are also still detected in the environment long after their input (legacy contamination; Carratalá et al., 2017).

The Spanish Mediterranean coast has a long history of OP generating activities such as agriculture, tourism or industry, and important riverine and atmospheric inputs (Castro-Jiménez et al., 2010; de Rosa et al., 2022; El-Maradny et al., 2023). Despite the relevance of the matter, few studies have delved into the OPs signature found in the endemic seagrass *P. oceanica* and related sediments (Vassallo et al., 2013).

Some investigations have performed analysis of priority OPs (mainly PAHs) in *P. oceanica* and sediments, without considering retrospective or non-target screenings (Apostolopoulou et al., 2014; Balcioğlu, 2021; Jebara et al., 2021; Mauro et al., 2013; Pergent et al., 2011; Renzi et al., 2022). Hence, the conducted monitoring programmes have not performed a comprehensive analysis, missing to achieve an accurate picture of the organic micropollutants signature in *P. oceanica* and related sediment. Moreover, these studies did not miniaturize the extraction and analytical methods. This step would help reduce the impact of research in the environment (Green Chemistry Principles; Gałuszka et al., 2013; López-Lorente et al., 2022). This is of special interest considering the key roles of the protected *P. oceanica* (by the European Union's Habitat Directive; Council Directive, 1992), and how relevant it is to guarantee its ecological integrity.

Given these points, the main aim of this chapter is to investigate the organic pollution (namely, 25 PCBs, 13 priority pesticides, 16 priority PAHs) present in the relevant *P. oceanica* seagrass and sediments using eco-friendly methods. For this purpose, multiclass extraction methods must be developed, optimized, validated, and applied in environmental samples of P. oceanica and marine sediments. Furthermore, to guarantee an accurate study, the analysis is to be conducted by GC coupled with HRMS (GC-Q-Orbitrap MS). Hence, applying the 3-ways analysis (i.e., target, suspected, and unknown) for the first time in this field of study.

The published articles encompassed in this chapter are:

- Scientific article II: Target and Suspect Analysis with High-Resolution Mass Spectrometry for the Exhaustive Monitoring of PCBs and Pesticides in *Posidonia oceanica* Meadows and Sediments.
- Scientific article III: Determination of PAHs, PAH-Derivatives and Other Concerning Substances in *Posidonia oceanica* Seagrass and Marine Sediments by High Resolution Mass Spectrometry.

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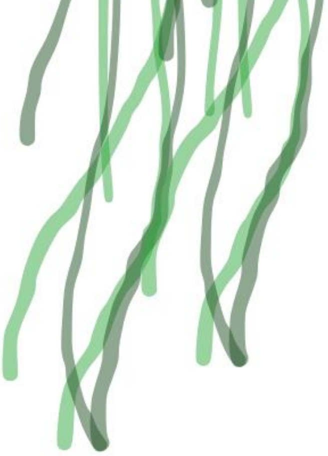

Scientific Contribution 2

Target and suspect analysis with high-resolution mass spectrometry for the exhaustive monitoring of PCBs and pesticides in *Posidonia oceanica* meadows and sediments

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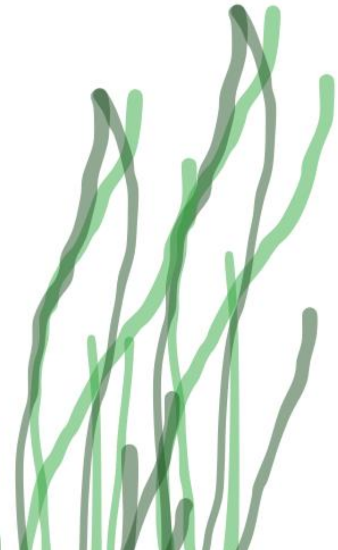

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Article

Target and Suspect Analysis with High-Resolution Mass Spectrometry for the Exhaustive Monitoring of PCBs and Pesticides in *Posidonia oceanica* Meadows and Sediments

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Abstract: This study enables the simultaneous monitoring of persistent organics pollutants (POPs) in the relevant marine seagrass *Posidonia oceanica* (L.) Delile (*P. oceanica*), without causing damage and preserving their ecological integrity and their key ecosystem services, and in marine sediments. Two classes of POPs that suppose a current threat to the environmental health status are investigated: polychlorinated biphenyls (PCBs) and pesticides. Comparisons between tissues and sediment compartmentation are studied for the first time. For these purposes, the sediments, *P. oceanica* leaves and, as a novelty, rhizomes, were studied. Samples were analyzed by gas chromatography coupled with high-resolution mass spectrometry (GC-Q-Orbitrap MS) for a comprehensive study. Eco-friendly methods were developed and validated for the determination of 38 POPs, 25 PCBs and 13 priority pesticides. The results showed that, when detected, regulated contaminants were localized mainly in the long-lived rhizomes, and 7 PCBs (the most abundant being PCB 44) and 4 priority pesticides (trifluralin, chlorpyrifos, isodrin and o,p′-DDT) were seen. Additionally, a retrospective analysis (suspect screening) was conducted, exhibiting up to 13 current-use pesticide residues in leaves and rhizomes alike. The results suggest that *P. oceanica* might be acting as a sink to contaminants in coastal areas and that rhizomes, due to their longer lifespan, reflect past and legacy contamination.

Keywords: seagrass; leaves; rhizomes; sediments; organic micropollutants; POPs; GC-HRMS; polychlorinated biphenyls

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1. Introduction

Persistent Organic Pollutants (POPs) represent a serious hazard for living beings. The nature of these mostly synthetic substances, such as their hydrophobic character, elevated bioaccumulation capacity, and long half-life, has facilitated their ubiquitous distribution in the environment. In fact, several remote ecosystems present background levels of POPs, and Background Assessment Concentrations (BACs) have been settled [1,2]. Aiming to control POPs production in industrial and agricultural activities and to reduce their presence in the environment, the Water Framework Directive (WFD) has regulated and cataloged some of them as priority substances [3], while others have been directly banned (Stockholm Convention) [4]. To ensure regulatory compliance and to fulfill the objective of achieving or maintaining the good status of the environment by 2020, the WFD has launched periodic monitoring programs. In the Barcelona Convention, several matrices were proposed for monitoring and considered in the Marine Strategy Framework Directive [5–7]. The proposed matrices were: surficial sediments acting as accumulation zones and POP reservoirs (thus feeding the water column and organisms) and sediment-hosting animals, such as the mussel *Mytilus galloprovincialis* (*M. galloprovincialis*) and the fish *Mullus barbatus* (*M. barbatus*).

The Mediterranean marine phanerogam, *Posidonia oceanica* (L.) Delile (*P. oceanica*), has been long known as one reliable indicator of disturbances in the coast and the presence of marine pollution [8–11]. Additionally, *P. oceanica* meadows are in regression, although they have been acknowledged as priority habitats due to the key ecosystem services provided, such as carbon sequestering [12–14]. It would be of great interest to develop an analytical method that allows for the sustainable monitoring of marine pollutants, such as polychlorinated biphenyls (PCBs) and pesticides, in this matrix. It is imperative to understand if the chemical content within *P. oceanica*, such as organic contaminants, might represent an additional threat to the health status of this relevant seagrass.

A recent study has shown that *P. oceanica* leaves possess a relatively higher residue accumulation than sediments, mussels or fish, which is recently arousing interest in this plant for monitoring programs [15]. However, similar to past studies which focused on other POPs, the extractions present clear disadvantages in terms of the amount of sample used [15], the solvent quantity [16,17] or the duration [18]. Likewise, articles mainly focus on the leaves, tissues with an average life span of about one year, meaning that the substances found reflect current pollution in the marine environment. On the other hand, the rhizomes, tissues that live for decades and are therefore potential providers of information related to legacy or inherited contamination, are often neglected [19,20]. Additionally, the published studies conducted their analysis using gas (GC) or liquid chromatography (LC) coupled with low-resolution mass spectrometry analyzers (LRMS) [15–18,21]. None of these studies have applied the current improvements in high-resolution mass spectrometry (HRMS) analyzers. The HRMS is fundamental to conducting ultrasensitive analysis and retrospective analysis to broaden the search to suspected and/or unknown compounds (even long after analyzing the samples) and is starting to be utilized in environmental studies [22–24]. The application of GC combined with HRMS allows for the exhaustive monitoring (searching for a large number of pollutants simultaneously) of POPs, their metabolites, sources or motion pathways.

Given the aforementioned information, this work aims to: (1) develop and validate eco-friendly methods for the simultaneous extraction of PCBs and pesticides in *P. oceanica* leaves, rhizomes and surficial sediments, using, for the first time, GC combined with HRMS (GC-Q-Orbitrap MS), and (2) analyze the presence and compartmentation of POPs residues, such as PCBs, priority pesticides and current-use pesticides, in the biotic and abiotic matrices, thanks to the combination of target and suspect screenings, which have not been previously addressed.

2. Materials and Methods

2.1. Chemical Reagents

Pesticide residue analysis grade solvents (PAR), such as acetone and ethyl acetate, were obtained from Panreac (Barcelona, Spain) and Riedel-de Haën^{TM}, respectively. Additionally, *n*-hexane and dichloromethane (DCM) were purchased from Riedel-de Haën^{TM} and Fluka, respectively. LC/MS-grade water was acquired from Supelco (Darmstadt, Germany).

The employed certified PCB standards were retrieved from Dr. Ehrenstorfer GmbH (Ausgburg, Germany). Pesticide standards, cataloged as priority substances, were obtained from Dr. Ehrenstorfer GmbH, Riedel de Haën (Seelze, Germany) and Fluka (Steinheim, Germany). A second batch of 246 pesticide standards was purchased from Dr. Ehrenstorfer GmbH and Sigma-Aldrich (St. Louis, MO, USA). The isotopically labeled standards (IIS), PCB 28F and hexachlorobenzene-13C, were purchased from Dr. Ehrenstorfer GmbH and Supelco (Bellefonte, PA, USA), respectively. These compounds were used as injection internal standards (IIS) and allowed for the correct normalization and quantification of the detected pollutants. A detailed description of the employed standards and IIS can be found in the supplementary material (*Chemical reagents: Standards* and Table S1).

2.2. Study Area and Sampling

In this study, as shown in Figure S1, a total of 28 sampling areas from the Mediterranean Spanish Coast (Almeria, Murcia, Alicante) were selected. The selection was conducted considering their environmental characteristics and potential (point and diffuse) sources of human-driven pollution [25]. Further information on the sampling areas is shown in the supplementary material (Table S2).

Where the sampling area allowed for it, divers manually collected: (1) the five uppermost cm of non-vegetated sediment from the front line of the meadow, (2) one *P. oceanica* specimen and (3) five surficial cm of vegetated sediment inside the meadow. In the areas where *P. oceanica* meadows were not present, such as harbors, two replicates of surficial sediment were taken instead. Seagrass samples were stored in sterile plastic bags, and sediments were stored in plastic containers. Both matrices were kept at low temperatures (from 1 to 4 °C) during transport. On all occasions, *P. oceanica* sampling was carried out without altering the ecological integrity of the meadows and with the permission of the Regional Environmental Administration.

2.3. Sample Pretreatment

P. oceanica was processed as previously described in Astudillo-Pascual et al. [26]. Briefly, sand and salt were removed using distilled water. Afterwards, the samples were divided into two parts: the leaf and rhizome. Note that roots, young leaves, basal sheath and epiphytes were removed and not considered in this study. Later, the samples were stored at −20 °C (48 h) and dried at −50 °C (48 h) in a Thermo Electron Corporation Heto PowerDry LL3000 freeze-dryer (Thermo Fisher Scientific, Bremen, Germany). Subsequently, the samples were homogenized (Mixer Mill MM 200) and stored in desiccators until extraction.

In the case of sediments, these were air-dried at room temperature. During the process, samples were maintained in the dark at low temperatures and covered to avoid aerosol-driven contamination. Dried sediment was then sieved (2 mm stainless steel sieve), and the fine fraction was homogenized using a glass mortar. Samples were stored in the darkness and at room temperature until extraction.

2.4. Extraction Procedure

For the extraction of the organic pollutants from *P. oceanica* tissues, 3 mL of hexane/ethyl acetate 9:1 *v/v*, a solvent previously used in Jebara et al. [15], was added to a 15 mL Falcon tube containing 150 mg of a dry weight (d.w.) powder sample (~1 g wet weight, w.w.). All mixtures were vortexed for 1 min. Afterwards, the samples were homogenized for 2 min using a polytron PT 2100 (Kinematica AG, Lucerne, Switzerland) at room temperature (~25 °C). Subsequently, the samples were centrifuged (5000 rpm, 10 min), and the supernatant was filtered with 0.22 µm nylon filters (LLG, Meckenheim, Germany).

For the sediments, 5 g d.w. samples were placed in a 50 mL Falcon tube and hydrated with 5 mL LC/MS-grade water, followed by the addition of 10 mL of hexane/ethyl acetate 9:1 *v/v*, and they were vortexed for 1 min. The samples were then taken to the ultrasound for 10 min at room temperature and were finally centrifuged at 2700 rpm for 5 min. The resulting supernatant was filtered using 0.22 µm nylon filters.

In all cases, 1 mL of each extract (leaf, rhizome and sediment) was poured into 2 mL vials, spiked with 20 µL IIS mix and analyzed. The PCB 28F and hexachlorobenzene-13C IIS were chosen for the PCB congeners and pesticide analytes, respectively.

2.5. GC-Q-Orbitrap MS Parameters

A GC-Q-Orbitrap system made up of a TriPlus RSH autosampler, a Trace 1300 gas chromatograph and a Q-Exactive Orbitrap mass analyzer (Thermo Fisher Scientific, Bremen, Germany) was used in the present study. The injector was composed of a single taper liner of 78.5 mm × 4 mm ID (Thermo Fisher Scientific), performing hot spitless injections of 1 µL at 280 °C, and 1 min of spitless time. The carrier gas (Helium, 99.999%) flow was set at 1 mL min^{-1}. The GC separation was carried out on a VF-5 ms column of 30 m × 0.25 mm

ID and 0.25 µm. The oven program was set as described in Table S3. Positive electron ionization (EI) with a 50 uA emission current and 70 eV electron energy was chosen for the Q-Exactive Orbitrap mass analyzer. Further information on the analytical conditions and acquisition parameters can be found in the supplementary material (*Analysis of organic contaminants: GC-Q-Orbitrap MS parameters*).

2.6. Method Validation

The final methods, based on polytron extraction for *P. oceanica* and ultrasound-assisted extraction (UAE) for sediments followed by GC-Q-Exactive MS analysis, were validated following the DG SANTE guidelines, with minor modifications [27]. Hence, matrix-matched calibration points spiked with the standard mixture containing the target POPs were prepared at eight different concentrations, ranging from 2 to 2000 µg kg^{-1} in the case of *P. oceanica* tissues and from 0.2 to 200 µg kg^{-1} in the case of sediments. Since *P. oceanica* shoots washed up on the coast and the sediments retrieved from the sea shoreline were used as blanks, the POP signals observed before spiking were subtracted from the spiked blanks.

The parameters investigated were: (1) linearity, represented as the determination coefficient or R^2; (2) trueness in terms of recovery, obtained by spiking blanks at two different levels (VL1 = 20 and VL2 = 400 µg kg^{-1} for the leaf, VL1 = 10 and VL2 = 200 µg kg^{-1} for the rhizome and VL1 = 2 µg kg^{-1} and VL2 = 40 µg kg^{-1} for the sediment) after calculating the recovery values for each of the 38 target analytes; (3) the precision, estimated from the intraday and interday values. The intraday values were estimated at the same levels as the trueness, and the results were expressed as the relative standard deviations (%RSD); as for the interday precision, this was calculated like the intraday precision, but with repeating the process over three different days; (4) the limit of quantification, or LOQ, can be considered as the lowest level of the calibration curve offering suitable recoveries and RSD, but it was also calculated as 10 times the standard deviations obtained for the lowest level of the calibration curve [28]. To obtain all the mentioned parameters, each sample was analyzed in triplicate.

2.7. Analysis of Organic Contaminants: Target and Suspect Screenings

For the target analysis, a database with 38 POPs (25 PCBs and 13 priority pesticides), including their corresponding quantification ions, confirmation ions and retention times, (RT) was generated from the analysis of commercially available standards (Table S4).

The use of the HRMS analyzer operating in full scan mode allowed for conducting a retrospective analysis from the yet-generated raw data file. So, in addition to target analysis, a suspect analysis of additional pesticides of concern was carried out [29].

During the analyses, and to assure the results' reliability, a matrix-matched calibration point and a solvent-matched calibration point were injected as quality controls together with the environmental samples.

3. Results

3.1. Extraction Procedure Optimization and Validation

Before validation and analysis, an optimization step for each matrix (leaf, rhizome and sediment) was run to improve the efficiency of the extraction procedure. In the case of the *P. oceanica* tissues, five extractions systems were tested (polytron, agitation, UAE, QuEChERS and QuEChERS-UAE) using hexane/ethyl acetate 9:1 *v/v* as the solvent. Detailed information is shown in the supplementary material (*Extraction procedures*). However, for the sediments, a UAE method was tested, which exhibited satisfactory results in past studies, varying the solvent between DCM and hexane/ethyl acetate 9:1 *v/v* [30,31]. To evaluate the performance of each tested extraction strategy, blank samples were spiked with the standard mixtures of PCBs and pesticides at a final concentration of 2 mg kg^{-1}. Later, their corresponding recoveries were compared. The results revealed better recoveries for all the different analytes when using polytron with hexane/ethyl acetate for the leaves and rhizomes alike (as shown in Table S5), while UAE extraction with hexane/ethyl acetate proved to be more adequate for sediments (Table S6).

Then, the analytical methods were validated according to the parameters described above. The obtained values are shown in Table 1. Briefly, good linearity was obtained in all cases ($R^2 > 0.9728$ for the leaf, $R^2 > 0.9803$ in the case of the rhizome and $R^2 > 0.976$ for the sediment). Additionally, adequate recoveries were achieved (from 80% to 110%) at all concentrations inside their respective linear working ranges, as well as intraday and interday precision values, which showed satisfactory results (RSD < 18% for the leaf and sediments, and RSD < 20% in the case of the rhizome) for most of the analytes in the different matrices. Finally, the observed LOQs parameters ranged from 0.015 to 0.753 and from 0.076 to 5.348 μg kg^{-1} d.w. for PCBs and pesticides in leaves, from 0.009 to 0.534 and from 0.011 to 9.785 μg kg^{-1} d.w. in rhizomes and from 0.001 to 0.093 and from 0.001 to 0.205 μg kg^{-1} d.w. in sediments, showing the excellent sensitivity of the developed methods.

3.2. Application: Occurrence and Compartmentation of POPs

Under the extraction and analytical parameters employed here, several PCBs and pesticides were detected and identified as follows.

3.2.1. Target Analysis: PCBs

The chromatographic conditions employed in this study allowed for an optimal separation of all analytes, excluding the PCB 28 and PCB 31 congeners. Due to their similarities in terms of RT, mass and confirmation ions, it was not possible to differentiate them unequivocally. Therefore, isomers were counted as only one compound and expressed as PCB 28 + 31 [32].

Regarding the samples, no PCB congeners were detected in Almeria or Murcia in any of the matrices (*P. oceanica* or sediments). The PCBs were only located in three sampling sites in the Alicante Region (ALI5, ALI6 and ALI7; Table 2 and Figure S2). As for the distribution among matrices at these three sampling sites, PCB congeners were not detected in the leaves or in non-vegetated sediment, but they were in the rhizomes and vegetated sediment. Rhizomes showed residues of up to seven congeners (PCB 28+31, PCB 52, PCB 44, PCB 81, PCB 77, PCB 153 and PCB 167). Additionally, the ∑7 PCBs recommended by the International Council for the Exploration of the Sea (ICES) (i.e., the sum of PCB 28, PCB 52, PCB 101, PCB 118, PCB 153, PCB 138 and PCB 180) ranged from 2.4 to 14.3 μg kg^{-1} d.w. In rhizomes, PCB 44 and PCB 28 + 31 were usually the most abundant in all sites, as well as PCB 77 at site ALI5. Additionally, at ALI5, where the PCB presence in rhizome was higher, the vegetated sediment (collected during step 3 of the sampling strategy) showed a wider number of PCB congeners (up to 18; Table 2 and Figure S3) but lower concentrations, observing 8.3 μg kg^{-1} d.w. for ∑7 PCBs and 21.5 μg kg^{-1} for ∑PCBs. In sediments, PCB 44 was also the most abundant, followed by PCB 138 and PCB 170 > PCB 157, PCB 28 + 31, PCB 18 and PCB 167 > PCB 101 > PCB 123 and PCB 180 > PCB 128 > PCB 153 > PCB 114 and PCB 105 > PCB 118 > PCB 77 > PCB 81.

Table 1. Validation results for the analytical methods developed in this study for the studied matrices at two concentration levels [a].

	LEAF							RHIZOME							SEDIMENT						
Compounds	LOQ (µg kg^{-1})	Linear Working Range (µg kg^{-1})	Linearity (R^2)	Recovery R (%) [b]		Inter-Day Precision (RSD%)		LOQ (µg kg^{-1})	Linear Working Range (µg kg^{-1})	Linearity (R^2)	Recovery R (%) [b]		Inter-Day Precision (RSD%)		LOQ (µg kg^{-1})	Linear Working Range (µg kg^{-1})	Linearity (R^2)	Recovery R (%) [b]		Inter-Day Precision (RSD%)	
				VL1	VL2	VL1	VL2				VL1	VL2	VL1	VL2				VL1	VL2	VL1	VL2
PCBs																					
PCB 18	0.266	10–1000	0.9922	98(7)	108(10)	18	15	0.168	10–400	0.9986	97(9)	98(3)	17	6	0.017	1–200	0.9977	104(9)	98(3)	11	7
PCB 28+31	0.516	20–2000	0.9978	104(5)	99(0)	6	17	0.378	10–400	0.9988	93(3)	99(8)	1	7	0.023	1–200	0.9989	99(2)	108(3)	7	5
PCB 52	0.404	10–400	0.9966	110(5)	99(1)	17	13	0.378	10–400	0.9979	101(5)	100(5)	4	3	0.013	1–200	0.9991	111(4)	100(1)	5	3
PCB 44	0.015	20–2000	0.9998	99(0)	100(0)	8	17	0.127	10–1000	0.9982	105(4)	99(2)	5	6	0.009	1–200	0.9986	118(2)	98(3)	3	3
PCB 66	0.151	20–2000	0.9993	120(4)	101(1)	17	9	0.534	10–200	0.9930	115(17)	95(8)	18	5	0.027	1–200	0.9984	108(8)	98(4)	15	8
PCB 101	0.753	20–2000	0.9998	99(2)	100(0)	18	17	0.210	10–400	0.9894	94(11)	106(10)	2	4	0.040	1–200	0.9951	114(9)	97(8)	13	9
PCB 81	0.485	10–1000	0.9997	99(5)	99(2)	10	15	0.061	10–1000	0.9982	80(3)	99(2)	8	4	0.004	0.2–200	0.9926	102(7)	108(9)	10	10
PCB 77	0.018	10–2000	0.9997	106(7)	100(1)	8	14	0.162	10–200	0.9916	106(7)	101(1)	9	3	0.030	1–200	0.9964	94(10)	105(7)	12	10
PCB 123	0.363	20–1000	0.9996	104(1)	99(2)	8	15	0.009	10–400	0.9987	93(1)	95(13)	12	2	0.015	1–40	0.9975	103(7)	100(9)	8	9
PCB 118	0.121	20–1000	0.9979	101(2)	100(1)	5	14	0.037	10–400	0.9986	99(2)	91(8)	14	3	0.004	0.2–200	0.9926	102(5)	93(10)	7	6
PCB 114	0.032	10–400	0.9991	101(2)	102(3)	5	6	0.074	10–400	0.9984	98(8)	94(10)	18	9	0.046	1–200	0.9965	118(11)	96(7)	17	8
PCB 153	0.368	20–400	0.9951	100(6)	100(3)	11	18	0.035	10–400	0.9988	93(2)	99(10)	11	3	0.093	2–200	0.9989	97(10)	100(1)	15	5
PCB 105	0.608	20–1000	0.9973	97(4)	101(1)	10	18	0.075	10–400	0.9987	101(3)	98(10)	14	11	0.006	2–200	0.9936	102(13)	95(9)	15	9
PCB 138	0.267	10–1000	0.9970	99(0)	100(2)	3	14	0.261	10–1000	0.9803	93(15)	97(4)	10	5	0.004	1–200	0.9984	118(2)	99(2)	2	1
PCB 126	0.136	20–2000	0.9998	94(2)	100(0)	4	14	0.021	10–2000	0.9996	107(1)	100(1)	16	12	0.016	2–40	0.9760	85(5)	109(15)	15	7
PCB 128	0.022	20–400	0.9908	107(1)	101(1)	11	12	0.121	10–400	0.9979	99(12)	99(8)	1	3	0.019	2–200	0.9987	90(12)	100(5)	13	9
PCB 167	0.023	10–1000	0.9982	102(1)	100(1)	4	11	0.130	10–400	0.9988	92(7)	99(10)	3	1	0.046	1–200	0.9987	113(10)	100(1)	15	8
PCB 156	0.177	20–2000	0.9985	101(1)	109(2)	4	14	0.050	10–2000	0.9994	80(3)	100(1)	5	10	0.010	1–200	0.9990	85(7)	99(2)	18	7
PCB 157	0.265	20–1000	0.9934	100(1)	102(1)	6	15	0.035	10–400	0.9988	86(2)	99(10)	2	4	0.024	2–200	0.9995	84(4)	100(1)	18	10
PCB 180	0.206	20–1000	0.9965	108(9)	100(0)	9	17	0.182	10–400	0.9976	88(16)	98(4)	18	8	0.028	2–200	0.9997	101(16)	100(3)	18	5
PCB 169	0.171	10–1000	0.9977	100(1)	100(1)	10	15	0.052	10–2000	0.9996	102(1)	100(0)	13	8	0.048	2–200	0.9984	115(7)	101(0)	10	5
PCB 170	0.253	20–1000	0.9976	95(1)	99(2)	8	14	0.057	10–400	0.9987	91(4)	99(9)	5	4	0.001	1–200	0.9981	115(1)	99(3)	12	10
PCB 189	0.253	20–1000	0.9978	99(8)	100(1)	15	15	0.094	10–2000	0.9991	98(8)	101(2)	9	12	0.017	2–200	0.9981	82(6)	103(4)	10	10
PCB 194	0.078	20–2000	0.9986	97(5)	99(0)	5	16	0.069	10–2000	0.9993	90(8)	100(1)	10	5	0.070	2–40	0.9801	101(14)	102(7)	17	5
PCB 206	0.212	20–1000	0.9986	83(8)	99(2)	14	14	0.018	10–1000	0.9992	120(1)	100(1)	10	5	0.005	1- 40	0.9948	81(9)	98(5)	13	9

Table 1. *Cont.*

	LEAF								RHIZOME								SEDIMENT							
Compounds	**LOQ (µg kg^{-1})**	**Linear Working Range (µg kg^{-1})**	**Linearity (R^2)**	**Recovery R (%) [b]**		**Inter-Day Precision (RSD%)**		**LOQ (µg kg^{-1})**	**Linear Working Range (µg kg^{-1})**	**Linearity (R^2)**	**Recovery R (%) [b]**		**Inter-Day Precision (RSD%)**		**LOQ (µg kg^{-1})**	**Linear Working Range (µg kg^{-1})**	**Linearity (R^2)**	**Recovery R (%) [b]**		**Inter-Day Precision (RSD%)**	
				VL1	**VL2**	**VL1**	**VL2**				**VL1**	**VL2**	**VL1**	**VL2**				**VL1**	**VL2**	**VL1**	**VL2**
Pesticides																					
Pentachloro-benzene	0.286	2–1000	0.9993	85(17)	102(3)	18	5	0.070	2–400	0.9996	99(3)	100(1)	13	10	0.001	0.2–40	0.9995	104(0)	100(1)	5	2
Trifluralin	0.123	10–1000	0.9998	100(2)	102(0)	6	3	0.305	10–400	0.9980	106(5)	99(7)	7	2	0.050	1–40	0.9950	117(4)	101(1)	7	4
Hexachloro-benzene	1.131	10–2000	0.9985	97(10)	98(2)	5	18	0.359	10–200	0.9969	120(8)	101(0)	3	8	0.205	2–40	0.9966	114(10)	99(2)	4	5
Simazine	0.550	20–1000	0.9991	101(4)	102(2)	10	3	0.011	10–200	0.9978	119(8)	107(1)	5	2	0.019	2–40	0.9875	91(1)	103(6)	4	9
Atrazine	0.376	10–2000	0.9994	102(6)	102(2)	5	2	0.777	10–400	0.9981	108(8)	94(12)	4	10	0.047	1–40	0.9981	102(4)	101(4)	5	11
Chlorpyrifos	0.076	10–2000	0.9974	101(2)	97(4)	6	3	0.158	10–400	0.9863	96(5)	109(12)	3	5	0.103	2–40	0.9940	87(9)	101(3)	10	16
Aldrin	0.216	20–1000	0.9993	85(5)	101(1)	2	1	0.635	10–2000	0.9997	112(3)	99(2)	4	6	0.025	2–40	0.9958	81(4)	100(2)	6	9
Isodrin	0.756	10–2000	0.9976	97(15)	100(2)	5	1	0.221	10–1000	0.9997	90(5)	99(2)	20	10	0.098	2–100	0.9991	100(6)	100(2)	9	9
Dieldrin	0.126	20–1000	0.9959	82(3)	101(3)	4	5	0.621	10–200	1.0000	108(13)	101(3)	9	6	0.002	1–100	0.9989	87(1)	99(1)	14	6
Endrin	0.946	10–400	0.9805	89(1)	104(2)	9	10	1.819	10–200	0.9964	107(11)	99(1)	12	6	0.002	1–100	0.9873	87(1)	100(2)	13	4
o,p′-DDT	5.348	20–1000	0.9882	92(16)	100(1)	18	15	9.785	10–400	0.9991	115(15)	110(6)	17	10	0.130	1–40	0.9973	91(9)	102(3)	11	9
p,p′-DDD	0.482	10–400	0.9923	110(12)	106(9)	15	10	1.982	10–200	0.9991	107(13)	100(1)	16	8	0.048	1–40	0.9923	92(12)	94(9)	13	6
p,p′-DDT	4.725	10–400	0.9982	118(15)	100(9)	15	13	0.419	10–400	0.9996	110(13)	100(6)	19	8	0.015	2–100	0.9996	112(4)	99(2)	8	9

[a] The two validation levels (VL1 and VL2) correspond to 20 and 400 µg kg^{-1} for the leaf, 10 and 200 µg kg^{-1} for the rhizome and 2 and 40 µg kg^{-1} for the sediments. [b] Intra-day precision values (or repeatability, expressed as % RSD) are given in brackets ($n = 3$).

Table 2. Occurrence of PCBs (μg kg^{-1} d.w.) in the rhizomes of the marine plant *P. oceanica* and vegetated sediments (V-sed). Note that only the sampling areas where contamination was detected have been included in the table.

Matrix	Site	PCB 18	PCB 28+31	PCB 52	PCB 44	PCB 101	PCB 81	PCB 77	PCB 123	PCB 118	PCB 114	PCB 153	PCB 105	PCB 138	PCB 128	PCB 167	PCB 157	PCB 180	PCB 170	ΣPCBs	∑7 PCBs
Rhizome	ALI5	–	10.7	1.6	11.2	–	9.7	11.1	–	–	–	2.0	–	–	–	8.7	–	–	–	55.0	14.3
	ALI6	–	1.8	0.6	1.9	–	–	1.3	–	–	–	–	–	–	–	–	–	–	–	5.6	2.4
	ALI7	–	2.6	0.6	1.6	–	1.1	1.6	–	–	–	–	–	–	–	–	–	–	–	7.5	3.2
V-sed	ALI5	1.4	1.5	1.1	1.7	1.2	0.8	0.9	1.1	0.9	0.9	0.9	0.9	1.6	1.0	1.4	1.5	1.1	1.5	21.5	8.3

In this study the PCB 28 congener eluted together with its isomer PCB 31, and both were counted as only one compound; thus, the ∑7 PCBs value might be slightly overrated. (n.s.): not specified or not studied; (–): analyte not found or detected.

3.2.2. Target Analysis: Priority Pesticides

Regulated pesticides were not detected in any of the matrices (*P. oceanica* or sediments) from Almeria or Murcia. Instead, the priority pesticides were detected at the same stations of the Alicante Region where the PCBs were observed: ALI5, ALI6 and ALI7. In these stations, only rhizomes reflected the presence of these analytes; they were not seen in leaves and vegetated or non-vegetated sediment. The detected pesticides in the rhizomes were trifluralin, chlorpyrifos, isodrin and o,p′-DDT (Table 3). Some examples of extracted ion chromatograms are shown in Figure S4. The sum of all the priority pesticides considered in this study (ΣPesticides) ranged from 1.0 to 9.0 μg kg^{-1} d.w; the greater concentration was seen again at ALI5, followed by ALI6 and ALI7 (Figure S2).

Table 3. Occurrence of the priority pesticides (μg kg^{-1} d.w.) in the rhizomes of *P. oceanica*. Note that only the sampling areas where contamination was detected have been included in the table.

Matrix	Site	Trifluralin	Chlorpyrifos	Isodrin	o,p´-DDT	ΣPesticides
Rhizome	ALI5	3.9	3.2	1.9	*	9.0
	ALI6	2.0	1.3	1.7	–	5.0
	ALI7	–	–	1.0	–	1.0

(–) analyte not found or detected. (*) < LOQ.

3.2.3. Suspect Analysis: Current-Use Pesticides

Since the target screening of priority pesticides revealed the presence of four banned analytes in rhizomes (trifluralin, chlorpyrifos, isodrin and o,p´-DDT), and considering the historical records of agricultural activities on the Mediterranean Spanish coast, a supplementary retrospective analysis was conducted, aiming at an additional 246 pesticides of concern. From this wide array of compounds, several were considered emerging pesticides. As a result, several pesticides were tentatively identified in nine sampling areas of Almeria and seven from Alicante, while none were observed in Murcia (Table 4). In total, 16 sampling stations out of the 28 sampled showed these compounds in any of their matrices. The pesticide presence was major in the biotic compartment, i.e., leaves (revealing six compounds: 1,4-dimethyl naphthalene, 2-phenylphenol, terbutryn, tetraconazole, piperonylbutoxide and difenoconazole) and rhizomes (showing seven compounds: 1,4-dimethyl naphthalene, 2,4,6-trichlorophenol, lindane, pyrimethanil, penconazole, fludioxonil and fenbuconazole). Only the prallethrin insecticide was encountered in the abiotic compartment, i.e., vegetated and non-vegetated sediments. This analyte was detected at the RM6, C2 and V1 from Almeria and at the ALI1, ALI2, ALI3 and ALI7 from Alicante. Examples of extracted ion chromatograms are shown in Figure 1.

These tentatively identified structures were confirmed using analytical standards. Matching RTs, confirmation ions and the comparison of the spectra between real samples and the spiked blank extracts allowed for their confirmation (Table S7) [33,34]. For quantification, a standard addition procedure was followed, and concentrations were estimated [35]. The registered summatory values for leaves ranged from <LOQ to 366.50 μg kg^{-1} d.w., with the maximum at CG3 in Almeria (ΣCurrent-use pesticides; Figure S5). In the case of the rhizomes, concentrations varied from 0.13 to 47.15 μg kg^{-1} d.w.; higher concentrations were seen at ALM3 and CG4 and also in Almeria. On the other hand, sediments from several areas exhibited the prallethrin insecticide. The ΣCurrent-use pesticide concentrations ranged from 5.28 to 7.53 μg kg^{-1} d.w. in Almeria and from 4.27 to 6.01 μg kg^{-1} d.w. in Alicante, whereas non-vegetated sediments reflected 5.52–6.90 and 4.23–7.31 μg kg^{-1} d.w., respectively (Table 4 and Figure S5).

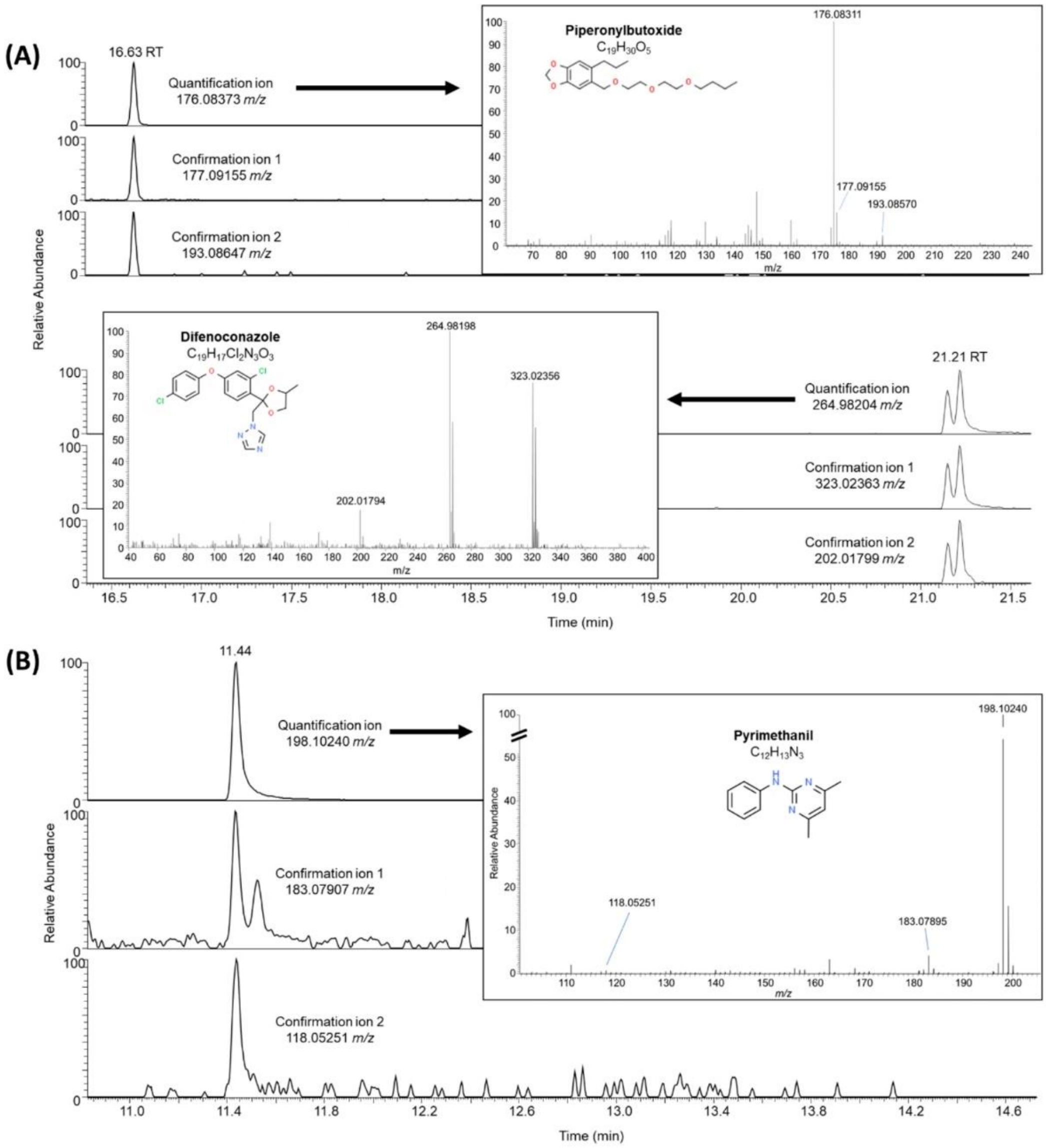

Figure 1. Extracted ion chromatograms and mass spectrum of some of the detected current-use pesticides: (**A**) piperonylbutoxide and difenoconazole in leaves at CG3, (**B**) pyrimethanil in the rhizome at station ALM3.

Table 4. Current-use pesticides (µg kg^{-1} d.w.) identified in this study in *P. oceanica*, non-vegetated sediments (NV-sed) and vegetated sediments (V-sed).

		Almeria Region												**Alicante Region**						
Matrix	**Compound**	**EE2**	**EE4**	**RM4**	**RM6**	**ALM1**	**ALM3**	**V1**	**V2**	**CG2**	**CG3**	**CG4**	**C2**	**ALI1**	**ALI2**	**ALI3**	**ALI7**	**ALI5**	**ALI6**	**ALI4**
Leaf	1,4-Dimethyl naphthalene	3.22	8.97	–	n.s.	–	–	n.s.	28.96	–	16.87	–	–	–	–	–	10.02	14.31	16.32	9.88
	2-Phenylphenol	–	–	2.89	n.s.	–	–	n.s.	–	–		–	–	–	–	11.03	8.43	9.18	7.16	9.6
	Terbutryn	–	–	–	n.s.	–	–	n.s.	–	–	–	–	–	0.21	0.11	–	–	–	–	–
	Tetraconazole	–	–	–	n.s.	–	–	n.s.	–	–	–	–	–	–	–	–	1.12	–	–	–
	Piperonylbutoxide	–	–	–	n.s.	–	–	n.s.	–	–	84.39	–	–	–	–	–	–	–	–	–
	Difenoconazole	–	–	–	n.s.	–	–	n.s.	–	–	265.24	–	–	–	–	–	10.05	–	–	–
	Mean regional values						82.11										15.35			
Rhizome	2,4,6-trichlorophenol	–	–	–	n.s.	–	0.88	n.s.	–	–	–	0.36	–	–	–	–	–	–	–	–
	1,4-Dimethyl naphthalene	–	–	–	n.s.	–	5.33	n.s.	–	–	–	4.46	–	–	–	–	–	–	–	–
	Lindane	–	–	–	n.s.	–	–	n.s.	–	–	–	–	–	–	–	–	0.22	0.51	0.11	–
	Pyrimethanil	–	–	–	n.s.	–	40.66	n.s.	–	–	–	29.29	–		0.13	–	–	–	–	–
	Penconazole	–	–	–	n.s.	–	0.28	n.s.	–	–	–	0.13	–	–	–	–	–	–	–	–
	Fludioxonil	–	–	–	n.s.	–	–	n.s.	–	–	–	–	–	–	–	–	–	–	2.85	7.93
	Fenbuconazole	–	6.16	8.73	n.s.	6.59	–	n.s.	–	6.84	7.11	–	–	4.08	–	–	3.15	–	–	–
	Mean regional values						16.69										3.63			
NV-Sed	Prallethrin	–	–	–	5.78	–	–	7.45; 5.52	n.s.	–	–	–	6.9	4.23	n.s.	n.s.	7.31	–	–	–
V-Sed		–	–	–	n.s.	–	–	n.s.	n.s.	–	–	–	5.28	5.01	6.01; 4.27	7.53; –	5.74	–	–	–

–: analyte not found. n.s.: not sampled.

4. Discussion

4.1. Extraction Procedure

The developed methods showed suitability for PCBs and priority pesticide detection in the different matrices (*P. oceanica* tissues and sediment). As a novelty, the premises of the sample-friendly techniques and the green chemistry were followed, i.e., diminishing the amount of the sample (~1 g w.w. for leaves and rhizomes) and solvent required [36]. This is of special interest when studying living organisms playing key roles in the environment. Therefore, this method would allow for conducting periodical analysis without compromising the meadows' ecosystem services. For minor interference with the meadow's health, sampling programs should be conducted during summer. In this period, leaves reach the maximum maturity and height, gathering chemical information for about 1 year.

Additionally, in all observed articles, extraction methods were time-consuming. In contrast, the extraction methods employed in this study proved to be faster (<15 min) compared to the others, such as the Soxhlet extraction (24 h; Table S8).

4.2. Target Analysis: PCBs

For the first time, the PCB congeners 44, 81 and 167 were detected in *P. oceanica*. In fact, PCB 44, the most abundant compound in rhizomes and sediments, was not considered before in *P. oceanica*-related studies. It is also important to highlight that, under our experimental conditions, PCBs were only noticed in three sampling areas from the Alicante Region (ALI5, ALI6 and ALI7). Here, all rhizomes showed PCB residues, and only ALI5 reflected contamination in the vegetated sediment. This distinction between vegetated sediment and non-vegetated sediment was also seen in other seagrass beds from Florida [37].

Clear compartmentation was spotted at ALI5, where PCB congeners showed a matrix-specific distribution based on their n-octanol/water partition coefficient (log K_{ow}). The PCBs with a low and high log K_{ow} were seen in the sediments (Table 2 and Table S1). On the contrary, only PCBs with a log K_{ow} lower than 7 were noticed in the rhizomes, except for PCB 167 (7.5 log K_{ow}), implying that the physicochemical properties of the PCBs might be influencing their fate and bioaccumulation, as observed in mangroves ecosystems [38].

For a primary evaluation of the Alicante PCB concentrations and their potentially harmful effects on the marine environment, the results were compared to the BAC, to the Effects Range–Low (ERL, i.e., concentrations in the sediments associated with biological effects) and the Environmental Assessment Criteria limits (EAC; below such concentration on marine species, chronic effects are not expected to occur). For instance, the ∑7 PCBs in sediments from ALI5 were above background levels or BAC but did not reach ERL (11.5 $\mu g\ kg^{-1}$ d.w.). On the other hand, rhizomes were compared to internationally recognized bioindicators, the mussel *M. galloprovincialis* and the fish *M. barbatus*. In general, several PCB congeners were above BAC, even exceeding EAC for mussels in the cases of the congeners PCB 28+31 and PCB 52 (Table S9). Note that no BAC or EAC for *P. oceanica* has been described yet, and, therefore, PCB concentrations in the rhizomes cannot be truly evaluated or classified as low, moderate or high.

In comparison to other studies, the ∑PCB values encountered in the rhizomes were similar to those reported in the leaves of the Central Eastern Tunisia Coast or inside the interval when considering ∑PCB7 [15]. Note that, to our knowledge, no other study delved into PCBs in *P. oceanica* rhizomes; thus, the values were compared to the only study conducted in leaves. Individual congener concentrations were generally greater in rhizomes than in fish and mussels from the Mediterranean Spanish coast and Tunisia but were surpassed by the values observed in the Adriatic Sea (Table S10) [15,39].

As for the ALI5 vegetated sediments, both ∑7 PCBs and ∑PCBs were of several orders of magnitude greater than those detected in the Central Eastern Tunisia Coast [15] and higher than those in other locations from the Mediterranean Spanish coast [40] and in the Rosseta Estuary in Egypt [41]. The values encountered in this study were surpassed by certain concentrations detected in the River Mouth Fiumicino Canal [42] or the Port of

Trieste in Italy (Table S10) [43]. The results obtained in the Alicante and Murcia regions were in line with previous studies conducted in these areas, where PCB congeners were not detected, or the levels were below EAC limits [44]. Likewise, the non-detection of PCBs in the sampling areas belonging to the Almeria region concurs with the results obtained in past published information on marine sediments [30,31] or on monitoring programs in sediment and mussels, where very low levels for the $\sum 7$ PCBs were observed ($<2.5\ \mu g\ kg^{-1}$ d.w.) [45].

4.3. Target Analysis: Priority Pesticides

The three priority pesticides detected and quantified in *P. oceanica* have been reported in this study according to the published data: trifluralin, chlorpyrifos and isodrin. Likewise, the analytes were observed only in rhizomes, and none were observed in *P. oceanica* leaves or sediments. On the other side, the priority pesticide levels detected in rhizomes cannot be assessed since a specific EAC for the *P. oceanica* marine plant or matrix is lacking, as well as for other marine vascular plants or biota. However, the estimated chlorpyrifos concentrations reported for *P. oceanica* rhizomes surpassed the predicted no-effect concentration of $0.032\ \mu g\ kg^{-1}$ d.w. established for marine sediment by the WFD, which gives an approximated idea of the magnitude [40].

Similar to the PCBs case, since no other studies have reported pesticides in rhizomes, the obtained data have been compared to those described for leaves, mussels, fish and other surficial marine sediments. The $\sum$Pesticides found in rhizomes from the Alicante Region were always of several orders of magnitude smaller than those observed in leaves and fish from the Central-Eastern Tunisia Coast, such as *Sparus aurata* and *Sarpa Salpa* (Table S11) [15]. Here, the presented values were also lower than those found in mussels along the Adriatic Sea coast [46]. Comparing the rhizomes to the sediments from other areas, ALI6 and ALI7 showed similar values to those encountered in the Rosseta Estuary in Egypt [41] but slightly higher values than those reported in Tunisia sediments, the River Mouth Fiumicino Canal or concentrations found along the Mediterranean Spanish Coast, as can be observed in Table S11 [15,40,42].

The findings reported here are supported by previous studies. For instance, in Alicante and Almeria, regions that are well known for their elevated intensive agricultural activities, no priority pesticides were detected during the initial evaluation of the Marine Strategy [44,45]. The agreement between the results presented here and the published literature is also attained in the case of the Murcia Region; according to past studies, no priority pesticides were found at concentrations higher than the LOQ at the exterior of the Mar Menor Lagoon in 2009. Nonetheless, two priority pesticides were detected later in autumn of 2010 (chlorpyrifos and simazine), and one was detected in spring of 2010 (chlorpyrifos), suggesting a potential seasonal factor altering the priority pesticides' presence at this location [47].

4.4. Suspect Analysis: Current-Use Pesticides

An unprecedented analysis aiming at current-use pesticides of concern has been conducted in *P. oceanica*. This analysis was also applied to surface sediments. Several areas from Alicante and Almeria showed some sort of pesticide residue. Overall higher mean values were observed in the eastern sampling areas of Almeria (Table 4). Additionally, a wider distribution was observed in terms of matrices compared to PCBs or the priority pesticides, being detected in leaves, rhizomes and sediments. However, these analytes gathered preferably in the biotic compartment, where they seemed to follow a tissue-dependent distribution, except for 1,4-dimethyl naphthalene, which was detected in both matrices. This preference for the biotic compartment concurs with the published information about the elevated bioaccumulation efficiency of *P. oceanica* over the sediment [15,18].

Leaves' concentrations were especially remarkable at CG3, followed by V2, ALI7, ALI5, ALI6 and ALI4 (Figure S5, Table 4). The plant growth regulator 1,4-dimethyl naphthalene found at V2 can be ascribed to the closeness to the Almanzora watercourse, surrounded by agricultural activities. At the four Alicante sampling stations, 1,4-dimethyl naphthalene and 2-phenylphenol (fungicide) were detected, as well as difenoconazole and tetraconazole (fungicides) in ALI7. Several activities occur in these four sampling areas, which might be acting as potential contamination sources. For instance, the relatively urbanized areas are influenced by seasonal tourism or the closeness to watercourses and breakwaters (at ALI4 and ALI6), as well as urban stormwater runoff or septic tanks (at ALI6 and ALI5). Additionally, ALI4, ALI5 and ALI6 have a long record of non-irrigated and irrigated agriculture in their surroundings.

In the case of the rhizomes, ALM3 and CG4 gathered the greatest concentrations, represented by the pyrimethanil fungicide. Here, the potential sources also consist of seasonal watercourses and different agricultural activities. For instance, at ALM3, greenhouses and irrigated extensive crops are predominate, whereas in CG4, non-irrigated crops are found.

As for the sediment, several areas of Almeria (RM6, C2 and V1) and Alicante (ALI1, ALI2, ALI3 and ALI7), usually close to seasonal watercourses or crops, presented signals of the prallethrin insecticide commonly employed in household products for mosquitoes' control. At these stations, no pattern or differentiation was identified between the non-vegetated and vegetated sediment.

The current-use pesticide concentrations measured inside the MPA were unexpected. Part of the contamination may originate in external sources, similar to what occurred in Santa Maria, another MPA from the Mediterranean Sea which presented a surprisingly elevated amount of microplastics [48]. It is well known that POPs may undergo long-range transport through ocean currents or atmospheric movements [49], affecting places that were meant to be pristine.

In summary, neither PCBs nor priority pesticides were detected in the leaves. Considering that *P. oceanica* regenerates its leaves periodically (~1 year) and the greater accumulation capacity (compared to the sediments) [15], the non-detection in the leaves or in the relatively long-lived rhizomes (~30 years) might indicate their absence (or concentrations below LODs) in the near water column. This would correlate with the decline in their synthesis and usage since their prohibition. Therefore, rhizomes might have been sequestering coastal POPs for years. Its monitoring would be of great interest in delving into near-past chemical events. Nonetheless, considering the key role of *P. oceanica* in carbon sequestration and other ecosystem services, rhizome monitoring should be performed only when the background or baseline information of a coastal environment is lacking.

Additionally, in other studies, *P. oceanica* meadows have been seen to affect the coastal water fluxes and act as a barrier, enhancing deposition and trapping organic and inorganic particles, even plastic debris, for years [50,51]. Therefore, in the case of the PCBs at ALI5, their presence in vegetated sediments and rhizomes alike could be due to the *P. oceanica* canopies preventing land-based POPs (adsorbed to particles, organic matter or microplastics) from entering into marine dynamics [52–54]. This would explain, to an extent, their non-detection in the non-vegetated surficial sediments from shallow waters, which, without the *P. oceanica* protection, are exposed to marine motion, such as currents and waves, and, therefore, to a stronger resuspension and remobilization [55]. Contrarily, the current-use pesticides employed nowadays and probably in the water column are found in leaves, rhizomes, non-vegetated sediment and those covered by the meadow.

5. Conclusions

Novel, sustainable and eco-friendly methods were optimized and validated to simultaneously detect PCBs and priority pesticides in the protected *P. oceanica* (rhizomes and leaves) and surficial sediments. These practical techniques allow for a reliable extraction without compromising the organism's integrity. This leaves open the possibility to conduct sustainable monitoring programs in this interesting and unique matrix. Additionally, for the first time, an ultra-sensitive analysis using the advantages provided by the GC combined with HRMS (sensitivity, selectivity and retrospective analyses) was conducted, saving time and resources.

These methods allowed for the satisfactory monitoring of several pollutants in marine samples from near-shore environments of the Mediterranean Spanish Coast. Overall, PCB contamination appeared preferentially in the rhizomes, followed by the sediments. For the first time, the PCB congeners 44, 81 and 167 were detected in *P. oceanica*. The values reflected the concentrations of PCBs in rhizomes exceeding the BAC for biota, even surpassing the EAC for mussels in some cases. Likewise, priority pesticides were also found in the rhizome of one sampling area of Alicante, detecting, for the first time, trifluralin, chlorpyrifos (which surpassed the predicted no-effect concentration for sediments) and isodrin.

An additional screening of current-use pesticides was conducted in leaves, rhizomes and sediments. To our knowledge, such screening was not performed before in *P. oceanica* and permitted the identification of another 13 compounds in the study areas of Almeria and Alicante. The current-use pesticides of concern presented a wider distribution compared to the banned or regulated compounds (PCBs and priority pesticides), likely due to the current usage in agriculture activities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemosensors10120531/s1, Table S1: The target PCBs, pesticides and IIS included in this study with their corresponding CAS number and n-octanol/water partition coefficient (log K_{ow}); Table S2: Sampling areas description, from west to east orientation. Non-vegetated sediment (▒, n = 23), leaves (◊, n = 21), rhizome (■, n = 20), vegetated sediment (▓, n = 20); Table S3: Oven program; Table S4: Retention time and *m/z* ions selected for the quantification and confirmation of the PCB and pesticide compounds in the present study and the IIS; Table S5: Minimum and maximum recoveries (%) per class of POP regarding the extraction procedures in *P. oceanica* leaves and rhizomes; Table S6: Minimum and maximum recoveries (%) per class of POP regarding the extraction solvent employed during the UAE of surficial sediments; Table S7: Current-use pesticides identified in this study and their corresponding RT, molecular weight, log K_{ow} and quantification and confirmation ions; Table S8: Summary of the known POPs extraction methods in *P. oceanica*. PLE: Pressurized Liquid Extraction; Table S9: PCBs concentration in rhizomes (µg kg^{-1} w.w.) compared to BAC and EAC for the fish (*M. barbatus*) and mussels (*M. galloprovincialis*) used by OSPAR and the Marine Strategy Framework Directive (MSFD). Wet weight values were obtained by considering the moisture content (%) of each sample; Table S10: PCBs (µg kg^{-1} d.w.) concentrations detected in the Mediterranean Sea[a,b,c,d]. Only the sampling areas where contamination was detected have been included in the table; Table S11: Priority pesticide concentrations (µg kg^{-1} d.w.) detected in the biota and sediments from other studies; Figure S1. Maps with the three regions belonging to the Mediterranean Spanish coast surveyed in this study: (A) Almeria region, (B) Murcia and (C) Alicante: stations ALI1, ALI2, ALI3, ALI4, ALI5, ALI6 and ALI7. Figure made by the author using Ocean Data View (Schlitzer, R., Ocean Data View, https://odv.awi.de, 2021); Figure S2: Location of ALI5, ALI6 and ALI7 sampling areas from Alicante and their corresponding ΣPCBs and ΣPriority Pesticides values (µg kg^{-1}) in rhizomes (RHIZ) and vegetated sediment (V-SED). Images retrieved from Google (©Images 2022 CNES, NOAA, U.S. Navy. NGA, GEBCO, TerraMetrics, Landsat, Copernicus); Figure S3: Extracted ion chromatogram and mass spectrum of PCB 153, 138, 128, 167 and 157 isomers detected in the vegetated sediment at ALI6; Figure S4: Extracted ion chromatogram of trifluralin, chlorpyrifos and isodrin detected in the rhizomes at ALI5; Figure S5: ΣCurrent-use pesticides of concern (µg kg^{-1}) seen in the different matrices of the aquatic plant *P. oceanica*, vegetated sediments (V-sed) and non-vegetated sediments (NV-sed) among different sampling stations.

Author Contributions: M.A.-P.: Conceptualization, Software, Validation, Investigation, Writing—Original Draft. P.A.A.: Conceptualization, Resources, Writing—Review & Editing. A.G.F.: Conceptualization, Validation, Resources, Writing—Review & Editing, Supervision. I.D.: Conceptualization, Methodology, Validation, Investigation, Writing—Review & Editing, Supervision. All authors have read and agreed to the published version of the manuscript.

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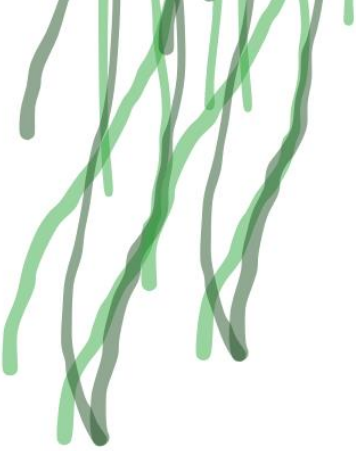

Scientific Contribution 3

Determination of PAHs, PAH-derivatives and other concerning substances in *Posidonia oceanica* seagrass and marine sediments by high resolution mass spectrometry

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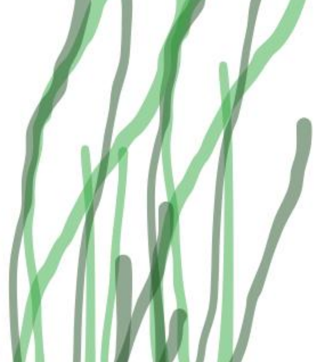

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Determination of PAHs, PAH-Derivatives and Other Concerning Substances in *Posidonia oceanica* Seagrass and Marine Sediments by High Resolution Mass Spectrometry

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Abstract: The *Posidonia oceanica* decline due to climate change and other anthropogenic pressures, such as chemical pollution, is well known in the scientific community. However, a comprehensive study of the full content of the organic micropollutants found in this significant seagrass has not yet been carried out. Second, an eco-friendly extraction procedure that does not require a large sample, preserves the seagrass's ecological integrity and functions, and follows green-chemistry principles, is lacking. These information gaps represent the aims of this study. For this purpose, trials with diverse simple and affordable extraction methods to detect one of the most ubiquitous contaminants (polycyclic aromatic hydrocarbons or PAHs) were conducted. As a result, the use and validation of a polytron homogenizer and an ultrasonic bath were proposed for the extraction of priority PAHs from tissues of *P. oceanica* and marine sediments, respectively. Tissues (leaves and rhizomes) of *P. oceanica* and sediment samples were collected, extracted, and subjected to a thorough analysis, i.e., target, suspect, and unknown screenings, using gas chromatography coupled to high resolution mass spectrometry (GC-Q-Orbitrap MS). Target analysis revealed seven priority parent-PAHs, whilst during the suspect screening, four PAH-derivatives and three other parent-PAHs were tentatively identified. In the additional third unknown analysis, 11 structures, several with concerning toxicity, were also tentatively identified. Numerous of the identified compounds showed elevated detection frequency in the environmental samples, even reaching 100%, such as the cases of the parent-PAHs (naphthalene, phenanthrene and retene), some PAH-derivatives, one UV stabilizer, and plastic additives along with pesticides. The methods proposed here should be considered for future monitoring of *P. oceanica*, as well as the three-way analytic approach (target, suspect and unknown), to obtain a more real and accurate idea of the organic micropollutants content in the environment.

Keywords: seagrass; sediments; GC-HRMS; suspect analysis; unknown analysis; organic micropollutants

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1. Introduction

Posidonia oceanica (L.) Delile, a seagrass endemic to the Mediterranean Sea, is considered one of the natural tools to tackle climate change [1,2]. This plant is a major carbon sink of enormous significance, reaching sequestration estimation of 11–42% of the total carbon emitted since the Industrial Revolution by the Mediterranean countries [1,3]. *Posidonia oceanica* beds also promotes millennial-scale services in coastal defense against erosion, biodiversity and fish nursery functions, rating its ecosystem services from 57 to 184 thousands €/ha/year [4,5], even though their area represents less than 2% of the Mediterranean basin. Largely due to the contribution of *P. oceanica*, a recent study suggests considering the Mediterranean basin as a priority candidate for conservation [6].

However, the areas of *P. oceanica* meadows are receding. In 2015, an article highlighted that meadows lost one-third of their area in the last 50 years [7]. The recession has been associated with numerous pressures, such as anchoring, illegal fishing, artificial beach nourishment, coastal and physicochemical changes (for instance, temperature and chemical pollution), impacting ecosystems and the Mediterranean economies [7–9]. One of these pressures has been poorly studied in *P. oceanica*, namely, the organic micropollutants. To our knowledge, only a few studies have been published since the first one in 2006 [10].

Polycyclic aromatic hydrocarbons (PAHs) are one of the most widespread organic pollutants. These types of highly toxic and complex organic compounds have been associated with toxicity, endocrine disruptions, reproductive impairment, genotoxicity, impacts on sexual differentiation, deformities in both young and adult fish, neoplasia, cancer and other mutations that can lead to Genotoxic Disease Syndromes (GDS) in marine biota [11–15]. In the case of human exposure to PAHs, it leads to generally increased morbidity, due to for example, allergies or asthma, cardiovascular and immunological disorders, placenta malfunction, and severe effects on the fetus [16,17].

This, added to their persistence, makes them a risk not only to human health but also to the environment. Consequently, several PAHs have been listed as priority pollutants and banned by the Water Framework Directive (WFD) [18].

The PAHs are the result of burning organic material. Although this combustion might occur naturally (volcanic eruptions and forest fires), PAHs found in the environment originate mainly from human-made processes. Some examples are the incomplete combustion of crude oil and petroleum derivatives in industrial activities and vehicles, or the burning of waste (such as agricultural organic matter, household waste, biomass, or cigarettes). Due to their natural and anthropogenic sources, the Background Assessment Concentration (BAC) must be considered to determine if the assessed pollution is close to background values, i.e., natural contamination at a pristine site based on contemporary or historical data, or whether the contamination levels are higher [19,20].

From the sources, PAHs move through atmospheric deposition, industrial flows, waste and river discharges or via runoff, towards the coastal environments. Once in the sea, the most common pathway is the intake by marine organisms or sedimentation, affecting the benthic community [21–23]. The fate or compartmentation of PAHs is believed to be closely linked to their physicochemical properties. High molecular weight (HMW) organic micropollutants with an elevated number of rings (>3 rings) or partition coefficient octanol-water (log K_{ow}) are expected to precipitate and attach to the sediment. In contrast, the most soluble and light (low molecular weight, LMW, i.e., $\leq$3 aromatic rings or approximately <200 g mol^{-1}) are likely to remain in the water column and be taken up by marine organisms [24].

The precipitation of pollutants in the Mediterranean Sea might be enhanced by *P. oceanica*, which inhabits the shallow coastal waters in ~0–40 m depth. Their long canopies, and often their barrier shaped meadows, promote the flocculation of floating particles (which frequently act as vectors for other substances) by attenuating the marine dynamics [25,26]. This is also the case of PAHs, which have been previously detected in leaves of *P. oceanica* [27–29]. However, only one article has delved into the compartmentation of the PAH inside *P. oceanica*, broadening the research to the rhizomes [30]. Additionally, green-chemistry principles have not been applied in the currently available publications (reduced amounts of sample and solvents, and usage of less hazardous chemicals), nor has the study of PAH breakdown structures been contemplated [31]. The paper by Apostolopoulou et al. [30] was the first and only study including certain PAH-derivatives in their assessments, although it did not report which individual analytes were encountered, their concentrations or the apportioning to the total measured contamination. The PAH derivatives exhibit greater solubility, mobility and bioavailability, consequently larger adverse effects on the environment. These derivatives usually get formed because of abiotic or biotic (metabolites) degradation of parent-PAHs. Additionally, there is the possibility of a synthetic apportioning of derivatives, for instance as co-formulants used in pesticides. Hence, the absence of parent PAHs in environmental samples might not directly correlate with pristine or uncontaminated *P. oceanica*, as PAH transformations might have taken place. Both parent PAHs and degradation byproducts should be therefore considered in pollution studies [32–34].

To fulfill these information gaps, the present study developed, validated, and successfully applied ecofriendly extraction methods for the target analysis of 16 priority parent PAHs in leaves and rhizomes of the seagrass *P. oceanica* and marine sediments. The analysis was run by means of gas chromatography (GC) combined with high resolution mass spectrometry (HRMS). Additionally, to maximize the obtained information, a complete analysis of each environmental sample was conducted by applying the potential offered by the Q-Exactive Orbitrap analyzer, and its capability to perform retrospective analyses, such as a suspect analysis (in search of PAH-derivatives, other parent PAHs, and linear alkylbenzenes or LABs) and an unknown analysis to determine additional organic micropollutants present in the samples.

2. Materials and Methods

2.1. Chemical and Reagents

A PAH standard mixture (QTM-Standard; 2 mg mL^{-1} for each compound in dichloromethane) containing 16 USEPA priority PAHs was purchased from Sigma–Aldrich (St. Louis, MO, USA). The isotopically labeled fluoranthene-d10, used as injection internal standard (IIS), was purchased from Supelco (Bellefonte, PA, USA). In all cases, the purity of the analytical standards was above 97%. Further information on the purchased standards can be found in Table S1.

The standard mixture was diluted in acetone until reaching 100 µg L^{-1}. The same occurred with the IIS fluoranthene-d10, in this case reaching a final concentration of 500 µg L^{-1}. Both standard working solutions were stored at −20 °C inside amber screw-capped glass vials.

Solvents of PAR quality (Pesticide residue analysis grade solvent) were employed, such as acetone and ethyl acetate from Panreac (Barcelona, Spain), dichloromethane (DCM) and *n*-hexane, obtained from Fluka and Riedel-de Haën^{TM} (Seelze, Germany) respectively. Additionally, LC/MS-grade water was obtained from Supelco (Darmstadt, Germany).

2.2. Study Area and Sampling

In this study, three regions from the southeast coast of Spain, exposed to a diverse range of pressures, were selected for sampling: Almeria (18 sampling sites), Murcia (3 sites), and Alicante (7 sites; Figure 1). A detailed description of the sampling sites, locations and sampling time are given in Table S2 (Supplementary Materials). In most cases, the sampling was carried out by scientific scuba divers, proceeded in three steps. First, a sediment sample was taken in front of the beginning of the meadow upper limit (non-vegetated sediment, NV-sed) taking manually the 5–7 uppermost cm with a 100 mL polystyrene container. Consequently, a plant of *P. oceanica* was carefully extracted from the meadow by hand, near to the upper limit. Finally, a few meters inside the meadow, a vegetated sediment sample (V-sed) was taken. An additional type of sediment, far from any influence of a *P. oceanica* meadow or any other vegetated area (non-meadow-related or NMR-sed), was retrieved on certain occasions. In total, during several campaigns, numerous tissues of *P. oceanica*, such as leaves (n = 22) and rhizomes (n = 20), along with V-sed, NV-sed and NMR-sed (n = 23, n = 18 and n = 12 respectively) were collected (Table S2). Note that the sampling was authorized in each case by the Regional Environmental Administration.

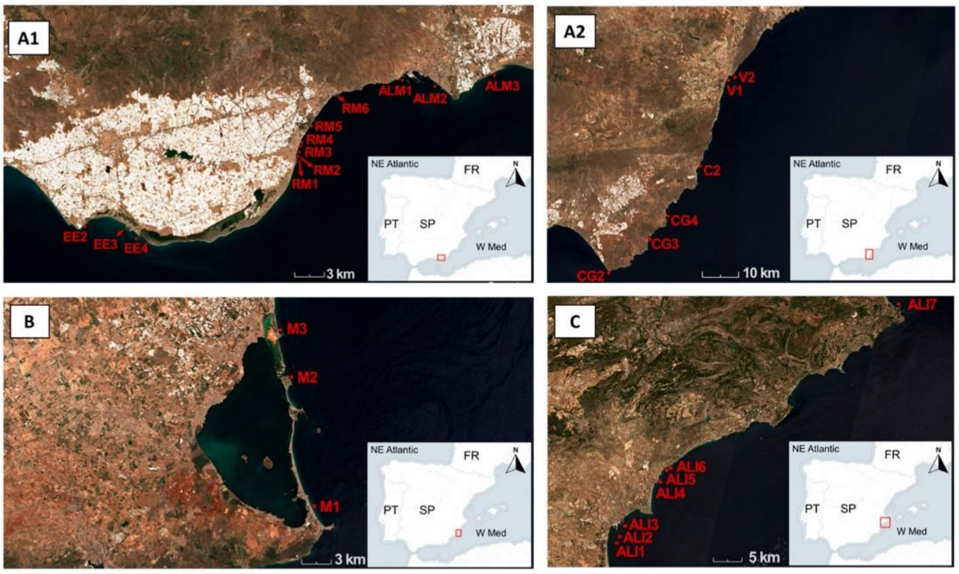

Figure 1. Maps of the surveyed points on the Mediterranean Spanish coast: (**A1**) Western Almeria, (**A2**) Eastern Almeria, (**B**) Murcia and (**C**) Alicante stations. White areas seen on land correspond to greenhouses. Figure made by the authors using Copernicus Sentinel data (2022) and processed with EO Browser.

2.3. Sample Pretreatment

Following a procedure already described in Astudillo-Pascual et al. [35], *P. oceanica* seagrasses were cleaned from sand and salt using distilled water. Afterward, adult leaves and rhizomes were selected for further procedures, while roots, young leaves, basal sheath, and epiphytes were removed and not considered in this study. Adult leaves and rhizomes were frozen (48 h) and subsequently freeze-dried (48 h) using a Thermo Electron Corporation Heto PowerDry LL3000 freeze-dryer (Thermo Fisher Scientific, Bremen, Germany). Finally, samples were ground with a Mixer Mill MM 200 (Retsch, Asturias, Spain) and stored in desiccators.

In the case of the sediment samples, these were air-dried. To avoid aerosol-driven contamination and photodegradation, all samples were covered with 50 × 50 cm filter sheets and kept inside a fume hood at room temperature (~20 °C). Afterward, samples were sieved using a 2 mm stainless steel sieve, placed in polystyrene containers, and stored in darkness until extractions.

2.4. Extraction Procedure

Tissue-specific extraction procedures, polytron for leaves and rhizomes, and ultrasound-assisted extraction (UAE) for sediments, were conducted for analysis of PAHs. For this matter, 150 mg dry weight (d.w.) of tissues of *P. oceanica* were extracted with 3 mL of hexane/ethyl acetate (9:1 v/v) in a 15 mL Falcon tube, mixed in a vortex for 1 min and homogenized for 2 min using a polytron PT 2100 (Kinematica AG, Lucerne, Switzerland). Samples were then centrifuged at 5000 rpm for 10 min. On the other hand, 5 g d.w. of sediment was hydrated with 5 mL LC/MS-grade water in a 50 mL Falcon tube. Afterward, 10 mL of hexane/ethyl acetate (9:1 v/v) were added and vortexed for 1 min. Falcon tubes were left in the ultrasonic bath (10 min, room temperature) and centrifuged (2700 rpm, 5 min). In all cases, the resulting extraction supernatant was filtered using 0.22 µm nylon filters (LLG, Meckenheim, Germany). Finally, 1 mL of the extract was taken into 2 mL vials with 20 µL of the IIS solution.

2.5. GC-Q-Orbitrap MS Parameters

In all cases, a GC-Q-Orbitrap MS system with a TriPlus RSH autosampler, a Trace 1300 gas chromatograph and a Q-Exactive Orbitrap mass analyzer (Thermo Fisher Scientific, Bremen, Germany) were used. The HRMS analyzer ran in full scan mode, producing a raw file that permitted also retrospective or non-target analyses (i.e., suspect, and unknown) by running a computerized data processing. Additional parameters regarding the analytical conditions and acquisition are described in the Supplementary Materials (GC-Q-Orbitrap MS parameters (Tables S3 and S4).

2.6. Method Validation

For validation of the methods, several parameters were evaluated, such as linearity, accuracy, precision (intraday and interday), limit of detection (LOD) and quantification (LOQ). Linearity was given by the determination coefficient (R^2) of the calibration curves. Accuracy was evaluated in terms of recovery ($n = 3$) at two validation levels (VL1 and VL2) within the linear range. Precision (defined as relative standard deviation, RSD %) was estimated by injecting three replicates per VL on the same day (intra-day precision) and along three different days (inter-day precision). The LODs were determined as three times the standard deviation of the lower calibration point. Meanwhile, the LOQs were established as 10 times such a value [36–38]. A more explanatory description of the process can be found in the Supplementary Materials (Validation).

2.7. Analysis of the Environmental Samples: Target and Non-Target Approaches

Three different analyses were carried out: an initial target analysis for the monitoring of 16 parent-PAHs; a suspect analysis, for the detection of PAH transformation products (i.e., nitro-, oxy-, methyl-, and hydroxy- PAH-derivatives, quinones and heterocyclic-PAHs), not-priority parent-PAHs and LABs; and an unknown analysis to complete a thorough characterization of the studied environmental samples.

2.7.1. Target Analysis

Information regarding the quantification and confirmation ions, retention times (RT) or analyte spectrum were obtained directly from the analysis of commercially available standards and gathered in Table S4. Note that benzo[b]fluoranthene and benzo[k]fluoranthene coeluted, sharing a common RT, mass, and confirmation ions, hence they were quantified as one compound. All other target compounds showed defined and well-separated peaks allowing for accurate quantification.

2.7.2. Suspect Analysis

Data were retrieved from research articles, online open-source databases and the NIST library. Later, the gathered information was used to develop compound databases inside the Software TraceFinder 4.1 (Thermo Fisher Scientific, Les Ulis, France) for rapid data acquisition and processing of the environmental samples. The database included a total of 143 compounds, 103 of them being PAH-derivatives (oxy-, nitro-, methyl and hydroxy-metabolites, plus quinone derivatives), 14 additional parent-PAHs and 26 LABs. The latter are high hydrophobic compounds, present in household detergents and therefore indicators of wastewater discharges [39–41].

As standards for the suspect analytes were not available, a semi-quantification was performed. For this purpose, the method described in Pieke et al. [42] was followed. Briefly, the IIS fluoranthene-d10 was selected as the quantification marker (QM), assuming a similar behavior (selection due to similarity) because it belongs to the same class as the suspect compounds. A response factor, hereafter RF, was calculated by dividing the fluoranthene-d10 measured signal by its actual concentration. Subsequently, the signals of the suspect analytes were divided by the previously calculated RF.

2.7.3. Unknown Analysis

This analysis was carried out using the Compound Discoverer software (Thermo Fisher Scientific, Les Ulis, France). This software performed a deconvolution, separating coeluting compounds and generating clean spectral information for a subsequential structural analysis and metabolite profiling. The information obtained was rapidly compared by the software to an in-house database and online spectral reference libraries, such as the NIST. To reach an accurate tentative identification several filters and criteria were applied for the potentially present compounds: (1) only well-defined peaks were chosen, (2) peak areas should be equal or greater than 1×10^6, (3) mass error (considering to the fifth decimal) between theoretical and candidate compounds had to be $\leq$5 ppm, (4) similarity index (SI) and reverse SI (RSI) should be $\geq$800, and (5) high resolution filtering tool (HRF) $\geq$ 80% [43]. Additionally, the recorded signal should be at least five times greater than the noise or signal found in the clean-ups, to differentiate laboratory-driven contamination during the extraction procedure and real environmental contamination.

3. Results and Discussion

3.1. Extraction Procedure Optimization and Validation

Before running the analysis, simple and eco-friendly techniques (polytron, agitation, UAE, QuEChERS and QuEChERS-UAE) were tested to optimize the extraction of 16 priority PAHs from tissues of *P. oceanica* in a hexane/ethyl acetate 9:1 *v*/*v* media [27]. Further explanation of the techniques can be found in the Extraction Procedures section (Supplementary Materials). For sediments, a UAE method, which previously yielded good results, was employed varying the extractant solvent from DCM [44] to hexane/ethyl acetate 9:1 *v*/*v* [27]. Each extraction method was subject to recovery trials, spiking samples (n = 3) at 100 µg L^{-1} (2 mg kg^{-1}) with the PAH standard mixture. The resulting recoveries from each extraction method were then compared. Results pointed out polytron extraction as the most suitable for the leaves and rhizomes, whereas the UAE method showed relatively better results for sediments (Table S5).

Consequently, a validation process was conducted for polytron and UAE analytical methods, to ensure method reliability and consistency. Obtained results are shown in Table S6, Supplementary Materials. Linearity values were $\geq$0.99 in 15 out of 16 compounds (i.e., in 94% of the cases): $R^2 > 0.9944$ in the leaf, $R^2 > 0.9819$ in the rhizome and $R^2 > 0.9877$ in sediment. Recoveries fell inside the range of 80–120% at each calibration point inside the linear working ranges. The intra-day as well as inter-day precision yielded RSD values < 20% for most of the compounds in the diverse matrices. Additionally, the generated LODs and LOQs are shown in Table S7. In this study, LOQs oscillated between 0.001–1.701 µg kg^{-1} d.w., 0.091–4.363 µg kg^{-1} d.w, and 0.007–1.056 µg kg^{-1} d.w. for leaves, rhizomes, and sediments, respectively. These LOQs were lower or similar to those presented in analogous studies [45,46]. The LODs obtained in this study varied from 0.0003 to 0.5103 µg kg^{-1} d.w., 0.0273 to 1.3089 µg kg^{-1} d.w., and from 0.0021 to 0.3168 µg kg^{-1} d.w. for leaves, rhizomes, and sediments respectively. In most cases, sedimentary LODs were smaller than those reported in previous studies [30,45]. A more exhaustive comparison, including tissues of *P. oceanica*, is hampered due to the scarce or not available information.

3.2. Application to Environmental Samples

3.2.1. Target Analysis

All sampling sites (n = 28) showed PAH signals in all their matrices, reflecting the well-known PAH ubiquity. A total of seven priority-target PAHs were detected in the analyzed samples (Table 1). Fluoranthene, naphthalene, phenanthrene, and pyrene were recurrent, being detected in more than 50% of samples. On the other hand, anthracene, benz[a]anthracene or fluorene were less commonly found and therefore cataloged as "rare" (<50% detection). As an example, the extracted ion chromatograms of the recurrent detected compounds are shown in Figure S1.

Table 1. PAH congeners (μg kg^{-1} d.w.) observed in leaves, rhizomes, and sediments (V-sed. NV-Sed and NMR-Sed) from the Almeria, Murcia and Alicante coastal regions compared to BAC [a], EAC [b] and ERL [c] concentrations. Detection is expressed in %.

Matrix	**Analyte**	**EE2**	**EE4**	**RM1**	**RM2**	**RM3**	**RM4**	**RM5**	**ALM1**	**ALM2**	**ALM3 [d]**	**CG2**	**CG3**	**CG4**	**C2**	**V2**	**ALI1**	**ALI2 [d]**	**ALI3**	**ALI4**	**ALI5**	**ALI6**	**ALI7**	**Detection**
Leaf	Naph	0.7	0.9	0.7	1.2	0.9	0.9	0.8	1.0	1.2	0.7	0.9	0.8	0.9	1.0	0.9	*	1.1	0.9	1.2	1.2	1.0	1.2	100
	Flu	–	–	–	–	–	–	–	–	–	–	–	7.3	–	–	–	–	–	–	–	–	–	–	5
	Phe	7.2	9.2	6.7	9.9	18.1	17.2	12.9	11.9	12.6	17.3	6.1	21.2	13.6	15.9	4.9	11.4	23.9	15.5	6.6	9.5	5.3	12.1	100
	Ant	*	–	*	–	–	–	–	–	–	–	*	–	–	–	–	*	–	–	*	*	*	–	32
	Flt	2.9	–	–	–	–	**9.4**	3.9	–	**7.8**	*	–	**8.1**	3.1	–	–	3.2	**10.1**	–	–	**7.5**	**7.7**	3.0	55
	Pyr	2.9	3.1	**8.3**	**17.1**	**8.8**	**205.8**	**10.8**	**13.8**	**8.3**	**8.7**	3.8	**10.2**	**7.3**	**8.7**	–	3.8	**7.8**	3.1	**10.3**	**10.2**	3.0	2.1	95
	BaA	–	–	–	–	–	–	–	–	–	–	–	–	**9.7**	–	–	–	–	–	–	–	–	–	5
	ΣPAHs	13.7	13.2	15.7	28.2	27.8	233.0	28.4	26.7	29.9	26.7	10.8	47.6	34.6	25.6	5.8	18.4	42.9	19.5	18.1	28.4	17.0	18.4	
Rhizome	Naph	–	20.2	15.1	n.s.	–	17.8	–	25.9	17.4	17.6	11.3	19.0	–	12.7	n.s.	8.3	15.8	–	18.6	18.6	19.9	12.2	73
	Phe	–	12.5	10.7	n.s.	10.7	*	*	18.6	4.5	33.7	3.7	4.5	18.7	15.2	n.s.	8.1	5.6	–	18.1	9.7	22.0	15.1	82
	Ant	–	–	–	n.s.	–	–	–	–	–	–	–	–	–	–	n.s.	–	–	–	–	–	**5.0**	–	5
	Flt	–	–	–	n.s.	–	–	–	–	–	0.8	–	–	0.6	–	n.s.	–	–	–	–	–	–	–	9
	ΣPAHs	–	32.7	25.8	–	10.7	17.8	–	44.5	21.9	52.1	15.0	23.5	19.3	27.9	n.s.	16.4	21.4	–	38.1	28.3	46.9	25.9	
V-Sed	Naph	4.2	3.6	–	2.0	2.1	1.4	2.2	2.6	3.9	4.2	4.3	4.8	2.3	95.4	n.s.	85.7	104.0	88.8	2.8	2.9	0.4	39.4	91
	Phe	1.5	–	–	–	–	–	–	–	–	–	–	1.0	–	**8.4**	n.s.	**12.9**	**23.0**	–	–	1.1	–	–	27
	Flt	–	–	–	–	–	–	–	4.4	–	–	–	–	–	–	n.s.	–	–	–	–	–	–	–	5
	Pyr	–	–	–	–	–	–	–	4.3	–	–	–	–	–	–	n.s.	–	–	–	–	–	–	–	5
	ΣPAHs	5.7	3.6	–	2.0	2.1	1.4	2.2	11.3	3.9	4.2	4.3	5.8	2.3	103.8	n.s.	98.6	127.0	88.8	2.8	4.0	0.4	39.4	

Table 1. *Cont.*

Matrix	Analyte	EE2	EE4	RM1	RM2	RM3	RM4	RM5	ALM1	ALM2	ALM3 [d]	CG2	CG3	CG4	C2	V2	ALI1	ALI2 [d]	ALI3	ALI4	ALI5	ALI6	ALI7	Detection
NV-Sed	Naph	2.1	3.4	2.1	2.5	3.9	1.0	1.6	4.3	6.3	n.s.	4.4	4.5	1.2	81.4	n.s.	90.0	n.s.	n.s.	2.3	0.8	1.3	79.1	82
	Phe	–	–	–	–	–	–	–	**47.0**	**10.1**	n.s.	–	–	–	*	n.s.	**15.5**	n.s.	n.s.	–	–	–	–	18
	Ant	–	–	–	–	–	–	–	**3.0**	–	n.s.	–	–	–	–	n.s.	–	n.s.	n.s.	–	–	–	–	5
	Flt	–	–	–	–	–	–	–	**40.7**	13.3	n.s.	–	–	–	–	n.s.	–	n.s.	n.s.	–	–	–	–	9
	Pyr	–	–	–	–	–	–	–	**30.8**	**11.7**	n.s.	–	–	–	–	n.s.	–	n.s.	n.s.	–	–	–	–	9
	ΣPAHs	2.1	3.4	2.1	2.5	3.9	1.0	1.6	125.8	41.4	n.s.	4.4	4.5	1.2	81.4	n.s.	106	n.s.	n.s.	2.3	0.8	1.3	79.1	

Matrix	Analyte	EE3	RM6	V1	M1	M2	M3	Detection
NMR-Sed	Naph	5.0	32.4	86.4	72.7	70.7	79.0	100
	Flu	–	3.4	–	–	–	–	17
	Phe	3.7	**7.5**	4.2	–	–	–	50
	Flt	3.9	–	–	–	–	–	17
	ΣPAHs	12.6	43.3	90.6	72.7	70.7	79.0	

Naph: naphthalene, Flu: fluorene, Phe: phenanthrene, Ant: anthracene, Flt: fluoranthene, Pyr: pyrene and BaA: benz[a]anthracene. [a] Concentrations > BAC levels established for mussels are highlighted in bold. [b] Concentrations > EAC levels established for mussels are underlined. [c] Concentrations > ERL levels established for sediments are underlined. [d] Sediment samples were taken in duplicates and expressed as mean values. (n.s.): not specified or not studied; (–) analyte not found or detected. (*) < LOQ.

Biotic Compartment: Leaves and Rhizomes

When observing minimum and maximum ΣPAHs values in the biotic compartment, several differences could be observed. In the case of the leaves, there is a wider range of concentrations (from 5.8 to 233.3 μg kg^{-1}). This range is narrower in the rhizomes (from 10.7 to 52.1 μg kg^{-1}; Table S8).

As for where the highest concentrations were found, this also differed depending on the tissue. For instance, concerning the leaves, the highest level of ΣPAHs was observed in station RM4, being 88% merely pyrene apportioning (Table 1). This sampling point lies on the exit of a watercourse nursed by an artificial wetland bordered by greenhouses. The rest of the stations had concentrations between 5 and 40 times lower. On the other hand, the measurements from the rhizomes showed the greatest concentration of ΣPAHs at the ALM3 and ALI6 sample points (52.1 and 46.9 μg kg^{-1}), followed by ALM1 (44.5 μg kg^{-1}) > ALI4 (38.1 μg kg^{-1}), as shown in Table 1.

In leaves of *P. oceanica*, the BAC settled for mussels in the Marine Strategies were surpassed on several occasions by the concentrations here found (Tables 1 and S10). Such is the case of fluoranthene (surpassing the BAC in 6 stations out of 22), pyrene (13 out of 21) and benz[a]anthracene (1 out of 1) in leaves. Even in the case of the pyrene at the sampling point RM4, the Assessment Criteria limit (EAC, over this concentration chronic effects on marine species are expected to occur) was surpassed by 2-folds (Tables 1 and S10). Regarding the rhizome, only anthracene at ALI6 overpassed the BAC.

When comparing the results to other studies, the ΣPAHs ranges in leaves resembled those values reported in the vicinity of a Tuscany port (Italy) [28], or those encountered in Corsica (France; Table S8) [29]. On the other hand, maximum pollution levels found in this study are above those reported on the Tunisian coast [27], but several orders of magnitude lower than those observed in the Alexandroupolis Gulf in Greece [30], or in the Italian Arno and Ombrone estuarine environments [10].

Due to the scarce information on rhizomes, the obtained values could only be compared to the study conducted in Greece [30] (Table S8). In that study, the authors examined the rhizomes of *P. oceanica*, providing ΣPAH information, but not distinguishing between parent-PAHs and PAH breakdown products. As occurred with the leaves, the concentrations found in Greece were of greater magnitude than the ones reported in the present study.

Abiotic Compartment: Sediment

The observed sedimentary ΣPAHs varied regarding the type of sample, observing the maximum PAH pollution in V-sed and NV-sed: not detected (n.d.) to 127.0 in V-Sed, from n.d. to 125.8 μg kg^{-1} in NV-sed and from 12.6 to 90.6 μg kg^{-1} in NMR-sed (Table S10).

Overall higher concentrations were localized in coastal environments subject to industrial activities (ALM1, C2, ALI1, 2, 3, ALM2 for V-sed or NV-sed, and V1 for NMR-Sed; Tables 1 and S2). The sediment at ALI7, in Alicante, was the only location showing elevated ΣPAHs without being industrialized, being subject to strong seasonal tourism and recreational boat traffic instead. In most of the mentioned stations, the main contributor to the ΣPAHs was naphthalene, except for ALM1 and AML2, where phenanthrene, fluoranthene and pyrene showed high concentrations (Table 1). In contrast, the sampling points relatively far from industrial activities reflected lower ΣPAHs (Tables 1 and S2).

Each analyte's concentration seen in the V-sed and NV-sed was compared to the BAC values (Table S9). Up to five stations had at least one analyte exceeding the BAC levels. These points were again the stations with the greatest ΣPAHs in the nearness of industrialized harbors (ALM1, ALM2 and C2 in Almeria, and ALI1 and ALI2 in Alicante; Table 1). One example was phenanthrene, which although it was not as recurrent as in the biotic compartment, had values that exceeded in most of the cases the BAC (Table 1). Similarly, anthracene, fluoranthene and pyrene concentrations in the NV-sed were over BAC values.

As observed in Table 1 and Table S9, NMR-Sed slightly exceeded the BAC levels only in the case of the phenanthrene at site RM6, but not surpassing the Effects Range-Low

(ERL). The ERL is described by OSPAR convention as the lowest 10th percentile of sediment concentration that is linked to biological effects, below which concentration adverse effects in biota are rarely found.

In comparison to other studies, overall values were relatively lower than those reported in Italy [28,45]. In fact, except for the data reported for Tunisia [27], the ΣPAH ranges found in this study were several folds lower than those observed in some coastal sediments from Libya, Turkey, Greece, or other points of Spain (Table S10) [46–48].

Compartmentation of the Target PAHs

As can be appreciated from Table 1, no clear pattern in terms of the distribution of the analytes was observed between leaves, rhizomes, and sediments. Based on the physicochemical properties of PAHs, low molecular weight (LMW) molecules would be expected to be more commonly found in the water column and hence in the foliar tissues. Contrary, high molecular weight (HMW) compounds are usually linked to the sediment and therefore would lead to a preferential uptake by the sediment-related tissues, such as rhizomes and roots [45].

Observing past results based on trace element analyses, a potential explanation for finding HMW compounds in leaves is the migration of the contaminants from the long-lived tissues to temporary organs as a removal strategy [49]. Further processes that might be altering the parent-PAH compartmentation, are the PAH metabolization inside the organism or the life-span differences tissues between the tissues (~1 year for leaves and ~30 years for rhizomes) [50,51]. Additionally, due to their age, rhizomes could also exhibit historical PAH pollution.

The presence of both, LMW and HMW PAHs in sediments could be ascribed to some extent to the frequent sedimentary resuspension episodes in the shallow waters provoked by the currents and wave action. During these episodes, the sediments and the organic matter mix temporarily with the overlaying water column, promoting water-sediment interactions such as sorption and desorption of pollutants [52].

PAH Source Identification

To identify the likely priority-PAH sources, and considering the obtained data, the LMW/HMW diagnostic ratio was selected among others options [53]. This rate is based on the assumption that LMW PAHs suggest a petrogenic origin (petroleum derivatives or crude oil) whilst HMW PAHs involve pyrogenic sources (burning of organic matter) [54–57]. The obtained results are represented in Table S11. In general, the ratios depicted a mixture of contamination sources along the study area. In summary, from the 22 points, 12 sites reflected petrogenic sources (>1; EE2, EE3 and EE4, RM3, ALM3, CG2, CG3 and C2), and merely six showed pyrogenic categorization (<1; RM1, RM2, RM4, RM5, ALM1 and ALM2). Additionally, the other four sampling sites presented mixed contributions depending on the type of matrix observed (CG4, ALI4, ALI5 and ALI6). Such sites share the same arrangement, observing pyrogenic sources in the leaves and petrogenic in the rhizome. No clear pattern or distribution was observed, which could be ascribed to the fact that other sources, different than the burning of organic matter, might be contributing to the PAH stock. This could be the case for the PAHs employed in pesticides as additives or co-formulants [58,59]. Consequently, conventional ratios that simplify the sources into pyrogenic or petrogenic might not be appropriate for certain areas, for example with elevated agricultural activity (such as the here studied regions).

3.2.2. Suspect Analysis

After conducting the suspect analysis, seven compounds were tentatively identified in the environmental samples, finding a greater number in the leaves (six), whereas in rhizomes and sediments only two were detected (Table S12). These were: PAH-derivatives such as dimethylnaphthalenes (DMN), 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), 1,6-dimethyl-4-(1-methylethyl)naphthalene (DMMN) or cadalin; quinones such as 2,3-

dimethylanthraquinone (2,3-DMAQ); and other parent-PAHs such as 1-methyl-7-(propan-2-yl)phenanthrene or retene (Ret), 2-bromonaphthalene (2-BrN) and dibenzofuran (DBF). Their ubiquity was assessed and described as detection (%). The greatest detection frequencies were observed in leaves (23–100%), while in rhizomes and sediments the detection of these pollutants was relatively low (Table S13).

As mentioned above, because commercial standards for these compounds were not available a semi-quantification was performed according to a procedure previously described [42]. To evaluate the semi-quantification accuracy, these calculations were also applied to two target compounds whose true concentrations were obtained using matrix-matched calibration curves (naphthalene and pyrene). The ratios between the true and the predicted concentrations were calculated and expressed as a fold-base prediction error [60]. The obtained prediction errors fall within 1.1 and 2.7, and a mean value of 1.6 (Table 2). Accepted prediction errors in literature are usually higher, reaching in some cases errors of 29 or 88 [60,61]. Hence, the values here attained depict an accurate semi-quantification.

Although it is a prediction or estimation, information derived from the semi-quantification identifies which of the tentatively identified compounds present a more interesting behavior and might be of more significant importance for further studies as reflected before [42,62]. In this study, this would be the case of the compounds TDN, DMN, and DMMnapth, which showed a greater presence and abundance in *P. oceanica* (Figure 2).

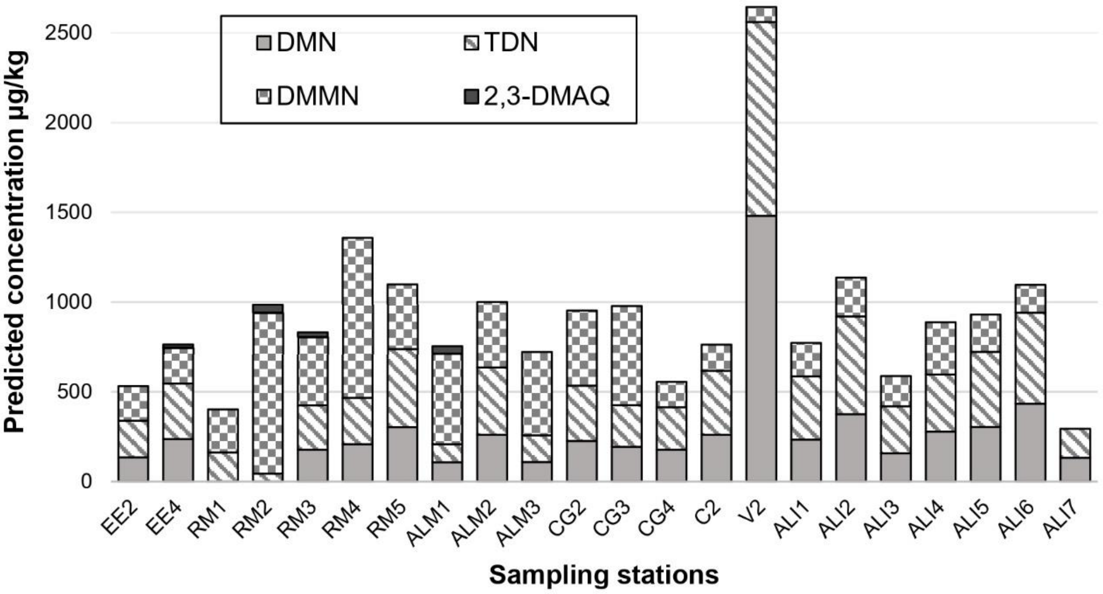

Figure 2. Semi-quantification results for PAH-derivatives: the example of the leaves of *P. oceanica*. Detections under LOQ were not included in this chart figure.

The DMN compounds detected in leaves and rhizomes (such as the 2,6-dimethy lnaphthalene) are usually linked to the degradation of α-methyl-naphthalene [63]. They are also found in pesticides as well as pharmaceuticals, being employed as precursors in their manufacture [58]. In the aquatic environment, these metabolites have been reported in sediments of the northern Persian Gulf [64], or the Northeast Aegean [30], as well as in rivers such as the case of the Selangor River in Malaysia [65]. Additionally, the 2,3-DMAQ is a PAH quinone, here detected in leaves, that derives from anthracene [66,67]. Previously, this compound has been detected in Lake Macquarie, Australia [68], or sediment and oysters belonging to an estuary also in Australia [69], as well as in the atmosphere [70].

Table 2. Results of the semi-quantification evaluation (µg kg^{-1}) in the target matrices. V-sed (▓), NV-sed (░) and NMR-sed (▒).

Matrix	Analyte	Concentration	EE2	EE4	RM1	RM2	RM3	RM4	RM5	ALM1	ALM2	ALM3	CG2	CG3	CG4	C2	V2	ALI1	ALI2	ALI3	ALI4	ALI5	ALI6	ALI7
Leaves	Pyr	Predicted	7.2	7.7	11.8	25.6	12.5	223.7	15.3	20.7	11.8	12.4	9.5	14.5	10.3	12.3	-	9.4	11.0	7.6	14.7	14.5	7.3	5.2
		True	2.9	3.1	8.3	17.1	8.8	205.8	10.8	13.8	8.3	8.7	3.8	10.2	7.3	8.7	-	3.8	7.8	3.1	10.3	10.2	3.0	2.1
		Error Factor	2.5	2.5	1.4	1.5	1.4	1.1	1.4	1.5	1.4	1.4	2.5	1.4	1.4	1.4	-	2.5	1.4	2.5	1.4	1.4	2.4	2.5
Rhizomes	Naph	Predicted	-	13.8	-	-	-	10.6	-	17.4	12.1	12.2	8.3	13.1	-	9.1	12.3	6.4	11.0	-	12.8	12.8	13.6	8.8
		True	-	20.2	-	-	-	15.1	-	25.9	17.4	17.6	11.3	19.0	-	12.7	17.8	8.3	15.8	-	18.6	18.6	19.9	12.2
		Error Factor	-	1.5	-	-	-	1.4	-	1.5	1.4	1.4	1.4	1.5	-	1.4	-	1.3	1.4	-	1.5	1.5	1.5	1.4

Matrix	Analyte	Concentration	EE4 ░	EE4 ▓	RM2 ▓	RM5 ▓	RM6 ▒	ALI3 ▓
Sediment	Naph	Predicted	4.5	4.7	3.2	2.8	2.4	223.3
		True	3.4	3.6	2	2.2	1.9	81.3
		Error Factor	1.3	1.3	1.6	1.3	1.3	2.7

Concerning the group "other parent-PAHs", the 2-BrN was only observed in rhizomes of the Alicante stations (from ALI4 to ALI7; Figure S2). The DBF was also tentatively identified, being seen mainly in the leaves from three stations (RM4, RM2 and CG3; Figure S3). This compound has been previously reported in the aquatic system of Lake Macquarie, Australia [68], as well as in the atmosphere of North China [71] and can be utilized as a PAHs feedstock for optoelectronic devices and materials [72]. In the case of Ret, its presence was widespread in leaves but reduced in the sediments, being noticed in the ALI3 V-Sed (Table S13 and Figure S3). As observed in other studies, Ret is frequently found in different environmental compartments, from air samples [73] to sediment from the Selangor River in Malaysia [65] or sediment cores from lakes in Alaska [74]. The compound Ret is a common biogenic/pyrogenic-PAH usually originated in forest fires [74], but it has been also associated with the microbial metabolism of abietic acid in wood resin that usually takes place inside pulp and paper mills [75]. Regardless of its origin, Ret has been observed to be toxic to certain aquatic life, as occurs with the alevin of fish [76].

For the first time, concentrations of PAH-derivatives were estimated in tissues of *P. oceanica*. The data suggested elevated concentrations existed in leaves (Figure 2) compared to from any other matrix (Figure S2). Interestingly, at V2, where the highest values of PAH-degradation by-products were recorded (~2600 μg kg^{-1}; Figure 2), the Σparent-PAH value was the lowest (Table S9).

This might suggest an elevated PAH transformation rate capacity or detoxification in leaves, explaining the reduced amount of parent-PAH but the increase in their metabolites. Therefore, for actual contamination assessment of environmental samples, parent-PAH analysis should be accompanied by the study of their derivatives.

3.2.3. Unknown Analysis

After applying the aforementioned filters and criteria, the analysis allowed for the tentative identification of up to 11 compounds not seen during the previous two analyses. Of these compounds, seven were found in leaves, one in the rhizomes and six in the sediment (Table 3). The tentatively identified compounds, although their sources and usages were variable, were broadly classified into four different categories: pesticides or agro-chemicals, plasticizers, UV stabilizers, and PAH metabolites. In the case of the pesticides, dimethyl benzaldehyde (DBAL), dodemorph (Dodem), and isoprocarb (Isop) seemed to be observed. Plastic additives, such as 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (7,9-DTBO), diallyl isophthalate (DAIP), dicyclohexyl phthalate (DCHP), irgafos 168 (Irg 168), and tributyl phosphate (TBP) were tentatively identified. Furthermore, a UV stabilizer, 2,4-di-tert-butylphenol (2,4-DTBP), and a PAH metabolite not included in the suspect analysis (1-methoxymethylfluorene, 1-MOF) were also tentatively identified.

Table 3. Compounds tentatively identified by unknown analysis in leaves of *P. oceanica* (◇), rhizomes (■) and marine sediments such as V-sed (▓), NV-sed (░) and NMR-sed (▒).

Compound	CAS	Molecular Formula	Molecular Mass	RT ± SD	HRF (%)	Matrix
TBP	126-73-8	$C_{12}H_{27}O_4P$	266.16415	5.84 ± 0.01	100.0	■
DBAL	5779-94-2	$C_9H_{10}O$	133.06473	6.73 ± 0.00	87.4	▓ ░ ▒
Isop	2631-40-5	$C_{11}H_{15}NO_2$	193.10973	8.29 ± 0.01	89.2	◇
2,4-DTBP	128-39-2	$C_{14}H_{22}O$	206.16706	8.89 ± 0.00	95.7–96.5	◇ ▓ ░ ▒
7,9-DTBO	82304-66-3	$C_{17}H_{24}O_3$	276.17254	12.39 ± 0.01	95.8–99.9	◇ ▓ ░ ▒
1-MOF	139552-06-0	$C_{15}H_{14}O$	210.10446	12.42 ± 0.01	90.3	▓ ░ ▒
DAIP	1087-21-4	$C_{14}H_{14}O_4$	246.08921	14.06 ± 0.01	94.1	◇
Dodem	1593-77-7	$C_{18}H_{35}NO$	281.27186	16.23 ± 0.01	97.5–97.7	◇ ▓ ░ ▒
DCHP	84-61-7	$C_{20}H_{26}O_4$	330.18311	17.61± 0.01	100.0	◇
Irg 168	31570-04-4	$C_{42}H_{63}O_3P$	646.45148	24.22 ± 0.00	92.9–97.7	◇ ▓ ░ ▒

Several of these compounds identified by the unknown analysis show concerning properties according to the Pesticide Properties Database (PPDB, University of Hertfordshire) and the European Chemicals Agency (ECHA, http://echa.europa.eu/; accessed on 12 July 2022) because the great majority are either toxic to reproduction (DCHP and Dodem) or toxic to aquatic life with long-lasting effects (Isop, TBP, 2,4-DTBP, DAIP and Dodem). Various of these organic micropollutants reached in some matrices a 100% detection, such as the case of 1-MOF, 2,4-DTBP, 7,9-DTBO, DBAL, Dodem, Irg 168, and Isop. More information on the unknown compounds can be found in Table S14, Supplementary Materials.

The signals of each tentatively detected compound were normalized and compared between matrices and sampling sites. The pollutant Dodem stands out, which in leaves had its greatest relative abundance at EE2, a site situated approximately 350 m far from a watercourse and greenhouses (Figure S4). Likewise, at EE2 the analyte TBP (85% detection in rhizomes) showed an abundance twelve times higher than the average (Figure S5). Interestingly, in the sediment, where the greatest 1-MOF or DBAL abundances were found, the compounds 7,9-DTBO, Irg 168, and Dodem were not detected, or their concentrations decreased sharply (Figure S6).

Overall, each analysis brought unique information, proving that for a comprehensive study of environmental samples, the combination of the three searching modes is required, in agreement with a previous study [39].

Additionally, the effects that the pollutants (found in *P. oceanica* and marine sediments) might signify are not certain due to the lack of studies. Considering the known effects of POPs, it is possible to speculate, in the case of *P. oceanica*, that above a certain concentration, its functions would be compromised, causing damage to the metabolic process, and potentially reducing its probability of survival.

As for the polluted sediments, these might affect the associated micro and macro organism communities via ingestion of sedimentary POPs stock, being then either metabolized by microbiota or propagated throughout the food web [77,78]. The metabolization would generate more soluble and bioavailable molecules, leaving the sediment and re-entering into the water column. Additionally, the *P. oceanica*-associated microbiota found in the V-sed could be potentially altered, consequently affecting their symbiosis and reducing the nutrient uptake through the roots [79,80]. As for the propagation into the food chain, it is also important to consider that fish, mussels, and crustaceans surpassing certain POP levels, are not allowed for consumption according to the European Commission [81]. This would cause the discard of marine food with its consequent damage to the economic benefits of the fisheries, catering and tourism sectors (the main economical pillars in the coastal Mediterranean), as already observed in other areas [82,83].

4. Conclusions

Answering the first aim, simple, fast, and ecofriendly methods were developed and satisfactorily validated for the extraction of 16 priority parent-PAH in tissues of *P. oceanica* and marine sediments. Second, these tissue-specific extractions, together with a three-way analysis (target, suspect, and unknown approaches) by the usage of a GC-Q-Orbitrap MS allowed for the so far most complete screening ever conducted in *P. oceanica*. As a result, up to seven priority parent-PAHs were detected, as well as various non-priority PAHs, PAH-derivatives, several pesticides, plasticizers, and one UV-filter with concerning toxic characteristics, were tentatively identified. Some parent-PAH concentrations in *P. oceanica* were found to be above the mussels BAC (anthracene, fluoranthene and pyrene), even surpassing on one occasion the EAC for the pyrene. Likewise, sediment near industries or industrialized harbors also reflected PAH concentrations above sedimentary BAC. Moreover, the fact that some compartments showed low parent-PAH levels but an elevated presence of toxic PAH-derivatives, proves that the current approach of the monitoring programs only considering priority PAHs might be underestimating the organic contamination in the marine environment.

Finally, although *P. oceanica* is a bioindicator of water quality, here it has been observed that this seagrass can hold elevated concentrations of harmful substances. The effects that such substances may exert on *P. oceanica* remains unknown, as well as how this might affect its carbon storage capacity, in consequence, other ecosystem services, and ultimately the economy. It would be advisable to apply the method here described in a greater set of samples and over a longer time trend, while investigating whether the organic pollutants might be triggering a defensive response in *P. oceanica* that can be used as an early warning.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/jmse11020369/s1. Figure S1: Examples of extracted ion chromatograms of some the recurrently detected target priority parent-PAHs, their molecules, and their spectrum (*m/z*), in the leaves (sample ALI2), rhizomes (ALI6) and sediments (ALM1 NV-sed). n.d.: not detected; Figure S2: Semi-quantification results obtained for the PAH-derivatives and other parent-PAH tentatively identified such as the (A) DMN (grey) and 2-BrN (black) in rhizomes of *P. oceanica*, and (B) TDN (grey) and Ret (black) in marine sediments (NV-sed and V-sed); Figure S3: Semi-quantification of the parent-PAHs (retene and dibenzofuran) observed in leaves of *P. oceanica*; Figure. S4: Normalized relative abundances of each tentatively identified compound by unknown analysis in leaves of *P. oceanica* per sampling point; Figure S4: Normalized relative abundances of each tentatively identified compound by unknown analysis in leaves of *P. oceanica* per sampling point; Figure S5; Normalized relative abundance of the tentatively identified compound by unknown analysis in rhizomes of *P. oceanica* per sampling point; and Figure S6: Normalized relative abundances of each tentatively identified compound by unknown analysis in marine sediments (V-sed, NV-sed and NMR-sed) per sampling point; Table S1: Target PAHs and the IIS included in this study with their corresponding CAS number; Table S2: Sampling points from the west to east regions, the site descriptions, pressures and samples taken such as NV-sed and NMR-sed (░), leaves (◊), rhizome (■) and V-sed (▓); Table S3: Characteristics of the oven program; Table S4. GC-Q-Orbitrap MS parameters employed for the identification of the 16 priority PAHs and IIS.; Table S5: Range of recoveries (%) in *P. oceanica* and surficial sediment for the target priority PAHs depending on the extraction procedures; Table S6: Method validation results for the different priority PAHs assessed in the target matrices at two levels; Table S7: Obtained LODs and LOQs, per matrix and target analyte, compared to other studies; Table S8: ∑PAHs ranges (μg kg^{-1} d.w.) seen in the leaves and rhizomes of *P. oceanica* from the Spanish coast in this research, compared to previous published studies; Table S9: BAC, EAC and ERL concentrations (μg kg^{-1} d.w.) established for the mussel *Mytilus galloprovincialis* and sediments concerning the PAH congeners found in this study; Table S10: ∑PAHs records (μg kg^{-1} d.w.) seen in this research in marine sediments from the Spanish coast, compared to previous published studies; Table S11: Color chart indicating the result obtained when applying the LMW/HMW PAH ratios. In purple petrogenic (>1), in blue pyrolytic sources (<1) and white when the ratios could not be applied. Only sites where ratios could be applied are shown; Table S12: Suspect compounds tentatively identified, their ions, RTs, CAS number and the compartment where they were found: (◊) leaves, (■) rhizomes, (▓) V-Sed, (▒) NV-Sed, and (░) NMd-Sed; Table S13: Detection (%) per matrix of the suspect compounds tentatively identified in this study; and Table S14: Description of the compounds tentatively identified by unknown analysis and their detection frequency in each target matrix.

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Chapter IV

Comprehensive discussion

The efficacy of an environmental study is contingent upon meticulous selection of the study area, an appropriate sample collection, and pre-treatment protocols. These steps can significantly impact the information obtained during the subsequent analytical process, such as the relative abundances of the PhCs, which may be influenced by sample handling and therefore require consistent treatment across all samples. Given the significance of these steps, a comprehensive description of the entire process is presented in detail in subsequent sections. Furthermore, the conducted method optimization, which also plays a pivotal role, is comprehensively described upon in the following paragraphs.

1. Pre-sampling

1.1. Task I: Selection of the regions of study

Regions with historical and current sources of pollution were identified and listed. A preliminary filter was applied, limiting the selection to regions where coastal economic activities were predominantly based on agriculture, industrial production, and/or tourism. The selection also considered the distance from the base laboratory located at the University of Almeria, ensuring appropriate sample conservation during transportation. From this preliminary screening, three coastal regions were selected for further investigation: Almeria, Murcia, and Alicante.

1.2. Task II: Selection of sampling points

A theoretical study was conducted on the three selected regions, Almeria, Murcia, and Alicante, using an atlas published by Ruiz et al. (2015) and Geographical Information System (GIS) tools, specifically ArcGIS. The objective of this study was to identify potentially polluted sampling points and others that could serve as blanks, such as Marine Protected Areas (MPAs). Fourteen variables that have been observed to negatively impact *P. oceanica* meadows were considered, including extensive non-irrigated crops, irrigated extensive crops, greenhouses, discontinuous and continuous urban areas, industrial or commercial areas, nautical, fishing, and industrial or commercial harbors, urban and non-urban spills, desalination plants, watercourses, and fish farms, as reported in previous studies (Marín-Guirao et al., 2011; Pérez et al., 2007; Pergent-Martini et al., 2002; Roca et al., 2014).

To identify the potentially contaminated sampling points where *P. oceanica* meadows were also present, a GIS study has been conducted. Different satellites and layers have been employed. For instance, to explore the land coverage, satellite data has been used, such as the CORINE inventory (2018) from the Copernicus Services, SIOSIDE 2013 and 2015, and the *Valencia* Government Cartographic Viewer (URL: https://visor.gva.es/visor/?idioma=es) have been utilized. To localize the pipelines the *Andalusian Environmental Information Network* (REDIAM, Government of Andalusia, Spain, URL: http://www.juntadeandalucia.es/medioambiente/site/rediam) was employed, together with the atlas published in Ruiz et al. (2015). Finally, cartography brought by the Life Blue Natura Project (Carreto et al., 2021) and information obtained from the Department of Marine Sciences and Applied Biology (University of Alicante) was considered for the location of the *P. oceanica* meadows.

From the potential sampling points, only those reachable by the divers were selected, considering the distance from the coast and depth of the meadow's upper limit. The diving team consisted of the present PhD candidate and a volunteer bachelor student. The volunteer was carefully trained, previously to the sampling, by the PhD candidate who owns the required diving instructor license and permits.

It should be also considered that the set of samples had to be further reduced due to the beginning of the COVID-19 pandemic and the strong and changing mobility restriction endured in Spain between regions. As a result, 28 sampling points were selected along the three regions, collecting leaves (n = 21), rhizomes (n = 20), vegetated sediment (n = 20), and non-vegetated sediment (n = 23). Table 4.1 includes information regarding sampling points.

Table 4.1. Sampling regions and their corresponding sampling points, location as well as type of collected samples. Leaves (◊), rhizomes (■), roots (•), vegetated sediments (▓), and non-vegetated sediments (░).

Region	ID	Sampling point name	Location (WGS84)	Samples				
Almeria	EE2	Guardias Viejas water course	36.695692, -2.842181	◊	■	•	▓	░
	EE3	Almerimar marina	36.698113, -2.789602					░
	EE4	Almerimar Punta Entinas	36.682722, -2.781789	◊	■	•	▓	░
	RM1	Roquetas de Mar Port	36.759793, -2.605386	◊	■	•	▓	░
	RM2	Roquetas de Mar	36.765929, -2.601214				▓	░
	RM3	Cura Ravine (W)	36.769575, -2.600480	◊	■	•	▓	░
	RM4	Cura Ravine (E)	36.772200, -2.600321	◊	■	•	▓	░
	RM5	Algaida	36.783936, -2.591814	◊	■	•	▓	░
	RM6	Aguadulce Harbor	36.815391, -2.561088					░
	ALM1	San Telmo	36.830635, -2.487396	◊	■	•	▓	░
	ALM2	Almeria Promenade	36.824700, -2.452053	◊	■	•	▓	░
	ALM3	Costacabana	36.828550, -2.385822	◊	■	•	▓	
	CG2	Cabo de Gata beach	36.723349, -2.194234	◊	■	•	▓	░
	CG3	Los Escullos	36.796096, -2.061859	◊	■	•	▓	░
	CG4	El Playazo	36.862794, -2.003661	◊	■	•	▓	░
	C2	Isla de San Andrés	36.995175, -1.889448	◊	■	•	▓	░
	V1	Villaricos 1	37.243813, -1.772810					░
	V2	Deretil	37.255522, -1.758341	◊				
Murcia	M1	Mar Menor North Chanel	37.653726, -0.716768					░
	M2	Mar Menor Harbor Chanel	37.778983, -0.74429					░
	M3	Mar Menor Encañizadas Chanel	37.823707, -0.756447					░
Alicante	ALI1	Urba 1	38.291326, -0.494084	◊	■	•	▓	░
	ALI2	Desal	38.302493, -0.488727	◊	■	•	▓	
	ALI3	Alicante Harbor	38.326396, -0.477619	◊	■	•	▓	
	ALI4	Campello	38.414186, -0.391352	◊	■	•	▓	░
	ALI5	Cala Nostra	38.443518, -0.370742	◊	■	•	▓	░
	ALI6	Cala Morro Blanc	38.445734, -0.364306	◊	■	•	▓	░
	ALI7	Cala Sardinera	38.762431, 0.219156	◊	■	•	▓	░

2. Sampling procedure

To develop the sampling strategy, a map per sampling point was built. An example is found in Figure 4.1-A, displaying the C2 sampling point. In most cases, divers started swimming from land, until reaching the meadow's upper limit. To accurately localized the actual sampling point, the distance from coast was measured as the number of kicks cycles. Later this information was translated into meters and GPS coordinates. In this process, a steady direction had to be followed using a compass, while controlling the exact depth (using a diving computer). A visual description of the process can be found in Video 4.I).

Video 4.I. Measuring distances.

Per meadows, a total of 3 shots were uprooted by hand (one on the limit and the other two further to the inside of the meadow; Figure 4.1-B) and 2 sediment samples, in and outside the meadow (i.e., vegetated and non-vegetated sediments; Figure 4.1-C). On certain occasions, sediment of a different kind, found far away from any type of meadow (non-meadow-related sediments), such as inside harbors, was also collected. All plant samples were carefully packed underwater in labeled plastic bags always adding seawater to help maintain the temperature and reduce the stress of the seagrass. Note that permits from the regional government allowing the sampling of *P. oceanica* were requested beforehand. As for the sediments, these were kept in polystyrene (PE) containers right after retrieval. As an example of the process, in Video 4.2 it can be observed how divers collect vegetated sediment. Once on land, all samples were placed in portable fridges and transported to the laboratory.

Video 4.2. Sediment sampling.

Figure 4.1. Graphical representation for the pre-sampling and sampling conducted in this Thesis: (A) Digital cartography for the sampling point C2 using GIS and different layers of information, (B) *P. oceanica* sampling and data collection, and (C) sampling of non-vegetated sediment.

3. Sample pre-treatment

In the case of *P. oceanica*, a picture per sample was taken upon arrival at the laboratory. Subsequently, samples were washed with tap water to remove sand and salt and rinsed with distilled water. Later, tissues were separated into leaves, rhizomes, and roots, removing the young leaves (Figure 4.2-A, B, and C). All tissues were kept in labeled Petri dishes and weighed to obtain the wet weight (w.w.) and maintained at – 20 ˚C for 48 h. After, the tissues were freeze-dried (at – 30 ˚C). In the case of the rhizomes and roots, the process lasted 48 h. Later, rhizomes, previously peeled by removing the sheaths, and roots were separately weighted to obtain the dry weight (d.w.). For leaves, the freeze-drying process was stopped after 24 h to carefully remove the dried crust of epiphytes with a brush before resuming the process until reaching 48 h and being weighed. All tissues were then ground and homogenized for 2 min at 25 rps in a ball mill. The resulting homogenized powder was kept in desiccators until further analyses.

On the other hand, each sediment sample was placed in individual pre-cleaned trays to let dry inside an extraction hood at room temperature (20 ± 2 ˚C). To protect from light and to avoid air-borne pollution samples were covered with filter sheets. Once dried, samples were pictured, weighted, and sieved with a 2 mm stainless steel sieve (Figure 4.2-D). The portion < 2 mm was then ground using a glass mortar and stored in PE containers in darkness.

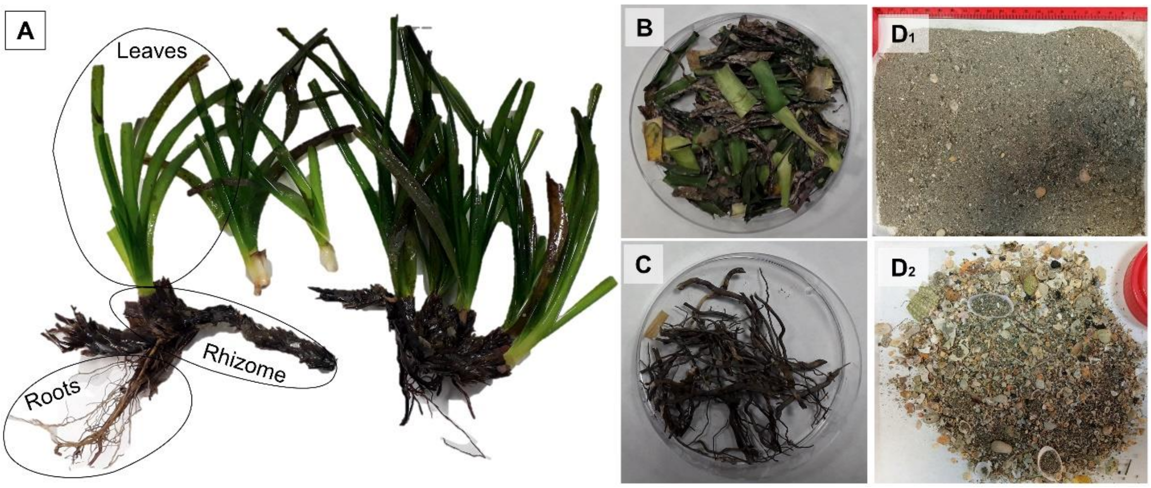

Figure 4.2. Pictures of: (A) *P. oceanica* seagrass and its different tissues, (B) leaves and (C) roots after being freeze-dried, and (D) two different air-dried sediment samples.

4. Target compounds

Two different types of organic compounds were aimed in this Thesis. One of them, the PhCs, are compounds naturally formed inside plants, and therefore classified as a type of endogenous compound in land and marine plants. The other group, OPs, are not generated inside *P. oceanica* and therefore belong to the exogenous compound group, their presence being associated with human-driven activities. These OPs are transported into the sea and bioaccumulated in marine organisms such as the case of *P. oceanica.* Both, endogenous and exogenous compounds aimed in this Thesis are shown in Figure 4.3.

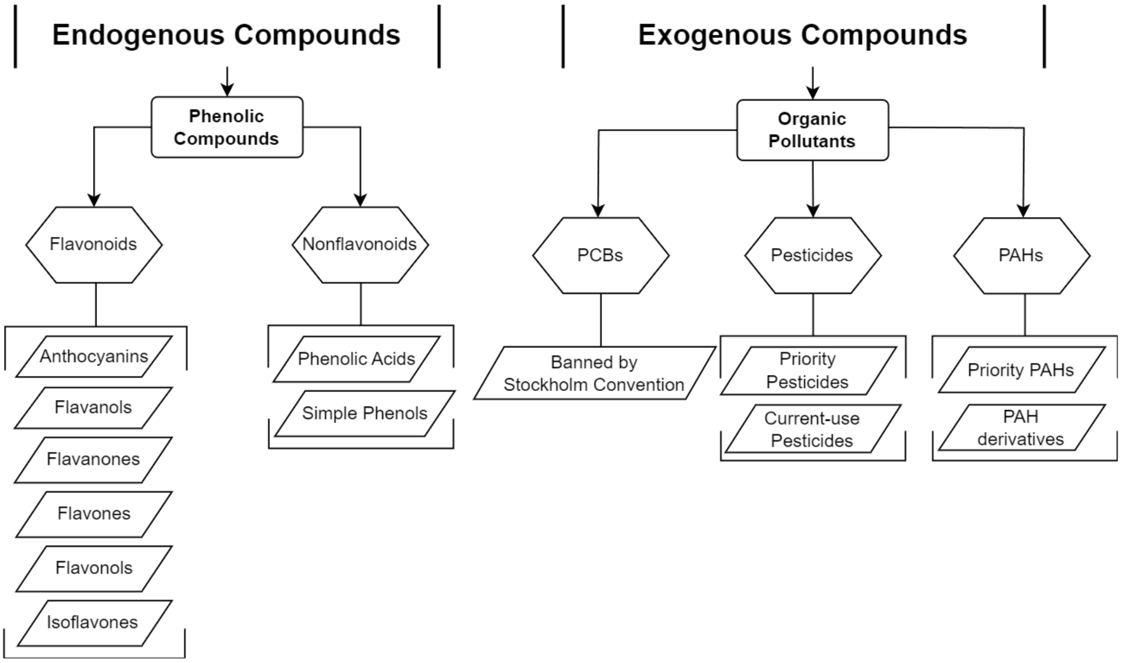

Figure 4.3. Diagram of the studied endogenous and exogenous compounds in this Thesis.

5. Optimization of the methods

To investigate PhCs and OPs in the matrices under study, certain procedures were assessed and optimized to develop the most suitable methods. To evaluate the different performances, several databases, with the most common or relevant compounds, were utilized to run target screenings and compare the yielded results. Only those parameters exhibiting greater sensitivity, such as a higher number of compounds, higher peak intensities or recoveries, better peak shape, or a greater presence of confirmation fragments, were later considered for validation. Nonetheless, the databases for the mentioned target analyses, as well as for the subsequent retrospective analyses, had to be either improved or created from scratch and optimized before the evaluation of the extraction and analytical procedures.

5.1. Analytical method

Certain chromatographic and mass conditions were optimized in the Article I regarding PhCs. These variables were the chromatographic column and the employed mobile phases. Two commonly employed sub-2-µm columns in PhCs studies by LC were selected: Acquity C18 and Hypersil GOLD™ C18. For this purpose, a mix of several available standards (i.e., multicomponent solution) was injected in the ultra high performance LC (UHPLC) coupled to HRMS (Orbitrap MS) and a target analysis was run. Results showed that, although the chromatographic separation did not differ, the achieved intensities per compound were relatively higher with Hypersil GOLD™ C18. Regarding the aqueous mobile phases, a 30 mM ammonium acetate solution (pH 5) and a 4 mM formic acid solution (pH 3), also common in these sorts of investigations, were tested. Overall, more intense signals were seen when using the formic acid mobile phase.

The injection and analysis of standards was also conducted in the OP study, to assure the correct chromatographic separation of the analytes and to obtain the corresponding RTs, among other variables. In this case, the chromatographic and mass conditions were those previously employed in the research group. As examples, 29 min was the runtime, and the injector temperature was set at 280 °C. The split flow rate was 50 ml min^{-1} and the spitless time was 1.0 min. A VF-5 MS column of 30 m × 0.25 mm ID × 0.25 µm film thickness and, as gas carrier, He (1 ml min^{-1}) was used. MS was performed in positive electron ionization (EI) at 70 eV. The target analysis proved that the chromatographic separation was satisfactory and therefore no changes were applied.

5.2. Databases development

5.2.1. Databases for target analysis

In the case of the PhCs study, a previously developed *in-house* database containing 93 commonly seen compounds in agri-food products was selected. Part of the research effort exerted on this Thesis was to fine-tune such a database by analyzing standard mixes, as mentioned before. The yielded compound-specific information was then incorporated into the *in-house* database: *m*/*z* of the molecular ions and characteristic fragments, negative and positive heated electrospray ionization mode (HESI− or HESI+, respectively), and RT. The relevance of this update lies in the specific behavior of the compounds depending on the analyzed matrix and the analytical conditions.

For OPs, a database was built from scratch instead. In this sense, 25 PCBs, 13 priority pesticides, and 16 priority PAHs standards were injected and analyzed via GC couple to a quadrupole Orbitrap MS (GC-Q-Orbitrap MS). The generated compound-specific information was gathered in the database, such as quantification ions, confirmation ions, and RTs.

5.2.2. Databases for suspect analysis - retrospective analyses

To broaden the search for PhCs, a second phenolic database was built. This database contained not only previously identified PhCs in *P. oceanica* but also those reported in other Mediterranean seagrasses. Additionally, information regarding their theoretical molecular formula and theoretical mass (of the parent ion and confirmation/characteristic fragments) for each ionization mode was also incorporated. The data was gathered by conducting a literature review and using open sources, such as PubChem, ChemSpider, and Human Metabolomics. In total, the database for the suspect analysis comprised 38 extra PhCs.

For the suspect screening of OPs, two additional databases were used. Firstly, an *in-house* database, commonly employed in the laboratory (with 246 pesticides of concern) was fine-tuned. The approach was identical to the one followed for the target database of PhC, i.e., by analyzing available standards. Secondly, a database with 143 OPs was built: 103 PAH-derivatives (oxy-, nitro-, methyl and hydroxy- metabolites, and quinone derivatives), 14 non-priority parent-PAHs, and 26 linear alkylbenzenes (LABs). The latter are compounds commonly found in household detergents, so their occurrence would further confirm the influence of the discharges on the studied coastal ecosystems. Regarding the procedure, this was done as described in the development of the suspect database for PhCs. More in detail, compound names and specific information were gathered from research articles, online open-source databases, or spectral reference libraries (e.g., the NIST library). A compilation of the here developed or updated databases for target and suspect analyses is found in Table 4.2.

It also important to mention that, to broaden the search of additional OPs unknown analyses were run. In this case, an internal database previous optimized in the research group was used, as well as several online databases supported by Compound Discoverer software (Thermo Fisher Scientific, Les Ulis, France).

Table 4.2. Compilation of the generated databases or improved *in-house* databases used in this Thesis.

Study	Target screening		Suspect screening		Total compounds
	Compounds	**Description**	**Compounds**	**Description**	
Article I (PhCs)	*n* = 93	PhCs usually found in agri- food products	*n* = 38	Reported PhC in *P. oceanica* and other Mediterranean seagrasses	127
Article II Article III (OPs)	*n* = 54	Concerning PCBs, priority pesticides, and priority PAHs	*n* = 246	Non-priority pesticides	443
			n = 143	PAH-derivatives, quinone derivatives, non-priority parent-PAHs, and LABs	

LABs: linear alkylbenzenes; OP: Organic Pollutants; PAHs: polycyclic aromatic hydrocarbons; PCBs: polychlorinated biphenyls; and PhCs: Phenolic Compounds.

5.3. Extraction method

In this section, variables such as solvent type, solvent volume, and extraction techniques were evaluated. The following is an explanation of the different extraction optimizations followed in each article and compound type.

5.3.1. Extraction of endogenous compounds: Phenolic compounds

Based on the available bibliography, two published extraction procedures were chosen, and the composition of the extraction solution was evaluated. This varied between a methanol/water solution (pH 4) with a ratio of 8:2 *v/v* and 5:5 *v/v*. Overall, the first mixture was seen as more adequate for rhizomes and roots, while the second mixture worked better for leaves (Table 4.3).

Table 4.3. Number of PhCs observed regarding the type of tissue and the ratio of the solvent mixture.

Tissue	Solvent mixture (*v/v*)	
	8:2	**5:5**
Leaf	16	18
Rhizome	19	16
Root	18	14

5.3.2. Extraction of exogenous compounds: Organic pollutants

When assessing OPs in *P. oceanica* tissues, several mechanical extractions and a set of cleaning salts were tested. Regarding the extraction techniques, the chosen procedures were agitation by rotary shaker, polytron homogenizer, ultrasound-assisted extractions (UAE), Quick, Easy, Cheap, Effective, Rugged & Safe extraction method (QuEChERS), and QuEChERS-UAE. In all cases, hexane/ethyl acetate (Hx/EtAc) 9:1 *v/v* was used as the extraction solvent. Additionally, for the QuEChERS, magnesium sulfate and sodium chloride were selected as the extraction salts. Results showed that, for a multiclass method, polytron, followed by UAE, seemed to be more suitable in all cases without the need for an elevated amount of sample material and extraction time, as supported by the recovery trial shown in Table 4.4. Their efficiency may lie in their capability to break the bonds in plant tissues (with elevated lignin content), facilitating the extraction of contaminants. Surprisingly, these two simple and affordable methods were not tested in *P. oceanica* before this Thesis. Figure 4.4 is shown a graphical example of the workflow for the OPs extraction.

Table 4.4. Observed recoveries (%) per extraction type and solvent (*n* = 3), for the determination of PCBs, pesticides, and PAHs at concentration level of 100 µg l^{-1} in *P. oceanica* tissues.

Matrix	OPs	Polytron	Agitation	UAE	QuEChERS	QuEChERS-UAE
Leaves	PCBs	106-117	60-80	29-108	12-22	3-15
	Pesticides	109-119	46-152	73-140	12-157	11-67
	PAHs	96-119	41-91	57-102	2-93	1-91
Rhizomes	PCBs	107-120	61-80	78-108	12-22	3-15
	Pesticides	110-117	61-189	77-126	14-83	50-140
	PAHs	74-115	50-137	44-144	38-124	31-551

OPs: Organic Pollutants; PAHs: Polycyclic Aromatic Hydrocarbons; PCBs: Polychlorinated Biphenyls; QueChERS: Quick, Easy, Cheap, Effective, Rugged & Safe extraction method; UAE: Ultrasound-Assisted Extraction.

On the other hand, to investigate the presence of OPs in marine sediments, various extraction solvents were tested, Hx/EtAc 9:1 *v/v* and dichloromethane (DCM). It was found that the Hx/EtAc mixture was the most effective for detecting multiple classes of OPs, as observed in the resulting recoveries (Table 4.5).

Concerning the cleaning salts, five trials of samples were carried out: (1) no clean-up, (2) primary secondary amine (PSA), (3) Florisil, (4) active carbon, and (5) octadecylsilane (C18). Each clean-up was chosen based on their contribution. For instance, PSA facilitated the removal of organic acids, fatty acids, and sugars. Florisil is used for the purification of analytes from samples rich in fats due to its ability to selectively retain certain lipids. Polar matrices such as triglycerides and phytosterols can also be easily removed from the sample extract by associating with the surface of Florisil. On the other hand, C18 retains sterols and lipids, while active carbon retains sterols and pigments. The cleaned up from interfering matrix components is necessary in most cases and should be considered.

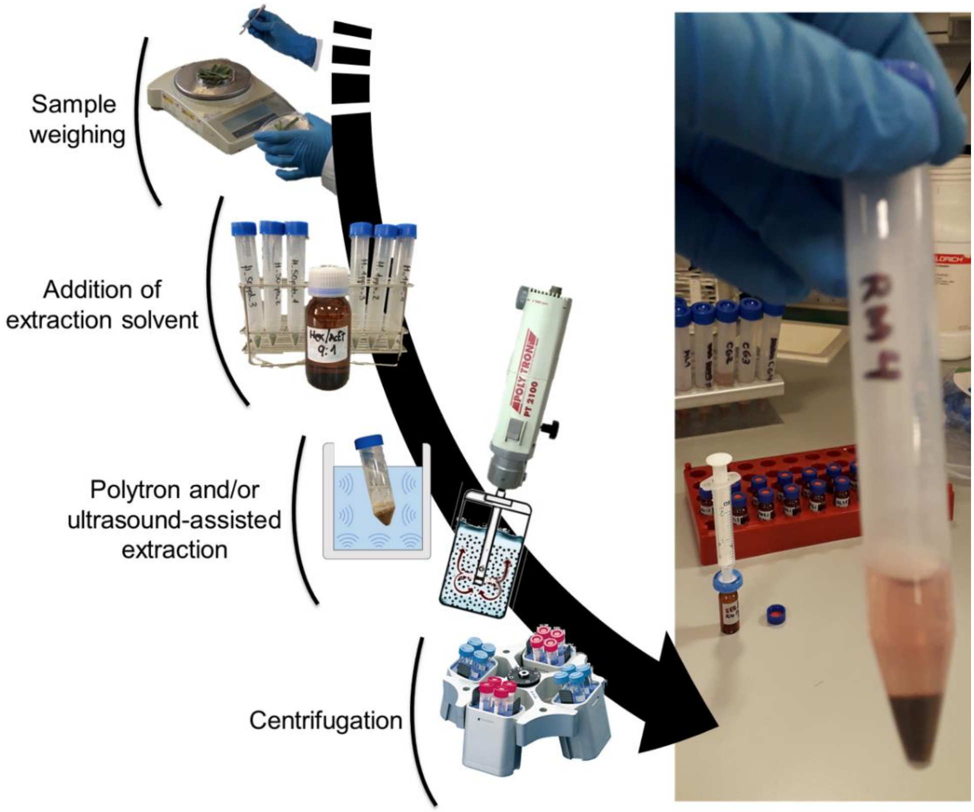

Figure 4.4. Phases of the extraction process for the detection of OPs in *P. oceanica* and sediments.

Table 4.5. Recoveries (%) regarding the employed solvent (n = 3) for the determination of PCBs, pesticides, and PAHs at a concentration level of 100 µg l^{-1} in marine sediments.

Matrix	Compound	Solvent	
		Hx/EtAc	DCM
Sediment	PCBs	101-115	53-75
	Pesticides	84-119	65-103
	PAHs	71-119	18-165

DCM: Dichloromethane; Hx/EtAc: Hexane/Ethyl acetate; OPs: Organic Pollutants; PAHs: Polycyclic Aromatic Hydrocarbons; PCBs: Polychlorinated Biphenyls.

Contrary to the aforementioned parameters, the matrix effect was evaluated instead of sensibility or recovery. For this purpose, 1.5 ml sample aliquots were spiked after the extraction (5 µg l^{-1}). Subsequently, 150 mg of cleaning salts were added, centrifuge (10 min, 5000 rpm), and filtered (0.22 µm nylon filter). After analysis, results showed that the mean matrix effect did not seem to notably change after the addition of cleaning salts

(Table 4.6). Therefore, this step was not incorporated into the final method, nor considered for validation.

Table 4.6. Matrix Effect (%) obtained per treatment and compounds' type in *P. oceanica* tissues.

Tissue	Compound		No cleanup	PSA	Florisil	Active Carbon	C18
Leaf	**PCBs**	min	93	61	112	5	90
		max	139	211	278	954	212
		mean	128	171	216	169	175
	Pest	min	94	107	84	41	90
		max	190	223	294	231	232
		mean	140	160	207	149	177
	PAHs	min	80	198	259	12	178
		max	226	273	850	295	297
		mean	159	236	377	146	245
Rhizome	**PCBs**	min	116	21	88	48	68
		max	184	166	216	173	192
		mean	155	134	159	130	154
	Pest	min	99	80	111	32	120
		max	226	181	215	203	213
		mean	147	126	155	131	162
	PAH	min	34	17	167	17	129
		max	149	167	337	527	259
		mean	72	56	237	318	199

C18: Octadecylsilane; PAHs: Polycyclic Aromatic Hydrocarbons; PCBs: Polychlorinated Biphenyls; Pest: Pesticides; and PSA: Primary Secondary Amine.

6. Validation of the proposed methods for the monitoring of organic pollutants in *P. oceanica*

Various parameters were assessed to validate the analytical methods used for the analysis of OPs and ensure their reliability. This included linearity, trueness, precision (both intraday and interday), limit of detection (LOD), and limit of quantification (LOQ). Linearity was determined using the determination coefficient (R^2) of the calibration curves, while trueness was measured by recovery (*n* = 3) at two validation levels (VL1 and VL2) within the linear range. Precision was estimated by injecting three replicates per validation level on the same day (intraday precision) and across three different days (interday precision), expressed as relative standard deviation (RSD %). Yielded results are summarized in Tables 4.7 and 4.8. Overall satisfactory results were observed for most of the analytes in the different matrices (e.g., RSD < 20%, and recoveries between 80 and 120%).

Table 4.7. Compilation of the validation data (*) obtained in this Thesis per matrix and compounds.

Matrix	Compound	Linearity (R^2)	Recovery (%)** VL1	Recovery (%)** VL2	Inter-day precision (RSD%) VL1	Inter-day precision (RSD%) VL2
Leaf	**PCBs**	0.9908-0.9998	83-120 (0-9)	99-109 (0-10)	3-18	6-18
	Pesticides	0.9805-0.9998	82-118 (1-17)	97-106 (0-9)	2-18	1-18
	PAHs	0.9944-0.9999	88-120 (1-18)	97-102 (0-9)	10-18	3-15
Rhizome	**PCBs**	0.9803-0.9996	80-120 (1-17)	91-106 (0-13)	1-18	1-12
	Pesticides	0.9863-1.0000	90-120 (3-15)	94-110 (0-12)	3-20	2-10
	PAHs	0.9819-0.9999	85-120 (2-17)	82-115 (1-19)	6-18	9-20
Sediment	**PCBs**	0.9760-0.9997	81-118 (1-16)	93-109 (0-15)	2-18	1-10
	Pesticides	0.9873-0.9996	81-117 (0-12)	94-103 (1-9)	4-14	2-16
	PAHs	0.9877-0.9999	81-118 (0-17)	95-102 (0-12)	7-19	5-19

(*) Two validation levels (VL1 and VL2). For leaves 20 and 400 μg kg^{-1}, for rhizomes 10 and 200 μg kg^{-1}, and for sediment 2 and 40 μg kg^{-1}.
(**) Intra-day precision values, expressed as % RSD, are given in brackets (*n* = 3).

Table 4.8. LOD and LOQ (μg kg^{-1}) per compound class and matrix.

Compound		Leaf LOD	Leaf LOQ	Rhizome LOD	Rhizome LOQ	Sediment LOD	Sediment LOQ
PCBs	min	0.0045	0.015	0.0027	0.009	0.0003	0.001
	max	0.2259	0.753	0.1602	0.534	0.0279	0.093
Pesticides	min	0.0228	0.076	0.0033	0.011	0.0003	0.001
	max	1.6044	5.348	2.9355	9.785	0.0615	0.205
PAHs	min	0.0003	0.001	0.0273	0.091	0.0021	0.007
	max	0.5103	1.701	1.3089	4.363	0.3168	1.056

LOD: limit of detection; LOD: limit of quantification; PAHs: polycyclic aromatic hydrocarbons; and PCBs: polychlorinated biphenyls.

7. Sample analysis

The developed, sample-friendly, miniaturized and tissue-specific methods for the detection of PhCs and OPs (PCBs, pesticides, and PAHs) were applied in the study of environmental samples. In both studies, PhCs and OPs, a target analysis mode combined with retrospective analyses were performed for the first time using LC or GC coupled to HRMS (Q-Orbitrap MS).

In the case of the PhCs study, 22 compounds were detected during target screening, 9 tentatively identified in the suspect analysis, and another 11 were tentatively identified during the unknown mode. Therefore, a total of 42 PhCs were detected, from which 24 were seen in *P. oceanica* for the first time. All these compounds are gathered in Table 4.9, and an example of the isorhamnetin-3-O-malonylglucoside extracted ion chromatogram is given in Figure 4.5.

The PhCs distribution among *P. oceanica* tissues revealed a relatively higher number in leaves than in the other two compartments. Such difference was mainly due to the cinnamic acid subclass. Since leaves are more exposed to UV radiation, water motion, shifts in temperature, epiphytes, and so on, this might imply that cinnamic acids respond to stressors specific to the leaves.

Moreover, during OPs analysis, a diverse range of compounds were detected. The target analysis identified 25 PCBs, 13 priority pesticides and 7 priority PAHs (Table 4.10). In the suspect analysis 13 current-use pesticides, 3 non-priority parent PAHs, and 4 PAH-derivatives were tentatively identified (Table 4.11). These suspected current-use pesticides were later confirmed using their corresponding analytical standards. Furthermore, the unknown analysis tentatively identified 11 other compounds with a relatively higher detection frequency (Table 4.12). Moreover, none of the compounds identified during suspect and unknown screening had been previously reported in *P. oceanica*.

Table 4.9. List of PhCs detected in *P. oceanica* tissues in this Thesis.

Target		**Suspect**	**Unknown**
Catechin (+)	Quercetin	Protocatechualdehyde	**Gambiriin A1**[1]
Caffeic acid	**Naringenin**[1]	**Zosteric acid**[1]	**Mascaroside**[1]
Epicatechin (-)	**Luteolin**[1]	p-Anisic acid	**Astilbin**[1]
Baicalein[1]	**Isorhamnetin**[1]	Caftaric Acid	Tracheloside
Genistein[1]	**Apigenin**[1]	Chicoric acid	**Quercetin 3-O-sulfate**[1]
Eriodictyol[1]	**Naringenin Chalcone**[1]	Fertaric acid	**Glabridin**[1]
p-Coumaric acid	**Pinocembrin**[1]	Cinnamic acid	**Sophoraflavanone B**[1]
Ferulic acid	**Biochanin A**[1]	Quercetin-3-O-malonylglucoside	**Piceatannol**[1]
Quercetin-3-O-glucoside	**Glycitein**[1]	Isorhamnetin-3-O-malonylglucoside	**Tetrahydrocurcumin**[1]
Kaempferol-3-O-glucoside[1] + 4, Luteolin-4'-O-glucoside[1]	**Galangin**[1]		**Demethoxycurcumin**[1]
Isorhamnetin-3-O-glucoside	**Sakuranetin**[1] **+ Isosakuranetin**[1]		**Xanthohumol**[1]

[1] Compounds detected for the first time in *P. oceanica*.

Concerning the target analysis, and under the experimental conditions used in this study, PAHs were found to be the most ubiquitous compounds, being present in all sampling sites and matrices. In contrast, PCBs were only detected in rhizomes and marine sediments from three sampling points, where priority pesticides were also found, but only in the rhizomes. Furthermore, several compounds exhibited residue levels above limits proposed by the OSPAR Commission and the WFD, such as the BAC, the Assessment Criteria limit (EAC), or the No-effect concentration (Table 4.10). The distribution of PCBs among matrices seemed to follow a pattern based on their n-octanol/water partition coefficient (log K_{ow}). Generally, compounds with smaller log K_{ow} were seen in rhizomes, while greater log K_{ow} were present in sediments. The absence of a clear pattern when observing PAHs and pesticides could be attributed to various processes, including migration of these compounds to temporary compartments (plants detoxification strategy), metabolism, differences in tissue age (older tissues accumulate for longer periods, being possible to observe legacy contamination), and sedimentary resuspension (mixing low molecular weight compounds, or LMW, from the water column, with high molecular weight compounds, HMW, from the sediment).

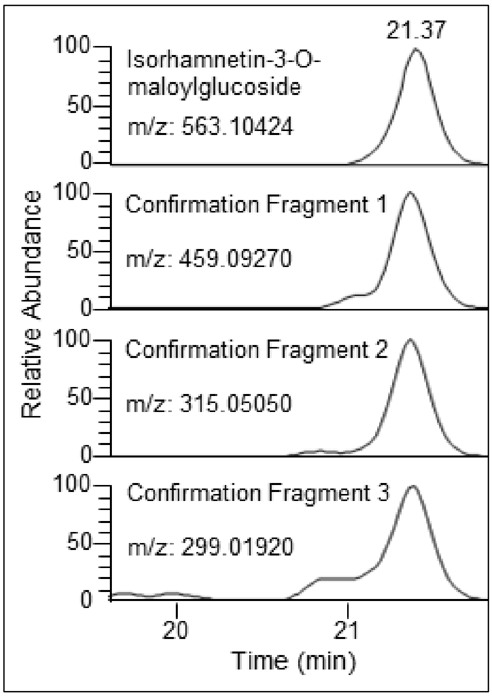

Figure 4.5. Extracted ion chromatogram of the compound Isorhamnetin-3-O-malonyl-glucoside, detected in *P. oceanica* leaves in ALM2.

In the suspect screening phase, a considerable number of current-use pesticides were detected (Table 4.11), exhibiting a wider distribution across matrices and sampling points when compared to the banned or regulated compounds (Table 4.10). As an example, Figure 4.6 shows the extracted ion chromatogram for the pesticide pyrimethanil, with their corresponding confirmation ions. Additionally, several PAH-derivatives, including some that were not previously reported in *P. oceanica* literature, were tentatively identified. Surprisingly, after the semi-quantification, elevated concentrations of PAH-derivatives were observed in some samples where the priority parent-PAHs were below the LOD or not notably present. These findings highlight the importance of considering the derivatives of ubiquitous OPs, such as PAHs, as they may exhibit greater toxicity, and thus should not be overlooked in the assessment of environmental samples.

Table 4.10. Concentration ranges of priority compounds (µg kg^{-1} d.w.) observed during the target analysis in leaves, rhizomes, vegetated sediments (V-sed), non-vegetated sediments (NV-sed), and not-meadow-related sediments (NMR-sed). In this case, the compound classes are shown in order from more widely detected to less detected (PAHs > PCBs > Pesticides).

Class	**Analyte**	**Matrix**				
		leaf	**rhizome**	**V-sed**	**NV-sed**	**NMR-sed**
PAHs	Naphthalene	0.7-1.2	n.d.-25.9	n.d.-104.0	0.8-90.0	5.0-86.4
	Fluorene	n.d.-7.3				n.d.-3.4
	Phenanthrene	4.9-23.9	n.d.-33.7	n.d.-23.0$^{(2)}$	n.d.-47.0$^{(2)}$	n.d.-7.5$^{(2)}$
	Anthracene	n.d.-<LOQ	n.d.-5.0$^{(2)}$		n.d.-3.0$^{(2)}$	
	Fluoranthene	2.9-10.1$^{(2)}$	n.d.-0.8	n.d.-4.4	n.d.-40.7$^{(2)}$	n.d.-3.9
	Pyrene	2.9-205.8$^{(2,3)}$		n.d.-4.3	n.d.-30.8$^{(2)}$	
	Benz[a]anthracene	n.d.-9.7				
	∑PAHs	5.8-233	n.d.- 52.1	n.d.-127.0	0.8-125.8	12.6-90.6
PCBs	PCB 18			n.d.-1.4		
	PCB 28+31		n.d.-10.7$^{(2,3)}$	n.d.-1.5		
	PCB 52		n.d.-1.6$^{(2,3)}$	n.d.-1.1		
	PCB 44		n.d.-11.2$^{(1)}$	n.d.-1.7		
	PCB 101			n.d.-1.2		
	PCB 81		n.d.-9.7$^{(1)}$	n.d.-0.8		
	PCB 77		n.d.-11.1	n.d.-0.9		
	PCB 123			n.d.-1.1		
	PCB 118			n.d.-0.9		
	PCB 114			n.d.-0.9		
	PCB 153		n.d.-2.0$^{(2)}$	n.d.-0.9		
	PCB 105			n.d.-0.9		
	PCB 138			n.d.-1.6		
	PCB 128		n.d.-8.7$^{(1)}$	n.d.-1.0		
	PCB 167			n.d.-1.4		
	PCB 157			n.d.-1.5		
	PCB 180			n.d.-1.1		
	PCB 170			n.d.-1.5		
	∑PCBs		n.d.-55.0	n.d.-21.5		
Pesticides	Trifluralin		n.d.-3.9$^{(1)}$			
	Chlorpyrifos		n.d.-3.2$^{(1,4)}$			
	Isodrin		n.d.-1.9$^{(1)}$			
	o.p'-DDT		n.d.- <LOQ			
	∑Pesticides		n.d.-9.0			

(n.d.): not detected

[1] Compounds detected for the first time.

[2] Concentrations > Background Assessment Concentration (BAC).

[3] Concentrations > Assessment Criteria limit (EAC).

[4] Concentrations > No-effect concentration.

Regarding the unknown screening, numerous compounds were tentatively observed, from pesticides or agrochemicals and PAH metabolites, to plasticizers, and UV stabilizers. Some of them showed concerning detection frequencies, being present in all analyzed samples. This information can be found, sorted by compartments, in Table 4.12. Other studies have also observed relatively high detection for these types of compounds. For example, the widely used 2,4-di-tert-butylphenol, which serves as a raw material for phosphite antioxidants and UV stabilizers, occurs naturally as PhC in bacteria and plants, and has been found at a frequency of 100% in surface waters, as reported by Xie et al. (2020). Similarly, the pesticides dodemorph and isoprocarb were detected in the fat and muscle tissues of loggerhead turtles (*Caretta caretta*) near our sampling areas, with frequencies of up to 56%, according to Novillo et al. (2017). Furthermore, Ding et al. (2023) identified isoprocarb as the second most abundant compound in water samples taken in the Arctic Ocean. Likewise, certain plasticizers, such as Irgafos 168, readily leach from plastics in turbulent seawater, making them of particular interest in sampling areas near coastal agriculture, where plastic greenhouses are prevalent (Suhrhoff and Scholz-Böttcher, 2016). Detection frequencies can be also high in compounds with multiple usages or sources, as the case of 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, dimethyl benzaldehydes and tributyl phosphate. In fact, León et al. (2020) previously reported a detection frequency of 96% for tributyl phosphate in marine sediments near our sampling areas.

Summarizing, the application of these methods together with the different analysis strategies (target and non-target), and HRMS, led to the detection of the most complete phenolic fingerprint described in Table 4.9, as well as the so far most accurate analysis of organic micropollutants ever conducted in *P. oceanica* (Tables 4.10, 4.11 and 4.12). The fact that a considerable number of compounds were detected during retrospective analyses, highlights the need for a 3-way analysis when attempting to properly analyze environmental samples. Finally, the novel development of miniaturized and more eco-friendly methods for *P. oceanica* and sediments, simplifies the laboratory effort while conducting a more sustainable investigation. This is done by reducing the amount of required sample material and solvents, in comparison to the previous studies.

Table 4.11. Concentration ranges (µg kg^{-1} d.w.), estimated by semi-quantification or standard addition procedure, of compounds detected or tentatively detected during the suspect analysis in leaves, rhizomes, vegetated sediments (V-sed), non-vegetated sediments (NV-sed), and not-meadow-related sediments (NMR-sed).

Class	Analyte	Matrix				
		Leaf	Rhizome	V-sed	NV-sed	NMR-sed
Current-used pesticides	2,4,6-trichlorophenol		n.d.-0.9			
	1,4-Dimethyl naphthalene	n.d.-29.0	n.d.-5.3			
	2-Phenylphenol	n.d.-11.0				
	Lindane		n.d.-0.5			
	Pyrimethanil		n.d.-40.7			
	Terbutryn	n.d.-0.2				
	Tetraconazole	n.d.-1.1				
	Penconazole		n.d.-0.3			
	Prallethrin			n.d.-7.5	n.d.-7.3	n.d.-7.5
	Fludioxonil		n.d.-7.9			
	Piperonylbutoxide	n.d.-84.4				
	Fenbuconazole		n.d.-8.7			
	Difenoconazole	n.d.-265.2				
PAH-deriv	DMNs	n.d.-1480.0	n.d.-47.0			
	TDN	43.8-1080.3		n.d.-9.0		n.d.-13.6
	DMMN	n.d.-898.1				
	2,3-DMAQ	n.d.-42.7				
Parent PAHs	Ret	9.8-95.8		n.d.-0.7		
	DBF	n.d.-16.6				
	2-BrN		n.d.-11.2			

2,3-DMAQ: 2,3-Dimethylanthraquinone; 2-BrN: 2-bromonaphthalene; PAH-deriv: PAH-derivatives; DBF: Dibenzofuran; DMMN: 1,6-Dimethyl-4-(1-methylethyl)naphthalene; DMNs: Dimethylnaphthalenes; Ret: Retene; and TDN: 1,1,6-Trimethyl-1,2-dihydronaphthalene.

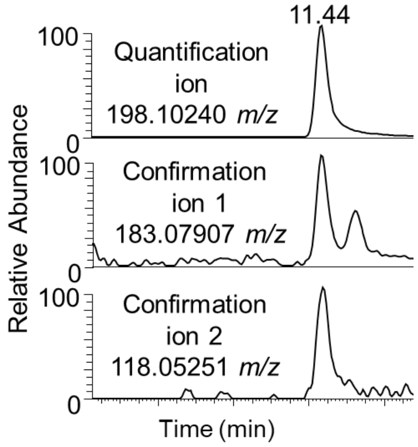

Figure 4.6. Extracted ion chromatogram of current-used pesticide pyrimethanil, detected in the rhizome in ALM3.

Table 4.12. Compounds tentatively identified during the unknown analysis of leaves, rhizomes, and sediments, together with their detection frequency per matrix (%).

	Matrix	**1-MOF**	**2,4-DTBP**	**7,9-DTBO**	**DBAL**	**DCHP**	**DAIP**	**Dodem**	**Irg 168**	**Isop**	**TBP**
Detection (%)	Leaf	-	100	100	-	41	86	100	100	100	-
	Rhizome	-	-	-	-	-	-	-	-	-	85
	Sediment	100	100	64	100	-	-	100	77	-	-
Category	Pesticide or agrochemical				▨	▨		▨		▨	
	Plasticizer			▨			▨		▨		▨
	Plant/algae metabolite			▨	▨						
	Medicals			▨	▨						
	Industrial chemical (hydraulic fluid, solvent, heat exchange agent)		▨								▨
	Flavor and fragrance agent				▨						
	Antioxidant			▨							▨
	UV Stabilizer		▨								
	PAH breakdown product	▨									

1-MOF: 1-Methoxymethylfluorene; 2,4-DTBP: 2,4-Di-tert-butylphenol; 7,9-DTBO: 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione; DBAL: Dimethyl benzaldehyde; DCHP: Dicyclohexyl phthalate; DAIP: Diallyl isophthalate; Dodem: Dodemorph; Irg 168: Irgafos 168; Isop: Isoprocarb; and TBP: Tributyl phosphate.

8. Implications of the findings

The newly identified PhCs, complete the phenolic fingerprint of *P. oceanica*. This finding might be of great interest in future investigations. For instance, the response of the 42 PhCs to different pressures could be assessed, as well as their potential as specific stress biomarkers. Additionally, in forthcoming correlation studies, it will be observed whether the OPs might be triggering any sort of phenolic early warning.

This study also brings new information about the OP content in the marine environment, by detecting several OPs for the first time in *P. oceanica*, and sediments. With this data, future research could delve into the effects that each of the detected OP might exert on *P. oceanica* (such as their CO_2 storage capacity, photosynthetic activity, or resilience against stressors). Likewise, this Thesis proves that the temporal and spatial range of mostly land-born OPs is considerably high in the marine coastal environment (plants and sediments). The fact that long-banned OPs are seen in *P. oceanica* shows the need to monitor this relevant seagrass, and its environment (e.g., surrounding sediment) while ensuring its ecological integrity, by using methods that required reduce quantities of sample material and solvents, such as the ones here proposed.

Furthermore, it is important to highlight that OPs were seen in shallow aquatic environments, where the marine dynamic is usually strong. This means that OPs resuspension and solubilization events might be fostered, enhancing their bioavailability. This might negatively impact several key economic sectors in the Mediterranean Basin, such as fisheries, catering, and tourism, which partly rely on the trade of fish, mussels, and crustaceans. If these species surpass certain OP concentration thresholds established by the current legislation, their consumption would be prohibited, posing a serious risk for the sectors enumerated above. Therefore, OPs contamination is not only detrimental to the health status of the environment and humans but also to the economy.

Lastly, this Thesis has proved that a reduced amount of sample material (e.g., one single *P. oceanica* leaf) is sufficient to conduct PhCs and OPs detection studies. This, together with non-destructive sampling techniques, such as the recently introduced by Gobert et al. (2020), could set the baselines for sustainable monitoring of seagrasses. This is a key point in a scenario where global seagrasses' state is critical, such as *P. oceanica*, and considering their contribution to the fast recovery of coastal ecosystem services (Orth et al., 2020).

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Chapter V

Conclusions

This Thesis aimed to conduct an exhaustive study of the endogenous and exogenous compounds present in *P. oceanica* and related marine sediments, more specifically PhCs and OPs. Likewise, a second aim was intended, the distribution of the investigated compounds along the studied compartments (leaves, rhizomes, roots, and sediments). For these purposes it was required to develop several methods, considering the maximum number possible of the Green and White Chemistry Principles, and using the HRMS benefits. The subsequent conclusions are here highlighted:

1. Optimized, tissue-specific, ultrasound-based methods coupled to UHPLC-HRMS allowed for the comprehensive analysis of PhCs in *P. oceanica* tissues (leaves, rhizomes, and roots).
2. The PhCs' study applied for the first time the potential of HRMS in *P. oceanica*: retrospective analyses, elevated sensitivity, and selectivity. This helped reduce the required amount of sample material, compared to previously published studies, while maximizing data collection by using the 3-way analysis (target, suspect and unknown).
3. The methods developed for PhCs monitoring were applied to samples from 5 different sampling points, subject to diverse stressors, from the Almeria coast. This resulted in the satisfactory identification of 42 PhCs, from which 24 were detected for the first time in this matrix (including, among others, naringenin, naringenin chalcone, and pinocembrin).
4. The distribution of PhCs across different tissue compartments showed a comparatively higher abundance in leaves. This disparity was primarily attributed to a greater presence of compounds belonging to the cinnamic acid subclass.
5. For the OP study, miniaturized, multiclass, tissue-specific methods were developed and satisfactorily validated (in terms of recovery and precision) for the determination of 25 PCBs regulated by the Stockholm Convention, 13 priority pesticides, and 16 priority PAHs in *P. oceanica* leaves, rhizomes, and marine sediments. The methods consisted of a polytron-based extraction for *P. oceanica* tissues and UAE for sediments, followed by a GC analysis, coupled to HRMS as a novelty.
6. The validated methods were applied to 21 leaves, 21 rhizomes, 20 vegetated sediment, and 23 non-vegetated sediment samples. This resulted in the observation of 4 priority pesticides, 7 priority PAHs, and 7 PCBs.
7. Regarding the distribution, priority pesticides were observed merely in one rhizome from Alicante. Banned PCBs were seen in the long-live rhizomes and vegetated sediment of several points from the Alicante coast, potentially following a distribution based on their log k_{ow}. On the other hand, priority PAHs presented the greatest distribution,

being observed in all sampling points and compartments, but not following a clear pattern in distribution.

8. The application of retrospective analysis revealed current-use pesticide residues, non-priority PAHs, PAH-derivatives, and plasticizers. Thirty-one of these substances were seen for the first time in *P. oceanica* tissues, such as 2,4-di-tert-butylphenol, dicyclohexyl phthalate, and dodemorph. Additionally, various of these substances showed 100 % detection frequency in certain matrices, such as the case of the PAH retene, and the pesticides dodemorph and isoprocarb, among others.
9. The incorporation of analytical procedures into environmental studies, such as miniaturization of the method, optimization, and the benefits of HRMS (i.e., sensitivity and the 3-way analysis) provided the most accurate phenolic fingerprint and OP content while promoting a sustainable study.

Annex

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