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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 ad-

verse 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™ (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.

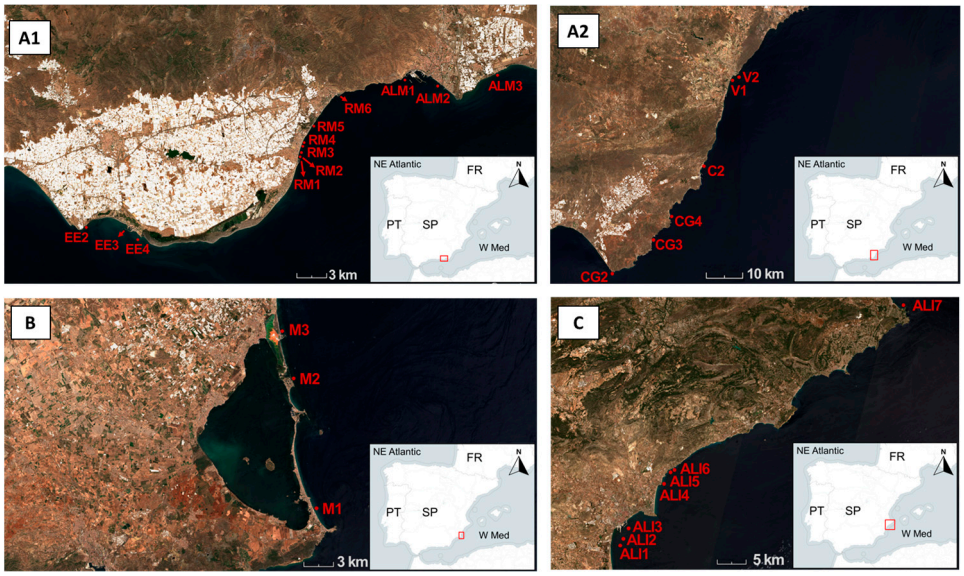

Figure 1. Maps of the surveyed points on the Mediterranean Spanish coast: (**A1**) Western Almeria, (**A2**) Eastern Almeria, (**B**) Murcia and (**C**) Alicante stations. White areas seen on land correspond to greenhouses. Figure made by the authors using Copernicus Sentinel data (2022) and processed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

tural 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).

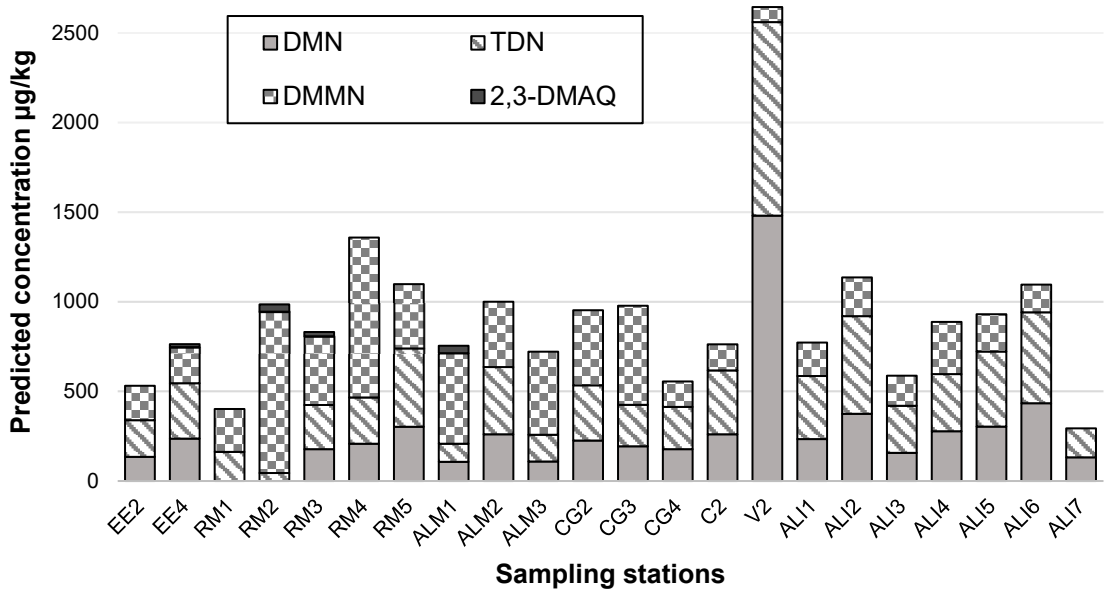

Figure 2. Semi-quantification results for PAH-derivatives: the example of the leaves of *P. oceanica*. Detections under LOQ were not included in this chart figure.

The DMN compounds detected in leaves and rhizomes (such as the 2,6-dimethylnaphthalene) 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	$C_{18}H_{35}N_O$	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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