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Nature-Based Solution for Lambda-Cyhalothrin Removal in Ria Formosa Contaminated Sediment: A Mesocosm Study

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Faculdade de Ciências e Tecnologia

2023

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Master in Applied Ecohydrology

Supervisor:

Prof. Dr. Luís Chícharo

Co-supervisor:

Dr. Olfa Ben Said

UNIVERSIDADE DO ALGARVE

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2023

Declaration of authorship of the work

I declare to be the author of this work, which is original and unprecedented. Consulted authors and works are rightfully cited in the text and are included in the list of references.

Faro, September 2023

Hornam Azanda

ACKNOWLEDGEMENT

I would like to express my profound gratitude to Dr. Olfa Ben Said for the invaluable technical assistance and mentorship rendered throughout the duration of this research work. Her insightful feedback and unwavering support played a significant role in the enhancement of this research's quality and credibility.

Special thanks to Prof. Luis Manuel Zambujal Chicharo, whose expert advice and constructive criticism guided the structuring and focus of this study. His comprehensive knowledge and academic expertise greatly contributed to the attainment of the research objectives.

My heartfelt appreciation goes to Pedro, whose assistance during the field campaigns was crucial for the seamless collection and analysis of the necessary data. His commitment and hands-on support ensured the timeliness and efficiency of the research process.

I am also deeply thankful to the consortium partners: the University of Algarve, University of Lodz, Technical University of Luebeck, and University of Antwerp for their co-facilitation of the master’s in applied Ecohydrology (MAEH) in advancing knowledge and innovation.

May this acknowledgment serve as a small token of my immense gratitude to everyone who contributed to the success of this research project. Your collective expertise, support, and commitment have been the driving force behind the timely and successful completion of this work. Thank you.

DEDICATION

This thesis is lovingly dedicated to my dearest parents, whose unwavering love, support, and sacrifice have been my guiding light throughout this academic journey. Your faith in me has been the pillar upon which my academic pursuits have rested, fueling my passion and resilience in the face of challenges. This accomplishment is not just my own, but also a testament to your enduring love, guidance, and belief in my potentials. Thank you for being my constant source of inspiration and for providing me with the foundation upon which all my successes are built. This milestone is as much yours as it is mine.

ABSTRACT

Nature-based solutions (NBS) harness natural tools for effective contaminant removal via organism-based processes like phytoremediation. However, in highly contaminated environments, phytoremediation effectiveness may be limited due to pollutant interactions. Microbe-assisted phytoremediation (rhizoremediation) offers a promising alternative for removing pollutants in challenging contaminated settings. The objective of the present project was to evaluate the biodegradation of Lambda-cyhalothrin (LC) of a Ria Formosa salt marsh plant (*Spartina maritima*) through biodegradation mesocosms of plant, and sediments experiments following the inoculation of contaminated sediments with a LC - degrading rhizospheric bacteria. Experimental setups encompassed various treatments, including phytoremediation, bioaugmentation, and rhizoremediation and different control conditions. Results from the study highlighted substantial LC degradation across all three treatments, indicating a reduction of 30% for Phytoremediation (Phyto), 27% for Combined Phytoremediation and Bioaugmentation (Phyt + BioA), and 22% for Bioaugmentation (BioA) after a 7-day duration. The utilization of *Spartina maritima* in phytoremediation demonstrated effectiveness in LC degradation within Ria Formosa sediment. Moreover, the combination of *Spartina maritima* with its rhizospheric bacterial consortium (rhizoremediation) also proved to be an efficient approach for LC degradation and has the potential to facilitate the degradation of the pesticide under highly stressed environment conditions. Future research endeavors should focus on further exploring the potential of these bioremediation techniques under varied environmental conditions and differing contamination levels. This comprehensive exploration will offer invaluable insights into the optimization and applicability of these strategies for LC degradation in diverse ecological contexts.

Keywords: Ria Formosa, nature-based solution, bioremediation, rhizoremediation, lambda-cyhalothrin

RESUMO

Soluções baseadas na natureza (NBS) representam um conjunto de ferramentas naturais existentes que são eficazes na remoção de contaminantes por meio das capacidades de biodegradação e biotransformação de organismos, como a fitorremediação baseada na capacidade de fitoextração-fitostabilização de plantas. No entanto, em ambientes estressantes (contaminação média a alta), a eficácia da fitorremediação pode ser limitada pela biogeoquímica e dinâmica dos poluentes. Assim, a fitorremediação assistida por microrganismos (rizoremediação) poderia representar uma alternativa confiável para a remoção de poluentes em ambientes estressados e contaminados.

O objetivo do presente projeto foi avaliar a biodegradação de Lambda-cyhalothrin (LC) de uma planta de sapal da Ria Formosa (Spartina maritima) por meio de mesocosmos de biodegradação de plantas e experimentos de sedimentos após a inoculação de sedimentos contaminados com uma bactéria rizosférica degradadora de LC. As configurações experimentais abrangeram vários tratamentos, incluindo fitorremediação, bioaumentação, rizoremediação e diferentes condições de controle.

Os resultados do estudo destacaram uma degradação substancial de LC em todos os três tratamentos, indicando uma redução de 30% para Fitorremediação (Phyto), 27% para Fitorremediação e Bioaumentação Combinadas (Phyt + BioA) e 22% para Bioaumentação (BioA) após um período de 7 dias. A utilização de Spartina maritima na fitorremediação demonstrou eficácia na degradação de LC nos sedimentos da Ria Formosa. Além disso, a combinação de Spartina maritima com seu consórcio bacteriano rizosférico (rizoremediação) também se mostrou uma abordagem eficiente para a degradação de LC e tem o potencial de facilitar a degradação do pesticida em condições de ambientes altamente estressados.

Futuras pesquisas devem focar na caracterização do consórcio bacteriano degradador de LC e na exploração adicional do potencial dessas técnicas de biorremediação em diferentes condições ambientais e níveis de contaminação. Essa exploração abrangente fornecerá insights valiosos para a otimização e aplicabilidade dessas estratégias na degradação de LC em diversos contextos ecológicos.

Palavras-chave: Ria Formosa, solução baseada na natureza, biorremediação, rizorremediação, lambda-cyhalothrin

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List of Abbreviations & Acronyms

ANOVA	Analysis of Variance
BioA	Bioaugmentation
CSC	Contaminated sediment control
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
LC	Lambda-cyhalothrin
LOI	Loss on Ignition
MSM	Mineral Salt Medium
NBS	Nature-based solution
OC	Organic Carbon
PGPR	Plant growth promoting rhizobacteria
Phyto	Phytoremediation
WFD	Water Framework Directive
WHO	World Health Organization

1 INTRODUCTION

1.1 Background

Coastal lagoons are ecologically significant due to their high productivity and their pivotal role in biogeochemical processes (Newton *et al.*, 2018; Sousa *et al.*, 2013). These shallow water bodies support a diverse array of habitats such as wetlands, mangroves, salt-marshes and seagrass meadows (Basset *et al.*, 2013). However, these ecosystems are facing significant anthropogenic pressures such as human encroachment, pollution, resource extraction, and global climate change (Basset *et al.*, 2013).

Recent research has established that coastal environments present a high risk of environmental contamination due to the presence of a wide range of urban and industrial chemicals, including metals, PCBs, PAHs, and emerging contaminants such as pharmaceuticals and pesticides (Capolupo *et al.*, 2017). Agricultural water quality is a significant environmental concern in most countries, with the primary agricultural sector being the main source of nitrate, phosphorus, pesticides, soil sediment, salt, and pathogen pollution from crop and livestock activities (Parris, 2011). As a result, complex mixtures of contaminants are continuously released into these systems, resulting in a deterioration of water quality and significant constraints on organisms, ultimately leading to a potential depletion of natural resources (Cravo *et al.*, 2012).

Aquatic ecosystems are facing a diverse range of pesticides, primarily originating from agricultural practices (Ribeiro *et al.*, 2005). Pesticides are substances, either chemical or biological, commonly utilized to manage pests and enhance crop yield (Kaur *et al.*, 2023). However, the detrimental impacts of pesticide use have surpassed their advantages. Pesticide contamination in estuaries and coastal lagoons is a frequent issue, with organochlorine compounds being commonly found in these areas (Ribeiro *et al.*, 2005). However, pyrethroids are now becoming a more concerning type of aqueous micropollutant (Cao *et al.*, 2022), due

to their harmful effects on human health (Sogorb *et al.*, 2004) and aquatic organisms. Lambda-cyhalothrin (LC), a synthetic pyrethroid insecticide, has been increasingly used for crop production in recent years, with its use increasing by 50 folds (Farag *et al*., 2011). As a result, residues of LC have been detected in runoff water and sediments from agricultural, public health, and residential applications (Whitacre, 2008). Pesticides are known to bind strongly to sediments in water, and their release to the surrounding water is largely determined by the pH, oxidation-reduction state, and organic matter content of the water (Neary & Carr, 2006). Recent studies have demonstrated that pyrethroids, including LC, are commonly found in aquatic sediments (Cruzeiro *et al.*, 2015b) and can cause toxicological issues for aquatic organisms (Amweg *et al*., 2005; Weston *et al*., 2004).

The presence of pesticides within the lagoon ecosystem represents a significant threat to both biodiversity and economic development (Krishna & Philip, 2011). The synthetic pyrethroids like LC are extremely toxic to fish and aquatic invertebrates and due to their lipophilicity, they have a high rate of gill absorption, which in turn would be a contributing factor in the sensitivity of fish to these pollutants (Xia, 2008). Moreover, LC potential in contaminating water and sediment has been reported to lead to toxicity in aquatic organisms such as mosquitofish, shrimps, crabs, and clams (Whitacre, 2008). Additionally, human exposure to LC may cause irritation to the skin, throat, nose, and other body parts (Guo et al., 2023).

In recent years, there has been a growing awareness of the importance of establishing standard guidelines for safeguarding coastal lagoons, which have traditionally been subjected to severe human-induced pressures due to their industrial, commercial, and agricultural significance (Capolupo *et al.*, 2017). To address this concern, a range of regulatory frameworks and guidelines have been implemented, including the World Health Organization's (WHO) Guidelines for ensuring the safety of recreational water environments, the Environmental Protection Agency's (EPA) water quality criteria, the European Water Framework Directive

(WFD), as well as other guidelines and regulations pertaining to water quality and management.

In coastal waters, conventional technologies are typically employed to mitigate pollution; however, these methods can be costly and environmentally detrimental (Kuiper et al., 2004). In contrast, bioremediation, an ecohydrological technique that employs microorganisms, plants, or a combination of both, has been shown to be a more sustainable, cost-effective and dependable alternative to chemical and physical methods (Klein, 2008). This nature-based solution (NBS) has demonstrated significant potential in degrading a variety of pollutants (Ben Said *et al*., 2019; Kaur *et al*., 2023), including pesticides, and may be an effective solution for mitigating LC contamination of coastal waters.

1.2 Problem statement and justification

The Ria Formosa Lagoon, located on the southern coast of Portugal, is a coastal lagoon of great ecological importance for tourism and fisheries, particularly shellfish (Bebianno et al., 2007). However, the lagoon has been subjected to a wide range of pollutants, including pesticides. Cruzeiro *et al.*, (2015) have reported that the levels of certain pesticides in the lagoon have reached critical levels, indicating poor ecological status on the ecosystem.

The synthetic pyrethroid insecticide LC has been extensively used in agriculture and has been detected in various environmental compartments, including aquatic ecosystems (Cao *et al.*, 2022). LC is one of the most used insecticides in the Algarve region due to its efficiency (SIFITO, 2023). The use of LC in agricultural areas near Ria Formosa may be negatively affecting the ecological integrity of the ecosystem. The potential accumulation of this pesticide in the lagoon system may pose risks to non-target organisms and threaten the economic viability of the region. This suggests that anthropogenic activities have significantly affected

the Ria Formosa Lagoon, emphasizing the need for effective management strategies to mitigate the negative effects of pollution on the area's ecology and economy.

Rhizoremediation is a promising bioremediation technique that employs plant-microbe interactions to degrade pollutants in soil or water (Kaur *et al*., 2023; Zalewski, 2004). This technique has demonstrated significant potential in degrading a wide range of pollutants, including insecticides, and may be an effective tool for the remediation of LC in the Ria Formosa sediment. However, there is limited research on the efficacy of rhizoremediation in degrading LC in aquatic environments, particularly in the Ria Formosa lagoon.

Therefore, this study aims to evaluate the potential of rhizoremediation as a NBS in degrading LC in the Ria Formosa sediment. This study will contribute to the understanding of the effectiveness of rhizoremediation in aquatic environments and provide insights into the potential of this technique for the remediation of pesticide-contaminated aquatic ecosystems. The results of this study will inform policymakers, environmental agencies, and stakeholders in developing effective strategies for the conservation and sustainable management of the Ria Formosa lagoon.

1.3 Research objectives

The objectives of this study were to:

i. Select LC- degrading rhizospheric bacterial consortium from Ria Formosa Sediment.
ii. Evaluate the biodegradation of LC of *Spartina maritima* following the inoculation of contaminated sediments with a LC- degrading rhizospheric bacterial consortium.
iii. Examine the effectiveness of this bioremediation approach in the removal of pesticides through biodegradation mesocosms of plant, and sediments experiments.

1.4 Research questions

i. How effective is *Spartina maritima* in the biodegradation of LC, and how does the presence of LC- degrading rhizospheric bacteria affect this process?

ii. What are the factors that influence the biodegradation of LC by the rhizospheric bacterial consortium and *Spartina maritima*?

iii. What is the potential for using this bioremediation approach in the removal of pesticides from contaminated sediments and water in coastal waters?

iv. How does the bioremediation approach compare to other traditional remediation approaches, such as chemical oxidation or physical removal of contaminated sediment?

2 LITERATURE REVIEW

2.1 Global Pesticide Problem

The utilization of pesticides as a means of pest management has witnessed a substantial upsurge in the previous decades on a global scale (Kaur *et al.*, 2023). The Food and Agriculture Organization (FAO) reported that the worldwide yearly application of pesticides in agricultural practices reached an estimated 2.7 million tonnes in 2020 (FAO, 2022). Moreover, in addition to their widespread usage in agricultural settings, pesticides are also commonly employed within residential environments in various forms, such as powders, sprays, and poisons, to effectively control infestations of rodents, fleas, cockroaches, ticks, mosquitoes, and other insects (Kaur *et al.*, 2023).

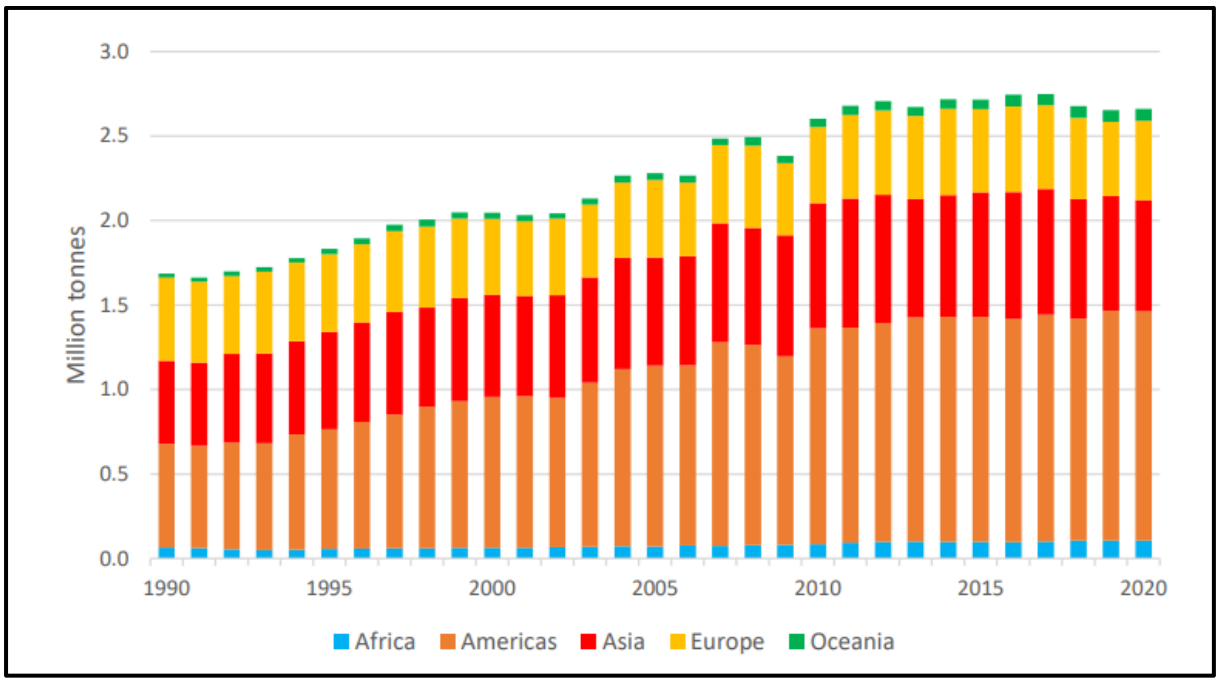

Figure 1: Total pesticides use by region (source: FAO, 2022)

The deposition of persistent pollutants into the environment is often perceived as temporary storage in sedimentary systems (Carvalho et al., 2009). Studies have documented the escalation of pesticide residues in environmental compartments due to excessive application of xenobiotics in soil (Krishna & Philip, 2011). Uncontrolled and haphazard application of

agricultural chemicals over the years has led to significant environmental challenges (Odukkathil & Vasudevan, 2013).

The global pesticide problem has been linked to numerous health issues and problems. The presence of agrochemical residues can have detrimental effects on the ecosystem, as they can enter the food chain directly or indirectly, posing potential health hazards to animals and plants (Krishna & Philip, 2011). Furthermore, many agricultural pesticides applied to soil may interact with non-target soil organisms, including microorganisms that can degrade pesticide compounds (Krishna & Philip, 2011). These residues may also have long-lasting effects, including carcinogenic, mutagenic, neurological, and reproductive impacts *(*Bassil *et al.*, 2007).

2.2 Classification of pesticides

Pesticides are a collective term referring to a diverse range of substances that can be further categorized based on their application methods and organism killed (Akashe *et al*., 2018; Yadav & Devi, n.d.). The primary classifications of pesticides encompass insecticides, herbicides, fungicides, and rodenticides, which are further subdivided into specific subcategories (Akashe *et al*., 2018).

2.2.1 Insecticides

Insecticides, as a subset of pesticides, are designed specifically to combat and manage insect pests (Cardoso & Alves, 2012). They play a crucial role in pest control and can be further classified into various groups based on their chemical composition, as illustrated in Figure 2 (Kaur *et al*., 2019).

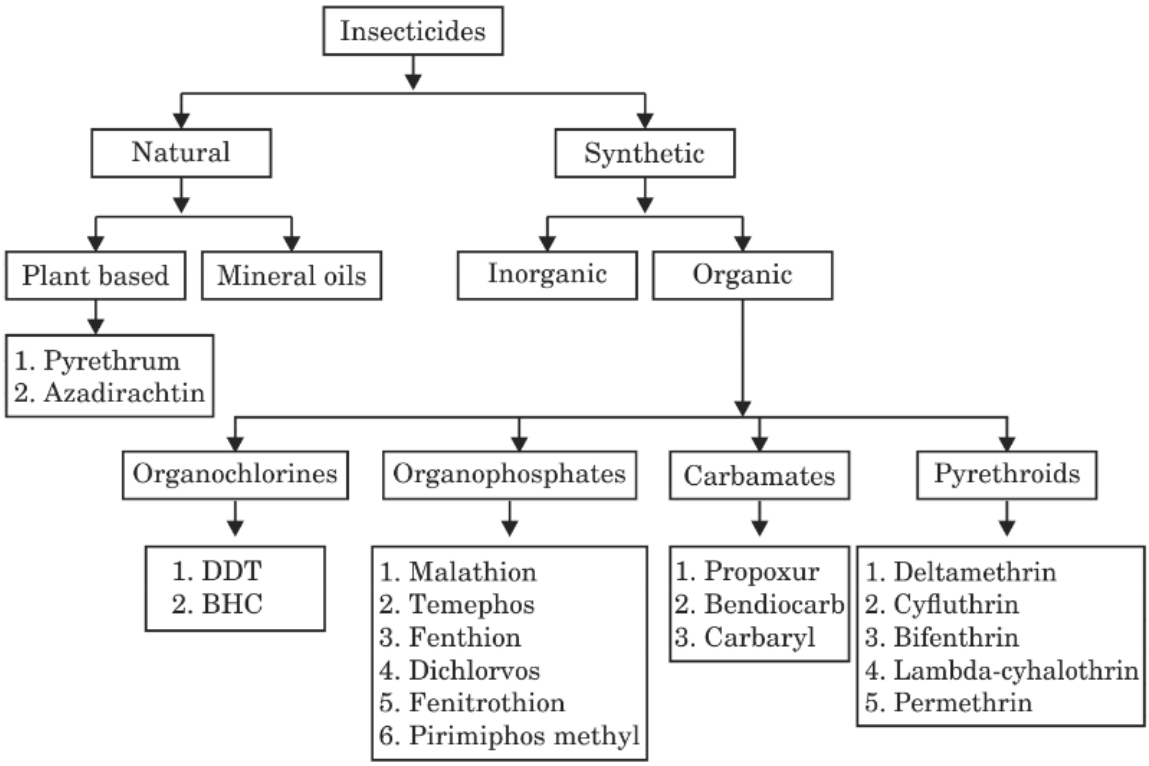

Figure 2: Classification of insecticide (Source: Kaur et al., 2019)

According to their chemical composition, insecticides can be categorized into distinct classes, including Carbamates (e.g., Carbaryl), Organochlorines (e.g., Endosulfan), Organophosphorus compounds (e.g., Monocrotophos), Pyrethroids (e.g., Permethrin), Neonicotinoids (e.g., Imidacloprid), and miscellaneous pesticides such as Spinosyns (e.g., Spinosad), Benzoylureas (e.g., Diflubenzuron), and Antibiotics (e.g., Abamectin) (Yadav & Devi, n.d.). Each class of insecticides employs distinct mechanisms to target insects, such as disrupting their nervous system, inhibiting enzyme activity, or interfering with their growth and development (Akashe *et al*., 2018).

2.2.2 Fungicides

Fungicides are a specific type of pesticides designed to eliminate fungi, encompassing various fungal pathogens such as blights, mildews, molds, and rusts (Yadav & Devi, n.d.). Fungicides

can be categorized into different classes, including aliphatic nitrogen fungicides (e.g., dodine), amide fungicides (e.g., carpropamid), aromatic fungicides (e.g., chlorothalonil), dicarboximide fungicides (e.g., famoxadone), dinitrophenol fungicides (e.g., dinocap), and others *(*Akashe *et al.*, 2018).

2.2.3 Herbicides

Herbicides are a class of pesticides specifically designed to eliminate unwanted weeds and other undesirable plant growth (Yadav & Devi, n.d.). These herbicides can be classified into various groups, such as anilide herbicides (e.g., flufenacet), phenoxyacetic herbicides (e.g., 2, 4-D), quaternary ammonium herbicides (e.g., Paraquat), chlorotriazine herbicides (e.g., atrazine), sulfonylurea herbicides (e.g., chlorimuron), and others (Akashe *et al*., 2018).

2.2.4 Rodenticides

Rodenticides are substances specifically designed to exterminate rats, mice, moles, and other rodent species. They can be categorized into different groups, including inorganic rodenticides such as Zinc phosphide and Aluminium Phosphide, as well as coumarin rodenticides which fall under the organic category, such as bromadiolone and coumatetralyl (Akashe *et al*., 2018).

2.3 Lambda Cyhalothrin (LC)

Lambda-cyhalothrin (LC) is a synthetic insecticide classified as a member of the pyrethroid chemical group. Pyrethroids are artificial pesticides that replicate the properties of naturally occurring pyrethrins found in the flowers of *Chrysanthemum cinerariaefolium* (Gu *et al.*, 2007). Within the pyrethroid family, there exist two categories: type I and type II pyrethroids. Type II pyrethroids generally exhibit greater potency compared to type I pyrethroids. LC, as a broad-spectrum type II pyrethroid insecticide, is employed for the control of various insect species (Santos *et al*., 2012). The mechanism of action for pyrethroids is rooted in their capacity to affect the sodium channels present in the cell membranes of nervous cells, which play a

crucial role in the initiation and propagation of nerve impulses. Consequently, exposure to LC via ingestion or contact results in paralysis and eventual mortality of the targeted organisms (Wang *et al*., 2007). LC is commercially available under different brand names such as Karate, Kungfu, Demand, and Warrior (Onuorah *et al*., 2020).

LC exhibits low vapor pressure and a low Henry's law constant, indicating its limited tendency to volatilize. However, it possesses a high octanol-water partition coefficient (K_{ow}) and water-solid-organic carbon partition coefficient (K_{oc}) values. In aquatic environments with a pH below 8, LC demonstrates considerable stability, while alkaline conditions lead to its hydrolysis, resulting in the formation of hydrogen cyanide (HCN) and aldehyde. Although LC is relatively resistant to degradation under natural sunlight exposure, with a half-life exceeding three weeks, it undergoes rapid photolysis under UV irradiation, with a half-life of less than ten minutes. The fate of LC within aquatic ecosystems depends on the presence of system components such as suspended solids (both mineral and organic particles) and aquatic organisms (such as algae, macrophytes, or aquatic animals). The dissolved residues of lambda-cyhalothrin in water decrease rapidly in the presence of suspended solids and/or aquatic organisms due to strong adsorption of lambda-cyhalothrin molecules by particulates and plants (Whitacre, 2008).

Table 1. Physical, chemical, and environmental properties of lambda-cyhalothrin

CAS Number	91465-08-6
Molecular formular	$C_{23}H_{19}ClF_3NO_3$
Molecular weight (g/mol)	449.9
Density (g/mL at 25 ^oC)	1.33
Melting point (^oC)	49.2
Boiling point (^oC at 2 mmHg)	187-190
Vapour pressure (mPa at 20 ^oC)	0.0002
Henry's Law constant (Pa-m^3/mole)	0.018
Water Solubility (mg/L at 20 ^oC)	0.005
Solubility in other solvents (e.g., acetone) (mg/L)	>500000
Octanol – water partitioning (log k_{ow} at 20 ^oC)	7.00
Hydrolysis half-life (d)	
pH 5	Stable
pH 7	Stable
pH 9	8.66
Photolysis half-life (d)	
Water at pH 5 and 25 ^oC	24.5
Soil	53.7
Bioconcentration factor (BCF) in fish	2240
Soil adsorption K_{oc} (cm^3/g)	247000-330000
Aerobic Soil degradation half-life (d)	42.6
Aerobic aquatic degradation half-life (d)	21.9

Source: (Whitacre, 2008)

2.4 Microbes used for contaminants degradation and bioremediation

Microbial communities are known to have a vital role in maintaining ecosystem functionality, particularly in the decomposition of organic matter, nutrient cycling, and the removal of pollutants (Bourhane *et al.*, 2022). Biodegradation, an efficient bioremediation technique, occurs in microorganisms present in various ecosystems, and through their symbiotic relationship with xenobiotics, they are capable of thriving even in unsuitable conditions

(Kafilzadeh *et al.*, 2015). The basic bioremediation methods involving microbes are biostimulation, bioattenuation, bioaugmentation, venting and piles (Abatenh *et al.*, 2017).

The microbial diversity in wetland sediments is significantly higher than that in water, and microorganisms from sediments are key biological actors for the transformation and migration of substances in wetland ecosystems, thus having an important impact on wetland ecosystems (Li *et al.*, 2020). Leveraging microorganisms as indicators for assessing soil contamination offers several advantageous attributes, including simplicity, expeditiousness, affordability, minimal sample consumption, and the simultaneous evaluation of the contamination status pertaining to multiple elements and other pollutants (Tang *et al*., 2019).

In recent years, certain microbial "specialists" such as *Bacteroidia* and *Nitrospira* have been utilized as bioindicators for pollution in wetland sediments (Li *et al.*, 2020). In addition, enriched bacterial consortia comprising of *P. aeruginosa*, *Bacillus sp.*, *C. joostei*, and *Klebsiella pneumonia* have been employed in the mixed degradation of pesticides (Krishna & Philip, 2011).

2.5 Phytoremediation and Rhizoremediation for pollutant removal

Bioremediation refers to the use of biological processes (Gianfreda & Rao, 2004) involving naturally occurring bacteria, fungi, or plants, to degrade or detoxify hazardous substances that are harmful to human health (Zalewski, 2004). This approach has several advantages over physicochemical remediation methods, such as being cost-effective, convenient, and leading to complete degradation of organic pollutants, without causing collateral damage to the site material or its indigenous flora and fauna (Klein, 2008; Timmis & Pieper, 1999). Biodegradation using microorganisms and phytoremediation using plants are two key biological methods employed for the removal and/or decomposition of pollutants, including metals, pesticides, and nutrients (Ben Said *et al.*, 2019; Chaudhry *et al.*, 2002).

Phytoremediation is a cost-effective and environmentally friendly technology that uses plants to extract, degrade or immobilize contaminants from soil, water and sediments (Zalewski, 2004). Phytoremediation of pollutants may take one of several forms such as phytoextraction, rhizofiltration, phytostabilization, phytovolatilization and phytodegradation (Ghosh & Singh, 2005; Zalewski, 2004). The efficacy of specific plant species to remove pollutant chemicals from the environment is well known, and some species such as *Sarcocornia fruticose* have been well studied for their ability to hyperaccumulate inorganic pollutants such as toxic heavy metals (Ben Said *et al.*, 2019).

However, the selection of plant species for phytoremediation purposes is contingent upon the intended objective, and it is preferable to utilize native plant species that are well-suited to the local environment and capable of withstanding soil pollutants (Zalewski, 2004). The capacity of plants to absorb metals and pesticides is influenced by a range of factors including physicochemical properties of the substances, mode of application, soil type, climatic conditions, and plant species (Ben Said *et al.*, 2019; Chaudhry *et al.*, 2002). Despite the demonstrated efficacy of phytoremediation in mitigating chemical hazards linked to diverse organic and inorganic pollutants, its extensive deployment in real-world situations is hindered by a number of significant limitations.

One of the major impediments to the widespread implementation of phytoremediation techniques is the prevalence of contaminant-induced stress that can significantly reduce the rate of seed germination, retard plant development, and decrease biomass production (Divya & Kumar, 2011). This issue can be addressed by employing plant growth promoting rhizobacteria (PGPR) (Glick, 2003). Rhizoremediation, a specialized form of phytoremediation that leverages plants and their associated rhizospheric microorganisms (e.g., bacteria and fungi), can occur naturally or be facilitated by inoculating soil with microorganisms capable of breaking down environmental pollutants (Divya & Kumar, 2011). Endophytic bacteria (non-

pathogenic bacteria that occur naturally in plants) and rhizospheric bacteria (bacteria that live on and near the roots of plants) are among the plant-associated bacteria that can aid in the biodegradation of toxic organic compounds in polluted soil and hold promise for enhancing phytoremediation (Divya & Kumar, 2011).

2.6 Studies in mesocosm

In recent years, mesocosms have gained popularity as an experimental approach due to their ability to bridge the gap between microcosm experiments, which lack realism, and natural systems, which are complex and often difficult to understand mechanistically (Stewart *et al.*, 2013). Aquatic mesocosms are now widely used in both coastal and freshwater systems (Guy-Haim *et al.*, 2017). In addition, benthic mesocosms have been developed for marine environments to investigate the effects of eutrophication and hypoxia on shallow coastal ecosystems, while allowing for natural fluctuations and increasing experimental realism (Wahl *et al.*, 2015).

The utilization of natural, biological interactions in mesocosm studies renders the findings more ecologically applicable than laboratory experiments (Widdicombe et al., 2010). Numerous mesocosm investigations have demonstrated the functional abilities of organisms, such as *Pistia stratiotes*, exhibiting remarkable efficacy in pollutant removal and productivity (Olguín *et al.*, 2017).

3 MATERIALS AND METHODS

3.1 Study Area

3.1.1 Site description and Hydrogeology

The Ria Formosa is a mesotidal coastal lagoon, covering 18,000 ha of area, and permanently connected to the sea through six channels (*Fig. 1*). These six inlets delimit three hydrodynamically distinct sectors: the eastern sector that includes Cacela; the central sector that includes Fuseta and Tavira inlets; and the western sector that is the most important one in terms of water circulation, encompassing Ancão, Faro-Olhão and Armona inlets (Aníbal *et al.*, 2019).

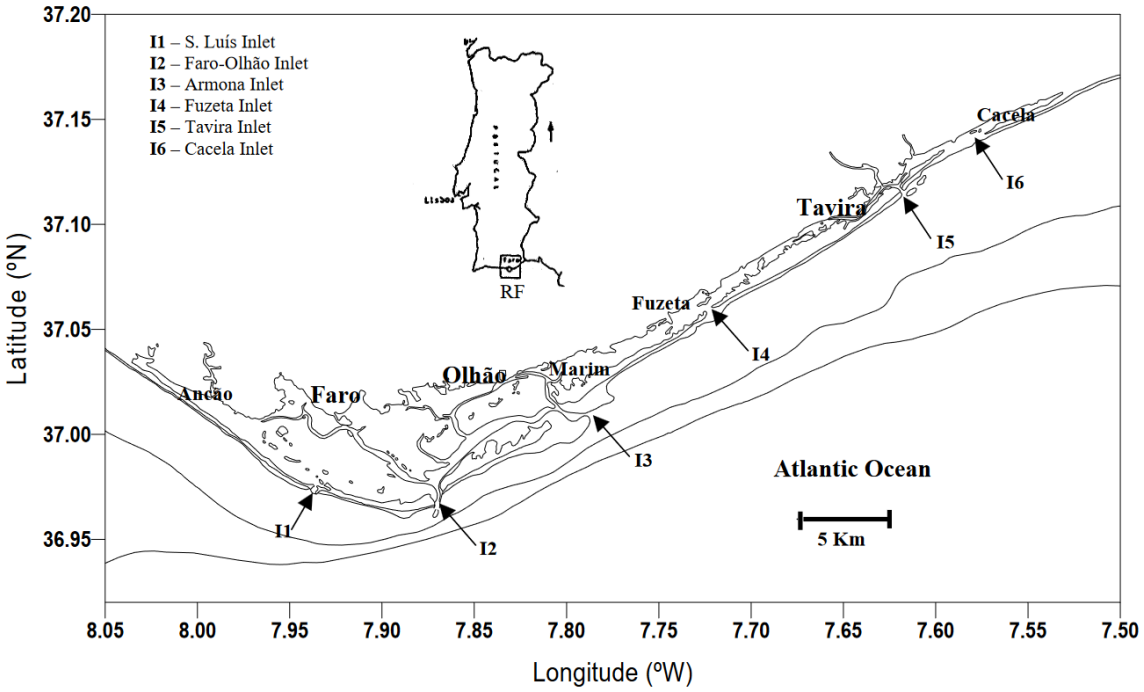

Figure 3: Geographic location of Ria Formosa and its inlets (Source: Falcão et al., 2003)

The catchment is approximately 864 km^2, with a maximum altitude of 522 m, and an average altitude of 112 m, with an average slope of 17% (Duarte *et al.*, 2008) and is protected from sea storms by large sand dunes and five barrier islands (Dionisio *et al.*, 2000). The lagoon possesses a significant intertidal region, which comprises approximately 50% of the total area and is predominantly characterized by sandy, muddy sand-flats, and salt marshes (Duarte & Azevedo,

2005). The intertidal area is exposed to the atmosphere for extended periods during each semi-diurnal tidal cycle due to its gradual slopes (Falcão *et al*., 2003). The lagoon receives minimal freshwater input, resulting in a salinity level that remains around 36 ppt, except for sporadic and brief episodes of winter run-off (Duarte & Azevedo, 2005). The tidal amplitude fluctuates between 1 to 3.5 meters and the mean water depth is 3.5 m, with an intense exchange of 50 – 75% of water mass during each tide (Falcão *et al*., 2003). The western sector of Ria Formosa represents approximately 90% of the total tidal prism of the entire lagoon (Pacheco *et al*., 2010).

3.1.2 Ecosystem services

The lagoon is important on a local, national, regional, and international scale (Newton *et al*., 2018; Newton & Mudge, 2003). Ria Formosa has been recognized as an important natural and permanent wetland with marine water on a national and international level, having been designated a Natura 2000 and a Ramsar site since 1987 (Newton *et al*., 2003). According to the European Environment Agency's European Nature Information System, the lagoon protects 121 species of the Nature Directives and 19 habitat types of the Habitats Directive (Newton *et al*., 2022). The Ria Formosa supports various economic activities such as agriculture, livestock raising, aquaculture, fisheries, tourism and urbanisation (Gari *et al*., 2014). Many fish species are caught in the lagoon, and the lagoon also serves as an important nursery for species caught in the nearby coastal waters (Newton & Mudge, 2005). Additionally, the lagoon provides abundant natural resources for artisanal fishing and shellfish harvesting, as well as housing several bivalve farms that cultivate highly valuable species such as *Magallana angulata* and *Ruditapes decussatus* (Falcão *et al*., 2003). The recreational and commercial harvest of polychaete worms (e.g. *Diopatra* spp. and *Hediste diversicolor*), mud shrimps (e.g. *Upogebia* spp.), crabs (e.g. *Carcinus maenas* and *Liocarcinus* spp.), and razor clams (e.g. Ensis siliqua) for bait is also a significant economic activity (Parreira et al., 2021). The Ria Formosa is a

valuable tourism resource in Algarve, as it is a popular destination for both domestic and international visitors, and the area of land used for tourism in the Ria Formosa is second only to urban development (Newton *et al*., 2003; Tett *et al*., 2003).

3.1.3 Pesticide pollution in Ria Formosa

The Ria Formosa is an extensively researched ecological system, with a large number of publications documenting its hydrological characteristics and the anthropogenic pressures it faces (Newton *et al.*, 2022). Over time, the system has been subjected to various human pressures, such as the construction of dams in water courses, consolidation of inlets, extensive agriculture, saltmarsh reclamation for salt-extraction and aquaculture ponds, as well as the development of major infrastructures such as the international airport of Faro, sewage treatment plants, and construction on the dunes (Newton *et al*., 2003, 2020). However, there is evidence of some undesirable changes occurring in the lagoon system, such as a decline in fish species like bivalves and these losses have been attributed to the deterioration in water quality (Newton *et al*., 2003). The Ria Formosa lagoon has been found to contain various contaminants, including metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organotin compounds such as pesticides in its water, sediments, and biota at specific locations (Bebianno *et al.*, 2007; Díez *et al.*, 2005). The Algarve region is recognized for its cultivation of citrus and other crops, including corn, almond, and hydroponic production of red fruits (Vaz *et al*., 2014). These agricultural activities have led to an increase in the use of pesticides (Cruzeiro *et al.*, 2015a). Cruzeiro, *et al.* (2015b) reported the levels of some pesticides in the Ria Formosa area including those that were regulated under the Stockholm Convention. Further investigation conducted by Cruzeiro, *et al*. (2015b) has revealed that the cumulative loads of some of these pesticides in the Ria Formosa lagoon surpass legal thresholds. Most of the pesticides that are above the limits were insecticides (79 %), followed by fungicides (17 %), and then herbicides (5 %), evidencing an extensive use of insecticides

above the other categories. These findings suggest that the region is experiencing anthropogenic pressure resulting from agricultural activities.

3.1.4 Management of Ria Formosa system

It is essential to maintain good water quality in the Ria Formosa in order to sustain the valuable economic activities, such as shellfish harvesting, fishing, water sports, beaches, and eco-tourism that are supported by the ecosystem services of the lagoon (Newton et al., 2018, 2022). Several environmental management measures have been implemented over the years to improve the water quality of the western part of the lagoon (Newton et al., 2022). Furthermore, the lagoon has garnered significant recognition as a noteworthy and enduring wetland of marine origin, both domestically and globally. This distinction has been bestowed upon it since 1987, with designations as a Natura 2000 site and a Ramsar site. The Ria Formosa is also the receiving environment of six Waste Water Treatment Plants (WWTP) for urban waste water treatment, in order to assure water quality standards for economic activities such as shellfish aquaculture (Aníbal *et al*., 2019).

3.1.5 Sampling stations

Along the extension of Ria Formosa, two strategic sampling stations (S1 (37.017728N, -7.955991E) and S2 (37.015054N, -7.988661E), Figure 3.2) were selected. S1 is situated in proximity to an agricultural area featuring orange farms, which is adjacent to ETAR de Faro Noroeste, a wastewater treatment plant. Conversely, S2 is situated along the Ludo hiking trail, in the vicinity of Faro beach. This particular area is devoid of any agricultural activities and exhibits a tranquil environment. It is worth noting that the Faro Airport lies between the two sampling stations, with S1 and S2 located on opposite ends of the airport.

Figure 4: Sampling stations (S1 and S2) along Ria Formosa

3.2 Field Survey

3.2.1 Sampling method

Two sampling campaigns were conducted at low tides in this study on the 03/04/2023 and 14/04/2023. The initial campaign involved the collection of sediment (collected from the rhizosphere of *Spartina maritima*) and water samples from site S1 for LC degrading bacterial consortium isolation. The second sampling campaign was conducted at both sites (S1 and S2), where sediment samples were collected for in-lab measurements and preparation of mesocosm experiments. In both campaigns, sediments were sampled with a core sampler and transported to the laboratory in clean zip-lock plastic bags to ensure the preservation of sample integrity.

3.2.2 Physico-chemical parameters measurement

In-situ measurements were performed to assess the physico-chemical characteristics of the water at the two sites. Parameters such as dissolved oxygen (DO), salinity, total dissolved solids

(TDS), pH, temperature, and electrical conductivity (EC) were investigated. The Hanna multiparametric probe (Model HI98194) was utilized as the instrument for conducting these measurements.

3.3 Pre-environmental studies

The sediment samples obtained at the two sites (S1 and S2) underwent comprehensive analysis at the ALS Czech Republic laboratory under work order number PR2338969 (refer to annex A for methods and detailed description). The analysis encompassed a spectrum of assessments, including physical parameters, inorganic parameters, metallic composition, major cations, and organochlorine pesticides present.

3.4 Laboratory measurements

Laboratory analyses were conducted on sediment samples collected from the two sampling locations, namely S1 and S2, within the Ria Formosa area. These analyses were further performed on the mixture of the sediments from both locations. The tests performed included sediment moisture content determination, organic matter content determination and granulometry test.

3.4.1 Sediment moisture content determination

The moisture content in sediments was determined based on gravimetric method adopted from (Black, 1965; DeAngelis, 2007). Initially, 30 g of wet sediment were carefully measured and placed into a crucible. This measurement was repeated two more times to ensure accuracy. Subsequently, the samples were labelled and oven-dried at a temperature of 105°C for a duration of 24 hours. After this period, the samples were allowed to cool within a desiccator for 30 minutes to eliminate any residual atmospheric moisture. The weight of the dried samples was then measured and recorded. By calculating the difference between the initial weight of the wet sediment and the final weight of the dry sediment, the water content within the sediment

was determined. The moisture content was expressed as a percentage of the dry mass using the equation below.

$w = (M_W / M_D) \times 100$…...…….(1)

Where w represents the moisture content (in percentage), M_W represents the weight of water and M_D represents the dry weight of sample (both in grams).

3.4.2 Organic carbon (OC) content determination

Determination of weight percent organic carbon (OC) in sediments by means of loss on ignition (LOI) was based on sequential heating of the samples in an oven and a muffle furnace (Heiri *et al*., 2001). Initially, 30 g of wet sediment were measured and placed into a crucible. This measurement was repeated two more times to ensure reliability and accuracy. The three samples were appropriately labelled and were oven-dried at a temperature of 105°C for a duration of 24 hours. After completion of the drying period, the samples were allowed to cool within the desiccator for 30 minutes to eliminate any residual atmospheric moisture. Subsequently, the dry weights of the sediment samples were measured and recorded.

To determine the organic matter composition, an additional step was undertaken. The sediment samples were subjected to further drying in a muffle furnace, this time at a higher temperature of 500 °C, for a duration of 4 hours. The purpose of this process was to facilitate the combustion of OC present within the sediment. Following the completion of this burning phase, the dry weights of the sediment samples were measured and recorded.

The LOI is then calculated using the following equation:

$$LOI_{500} = ((DW_{105} - DW_{500}) / DW_{105}) \times 100 \quad \text{.....(2)}$$

Where LOI_{500} represents LOI at 500 °C (as a percentage), DW_{105} represents the dry weight of the sample before combustion and DW_{500} the dry weight of the sample after heating to 500 °C (both in grams).

3.4.3 Granulometry test

The sieve analysis method was adopted to determine the particle size distribution of the sediments. Firstly, 200 g each of wet sediments were measured into three crucibles. The samples were labelled and oven-dried at 105 °C for 24 hrs to remove the moisture in the sediment. The dried sediment samples were pulverized as finely as possible using a mortar and pestle. 100 g of each of the sediment samples was obtained by weighing. Sieves with openings, 4, 2, 1, 0.5, 0.25, 0.125, 0.063 and 0.032 (mm), were obtained and their weights taken separately. The sieves were stacked together with larger openings placed above those with smaller openings and a pan was placed under the last sieve to collect the portion of soil passing through it. The pulverized sediment was poured from above into the stack of sieves and the upper sieve was covered. The stacked sieve was shaken mechanically by hand for 20- 30 minutes. After, the weight of each sieve and retained soil was measured and recorded. The percentage of sediment retained, the cumulative percentage retained, and the percentage finer by weight were calculated using the following equations:

$$P_R = ((W_{Ss} – W_S) / W_T) \times 100 \quad \text{.....................}(3)$$

Where P_R is percentage of sediment retained, W_{Ss} is weight of the sieve and retained sediment, W_S is weight of the sieve and W_T is total weight of the sediment (all in grams).

$$P_C = P_{Ri} + P_R \quad \text{.....................}(4)$$

where P_C is cumulative percentage and P_{Ri} is cumulative percentage of top sieve.

$$P_F = 100 - P_C \quad \text{.....................}(5)$$

Where P_F is percentage finer by weight.

The coefficient of uniformity (Cu) was also calculated for each sample with this equation:

$$C_u = D_{60}/D_{10} \quad \ldots\ldots (6)$$

Where D_{60} represents the particle size at 60% percentage finer by weight, and D_{10} represents the particle size at 10% percentage finer by weight.

3.5 Pesticide Used

The insecticide with the commercial name Karate Zeon® containing active compound LC (100 g/l or 9.5 % w/w) was obtained from a local farmer. LC treatment was calculated with the recommended field dose of Karate Zeon® (average dosage: 300 g/ha), detection limit of LC in sediment and toxicity levels in macrophytes and microbes (Whitacre, 2008).

3.6 LC- degrading Bacterial Consortium selection

3.6.1 Preparation of Mineral Salt Medium (MSM)

Two separate Mineral Salt Media (MSM) were prepared, one without dextrose and the other with dextrose. The non-dextrose MSM was prepared by dissolving K_2HPO_4 (1.5g), KH_2PO_4 (0.5g), NaCl (0.5g), $MgSO_4$ (0.2g), and NH_3NO_3 (1g) in 800 mL of distilled water in a 1 L volumetric flask. The volume was made up to 1 L by adding distilled water. The preparation of 1 L dextrose MSM solution followed the same procedure as the former. The only difference was that 1g of dextrose was added in the process. The pH of both solutions was adjusted to 7.0 by adding drops of concentrated HCl since the initial pH was alkaline. Both media were transferred into reagent bottles and sterilized by autoclaving at 121°C for 12 minutes. After, 30 mg and 15 mg of LC was added to non-dextrose MSM and dextrose MSM respectively and both media were stored in a refrigerator at -4°C for further use.

3.6.2 LC-degrading Bacterial Consortium enrichment

The microbial consortium selection underwent several enrichment steps. The procedures involved the enrichment of LC degrading bacteria from rhizosphere of *Spartina maritima*, on MSM.

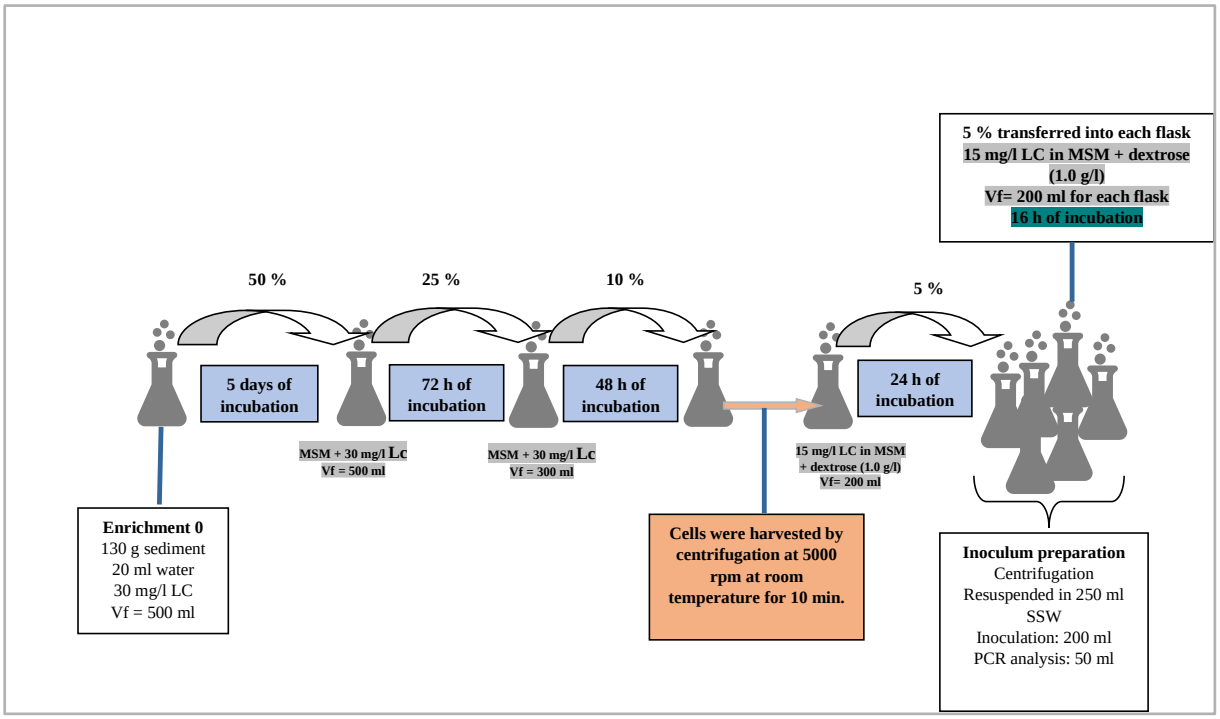

Figure 5: LC-degrading bacterial consortium enrichment. Schematic presentation of the enrichment strategy used to select the LC-degrading bacterial consortium from rhizosphere of *Spartina maritima* on MSM.

A sample with 180g of sediment and 20 mL of water from site S1, colonized by *Spartina maritima*, was prepared in an Erlenmeyer flask and the mixture was homogenized. LC-enriched non-dextrose MSM was added to the mixture, adjusting the final volume to 500 mL. The sample was then incubated at 30 °C with agitation at 100 rpm for five days to promote bacterial growth in response to LC.

After the initial incubation, 250 mL of the sample was transferred into a new flask, and an equal volume (250 mL) of LC-enriched non-dextrose MSM was added to reach a final volume

of 500 mL. This new sample was incubated for an additional three days under the same conditions.

Following the three-day incubation, 125 mL of the sample was transferred into another flask, and 175 mL of LC-enriched non-dextrose MSM was added to obtain a final volume of 300 ml. The sample was incubated for 48 hours under the same conditions.

After this period, the sample underwent centrifugation at 5000 rpm for 10 minutes to separate the microbial cells from the supernatant. The separated microbial cells were then resuspended in a solution containing 15 mg/L of LC and dextrose MSM. Aliquots of 200 mL of the resuspended cells were transferred into six separate flasks. These microbial cultures were incubated at room temperature with agitation at 100 rpm for 24 hours.

3.6.3 Inoculum preparation

Following the enrichment process, the specimens were subjected once again to centrifugation under same conditions. After centrifugation, the microbial inoculum was prepared by resuspending the microbial pellet obtained in 250 ml of saline seawater (SSW). The mixture was agitated to obtain homogeneity. From the prepared microbial inoculum, a volume of 200 ml was utilized for the establishment of the mesocosm set-up experiment, and 50 ml was dedicated for microbial identification with PCR analysis.

3.7 DNA extraction, library preparation and sequencing

3.7.1 DNA extraction

The initial step in DNA sequencing involves the extraction of DNA from the starting material (Inoculum). Various commercially available DNA extraction kits are tailored to different sample types. In this study, the Qiagen DNeasy®PowerSoil®Pro Kit was employed to extract DNA from approximately 1g of sample (inoculum), following the provided protocol. Small amounts of about 50 µl from each sample were extracted and preserved at -20°C. Post-

extraction, the concentration of the obtained DNA in each sample was measured using the Thermo Scientific™ NanoDrop™2000c Spectrophotometer (Thermo Fischer Scientific, USA). Specifically designed for quantifying DNA, RNA, and protein content in minute sample volumes (1-2 µl) with high precision, this instrument determined whether the DNA concentration was adequate for subsequent sequencing. The highly conserved 16S rRNA gene's hypervariable V4 region was sequenced using high-throughput Illumina sequencing after this confirmation.

3.7.2 Library Preparation and Sequencing

For library preparation and sequencing, a customized procedure based on Illumina's standard protocol (Illumina, 2015) was adopted. The primer pair 515FB [GTGYCAGCMGCCGCGGTAA] and 806RB [GGACTACNVGGGTWTCTAAT] was used to amplify the V4 region of the 16S rRNA gene. The resulting amplicon libraries underwent purification with CleanNGS SPRI beads (CleanNA, NL) following recommended methodology, with a bead-to-sample ratio of 4:5. Subsequently, the final sequencing libraries were generated from the purified amplicon libraries through a second PCR step, also purified as per the earlier protocol. These libraries were subjected to paired-end sequencing on a MiSeq (Illumina, USA) using a MiSeq Reagent kit v3 (Illumina, USA) according to recommended procedures.

3.8 Bioremediation of contaminated Ria Formosa sediment: mesocosm set-up

3.8.1 LC sediment spiking

A sample with 3.38 kg sediment from site S1 (contaminated site) was carefully prepared and duplicated, resulting in two separate samples labelled as R1 and R2. Subsequently, a quantity of 144.54 mg of LC was added to each sample. The two mixtures were gently agitated using a stirring rod at 8-hour intervals over a duration of 1 day, allowing ample time for the pesticide (LC) to attach to the sediment particles.

3.8.2 Mesocosm Set-up and Design

The mesocosm experiment was conducted following a systematic procedure. The design and implementation of the mesocosm setup involved several key steps. The experiment utilized a total of twelve (12) mesocosms (Figure 6). Each mesocosm treatment was replicated once within the 12 experimental units to establish a robust experimental framework. These treatments encompassed various approaches, including bioaugmentation (BioA), phytoremediation (Phyto), rhizoremediation (BioA + Phyto), biotic control, abiotic control, and LC contaminated sediment control (CSC). The mesocosms were assembled using plastic water bottles (6 L in size). The upper sections of the bottles were carefully removed by means of cutting techniques.

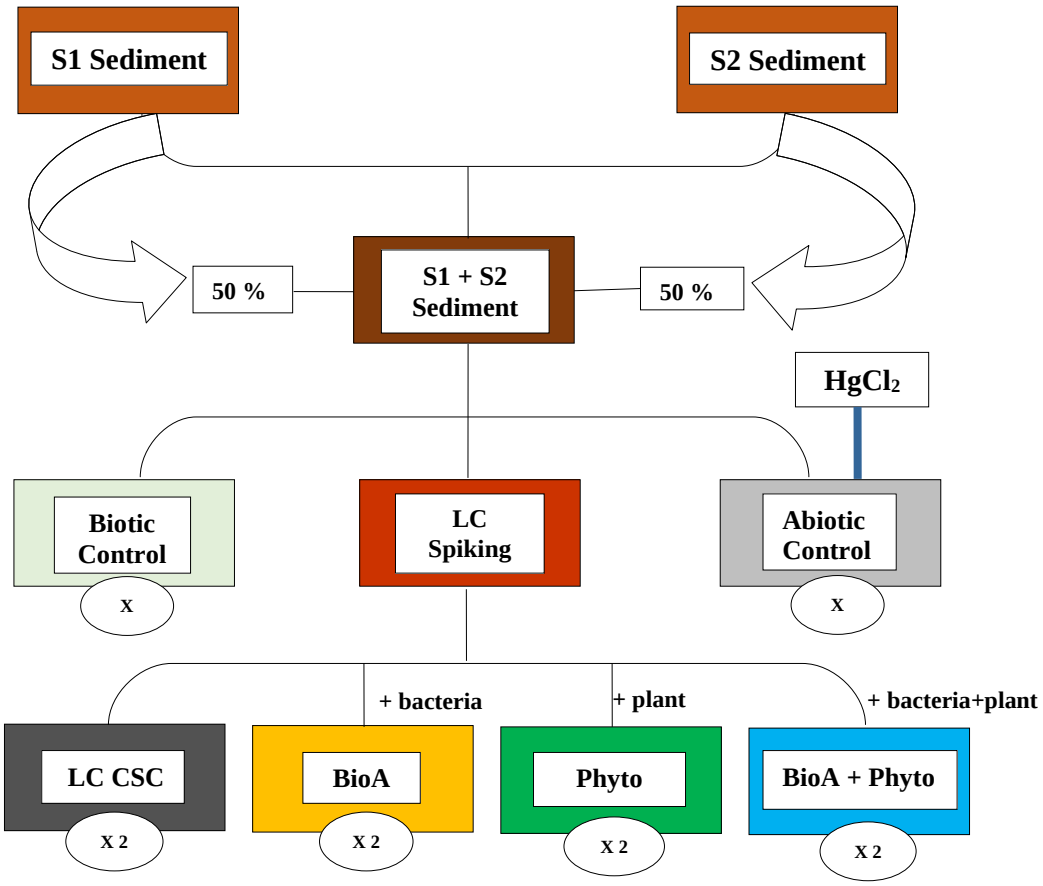

Figure 6: Mesocosm experimental design with different experimental conditions: Biotic control, Abiotic control, LC contaminated sediment control (CSC), bioaugmentation (BioA), phytoremediation (Phyto) and Rhizoremediation (BioA + Phyto).

Specific sediment compositions were prepared and added to the mesocosms to establish the desired treatments and controls. For the bioaugmentation, phytoremediation and rhizoremediation treatments, and the LC CSC, a total of 825g of sediment from R1 (contaminated with LC), along with 882g of natural sediment from S2, were added to four mesocosms. The same procedure was repeated for R2, resulting in a total of eight (8) mesocosms for these treatments.

To establish the biotic and abiotic controls, four mesocosms including two replicates each of biotic and abiotic controls were prepared. For each mesocosm, 825g of sediment from S1 and 882g of sediment from S2 were added and homogenized. The abiotic control mesocosms were prepared by sterilizing the bacteria in the natural untreated sediment. This was achieved by adding 4 mL of a 100 mg/mL solution of $HgCl_2$ to each abiotic control mesocosm.

Except for the bioaugmentation and rhizoremediation treatments, all the mesocosms were filled with 50 ml of SSW and homogenized. In the bioaugmentation and rhizoremediation treatments, 50ml of the microbial inoculum was introduced in each mesocosm and homogenized. Subsequently, all twelve mesocosms were transported to the designated field site (S2) in a basin.

For the phytoremediation and rhizoremediation treatments, each mesocosm received 40g of *Spartina maritima*, which was collected from the site. The plant material was carefully planted in the mesocosms, ensuring proper coverage and distribution within each unit.

Holes were strategically created at the sides and bottom of the basin, and approximately 3cm above the sediment level in each mesocosm. These openings allowed excess water to escape, preventing waterlogging and mimicking natural water flow dynamics.

The mesocosm experiments were placed in close proximity to the existing *Spartina maritima* community at the field site S2 (Non-contamited site). This placement aimed to mimic the natural environment and facilitate potential interactions between the planted vegetation and the surrounding ecosystem.

3.8.3 Mesocosm sampling

Mesocosm sampling was conducted at low tides to collect sediment samples for analysis within a two-time interval (day 0 and day 7). Sediment sampling involved collecting sediments from five different points within each mesocosm with a sterile sampling tube, ensuring that the samples were reliable and accurate. Separate tubes were used to collect sediments from each mesocosm in order not to contaminate samples. Approximately 10-20g of sediments were collected per sample. These samples were immediately transported to the laboratory and stored in a freezer at a temperature of -20°C.

3.8.4 Chemical Analysis

Chemical analysis for LC was performed using the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method (Ben Salem et al., 2016) followed by gas chromatography-mass spectrometry (GC-MS) as described by (Yang et al., 2010).

3.8.4.1 LC Extraction with QuEChERS method

Initially, 5g of sediment were mixed with 4 ml of ultrapure water and spiked with surrogate standards (phenanthrene d10, perylene d12, PCB 209, Atrazine d5). 20 ml of extraction solvents (hexane/acetone or dichloromethane/acetone) were added, followed by vigorous shaking for 1 minute. After this, a citrate buffer salt mixture was added, and further agitation occurred. The resulting mixture was centrifuged, and the supernatant was transferred to another tube containing specific reagents (900 mg of MgSO4 and 150 mg PSA). This mixture was then dried and re-dissolved in acetonitrile before analysis.

3.8.4.2 GC-MS determination

Pesticide analysis was conducted using an Agilent 6890N gas chromatograph coupled with an Agilent 5975B MSD mass spectrometer equipped with an electron impact ionization source (EI). Separation of compounds was achieved using an HP-5MSi capillary column (30 m × 0.25 mm, 0.25 µm film thickness) and helium with 99.9999% purity served as the carrier gas at a constant flow rate of 1.0 mL/min. The ion source and quadrupole temperatures were maintained at 230°C and 150°C, respectively.

In the analysis, 1 µL of the sample was injected in splitless mode at an inlet temperature of 300°C. The programmed column temperature sequence was as follows: starting at 55°C for 2 minutes, raised to 160°C at 20°C/min (held for 5 minutes), then increased at 2°C/min to 200°C, followed by a ramp of 4°C/min to 240°C (held for 3 minutes), and finally to 290°C (held for 5 minutes) at 5°C/min. The MS interface temperature was maintained at 280°C.

For qualitative analysis during method development, the full scan mode monitored the mass range from 50 to 600. Quantitative analysis was performed using selected ion monitoring mode (SIM).

3.8.5 Degradation

The degradation effectiveness of LC when exposed to the different treatment conditions was described by the percentage of degradation of the pesticide:

Degradation (%) = (initial conc. – final conc.) × 100/initial conc. ……(7)

3.9 Statistical analysis

The data obtained were evaluated on excel sheets. The statistical analysis was carried out with Excel and DATAtab. Boxplots were used to represent the data obtained from soil moisture content and OC content analysis. A one-factor analysis of variance (ANOVA) and

Bonferroni Post hoc test were then used to compare the mean soil moisture content and OC content across all groups.

4 RESULTS

4.1 Characterization of environmental parameters at the sampling sites

4.1.1 Water parameters

Considering the field survey conducted at both sampling sites (S1 and S2), there was not an evident difference among the water characteristics at any of the two sampling sites, except for DO, salinity and TDS values that showed some variations (Table 2).

Table 2. Water parameters measured at sites S1 and S2 during the sediment sampling campaign. Results are presented as mean and standard deviation (SD).

Parameters	**S1 (Mean ± SD)**	**S2 (Mean ± SD)**
Temperature (^{o}C)	27.35 ± 1.63	26.2 ± 0.86
pH	9.68 ± 1.32	8.68 ± 2.13
DO (mg/l)	13.74 ± 2.41	6.46 ± 3.23
Salinity (ppt)	25.4 ± 2.34	32.5 ± 1.45
TDS (ppm)	3018 ± 654.32	13163 ± 1252.38
EC (mS/cm)	50.12 ± 2.80	53.73 ± 2.21

Temperature was similar in both sites; the minimum was 26.2 ^{o}C in S2 and maximum 27.35 ^{o}C in S1. The pH was alkaline at both sites with an average pH of 9.68 (S1) and 8.68 (S2). The salinity was higher in S2 compared to S1, with maximum 32.5 ppt and minimum 25.4 ppt, respectively. DO levels were higher in S1 with an average of 13.74 mg/L and fairly lower in S2 with an average of 6.46 mg/L. TDS values were higher in S2 with average value of 13163 ppm and lower in S1 with average value of 3018 ppm. The EC at both sampling stations was typically of coastal waters with minimum value of 50.12 mS/cm (S1) and maximum value of 53.73 mS/cm (S2). The variations in parameters at both sites is due to tidal variability.

4.1.2 Sediment parameters

The findings from the sediment analysis conducted at the ALS Czech Republic laboratory are displayed in Table 3. Both sampling sites (S1 and S2) showed a diverse array of pollutants, encompassing heavy metals and organochlorine pesticides among others.

Table 3. Sediment parameters analysis at sampling sites S1 and S2: mean and standard deviation (SD) presentation of results.

Parameter	Units	Sampling Stations	
		S1	**S2**
Physical Parameters			
Dry matter @ 105°C	%	59.5 ± 3.03	72 ± 21.17
Inorganic Parameters			
Carbonates	% DW	6.55 ± 0.64	4.38 ± 0.1
Total Nitrogen	mg/kg DW	13800 ± 3260.37	1492.33 ± 2128.491
Total Organic Carbon	mg/kg DW	147666.67 ± 28360.77	26566.67 ± 42031.22
Total Inorganic Carbon	% DW	1.31 ± 0.13	0.88 ± 0.02
Extractable Metals / Major Cations			
Antimony	mg/kg DW	3.78 ± 0.38	<0.50
Arsenic	mg/kg DW	9.03 ± 0.23	1.06
Barium	mg/kg DW	238.33 ± 26.16	9.59 ± 9.12
Beryllium	mg/kg DW	0.65 ± 0.01	0.06 ± 0.03
Cadmium	mg/kg DW	0.96 ± 0.06	<0.40
Chromium	mg/kg DW	44.97 ± 1.37	120.05 ± 165.85
Cobalt	mg/kg DW	4.62 ± 0.26	1.03 ± 0.91
Copper	mg/kg DW	288.33 ± 10.69	29.07 ± 18.13
Iron	mg/kg DW	19733.33 ± 757.19	2476.67 ± 1227.86
Lead	mg/kg DW	103.67 ± 2.08	28.03 ± 28.47
Lithium	mg/kg DW	40.17 ± 3.37	2.9 ± 1.82
Manganese	mg/kg DW	225 ± 12.12	27.83 ± 15.34
Mercury	mg/kg DW	0.95 ± 0.14	<0.20
Molybdenum	mg/kg DW	2.69 ± 0.17	14.19 ± 23.4
Nickel	mg/kg DW	24.23 ± 0.40	87.5 ± 145.93
Phosphorus	mg/kg DW	14400 ± 1252.99	632.33 ± 603.93
Silver	mg/kg DW	16.93 ± 2.35	<0.50
Strontium	mg/kg DW	282.67 ± 25.42	24.17 ± 10.84
Thallium	mg/kg DW	<0.50	<0.50
Tin	mg/kg DW	45.63 ± 2.97	2.5
Vanadium	mg/kg DW	39.53 ± 3.38	3.53 ± 2.42
Zinc	mg/kg DW	1293.33 ± 30.55	60.6 ± 32.19
Organochlorine Pesticides			
Chlordane-cis	mg/kg DW	<0.010	<0.010

Table 3. *continued*

Parameter	Units	S1	S2
Organochlorine Pesticides			
Chlordane-trans	mg/kg DW	<0.010	<0.010
Endosulfan sulfate	mg/kg DW	<0.010	<0.010
Mirex	mg/kg DW	<0.010	<0.010
Nonachlor-cis	mg/kg DW	<0.010	<0.010
Nonachlor-trans	mg/kg DW	<0.010	<0.010
Oxychlordane	mg/kg DW	<0.010	<0.010
Hexachloroethane	mg/kg DW	<0.010	<0.010
Hexachlorobutadiene	mg/kg DW	<0.010	<0.010
1.2.3.5- & 1.2.4.5-Tetrachlorobenzene	mg/kg DW	<0.020	<0.020
1.2.3.4-Tetrachlorobenzene	mg/kg DW	<0.010	<0.010
Pentachlorobenzene	mg/kg DW	<0.010	<0.010
Trifluralin	mg/kg DW	<0.010	<0.010
Hexachlorocyclohexane Alpha	mg/kg DW	<0.010	<0.010
Hexachlorobenzene (HCB)	mg/kg DW	<0.0050	<0.0050
Hexachlorocyclohexane Beta	mg/kg DW	<0.010	<0.010
Hexachlorocyclohexane Gamma	mg/kg DW	<0.0100	<0.0100
Hexachlorocyclohexane Delta	mg/kg DW	<0.010	<0.010
Hexachlorocyclohexane Epsilon	mg/kg DW	<0.010	<0.010
Alachlor	mg/kg DW	<0.010	<0.010
Heptachlor	mg/kg DW	<0.010	<0.010
Aldrin	mg/kg DW	<0.010	<0.010
Telodrin	mg/kg DW	<0.010	<0.010
Isodrin	mg/kg DW	<0.010	<0.010
Heptachloroepoxide-cis	mg/kg DW	<0.010	<0.010
Heptachloroepoxide-trans	mg/kg DW	<0.010	<0.010
2.4-DDE	mg/kg DW	<0.010	<0.010
alpha-Endosulfan	mg/kg DW	<0.010	<0.010
4.4`-DDE	mg/kg DW	<0.010	<0.010
Dieldrin	mg/kg DW	<0.010	<0.010
2.4-DDD	mg/kg DW	<0.010	<0.010
Endrin	mg/kg DW	<0.010	<0.010
beta-Endosulfan	mg/kg DW	<0.010	<0.010
4.4`-DDD	mg/kg DW	<0.010	<0.010
2.4-DDT	mg/kg DW	<0.010	<0.010
4.4`-DDT	mg/kg DW	<0.010	<0.010
Methoxychlor	mg/kg DW	<0.010	<0.010
Sum of 3 tetrachlorobenzenes	mg/kg DW	<0.030	<0.030
Sum of 4 hexachlorcyclohexanes	mg/kg DW	<0.0400	<0.0400
Sum of 4 isomers DDT	mg/kg DW	<0.040	<0.040
Sum of 6 isomers DDT	mg/kg DW	<0.060	<0.060
PBBs			
PBB 153	mg/kg DW	<0.010	<0.010

4.2 Sediment properties characterization

The results of sediment properties of the sampling sites, S1 and S2, and the composite sample representing both sites (M) are presented in this section.

4.2.1 Moisture content

The moisture content analysis conducted on sediments at sites S1 and S2, as well as a composite sample representing both sites (referred to as M), revealed noteworthy differences. A one-factor analysis of variance (ANOVA) showed that there was a statistically significant difference between the samples, namely S1, S2, and M ($p < 0.05$), with a p-value of 0.004. A Bonferroni Post hoc test was used to compare the groups in pairs to find out which was significantly different. The Bonferroni post-hoc test revealed that the pairwise group comparisons of S1 - S2 ($p = 0.004$) and S1 - M (0.044) have a p-value less than 0.05 and thus, based on the available data, it can be assumed that these groups are each significantly different pairwise. However, there was no significant difference between the group pairs S2 - M as this group has a p-value ($p = 0.203$) greater than 0.05. To further visualize the differences among the various samples (S1, S2 and M), the soil moisture content from these samples was presented with boxplots (figure 7). The result from this figure shows that the moisture content in S1 sample was significantly lower than S2 and M.

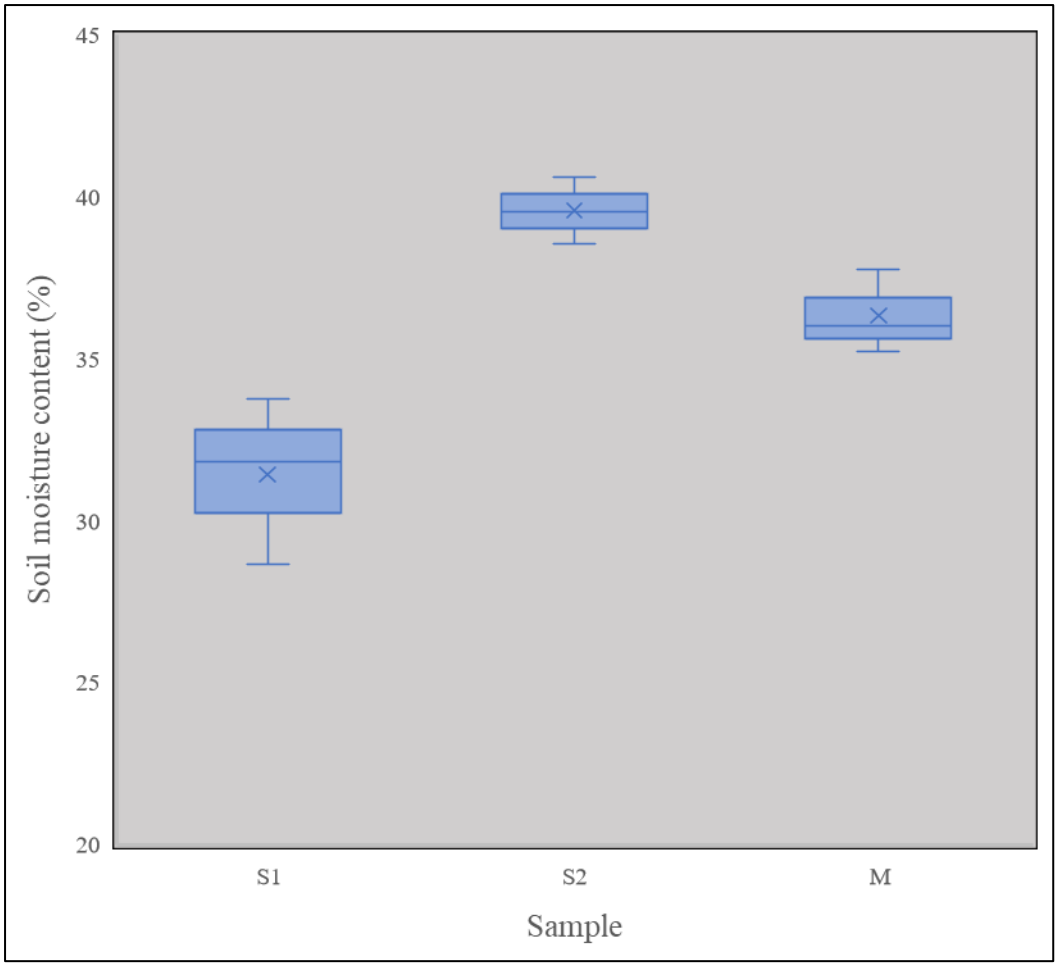

Figure 7: Boxplots representative of the moisture content (%) of the sediment samples, S1, S2 and M. The boxplot represent the mean, median, minimum and maximum LOI values for each sample type. The mean (x) and median (—) are located inside the shaded box (blue colour) whereas the whiskers located ouside the box are the minimum and maximum values. The shaded box represents the interquartile range with the lowest and the highest parts being the 25th and 75th percentiles of the data, respectively.

From figure 7, S2 has the highest average moisture content of 39.56% followed by M with an average moisture content of 36.33% and S1 has the least moisture content of 31.41%. These results show that the sample groups S2 - M show no significant difference compared to S1 - S2 and S1 - M groups as confirmed by the Bonferroni post-hoc test.

4.2.2 Organic carbon

The organic matter content by means of loss on ignition (LOI) were similar for all sample types. A one-factor analysis of variance (ANOVA) showed that there was no statistically

significant difference between the three sample groups, namely S1, S2 and M ($p > 0.05$) with a p-value of 0.773. Boxplots were used to represent the LOI of the different sample groups as shown in figure 8.

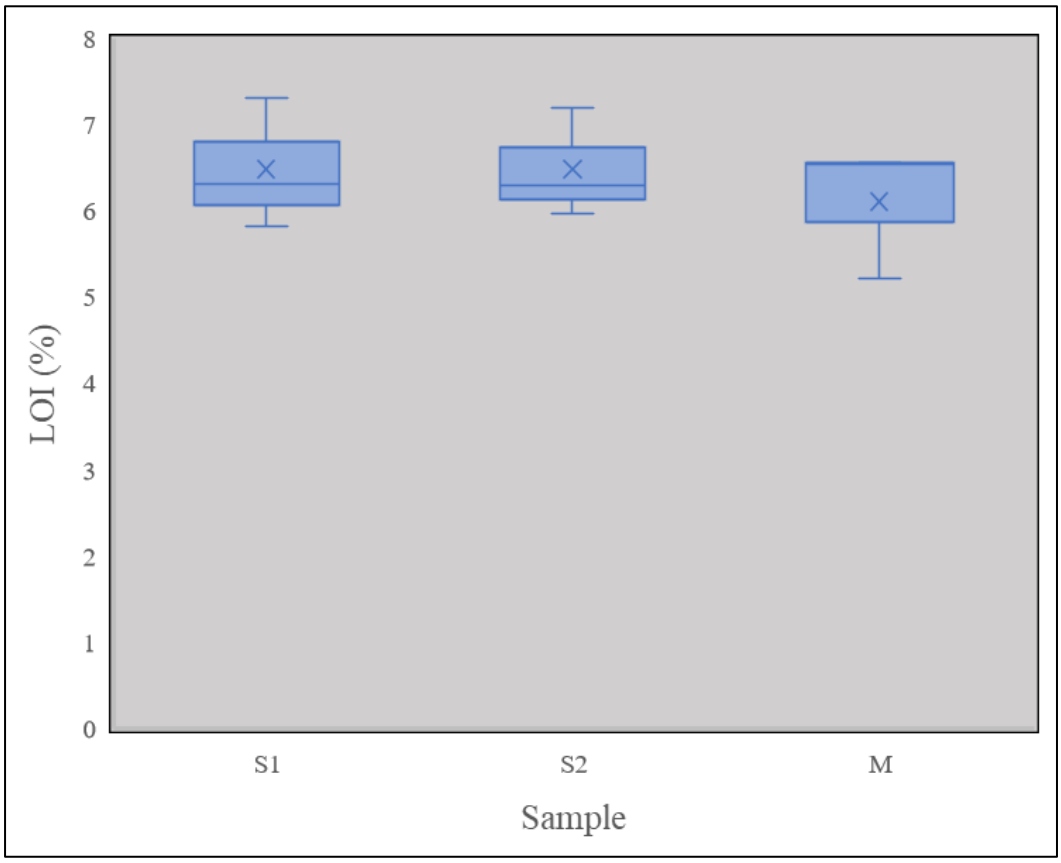

Figure 8: Boxplots representative of the organic carbon content by loss on ignition (LOI) (%) of the sediment samples, S1, S2 and M. The boxplot represents the mean, median, minimum and maximum LOI values for each sample type. The mean (x) and median (—) are located inside the shaded box (blue colour) whereas the whiskers located outside the box are the minimum and maximum values. The shaded box represents the interquartile range with the lowest and the highest parts being the 25th and 75th percentiles of the data, respectively. The values of the median, 75th percentile and maximum value are different for sample S1 and S2 but the same for sample M.

From figure 8, S2 presented a higher mean LOI of 6.50% followed by S1with LOI of 6.48% and M has the least LOI of 6.10%. There was no variation among the three samples. The high organic matter content at both sites could be attributed to the presence of biota and mineralization of pollutants by microbes at both sites.

4.2.3 Granulometry test

The result from the granulometry test was used to estimate the type and gradation of the different sediment samples, namely S1, S2 and M (table 3 and figure 9).

Table 4. Type and gradation of the different sediment samples (S1, S2 and M)

Sample	**D10 (mm)**	**D30 (mm)**	**D50 (mm)**	**D60 (mm)**	**C_u**	**C_c**	**Gradation**	**Soil type**
S1	0.05	0.1	0.2	0.27	5.40	0.74	Poorly graded	Coarse grained soil
S2	0.055	0.12	0.225	0.3	5.45	0.87	Poorly graded	Coarse grained soil
M	0.045	0.11	0.22	0.29	6.44	0.93	Poorly graded	Coarse grained soil

The values of D10, D30, and D60, which are the diameters that correspond to the percent finer of 10%, 30%, and 60%, respectively were determined from the particle size distribution curve and used to calculate the uniformity coefficient (C_u) and coefficient of gradation C_c. The values of C_u and C_c are used to classify whether the soil is well-graded or not. For a Sand is considered well-graded, if C_u is greater than 6 and C_c is between 1 and 3. C_u values for S1, S2 and M were 5.40, 5.45 and 6.44 respectively.

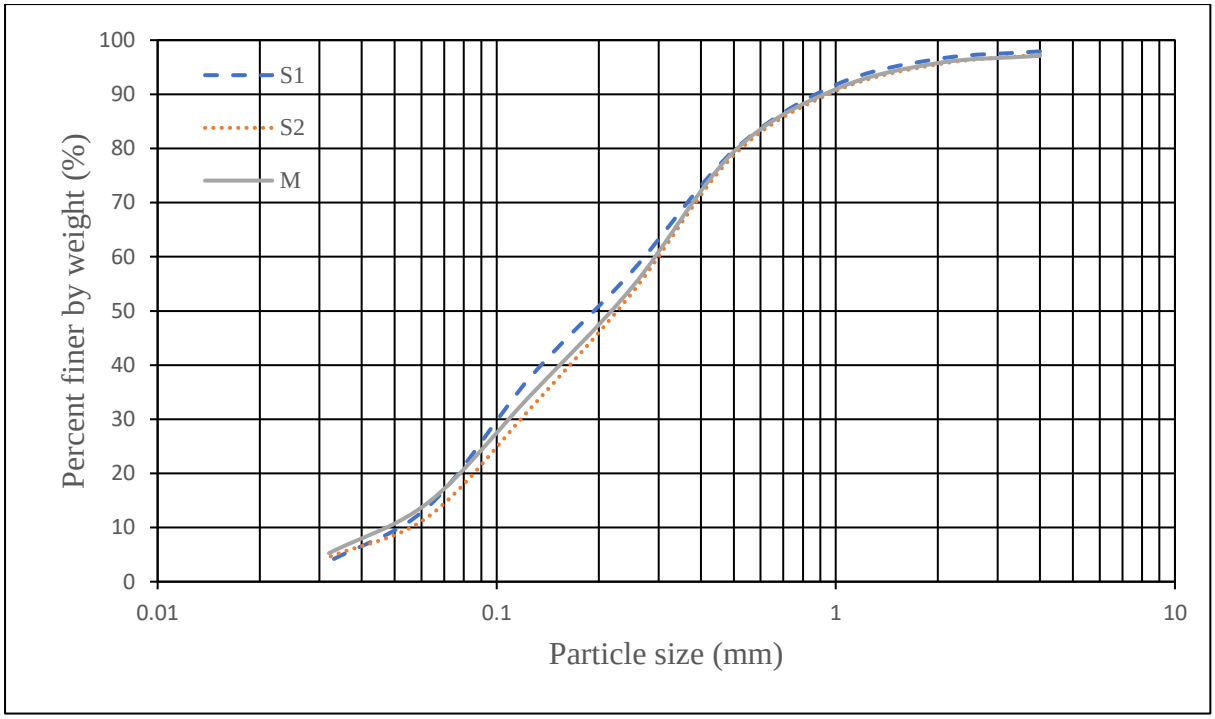

Figure 9: Particle size distribution curves for sediment samples S1, S2 and M. Each curve represents the percent finer by weight (%) of particles based on their size (mm).

The D50 values were determined by identifying the point on each curve where the cumulative percentage reached 50%. S1, S2 and M exhibited D50 values of 0.2 mm, 0.225 mm and 0.22 mm respectively, which were higher than 0.075 mm indicating coarse grained sediments.

4.3 Mesocosm treatments

4.3.1 Characterization of water parameters in mesocosms

The abiotic characteristics of the water in the mesocosms are depicted in Figure 10. Variability was observed across different parameters (DO, pH, TDS, and EC) in all the mesocosms. Dissolved oxygen (DO) levels ranged from 0 to 12 mg/L, pH levels varied between 7.73 and 8.43, total dissolved solids (TDS) showed a range of 14.6 to 30.14 ppt, electrical conductivity (EC) ranged from 29.43 to 60.2 mS/cm, salinity exhibited a range of 18 to 40.53 PSU, and temperature ranged between 23.45 and 24.8 °C.

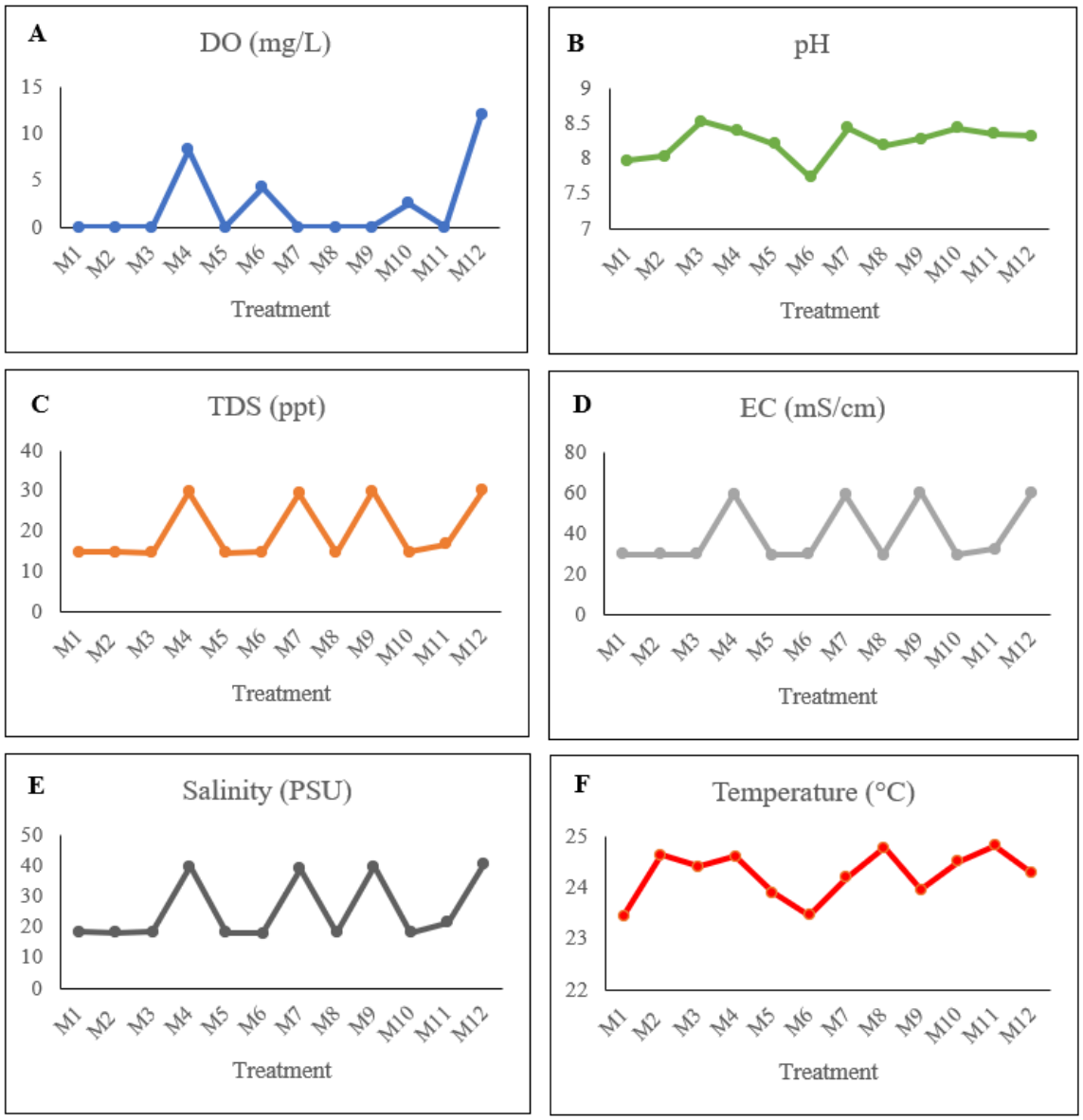

Figure 10: Physico-chemical parameters assessed in mesocosm treatments (M1 to M12) after 7 days, denoted by letters A to F for dissolved oxygen (DO), pH, total dissolved solids (TDS), electrical conductivity (EC), salinity, and temperature. Mesocosm treatments (M1 to M12) correspond to various experimental setups: biotic control R2, biotic control R1, abiotic control R2, abiotic control R1, phytoremediation treatment R1, phytoremediation treatment R2, CSC R1, CSC R2, rhizoremediation treatment R1, rhizoremediation treatment R2, bioaugmentation treatment R1, and bioaugmentation treatment R2. R1 and R2 represent experimental replicates.

4.3.2 Bioremediation treatments effects on sediment LC concentration

The LC degradation (%) was examined by comparing the LC concentration values of different experimental conditions with the LC concentration values of control conditions. In sediments with Phyto, Phyt + BioA and BioA conditions, a greater decrease of LC concentrations was

observed after 7 days. In this case, the sediment LC concentrations decreased more than 30% for Phyto, 27% for Phyt + BioA and 22% for BioA. There was a slight decrease (0.74 %) in LC observed for the LC contaminated sediment control (CSC) treatment. This may be due to hydrolysis, photolysis or leaching.

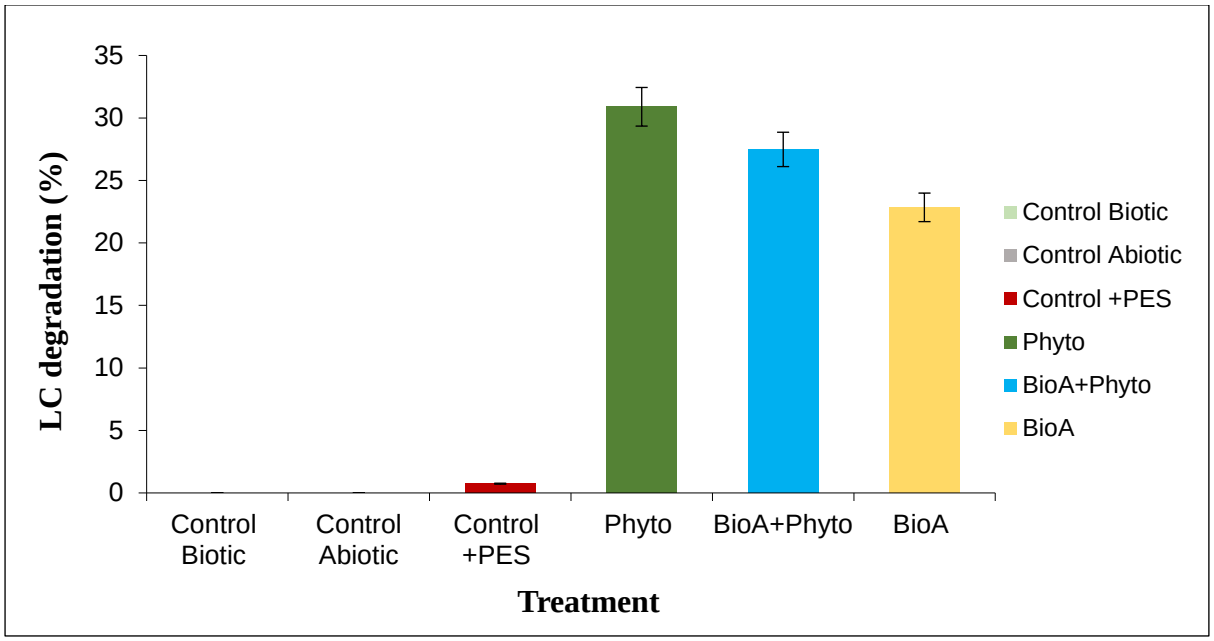

Figure 11: Sediment LC degradation (%) after 7 days in Ria Formosa sediment with different experimental conditions: biotic control; abiotic control; control + PES – LC contaminated sediment control (CSC); Phyto – phytoremediation treatment; BioA + Phyto – rhizoremediation treatment; and BioA – bioaugmentation treatment.

5 DISCUSSION

5.1 Characterization of environmental parameters at the sampling sites

In the comparison of water quality between sites S1 and S2 along Ria Formosa during the sediment sampling campaign, various parameters are considered to understand the aquatic environments of these areas effectively. Despite similarities in water parameters such as temperature and EC, significant variations in DO, salinity, and TDS between S1 and S2 were observed. The observed variations in DO, salinity, and TDS are attributed to the tidal variability, particularly at S2 with high tidal influence. This tidal influence emphasizes the dynamic nature of coastal water bodies, potentially leading to fluctuating and unstable environments as reported in studies conducted at Ria Formosa (Rosa *et al.*, 2022). In a general context, these analyzed parameters adhere to the acceptable thresholds established by the European Union Water Framework Directive (WFD). This is in line with previous studies conducted at Ria Formosa explaining that the water quality was increasing due to alterations of human activities along with hydrodynamical changes (Rosa *et al.*, 2022).

However, the analysis of sediment parameters at both sites reveals the presence of concerning environmental pollutants, including heavy metals and organochlorine pesticides. Previous reports on Ria Formosa indicate the occurrence of various contaminants—metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and organotin compounds—in water, sediments, and biota at specific locations (Bebianno *et al.*, 2007; Díez *et al.*, 2005), supporting these findings. Higher levels of heavy metals at S1 compared to S2 suggest that S1 acts as a recipient site for pollutants, likely due to its proximity to a wastewater treatment facility (ETAR de Faro). Although both sites contain organochlorine pesticides, their concentrations are relatively low. This suggests that agricultural impacts are reduced at both locations, although S1 is situated adjacent to an orange farm.

5.2 Sediment properties characterization

Sediment properties such as moisture, organic carbon (OC) content and particle size are responsible for the environmental fate of Lambda-cyhalothrin (LC). Although LC rapidly dissipates from water, hydrosoil (sediment) demonstrates significant LC adsorption capacity, functioning as a sink for this pyrethroid. The adsorptive behaviour of LC, underscored by elevated water-solid-organic carbon partition coefficient (K_{oc}) values, signifies its robust and rapid binding affinity to soil and sediment matrices. This adsorption is predominantly a surface-bound process, reliant upon the surface area and OC content of the adsorbent material (Whitacre, 2008).

In the present study, the observed increased OC content across the various soil samples underscores the preferential adsorption of LC onto particle surfaces. Empirical evidence from Ali and Baugh (2003) corroborates this observation. Their investigation into soils with an OC content between 1.15% and 2.46% revealed a minimal desorption rate of LC, with only 4.68% of the initially adsorbed LC released after successive desorption steps using deionized water. This finding highlights the near-irreversible adsorption of LC to soil matrices with high OC content in aquatic environments.

Furthermore, the intrinsic particle size within sediment plays a pivotal role in modulating LC adsorption. Finer sediment particles, including clay and silt, exhibit an augmented surface area conducive for enhanced LC adsorption compared to their coarser, sandy counterparts. In the present study, sediment samples (S1, S2, and M), characterized by poor gradation and coarse grain size, elucidate the propensity for reduced LC adsorption rates.

This differential adsorption rate concomitantly influences the bioavailability of LC to aquatic biota, thereby modulating its ecotoxicological impact. Enhanced LC adsorption mitigates its

bioavailability, potentially diminishing its aquatic toxicity. Contrarily, a reduction in adsorption rate augments LC bioavailability, enhancing its toxicological impact on non-target aquatic species. Additionally, LC adsorbed onto sediment particles exhibits a decreased degradation rate, attributed to its reduced accessibility for hydrolytic breakdown compared to unbound LC molecules within the aqueous column. However, the degradation of particle-adsorbed LC is facilitated by plant and microbial metabolic activities also known as bioremediation.

5.3 Bioremediation treatments effects on sediment LC concentration

Bioremediation, a technique harnessing biological processes to mitigate the effects of pollutants, has gained traction as an effective tool for addressing pesticide contamination in aquatic ecosystems. It encompasses various strategies, including phytoremediation, where plants aid in pollutant removal; bacterial biodegradation, where specific bacteria break down contaminants; and rhizoremediation, a combination of plant and microbial actions in the root zone.

Previous research underscores the efficiency of macrophytes in facilitating rapid LC degradation within aquatic sediments (Bennett *et al.*, 2005; Bouldin *et al.*, 2005). These plants, through their root systems and associated microorganisms, enhance the breakdown of contaminants like LC. Factors such as soil moisture, type, and organic carbon content play pivotal roles in this process, influencing LC adsorption to plant roots and sediments, thereby making it more amenable to degradation. Additionally, pH levels impact the rate of LC dissipation in water. LC remains stable at pH levels below 8; however, in alkaline conditions, it undergoes hydrolysis triggered by the nucleophilic attack of hydroxyl ions (Whitacre, 2008).

In the present study, various bioremediation techniques were specifically evaluated for their efficacy in degrading LC. Findings highlighted promising outcomes, showcasing a substantial

decrease in LC concentration over a relatively short duration of 7 days. Notably, phytoremediation treatment (Phyto) emerged as the most effective technique, demonstrating a 30% reduction in LC levels. This was followed by rhizoremediation treatment (Phyto + BioA) at 27% and bioaugmentation treatment (BioA) at 22%, indicating their considerable potential in mitigating LC contamination in Ria Formosa sediment.

These findings align with earlier investigations that have demonstrated the suitability of diverse plant species such as *Spartina maritima* for the application of phytoremediation and rhizoremediation techniques in addressing aquatic pollutants (Aníbal *et al.*, 2019; Kuiper *et al.*, 2002). Phytoremediation operates on fundamental principles involving the extraction of pollutants from the soil, subsequent translocation to aboveground plant tissues, sequestration within the root system to impede further dispersion and leaching into the soil or water, and potential conversion into less hazardous compounds.

Utilizing plants in conjunction with microorganisms offers several advantages, including the augmentation of microbial populations and metabolic activity within the rhizosphere. This synergistic approach can lead to improvements in the physical and chemical characteristics of contaminated soil, fostering enhanced contact between root-associated microbes and the contaminants present in the soil matrix.

The rhizosphere, enriched with exudates from plant roots, serves as a nexus for nutrient availability and root colonization (Kuiper *et al.*, 2004). Notably, the heightened degradation of Lambda-cyhalothrin (LC) observed in the rhizosphere is posited to stem from the escalated numbers and metabolic vigour of microbial communities. Root exudation of nutrients fosters a nutrient-rich environment that stimulates microbial activity, thereby contributing to the enhanced degradation of contaminants like LC in the vicinity of plant roots.

However, despite the promising results, the specific role of *Spartina maritima* in LC degradation within the Ria Formosa lagoon remains relatively unexplored. The present study sheds light on the potential of this plant species and its rhizospheric bacteria in LC degradation in Ria Formosa sediment. Both *Spartina maritima* and its associated microbial community demonstrated effectiveness in LC degradation, either independently or in combination (rhizoremediation). Nevertheless, for a comprehensive understanding of the full potential and efficacy of each bioremediation treatment, further investigation involving longer exposure periods to varying LC concentrations would be necessary.

6 CONCLUSION AND RECOMMENDATIONS

Bioremediation stands as a promising restorative method for the rehabilitation of Ria Formosa coastal areas, facilitating the degradation of pollutants like Lambda-cyhalothrin (LC). Combining bioaugmentation and phytoremediation into a cohesive rhizoremediation approach holds potential in addressing LC contamination within Ria Formosa sediment and beyond.

While existing studies emphasize the beneficial role of rhizoremediation in reviving pesticide polluted sites, scant literature delves into the specific selection criteria for an optimal rhizoremediation system, comprising *Spartina maritima* inoculated with a bacterial consortium displaying degradation proficiency. The present study established that *Spartina maritima*, whether employed alone or inoculated with a bacterial consortium, effectively contributed to LC degradation. The efficacy of bioremediation of LC using *Spartina maritima* is influenced by various factors such as soil moisture, organic carbon content, particle size, and pH, which significantly impact LC availability for remediation and its fate in water.

Nevertheless, comprehensive and extensive research remains imperative to corroborate the efficiency of the LC-degrading rhizospheric bacterial consortium and *Spartina maritima* in biodegrading LC, particularly under diverse environmental conditions and varying contamination levels. Focused optimization studies centering on the interplay between *Spartina maritima* and the rhizospheric bacterial consortium hold the promise of substantially enhancing rhizoremediation efficiency, offering robust and dependable bioremediation strategies.

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8 ANNEXES

8.1 Annex A

CERTIFICATE OF ANALYSIS

Work Order	: **PR2338969**	Issue Date	: 25-Apr-2023
Customer	: **Universidade do Algarve**	Laboratory	: ALS Czech Republic, s.r.o.
Contact	: Mr. Paul Pedro	Contact	: Client Service
Address	: Faculdade de Ciencias e Tecnologias - LAQ Laboratório Ánálises Químicas (Pav D6) Universidade do Algarve Campus de Gambelas 8005-139 Faro Portugal	Address	: Na Harfe 336/9 Prague 9 - Vysocany 190 00 Czech Republic
E-mail	: ppedro@ualg.pt	E-mail	: customer.support@alsglobal.com
Telephone	: +351 2898 00900	Telephone	: +420 226 226 228
Project	: UALG- LAQ	Page	: 1 of 6
Order number	: ----	Date Samples Received	: 18-Apr-2023
		Quote number	: PR2023UNIAL-PT0001 (PT-300-23-0128)
Site	: ----	Date of test	: 18-Apr-2023 - 25-Apr-2023
Sampled by	: customer	QC Level	: ALS CR Standard Quality Control Schedule

General Comments

This report shall not be reproduced except in full, without prior written approval from the laboratory.
The laboratory declares that the test results relate only to the listed samples. If the section "Sampled by" of the Certificate of analysis states: "Sampled by Customer" then the results relate to the sample as received.
Sample for the method S-TOC1-IR is dried at 105 °C and pulverized prior to analysis.
Sample for the method S-TIC-IR is dried at 105 °C and pulverized prior to analysis.

Responsible for accuracy

Testing Laboratory No. 1163
Accredited by CAI according to
CSN EN ISO/IEC 17025:2018

Signatories	Position
Lubomír Pokorný	Country Manager

L 1163

The company is certified according to ČSN EN ISO 14001 (Environmental management systems) and ČSN ISO 45001 (Occupational health and safety management systems)

The end of result part of the certificate of analysis

Brief Method Summaries

Analytical Methods	*Method Descriptions*
Location of test performance: Bendlova 1687/7 Ceska Lipa Czech Republic 470 01	
S-NTOT-PHO	CZ_SOP_D06_07_102 (CSN ISO 11261) Determination of total nitrogen by modified Kjeldahl method by spectrophotometry.
S-TIC-IR	CZ_SOP_D06_07_055 (CSN EN 13137:2002, CSN EN 15936, CSN ISO 10694) Determination of total carbon (TC) and inorganic carbon (TIC) by IR detection and calculation of total organic carbon (TOC), carbonates and organic matter from measured values.
S-TOC1-IR	CZ_SOP_D06_07_117 (Elementar Company methodology, CSN ISO 10694, CSN EN 13137:2002, CSN EN 15936) Determination of total carbon (TC), total organic carbon (TOC) by the combustion method with IR detection and calculation of total inorganic carbon (TIC), carbonates and organic matter from measured values.
Location of test performance: Na Harfe 336/9 Prague 9 - Vysocany Czech Republic 190 00	
S-DRY-GRCI	CZ_SOP_D06_01_045 (CSN ISO 11465, CSN EN 12880, CSN EN 14346:2007), CZ_SOP_D06_07_046 (CSN ISO 11465, CSN EN 12880, CSN EN 14346:2007, CSN 46 5735) Determination of dry matter by gravimetry and determination of moisture by calculation from measured values.
S-METAXHB1	CZ_SOP_D06_02_001 (US EPA 200.7, CSN EN ISO 11885, US EPA 6010, SM 3120) - Determination of elements by atomic emission spectrometry with inductively coupled plasma and stoichiometric calculations of compounds concentration from measured values. Sample was homogenized and mineralized by aqua regia prior to analysis.
S-OCPECD01	CZ_SOP_D06_03_169 except chap. 10.2 (US EPA 8081, ISO 10382) Determination of organochlorine pesticides and other halogen compounds by gas chromatography method with ECD detection and calculation of organochlorine pesticides and other halogen compounds sums from measured values
S-OCPECD04	CZ_SOP_D06_03_169 except chap. 10.2 (US EPA 8081, ISO 10382) Determination of organochlorine pesticides and other halogen compounds by gas chromatography method with ECD detection and calculation of organochlorine pesticides and other halogen compounds sums from measured values

Preparation Methods	*Method Descriptions*
Location of test performance: Bendlova 1687/7 Ceska Lipa Czech Republic 470 01	
*S-PPHOM.07	CZ_SOP_D06_07_P01 Preparation of solid samples for analysis (crushing, milling and pulverizing).
*S-PPHOM0.3	CZ_SOP_D06_07_P01 Preparation of solid samples for analysis (crushing, milling and pulverizing).
Location of test performance: Na Harfe 336/9 Prague 9 - Vysocany Czech Republic 190 00	
*S-PPHOM2	Drying and sieving of sample on the grain size < 2 mm

The symbol "*" for the method indicates a test outside the scope of accreditation of the laboratory or subcontractor. If the UNICO-SUB code is stated in the method table, this only informs that the tests have been performed by a subcontractor and the results are given in an annex to the test report, including information on test accreditation. If the lab used for matrix outside the scope of accreditation or non-standard sample matrix procedure specified in the accredited method and issues non-accredited results, this fact is stated on the title page of this protocol in the section "Notes". If the test report shows the results of subcontracting, the place of performance of the test is outside the laboratories of ALS Czech Republic, s.r.o.
The method for calculating of the summation parameters is available on request in the customer service.