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Alternative methods to pesticides coupled with biosensors to protect consumer health and the environment

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ABSTRACT

Pesticide contamination is one of the major issues of concern affecting the management of agriculture and environmental research today. Pesticides, including organophosphates and copper compounds, are very useful in controlling pathogens and pests to optimize agricultural production, but their use over time has caused lasting contamination of the environment. This contamination, particularly through residues in soil, water and food, poses a serious threat to humans, other living organisms and the sustainable use of crops in the future. In terms of pesticide detection methods, chromatographic techniques and spectrophotometry are used today; however, these methods are very accurate but also quite expensive and time-consuming and are not suitable for in-situ monitoring. These are limitations particularly evident in less developed countries that have limited access to laboratory instrumentation. As a result, a demand has arisen to develop methods for detecting pesticides in the environment that are efficient, inexpensive, and portable. The objectives of this Ph.D. thesis are to address these challenges through the presentation of innovative state-of-the-art technologies in pesticide detection and remediation, based on electrochemical biosensors. The research work involved the development of sensors (low-cost, small and non-invasive) for real-time monitoring of pesticides by using sustainable materials and developing novel sensor technology as a means to mitigate the problem of pesticide contamination while preserving the environment. The thesis is divided into five main chapters, each presenting specific knowledge about the development and diverse applications of

biosensors. The first chapter of this thesis describes the global pesticide pollution problem and will illustrate the intensity of pesticide application in agriculture, the dangers involved, and the current concern about the recurring nature of these chemicals in the environment. Chapter 1 emphasizes the importance of developing new methods for pesticide detection, particularly those that can be used in the field and provide immediate results. The objective of this study addresses the question of how to meet the demand for real-time monitoring of pesticides and, at the same time, to address the issues that have been raised about pesticide detection. In Chapter 2, the focus is on the basic concepts of electrochemical biosensors, with more emphasis on biosensor design and operation. In Chapter 3, the concept of bacterial biopolymers as new materials alternative to conventional nondegradable plastics in sensor production, is explained and tested. These are polymers produced by bacteria that are environmentally friendly and biodegradable, explores the possibilities of these new substrates, particularly their use in the construction of electrochemical sensors and incorporation into systems used for pesticide detection. By replacing conventional polymer-based materials with bacterial biopolymers, this research supports progress in the area of green technology for environmental assessment and conservation. Chapter 4 focuses on the development of wearable electrochemical sensors for pesticide detection. This new approach involves the use of sensors in the form of glove-like devices that enable real-time detection of pesticide residues on leaf surfaces and fruit skins. The chapter also presents the design, optimization and real-world application of these wearable sensors, focusing on a specific case study on grapevines at

the Quintodecimo winery. The biosensors prepared in this study were applied in the determination of copper ions in vineyards, to assess vine leaf pesticide residues. This wearable technology represents a significant advance in the field of precision agriculture because it is practical and can be easily used in the field to monitor pesticide residues. In Chapter 5, the remediation concept is further developed to demonstrate how pesticide detection can be linked to active remediation of polluted environments. This chapter presents an innovative strategy that incorporates electrochemical measurements and monitoring with remediation methods, such as the use of alginate beads and specific bacteria for the removal of copper ions in polluted environments. The system presented in this study allows pollutants to be detected and treated at the same time, thus offering an innovative and integrated approach to environmental monitoring and management. The integration of real-time sensor data with sanification techniques increases the efficiency of pollution control, especially in soils with high metal contamination, such as agricultural soils. In light of the research outlined in this thesis, it can be concluded that electrochemical biosensors, especially in the form of wearable devices, can revolutionize the process of pesticide detection and elimination in the agricultural and environmental context. This work integrated sensor technology with sustainable materials to provide solutions to problems caused by pesticide contamination. The connection between sensing and remediation technology improves management capacity, particularly for producers, scientists and food safety inspectors in the monitoring and decision making aimed to reduce the use of chemicals

such as pesticides in the agroecosystem, thus focusing on reducing future risks in the environment and to human health.

CHAPTER 1. GENERAL INTRODUCTION

To meet the growing demand for food, intensive industrial agricultural practices to improve productivity (e.g., use of synthetic chemical pesticides and fertilizers) have caused an increase in contamination of soil, water, and air resources that has reduced the quality of the environment.[1] Living organisms, such as animals or plants, that pose a threat to humans or animals are called pests, and microorganisms causing diseases in plants are called phytopathogens. Pesticides are crop protection products, principally based on chemical active substances, used to eliminate or control the proliferation of pests and pathogens.[2] In recent years, awareness of the negative impact of chemicals such as "pesticides" on both the environment and human health has grown worldwide. The use of "pesticides" is often used to describe various products, such as fungicides, insecticides and herbicides that aim to control pathogens-pests damaging plants and animals.[3,4] A practice that is used in traditional farming systems, but they also used in contemporary activities including management of public parks, urban and domestic areas . The Food and Agriculture Organization (FAO), a division of the United Nations (UN), describes pesticides as substances or combinations of substances used to manage and prevent nocive organisms, such as insects that damage plants or pathogens that cause disease in crops, humans and animals, that affect agricultural production during the cultivation and harvesting, distribution and storage stages.[5] Many pesticides consist of active substances developed to combat plant and insect pests and diseases or to regulate their growth, in order to improve crop yields and ensure

food safety in the agricultural sector. Agricultural pesticides, such as fungicides, insecticides and herbicides, are essential to safeguard crops from diseases, pests and competitors that can hinder productivity.[6]

The application of pesticides in agriculture has gradually increased to protect crops from pests, weeds and diseases; however, 80-90% of applied pesticides are noted to affect non-target organisms and biotas, remaining in the environment in the form of residues, which pose a potentially serious threat to the agroecosystem. Experts estimate that only 0.1% of probable pesticides have a real impact on expected pests.[7] Up to 1% actually controls the damaging insects, the rest remains in the environment. This results in pollution of air, water and soil ecosystems and exacerbates the severity of contamination. The need to improve agricultural performance has led to global pesticide use to over 2,000,000 tons per year, with the greatest users originating from China, USA and Argentina.[8] Although these pesticides are used to improve crop production and reduce the by-products from agricultural products, their widespread use becomes a serious challenge to the environment and human health.

When applied to crops, these pesticides directly interact with plants and the ecosystem.[9] These substances can penetrate the plant: root system, leaves and stems; interact with other organisms (insects and soil microorganisms) within the agricultural ecosystem, as well as plants and other animals in the vicinity, can also have, often inadvertently, contact with these pesticides. Although pesticides are aimed at crop protection, others may pose a risk to various targets, with their uptake and exposure passing through a series of interactions. These chemical residues can accumulate over time in surrounding plants and soil, in

turn be transported in the food chain and ultimately the cycle damages the ecosystem.[10]

Environmental pollution of soil and water is one of the consequences of pesticide use. Infiltration in the soil can alter its structure, reduce its ability to support plant growth, and negatively impact the soil-dwelling organisms.[11] Pesticide runoff, irrigation or stormwater runoff can pollute water bodies, such as rivers, lakes and aquifers, leading to the alteration of aquatic ecosystems,[12] and they can endanger the flora and fauna that reside there.[13] These changes in nature not only decrease biodiversity, but also alter the delicate biological, geobiochemical balance of ecosystems, making them less able to withstand other pressures. Furthermore, these residues enter the food chain by contaminating water bodies and accumulate in the aquatic flora and fauna, eventually entering in the food chain, reaching toxic levels to humans or animals when consumed.[14] Soil and water pollution can lead to contaminated food supplies, resulting in environmental hazards.

Today, the use of pesticides, which can devastate human health and the environment in the long run, is a real societal concern. Long-term exposure can cause serious health problems, whereby pesticide residues on fruits and vegetables and other agricultural products can enter the human body when consumed and affect bodily processes[15] such as cell damage, disruption of hormones, and organ dysfunction. Pesticide exposure is linked to several health problems, including nervous system toxicity and an increased risk of chronic diseases, including cancer.[16] These effects are particularly relevant to vulnerable populations, such as children and pregnant women, who are the most susceptible to the effects of chemical exposure.[17]

The flow process from pesticide use in agriculture is illustrated in Figure I.1, demonstrating the dispersion from pesticide applications to negative impacts on the environment and health risks to the consumer.

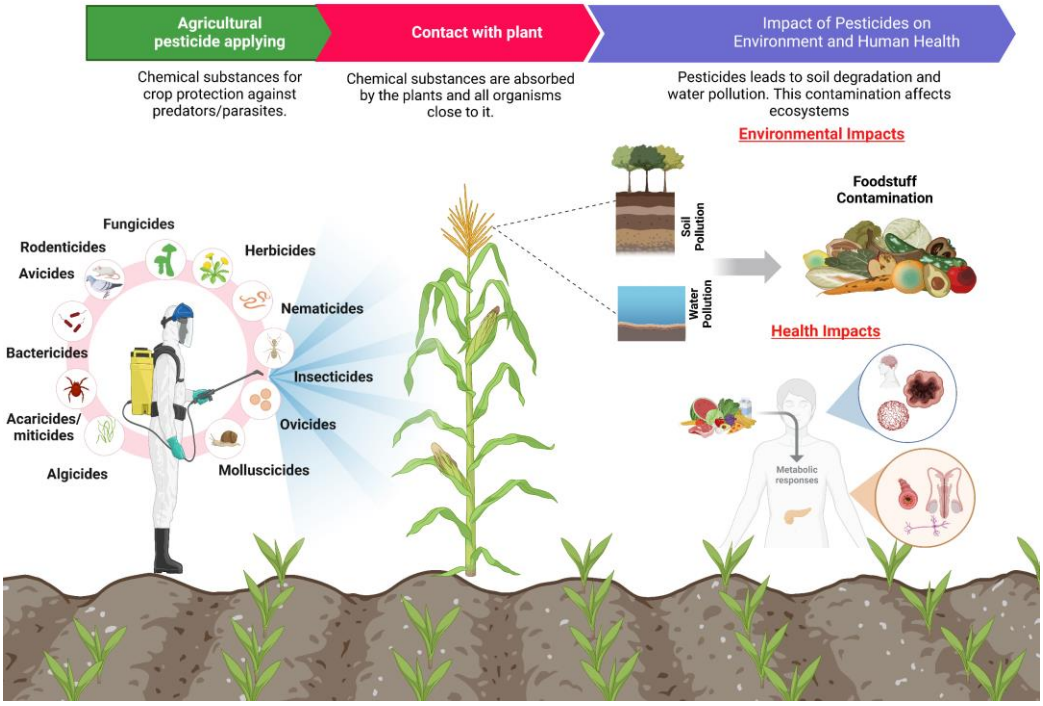

Figure I.1. Pathways and impacts of agricultural pesticides: From application to environmental contamination and human health risks.

The use of farm management practices must be carefully administered to balance the need to increase food supply with the necessity to protect public health and the environment. Pesticides are essential for disease and pest control and to increase crop yields, although there is growing concern about their effects on the environment and human health.

Thanks to ongoing scientific research, a thorough understanding of the health and environmental dangers of overuse of pesticides is being

recognized and focus on finding innovative and environmentally friendly pest control strategies as an alternative to chemical pesticides is the present challenge. Therefore, the use of new electronic and information technologies in crop production will enable agriculture to meet future challenges of rapidly monitoring the presence of pesticides in agriculture and improving environmental sustainability.

The development of portable analytical devices is an interesting topic in analytical chemistry because it allows the development of user-friendly and low-cost devices for use in situ. Sensors and biosensors, e-nose, optical-based sensors and wearable sensors provide indirect tests for plant disease management e-monitoring.[18]

Sensor systems can be classified into three main categories: physical, chemical, and biological. Chemical sensors are devices that respond selectively to a particular analyte through a chemical reaction, enabling both qualitative and quantitative determination of the analyte. These sensors consist of a transduction element covered by a recognition layer, which interacts with the target analyte, generating chemical changes that are then converted into an electrical signal by the transduction element.[19] Similarly, biosensors are analytical devices that convert a biological response into an electrical signal. These sensors integrate a bio-recognition element, such as an enzyme, nucleic acid or antibody, that binds or converts the target analyte, along with a transduction mechanism that displays the resulting biological event.[20]

In agriculture,(bio)sensors are used to detect pesticide residues in crops, water and soil effectively, quickly, inexpensively and with minimal equipment. They are useful tools for monitoring environmental conditions and predictors of food safety because they

provide “real-time information”. Unlike conventional laboratory methods, which include chromatography and mass spectrometry for analysis, [21,22] (bio)sensors are preferred for the rapid speed of obtaining samples and results, ease of use, and environmental friendliness due to the consumption of fewer resources and can be small in size, thus portable.[23–25] Details describing the characteristics of (bio)sensors will be provided in more detail in the successive section 1.2.1 below.

The transition to green technologies is critical for the development of next-generation (bio)sensors that are effective in measuring the key indicator factors in the environment but do not add to the problem with their composition or disposal. Integrating sustainable materials, such as “bio”plastics, into sensor design is a step toward reducing dependence on conventional petroleum-based materials. For example, bacterial polymers based on polyhydroxybutyrate-cohydroxyvalerate (PHBV) have shown promise as substrates for biosensors due to their biodegradability and compatibility with biological sensing elements.

To this end, wearable technologies represent another frontier for application in precision agriculture for the decisional management and practises used in the cultivation systems. For example, electrochemical sensors on gloves have been developed for real-time detection of pesticide residues on fruit surfaces and vine leaves. These sensors are integrated into gloves, allowing producers and farm workers to conduct on-site analysis with minimal equipment of the harvested produce. These innovations not only improve monitoring efficiency, but also equip users with easy-to-use tools in remote and resource-limited settings, i.e. in the field. By combining portability with advanced sensing capabilities, wearable sensors align well with the principles of

smart agriculture, which emphasize sustainable, data-driven agricultural practices.[18,26]

Another recent advancement in the field of environmental monitors is the remediate-and-sense architecture for removing and detecting contaminants. The integration of technologies enables a holistic method of dealing with the monitoring of a pollutant, particularly in an agricultural and industrial setting, by amalgamating remediation and detection capabilities. This methodology uses alginate beads and other materials for bioremediation by adsorption of the heavy metal contaminants, such as copper, to the support material that permits the monitoring of the bioremediation efficacy by analysis of the accumulated material by analysis with electrochemical sensors. The combination of these systems in small, transportable devices represents a major advance in pollution reduction strategies.

1.1. CLASSIFICATION OF PESTICIDES

Numerous plant protection products (also known as pesticides) are available in the global market for the control of various nocive organisms of crops, principally pests, pathogens and weeds that compromise production. These products are classified according to their active substances (synthetic chemicals, natural and proactive compounds, or living microbes) with specific modes of action that effect, inhibit or kill, the desired targeted agent. Depending upon the type of damaging organism that needs to be controlled, different pesticide types are selected for use: fungicides for pathogenic fungi, insecticides for insects, herbicides for weeds. It is critical to understand the mode of action of the active substance and the persistence of the pesticides to understand the impact on the environment, risks to health, and select the appropriate management of agricultural practices for their use.

The World Health Organization (WHO) has classified pesticides according to the pests they affect.[27] The best-known categories of pesticides are classified according to their functionality, including insecticides, herbicides, fungicides, and rodenticides.[28] A survey of the EU-27 member states indicated that, on average, fungicides accounted for more than 43 percent of total pesticide use between 2010 and 2019, making them a dominant category in crop protection practices (Figure I.3),[29] particularly difficult to phase out in conventional agricultural systems.

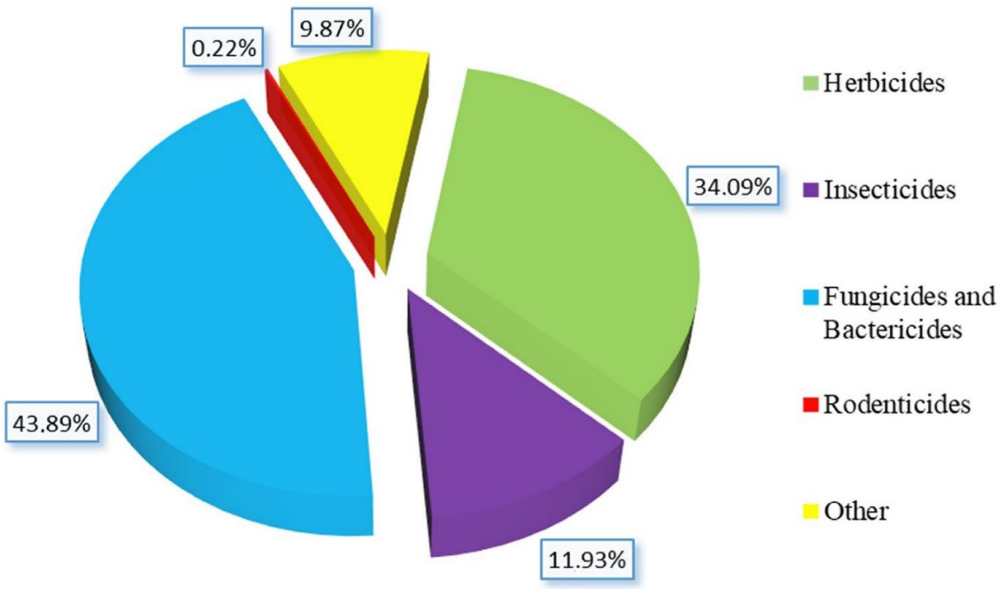

Figure I.2. Distribution of pesticide use in agriculture by class (%) in the EU-27, based on kg.[29]

1.1.1. Fungicides

Fungicides are chemical or biological agents used to prevent, suppress, or inhibit the growth of fungal pathogens responsible for plant diseases.[30] Their use is critical in modern agriculture because they prevent losses due to fungal infections, thus protecting and increasing yields.[31] Typical diseases targeted are powdery mildew, rusts, rots, blights, which can quickly destroy crops without proper management.[32]

Fungicides are classified according to their mode of action (systemic or contact), chemical composition (inorganic, organic or biofungicides) and specificity of the method of action (broad-spectrum or narrow-spectrum). Systemics penetrate the plant for internal protection; contacts act on the surface, preventing infection.[33,34]

The consequences of fungicide use are multiple. On one hand, they have a substantial impact on global food security by reducing crop losses and improving yield quality.[35] They also help control diseases that may cross regional boundaries, reducing the likelihood of outbreaks.[31] However, excessive and inappropriate use of fungicides can have environmental and ecological consequences.[36] Long-term use can allow pathogens to develop resistance to fungicides, reducing their effectiveness. In addition, fungicide residues can pollute soil, water, and nontarget organisms, threatening human health and biodiversity.[37,38]

Biofungicides are gaining ground as antifungal agents originating from living microorganisms and/or natural materials (such as bacteria, fungi, and plant extracts) to provide a sustainable alternative to chemical synthetic pesticides. These biocontrol agents are also environmentally friendly, with great potential for incorporation into disease management strategies to minimize dependence on chemical pesticides. However, biofungicides have limitations, including a low shelf life, slow action, and a host-specific pattern.[38] As agricultural paradigms change, judicious application of fungicides, along with IPM strategies, are a necessity not only to ensure agricultural productivity but also ecological sustainability. [39–41]

Fungicides come in different formulations, depending on chemical composition of the active ingredient, mode of action, health and environmental impacts as shown in Table I. 1 below. These classifications help identify the right measures to manage certain crop diseases without contributing to the development of resistance and with minimal effects on the environment.

Table I.1. Fungicides Classification with Mode of Action and Impacts.

Fungicide Class	Active Ingredient	Mode of Action	Acute and Chronic Health Impact	Environmental impact	Ref.
Copper-Based Fungicides	Copper sulfate, Copper oxychloride	Disrupts enzyme activity by denaturing proteins in fungal cells	Can cause irritation to skin, eyes, and respiratory system; kidney damage possible with prolonged exposure	Toxic to aquatic life and may accumulate in soil over time	[42]
Sulfur-Based Fungicides	Elemental sulfur	Interferes with fungal metabolism and spore germination	Skin and eye irritation; generally low toxicity to humans	Minimal impact; widely used in organic farming with low persistence	[43]
Azoles	Tebuconazole, Propiconazole	Inhibits ergosterol biosynthesis, compromising fungal cell walls	Chronic exposure may disrupt hormonal systems and liver function	Persistent in soil and can harm aquatic organisms	[44]
Strobilurins	Pyraclostrobin, Azoxystrobin	Inhibits mitochondrial respiration, leading to	Low acute toxicity; liver damage from long-	Potentially toxic to aquatic species; moderate degradation in soil	[45]

		fungal cell death	term exposure possible		
Carbamates	Mancozeb, Ziram	Disrupts enzyme activity, impairing fungal metabolism	Chronic exposure linked to thyroid damage; potential carcinogen	Persistent in the environment; toxic to aquatic life and birds	[46]
Dithiocarbamates	Thiram, Ferbam	Blocks fungal enzyme activity and spore germination	Skin and respiratory irritant; suspected carcinogen with prolonged exposure	Persistent in soil; toxic to fish and beneficial insects	[47]
Benzimidazoles	Carbendazim, Thiophanate-methyl	Interferes with fungal mitosis by disrupting microtubule formation	Potential reproductive toxicity; suspected carcinogen in long-term studies	Moderately persistent and toxic to aquatic invertebrates	[48,49]
Biological Fungicides	*Trichoderma harzianum, Bacillus subtilis/Bacillus Amyloliquefaciens*	Compete with pathogens and produce antifungal compounds	Minimal to no toxicity to humans	Environmentally safe; often used in organic farming	[50]

Copper-based fungicides

Fungicides, particularly copper-based fungicides, play a central role in this thesis work.

Copper (Cu) is one of the few chemicals frequently applied as an effective and inexpensive control against plant pathogens (bacteria and oomycetes) in conventional and organic agriculture.[14] It is classified as an essential trace element because it is involved in a number of metabolic and physiological processes in humans, animals, and plants. [51] Cu is essential for bone health, immune function, cardiovascular risk, and cholesterol metabolism.[52] It also plays a role in human brain growth and development and is involved in metabolic processes as an enzyme cofactor and in signalling and regulation. Cu ions are known to have a very significant effect on growth during infancy, host defense mechanisms, and brain development.[53,54]

Cu is a common bactericide and fungicide in agriculture because it is low cost and effective in disease control while having relatively low toxicity to humans.[42] One advantage of Cu is its wide range of activity against bacterial pathogens, including those that cause grape blight, late blight of potato, and secondary diseases in herbaceous areas such as fruit and vegetable crops. [55] For example, copper compounds are highly effective against a wide range of foliar fungal infections, particularly those caused by grapevine downy mildew, which is considered one of the most serious vine diseases worldwide.

However, overuse of Cu in agriculture has led to the emergence of Cu-tolerant microorganisms and adverse environmental effects due to its accumulation in soil and water. Prolonged exposure to high levels of Cu in soil can be toxic to plants, and chronic exposure to Cu, even if

moderate, can affect reproductive rates and vegetative growth (i.e., both aerial and underground plant biomass).[56] The extensive use of copper-based fungicides in the treatment of fungal infections in plant crops such as grapevine and tomato has played a role in the bioaccumulation of Cu in soil and water, which is threatening microbial ecosystems and coming into contact with animals, or plants, is toxic. Thus, the continued use of copper-based pesticides as plant protection products is no longer considered sustainable due to Cu toxicity to humans and animals, and alternative methods need to be found.

Recently, European legislation (Regulation 2018/1981)[57] has restricted the maximum quantity of copper allowed for plant protection to 28 kg per ha over a period of 7 years (status 2021), with a final objective to eventually phase out copper use.[58]

Detection and remediation of Cu contamination has become an urgent requirement for sustainable agriculture aimed to techniques for integration (by reducing doses) or substitution. Considerations can be given to innovative and portable sensors specifically designed to detect fungicide residues, including systems integrated into glove surfaces, are critical for safe and sustainable use of fungicides.[59] Our research on remediate-and-sense architectures[60] further exemplifies how this work engages fungicides as potential agents used in agriculture, enabling not only monitoring but also remediation of environments contaminated with such agents, overall, interfacing with the higher goals of agricultural sustainability and environmental protection.[61]

The development of new agricultural technologies is essential to sustainably increase agricultural production.[62] One promising strategy

is to use biological control agents (BCAs) and their products as an alternative to synthetic chemical pesticides.[63] BCAs are natural organisms or enemies, such as predators, that control pests, pathogens, and weeds and minimize the need for chemical interventions.[64] These agents offer a more environmentally friendly solution that corresponds to the growing demand for sustainable agricultural practices.

1.1.2. Insecticides

The inappropriate use of insecticides can lead to pest resistance in insect populations, reducing their effectiveness of control and causing negative environmental consequences. This has stimulated regulators and the public concerns about environmental and human health risks from pesticide use; including issues such as negative effects to non-target organisms, the presence of pesticide residues in the ecosystem and on produce.[65]

A major environmental concern related to pesticides is their potential to pollute water and soil. When pesticides are applied to crops, the products are subject to runoff into streams and rivers, they can leach into the soil and contaminate groundwater or be distributed in the atmosphere. This can harm aquatic life, wildlife and human health. In particular, insecticides can also harm important non-target species, such as bees and butterflies, insects are critical for pollination, and their decline can greatly impact agroecosystems. Insecticides have different origins, such as man-made chemicals and natural materials. Some origins of insecticides are listed below:[66]

- **Synthetic insecticides**: Substances created through industrial chemical processes to. Synthetic insecticides are meant to manage pests. They are often developed after research and

experimentation. Pyrethroids and neonicotinoids are among the insecticides produced also using organophosphates and carbamates as insect growth regulators.

- **Botanical Insecticides**: Insecticides made from plant origins or plant extracts are known as botanical insecticides. These organic substances often possess insecticidal qualities that make them useful against pests. Examples include neem oil (produced from the neem tree), rotenone (produced from the roots of specific plants) and pyrethrum (produced from chrysanthemum flowers).
- **Microbiological insecticides**: Microorganisms with insecticidal properties are the key components of microbiological insecticides. They can be viruses, bacteria, fungi or protozoa.
- **Insect growth regulators (IGRs)**: Insect growth regulators are elements that counteract the development and growth of insects, often focusing on particular stages of their life cycle. They can be created synthetically or naturally. IGRs have the power to prevent moulting, metamorphosis or reproduction, with the effect of reducing insect populations. Inhibitors of chitin synthesis and juvenile hormone analogues are examples.
- **Biopesticides**: Since biopesticides are made of natural products, it is commonly assumed that they are safer to the environment. Some examples of biochemical, botanicals and microbial insecticides are some examples and include substances such as pheromones that affect the behaviour or reproductive system of the pests.

Insecticides are very important in today's agriculture, as they help manage pests that pose a threat to crops and the food supply. However, their application has the potential to produce some effects that may not be desirable for the environment, plants and plant products that should be protected from insecticides.

Organophosphate Pesticides

In this thesis, research was focused on different methods to detect pesticides, in particular organophosphate (OP) pesticides were examined. Although these compounds represent some of the most widely used insecticides in agricultural practices, they are known to pose a serious risk to human health and the environment due to their neurotoxic effects and persistence. Therefore, the identification and control of OPs are critical for sustainable agriculture and consumer safety.

OPs are an important class of synthetic chemicals widely used in agriculture for pest management and crop yield improvement. Exposure to organophosphate pesticides has been linked to multiple adverse health outcomes. Acute exposure causes symptoms such as headaches, dizziness and nausea, while chronic exposure has been associated with increased rates of more serious outcomes, such as neurodevelopmental disorders and cognitive deficits. According to a systematic review, prenatal exposure to organophosphates poses risks for neurodevelopmental impairment in children, including cognitive deficits and behavioral problems.[67]

OPs act by inhibiting acetylcholinesterase (AChE), a vital enzyme in the nervous system that, when inhibited, can lead to neurotoxicity in humans and other organisms. The impact of OPs

on the environment is also of concern. These chemicals are also persistent and stable in soil and water, thus cause pollution of the environment and have adverse effects on wildlife. The study also shows that OPs have a negative impact on photosynthesis and mineral nutrition of plants and have been found in various environmental samples such as water and sediment.[68]

Their persistence in the environment, as well as their potential presence as residues on food, highlights the need for efficient detection methods.[69] Table I.2 summarizes all selected insecticides, their modes of action and health implications.

Table I.2. Globally applied insecticides, active substance, mode of action and negative health implications to humans.

Insecticide Class	**Active Ingredient**	**Mode of Action in Target insect**	**US EPA Toxicity Category**	**Acute and Chronic Impacts to Humans**	**Ref.**
Organophosphate	Chlorpyrifos	Inhibits AChe activity	II	Headache, dizziness, nausea, vomiting, endocrine disruption	[70]
Organophosphate	Malathion	Inhibits AChe activity	III	Muscle weakness, respiratory distress	[71]
Carbamate	Aldicarb	Blocks AChE, causing	I	Seizures, respiratory paralysis, fatal	[72]

		cholinergic crisis		poisoning if ingested	
Synthetic Pyrethroid	Permethrin	Disrupts sodium channel function, leading to paralysis	III	Burning sensations, nausea	[73]
Organochlorine	Endosulfan	Induces oxidative stress, disrupts DNA	II	Allergic reactions	[74]
Natural origin	Pyrethrins	Neurotoxic, causing nerve firing and death	IV	Skin and respiratory irritations	[75]
Synthetic Pyrethroid	Cyfluthrin	Disrupts sodium channels, causing paralysis	III	Numbness, burning sensations	[76]
Organophosphate	Diazinon	Inhibits AChe activity	II	Dizziness, vomiting, endocrine disruption	[77]
Neonicotinoid	Imidacloprid	Binds nicotinic acetylcholine receptors	II	Neurotoxicity, developmental delays	[78]
Carbamate	Carbaryl	Inhibits AChe activity	III	Dizziness, abdominal pain,	[79]

				respiratory issues	
Pyrethroid	Deltamethrin	Sodium channel disruption	II	Tremors, burning, numbness	[80]
Organophosphate	Phosmet	Inhibits AChe activity	II	Seizures, nausea, muscle cramps	[81]
Pyrethroid	Fenvalerate	Blocks sodium channels in neurons	II	Skin irritation, endocrine disruption	[82]
Neonicotinoid	Thiamethoxam	Nicotinic receptor agonist	II	Neurotoxicity, memory issues	[83]
Organophosphate	Parathion	Inhibits AChe activity	I	Convulsions, respiratory failure	[84]
Carbamate	Propoxur	Inhibits AChe activity	II	Nausea, respiratory issues	[85]
Pyrethroid	Lambda-cyhalothrin	Sodium channel modulator	III	Burning skin, respiratory irritation	[86]
Neonicotinoid	Acetamiprid	Nicotinic receptor agonist	III	Neurotoxic effects, tremors	[87]
Organophosphate	Methamidophos	Inhibits AChe activity	I	Paralysis, respiratory failure	[88]

1.1.3. Herbicides

These are a group of agrochemicals specifically developed to kill or inhibit the growth of unwanted plant competitors, usually weeds, but

also parasitic plants. By combating weeds that compete with crops for essential nutrients, water and sunlight, herbicides are a key tool in modern agriculture, which has increased crop yields and efficiency.[89] However, their extensive use has raised concerns about environmental contamination, impact on non-target species, and plant residues in food, underscoring the need for innovative and sustainable herbicide management strategies. Their application has significantly increased agricultural productivity; however, concerns have arisen about their environmental impact, the development of herbicide-resistant weed species, and potential health risks to humans and wildlife.[90,91]

Overuse of herbicides has resulted in negative impacts on the environment, such as soil and water pollution. Herbicides can seep into the soil and reach groundwater or be carried into streams and other water bodies by rain, adversely affecting aquatic life and eventually ending up in the human food chain. For example, glyphosate, one of the most widely used herbicides, has been found in several environmental samples, which has raised questions about its stability and environmental impacts.[92] The continued heavy use of herbicides has encouraged the emergence of resistant weed species, reducing the effectiveness of these products and creating problems in weed management. This requires the use of higher doses of herbicides or the use of different herbicides to combat resistance, resulting in worsening environmental and economic impacts.[93–95]

Herbicides can be classified according to their specificity into selective and nonselective. Selective herbicides are formulated to destroy specific types of plants while leaving others alone. For example, they determine plant species based on differential uptake, species-specific

translocation in the plant, or morphological and physiological properties. Two examples of selective herbicides are 2,4-D, mecoprop and dicamba. They kill broadleaf weeds and do not damage grasses. Non-selective herbicides, on the other hand, are nonspecific and destroy any plant material they come in contact with. These herbicides are mainly used in places where all vegetation needs to be eradicated and include industrial areas, railroad embankments and drainage systems. Non-selective herbicides include paraquat, glufosinate and glyphosate, of which plants are species. very effective in controlling a wide range of plants. [96]

Table I.3. Herbicide classification.

Herbicide Class	Active Ingredient	Mode of Action	Acute and Chronic Health Impact	Environmental impact	Ref.
Synthetic Herbicide	Glyphosate	Inhibits shikimate pathway (amino acid synthesis)	Potential links to cancer, endocrine disruption	Persistent in the environment; detected in water, soil, and some food products; impacts on microbial ecology	[97]
Synthetic Herbicide	Atrazine	Disrupts photosynthesis (PSII inhibitor)	Endocrine disruption, potential reproductive toxicity	Contaminates groundwater; linked to declines in amphibian populations	[98]

Synthetic Herbicide	Dicamba	Disrupts hormone action (synthetic auxin)	Respiratory and eye irritation, potential endocrine effects	High volatility causes unintended crop damage; contamination risk to nearby ecosystems	[99]
Synthetic Herbicide	2,4-D	Mimics plant growth hormones (auxins)	Skin irritation; long-term exposure under investigation	Potential for runoff into waterways; toxicity to aquatic plants and fish	[100]

1.2. THE DUAL ROLE OF PESTICIDES IN MODERN AGRICULTURE: SOLVING THE CHALLENGES THROUGH NEW AGE DETECTION TECHNIQUES

At the same time, pesticides in modern agriculture serve a dual purpose. On the one hand, they are critical for ensuring high crop yields, protecting plants from pests, diseases and weeds, and ensuring food security for an expanding global population. At the same time, their widespread use has caused significant environmental and health problems, such as contamination of food, soil, and water, depletion of biodiversity, and impacts on human health. As mentioned earlier, each class of pesticides (fungicides, insecticides and herbicides) has distinct advantages and disadvantages. To detect their use and reduce their negative impacts, new detection methodologies are needed to monitor pesticide use. To address these challenges, there are new sensing techniques that are being considered as a solution. These innovations include the integration of advanced analytical capabilities and

environmental sustainability to provide very accurate and environmentally friendly tools.

(Bio)sensors

The problem of pesticide contamination in agricultural and environmental matrices is very critical and alarming and must be overcome with innovative and effective detection methods. However accurate, conventional methods, such as chromatographic and spectrophotometric methods, can be expensive, time-consuming, and often require advanced equipment and specialized personnel. Conventional detection methods, while effective, are slow and not portable for on-site application. Consequently, they are not suitable for rapid, on-site testing.[101]

The development of new sensing techniques in pesticide biosensors is a viable solution to these challenges, providing rapid, specific, and cost-effective diagnoses.[18] Current biosensors are a revolutionary concept in that they enable real-time, portable and efficient pesticide measurement with the help of nanotechnology and biomimetic materials.[26]

These innovations contribute to the improvement of sustainable farming, food security, and the protection of the environment. This is illustrated in Figure I. 4 where the advanced biosensors are used in the fight against contamination.

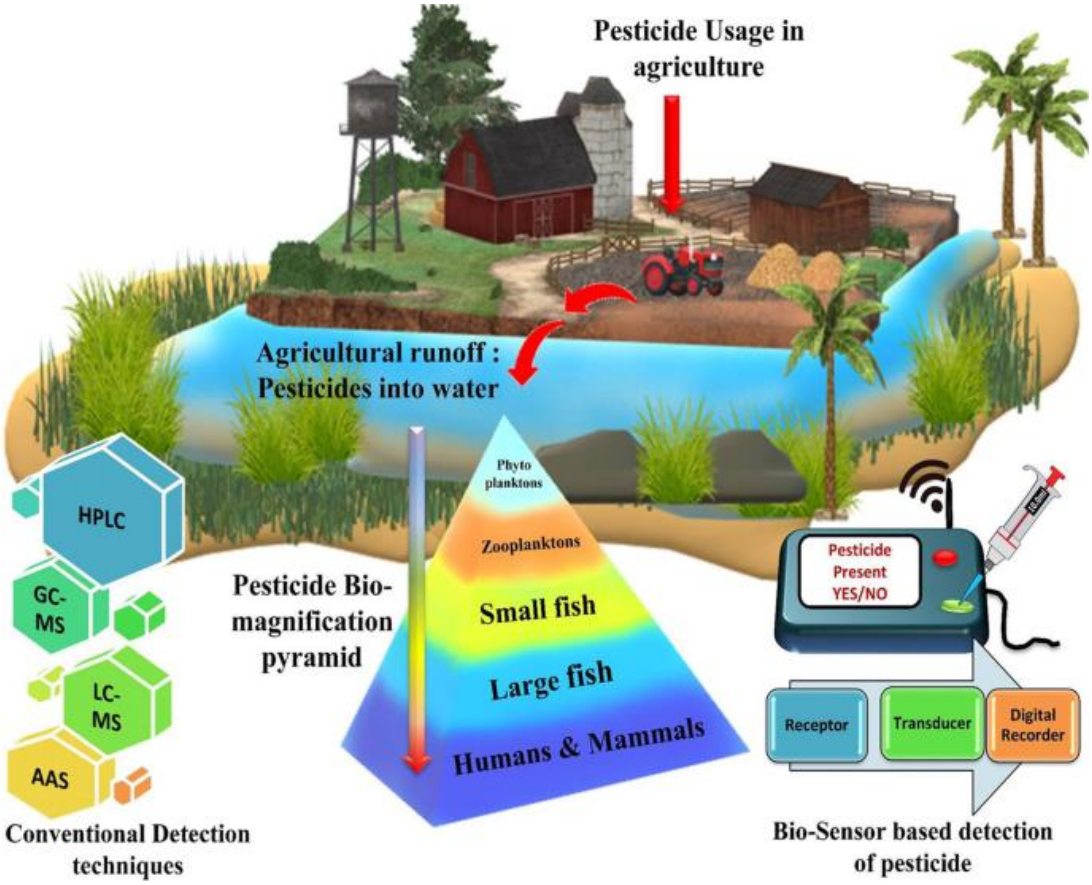

Figure I.3. Innovative biosensor technologies that combat pesticide contamination.[101]

Wearable sensor technologies are designed as non-invasive tools for on-site and real-time pesticide detection, combining portability, usability and sensitivity into one system. These devices, gloves, wristbands or patches equipped with sensors, enable agricultural workers, food safety inspectors and environmental researchers to directly monitor pesticide residues in the field. Wearable technologies that integrate the biosensor eliminate the need to bring samples to the laboratory, thus reducing the time and cost of pesticide testing. For example, a wearable glove-based electrochemical biosensor can detect

organophosphate pesticide residues on fruits, vegetables, or soil simply by physically touching the sample.[102] This interaction allows for on-site assessment, immediate response, and reduced risk of worker and environmental exposure to pesticides.

As shown in Figure I.5, wearable plant devices could be used in a variety of applications in agriculture. These sensors can be applied to various parts of the plant, such as stems, leaves, and roots, to accurately track biological processes within them and enable more accurate agricultural decision making.

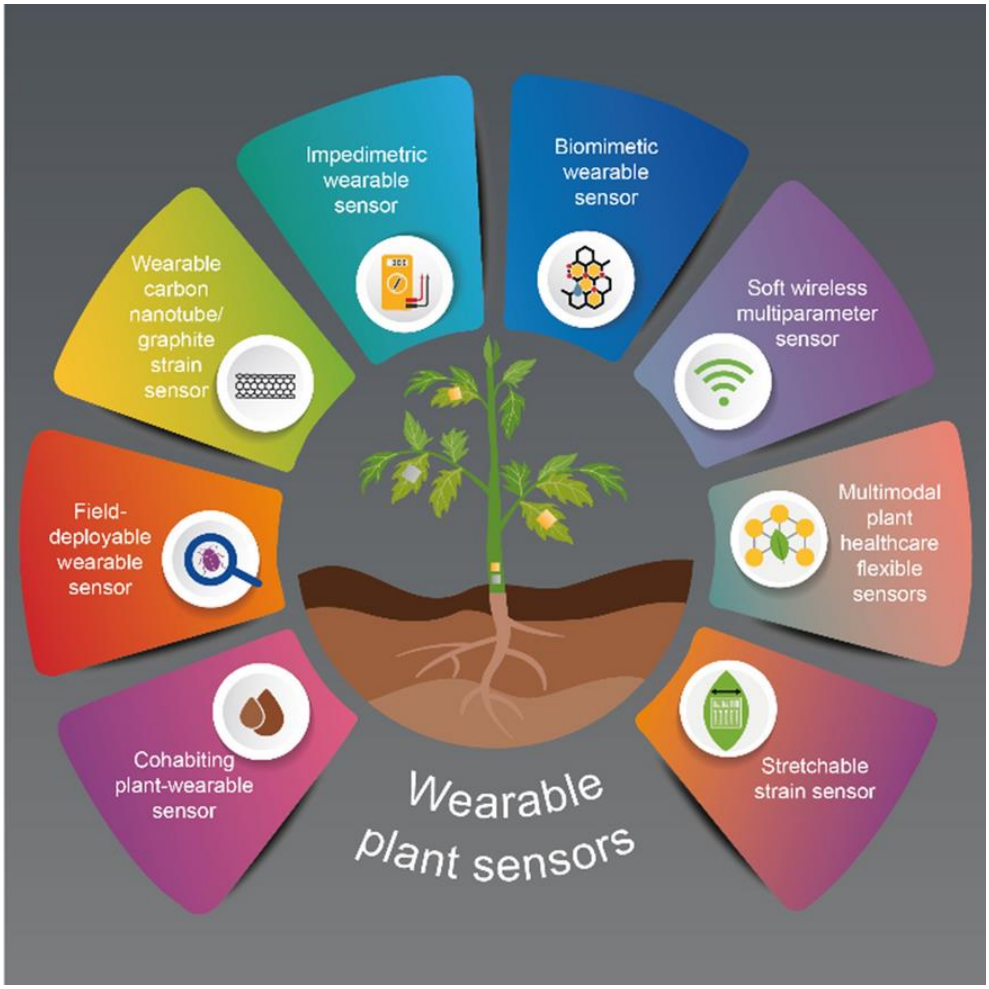

Figure I.4. Different wearable sensors and its applications in agriculture.[18]

Wearable sensing devices are portable, but they also include many features that can be seamlessly integrated with digital platforms. Most wearable sensors have wireless connectivity to send data to smartphones or cloud-based systems for processing, analysis, storage and decision making. Available information on pesticide contamination levels helps users accurately apply agrochemicals to

reduce environmental damage. In addition, the simplicity and ease of use of wearable sensors democratize pesticide monitoring, giving small farmers and resource-poor countries access to advanced sensing technologies.

Although biosensors and wearable sensors have improved the detection of agricultural contaminants, they alone are not sufficient to address the multifaceted problem of contaminants in agriculture. Remediate-and-sense systems bridge this important gap by integrating active detection and remediation to address the dual challenges of pesticide contamination and environmental pollution. These systems not only improve the efficiency of monitoring activities, but also encourage sustainable and proactive agricultural and environmental management practices, making them crucial to the future of precision agriculture projects and environmental conservation initiatives.

1.3. GENERAL OBJECTIVES

In In my Ph.D. program, my research focused on the problems associated with pesticides in agriculture, with an emphasis on investigating “quick” solutions for monitoring their presence and the need for finding alternatives to chemical pesticides to minimize the damage they cause to the environment. Building on the introductory theory and the problems identified in pesticide use, this thesis has a twofold objective:

1) The first goal is to develop advanced biosensor technologies with potentially new and user-friendly applications aimed at improving the detection of commonly used pesticides. This work explores the potential of bioplastics, wearable technologies and dual platforms for detection and remediation, aiming to integrate them into agricultural practices for a greener and safer future.
2) The second is to study an alternative approach to pesticides by testing selected microorganisms as biological control agents and plant biostimulants combined with the active substance of a pesticide commonly used in organic farming. The goal is to develop an integrated approach that improves environmental sustainability and human well-being by reducing recommended chemical doses for pest control.

In the context of this research, crucial agricultural and environmental needs are addressed using three innovative concepts: biobased electroanalytical platforms, wearable sensing technologies, and remediation and sensing architectures, as illustrated in Figure I.5. All three of these

approaches are designed to mitigate perceived disadvantages in traditional pesticide application by promoting sustainability, reducing consumer exposure to toxic compounds, and reducing harmful effects on the environment. These principles are designed to safeguard public health, protect the environment, and promote sustainability.

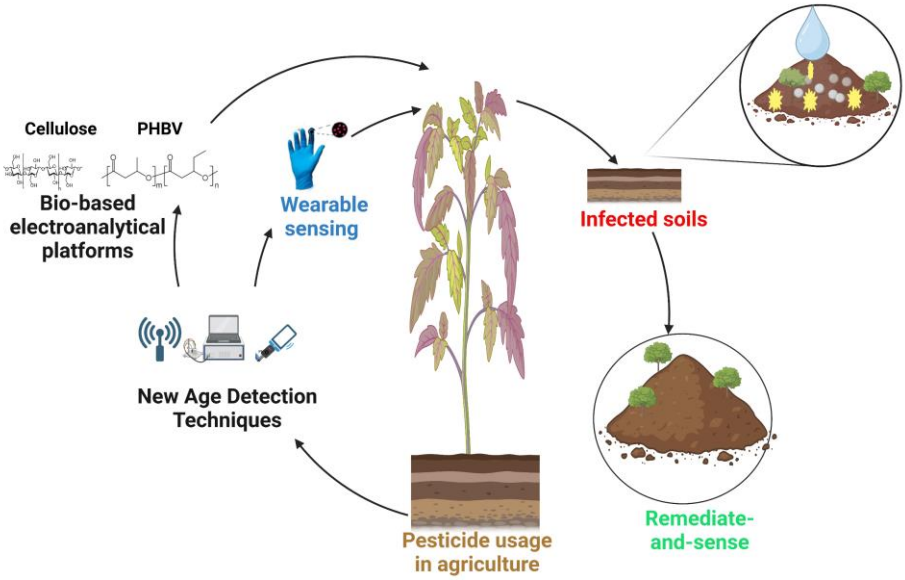

Figure I.5. Overview of sustainable agricultural strategies that integrate biobased electroanalytical platforms, wearable sensing technologies, and remediate-and-sense architectures to address pesticide use challenges, improve soil health, and promote environmental sustainability.

A central issue in this thesis concerns the need to develop efficient detection methods for persistent pesticides, such as OPs, and the risks they pose as residues in food. In this area, the design of electrochemical and biobased sensors for the detection of OPs has been an important

innovation. These methods have been adapted to ensure high sensitivity and specificity while respecting the principles of sustainability. For example, in the development of PHBV-based porous bacterial polymers, a new platform for electroanalytical detection was created that demonstrated excellent performance in detecting residual OPs, reducing the environmental impact of analytical devices.[103]

In addition, the continued use of copper pesticides is now considered unsustainable due to the toxicity of copper to humans and animals. Detection and remediation of copper contamination has become an urgent need for sustainable agriculture. In this context, innovative and portable sensors specifically designed to detect fungicide residues, including those embedded in glove surfaces, are crucial for the safe and sustainable use of fungicides.[59] Research on remediation and sense architectures[60] has also shown how the combination of monitoring and remediation can be applied to pesticides and fungicides, contributing to the achievement of more sustainable agricultural practices and environmental protection.[61]

Another crucial point is the exploration of BACs, such as *Bacillus amyloliquefaciens* and *Bacillus subtilis*, which have proven effective not only in controlling plant diseases but also in reducing environmental risks associated with contamination by heavy metals such as copper. These microorganisms are able to bioaccumulate copper, reducing its toxicity in soil and water, thus offering a solution that combines pest control with environmental remediation.

In summary, this thesis aims to propose innovative solutions to address the challenges of pesticide use in agriculture by integrating sustainable

technologies, advanced biosensors, and remediation strategies. The intent is to protect consumer health, promote agricultural sustainability and reduce environmental impact.

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CHAPTER 2. EXPERIMENTAL SECTION

2.1 ANALYTICAL METHODS FOR THE DETECTION OF AGRICULTURAL PESTICIDES

Analytical methods for pesticide residue detection play an important role in food safety and prevention of environmental pollution by hazardous/harmful residues. The processing technique depends on the chemical nature of pesticides, the sample matrix, and the level of sensitivity and selectivity of detection. The detection of agricultural pesticides is very important for food safety and the environment. Many analytical techniques have been developed and improved in recent decades. The high precision and accuracy in the determination of residual pesticides has traditionally ranked chromatographic and spectrometric applications as the most reliable options for residue determination.[1] High-performance liquid chromatography (HPLC) with mass spectrometry (MS) attached; gas chromatography (GC) with MS, is still widely used for chemical pesticide analysis due to its high accuracy and detection limits. When combined with other sample preparation techniques, such as solid-phase extraction that can help remove more interferences from the sample matrix, these methods become even more effective.[2] The limitation of GC, particularly its inability to separate non-volatile and polar pesticides, can be addressed by liquid chromatography (LC), particularly high-performance liquid chromatography (HPLC). The identification of additional pesticides with high sensitivity has been made possible by the integration of LC with mass spectrometry (LC-MS). [3] More advanced forms of analysis, including ultra-high performance liquid chromatography with triple

quadrupole MS, providing high sensitivity and throughput with low limits of quantitation, applicable to difficult samples such as cereal grains.[4] New methods, including enzyme-linked immunosorbent assays (ELISAs), that can provide sensitive and specific results while remaining simple, rapid, and inexpensive, making them suitable for initial screening and field use.[5] New methods, including surface-enhanced Raman spectroscopy (SERS), that boast high sensitivity with little or no sample preparation; however, these methods are still relatively new and under development for practical applications.[6] All of these approaches are very effective in detecting and quantifying contaminant levels found in the environment, especially in the case of pesticide residues in agricultural samples. However, their application is limited by several limitations, such as high operational costs, lengthy procedures with complex equipment, and the presence of technical experts. Hence, there is a growing demand for simpler, faster and cheaper methods for the detection of agricultural contaminants.

In recent decades, advances have been made in nanotechnology and biosensing (optical and electrochemical sensors) that have simplified and accelerated pesticide analysis.[7]

The comparison between traditional and advanced monitoring technologies for agricultural contaminants is shown in Figure II.1 and Table II.1.

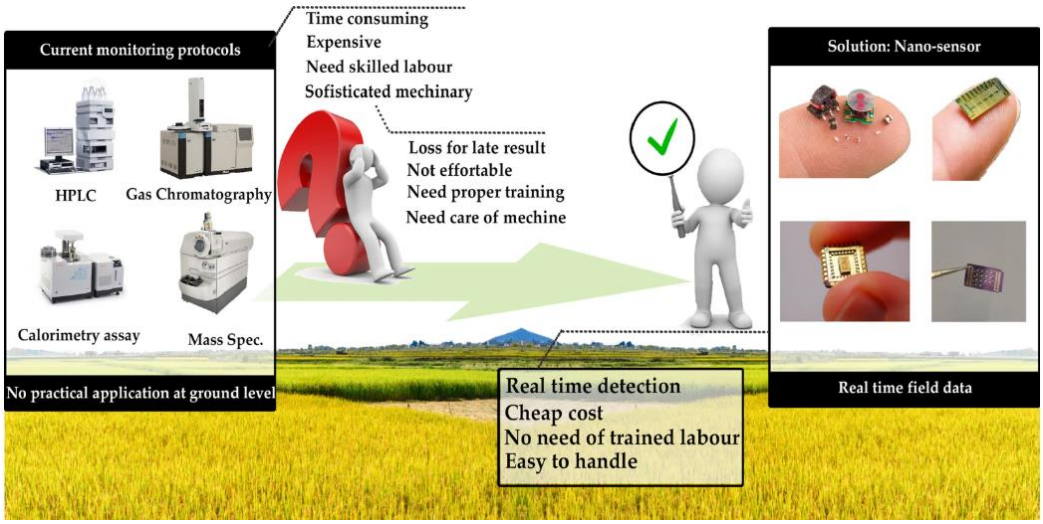

Figure II.1. A comparison between conventional monitoring technologies and advanced sensor-based solutions to analyzed samples from the field, showing variations in cost, efficiency, and feasibility of real-time field application.[7]

Table II.1. Comparison of traditional and advanced technologies for agricultural contaminants

Feature	Traditional Technologies	Advanced Technologies
Time Efficiency	Time-consuming	Real-time
Cost	Expensive machinery and operations	Cost-effective
Labor Requirements	Skilled personnel	No skilled personnel
Field Application	Not feasible for field use	Portable and deployable in the field
Sensitivity	High	High
Ease of use	Complex	Simple

2.2 SENSORS AND BIOSENSORS

By identifying the limitations of conventional analytical methods, sensors/biosensors have the ability to transform and simplify the process of monitoring contaminants in agriculture and the environment. This change could not only encourage the adoption of sustainable measures, but also advance the solutions to addressing the problem of world food security and environmental conservation. (Bio)sensors have become essential devices for pesticide residue detection because they offer a number of advantages over conventional analytical techniques.

Sensors and biosensors are fundamental devices for collecting and analyzing data from the environment. These tools are essential in

numerous fields, from industry to health care, from environmental monitoring to scientific studies. They can be considered the sensory organs of the technological universe, able to transmit real-world activities into data that can be understood by humans or managed by automated control systems. Sensors also respond to small changes in their surroundings and are therefore extremely useful in applications such as health care and environmental monitoring. A chemical sensor is a device that produces an analytical signal correlated with the concentration of a chemical species in the sample or the total chemical composition of the analyte, as specified by the International Union of Pure and Applied Chemistry (IUPAC).

Similarly, a biosensor can be defined as a type of chemical sensor in which the recognition system is based on a biochemical mechanism. Namely, a chemical (bio)sensor may be regarded as a tool for an analysis of the liquid or gas phase, returning the data on the chemical composition and concentration of the specific compounds.[8,9] A typical (bio)sensor system consists of: (a) a (bio)recognition element responsible for detecting a specific analyte; (b) a signal transducer that translates the analyte recognition event into a detectable signal; and (c) an electronic module to manage and operate the data. The design of (bio)sensors focuses on creating devices that are selective, sensitive, reproducible, fast, inexpensive, and easy to use to identify specific analytes. As a result, miniaturization and automation of these devices are increasingly important.[10,11]

(Bio)sensors can be classified according to several factors, such as the transduction mechanism, the type of recognition element employed,

and their specific application.[12] When considering the transduction mechanism, the main categories include:

- **Optical biosensors**, which detect changes in light characteristics (e.g., absorbance, fluorescence).
- **Electrochemical biosensors**, which monitor changes in electrical properties (e.g., current, voltage).
- **Piezoelectric biosensors**, which detect changes in mass on a quartz crystal.
- **Thermal biosensors**, which detect changes in heat production or absorption.

Currently, electrochemical (bio)sensors are a major area of research investigations due to their high sensitivity and stability. To this end, the focus of studies during this Ph.D. is on electrochemical biosensors, specifically on improving pesticide detection by enhancing biosensor interfaces with bioreceptors to achieve accurate and responsive measurements. The goal was to design portable and easy-to-use devices that can perform in situ monitoring, overcoming the limitations of traditional laboratory techniques.

According to IUPAC recommendation, an electrochemical (bio)sensor is a compact, integrated device designed to provide specific quantitative or semi-quantitative analytical data. This is achieved by a recognition element (chemical receptor) that is directly in contact with an electrochemical transduction component. Electrochemical biosensors detect the current generated by oxidation and reduction reactions. This current can be related to the concentration of the electroactive species or its production/consumption rate. The electrical signal generated corresponds to the recognition interaction between the

target and the analyte and is directly proportional to the analyte concentration.[13]

In dynamic electrochemical experiments, the most common configuration involves a three-electrode system consisting of a working electrode, a counter-electrode (auxiliary) and a reference electrode, all connected to a potentiostat. This configuration allows precise control of the potential difference between the reference and working electrodes. The reference electrode, often of the silver/silver chloride (Ag/AgCl) type, may be commercially available or manufactured in the laboratory. The counter electrode, typically made of inert materials such as platinum or carbon, supports the electrochemical process by balancing the current and maintaining system stability. The working electrode is the critical component where the electrochemical reaction takes place, and its material plays a key role in determining sensor performance. Common materials for the working electrode include noble metals such as gold and platinum, as well as carbon-based materials such as graphite and graphene. Recent research has focused on the use of graphene, both as an electrode material and as a surface modification to improve its properties.[14,15]

The functionality of electrochemical sensors is largely influenced by their three-electrode system, which ensures accurate and reproducible measurements. A potentiostat connects the electrodes, controlling the voltage applied between the working and reference electrodes and measures the resulting current or other electrical properties. The working electrode interacts directly with the target analyte, as in pesticide detection, and the signal generated is proportional to the analyte concentration. To improve the sensitivity and selectivity of the

sensor, nanomaterials are often incorporated into the electrode surface, enabling detection at trace or ultra-trace levels. The stability of the reference electrode, such as Ag/AgCl type or calomel, is critical to maintain precise control of the electrochemical potential, which is essential for reliable experimental results.[16,17]

Advances in instrument creation and in minimizing the number and quantity of samples are crucial in the field of electrochemical studies today. Achieving these criteria is essential for conducting tests both in the laboratory and in decentralized locations. In this context, screen-printed electrodes (SPEs) have become very popular because of their effectiveness in efficient, sensitive, and cost-effective detection. The use of SPEs has greatly improved the usability of electrochemical sensors by providing a convenient and customizable option that is compact and disposable.[18] Based on these advantages, this Ph.D. research adopted SPEs for broader analytical applications in real-world situations because of their portability and fast response times, making them ideal for on-site testing, especially in resource-limited locations such as remote areas or developing countries, where they can be very useful tools. Further integration with modern technologies, such as data collection via smartphones and wireless communication systems, broadens the efficiency of the application, making them more widely accepted in various fields.

The development of (bio)sensor involves three main steps:

1st step: screen-printed electrode fabrication

SPEs represent one of the most sophisticated and versatile solutions in the field of electrochemical sensors, combining simplicity of manufacture with highly reliable performance. These devices consist

of an inert substrate, usually rigid, on which three functional electrodes are printed by screen printing technique: the working electrode (WE), the reference electrode (RE) and the counter electrode (CE). This approach results in compact sensors that can be easily integrated into different applications without compromising their analytical capabilities. Screen printing, with its long industrial tradition, has proven particularly suitable for the fabrication of electrochemical devices because of its ability to deposit thin and precise layers of conductive material. The fabrication of a screen-printed electrode consists of well-defined steps: from the choice of the most suitable substrate (which may vary according to specific application requirements), to the fabrication of the electrodes, through the modification and optimization of their surface to improve their performance.[19–21]

The manufacturing process involves the application of a conductive paste or ink to the substrate, which, through the use of a screen-printing mesh, is transferred uniformly to the surface. At this stage, ink quality, curing temperature and heat treatment conditions play a crucial role in determining the robustness and reliability of the sensor performance. In addition, the roughness of the electrode surface and surface pretreatment techniques significantly affect the sensitivity, selectivity and stability of the device, making it necessary to carefully control all these parameters. The effectiveness of a screen-printed sensor depends, therefore, on a delicate balance between physical and chemical variables, which determine not only the analytical response of the device, but also its ability to operate in complex environments and withstand variable operating conditions over time. In this context, thin-

film technology, which has recently been the subject of increasing interest, represents a promising frontier, enabling significant improvements in miniaturization and customization of the sensing characteristics of SPEs.

- *Choice of substrates*. The choice of substrate plays a key role in the fabrication of single-use electrochemical (bio)sensors: paper and plastic are the most commonly used materials. Each type of substrate offers unique advantages and presents specific challenges depending on the intended application. For paper-based substrates, their inherent porosity can be a significant problem, as it can allow aqueous samples to diffuse beyond the designated test area, potentially introducing electrical disturbances and compromising the accuracy of sensor readings. To solve this problem, a hydrophobic barrier is applied around the test area to confine the liquid flow and ensure that it reaches only the intended region. This is typically achieved by wax printing, a process in which a wax ink is applied to paper to create a defined hydrophobic zone.[22] The wax printing procedure begins with designing the desired pattern using software such as Adobe Illustrator, PowerPoint or Paint. The wax ink is then printed on the paper substrate with a solid ink printer. Next, the printed wax layer is cured at a high temperature (above 100°C), which causes the wax to melt and form a three-dimensional hydrophobic barrier. In some cases, a second layer of wax can be applied, which further strengthens the barrier and improves the durability and robustness of the sensor. The paper typically used in this process is office paper (e.g., Copy 2, 80 g/m² from Fabriano, Italy), which is acid-free, ash-free and treated with an elemental chlorine-free bleaching process. In contrast, plastic-based

substrates possess inherent hydrophobic properties that eliminate the need for wax barriers. Instead, simple adhesive tape can be used to demarcate the test area, effectively isolating it and allowing the sample to flow only within the designated region. This approach is less complex and simpler and offers a practical and economical solution for some applications. The choice of substrate material is therefore a critical decision for optimizing the performance of electrochemical sensors. Paper-based, wax-printed substrates are particularly useful when precise control of liquid flow is required, while plastic-based substrates are more suitable for simpler applications where ease of use and cost-effectiveness are key factors. Both materials offer distinct advantages, and the choice depends largely on the specific requirements of the sensor design, application, and desired performance characteristics.

- *Screen-printing.* The production of SPEs begins with the application of a three-electrode system to a solid substrate using the screen-printing technique. This process, a type of thick-film deposition, relies on a mesh or screen to precisely define the geometry of the electrodes. Typically, the system consists of a WE, a CE and a RE, all printed with different specialized inks. Silver/silver chloride (Ag/AgCl) ink, such as Electrodag 477 SS (Acheson, Milan, Italy), is commonly used for the reference electrode. This ink is applied through a mask specifically designed for the reference electrode to create both the electrode and the necessary conductive connections. A carbon-based ink, such as Electrodag 421 (Acheson, Milan, Italy), is used for the working electrode and counter electrode. These inks provide the ideal properties for electrochemical activity and ensure electrode stability (Figure II.2).

Typically, the WE is about 0.4 cm in diameter, while the complete electrode system measures about 2.5 cm long and 1 cm wide. These compact dimensions allow for efficient performance without compromising sensitivity. After the printing process, thermal curing is performed at 100 °C and 60 °C for 30 and 15 min, respectively when plastic or paper are used as substrates, which is essential to make the printed ink stable for electrochemical measurements.

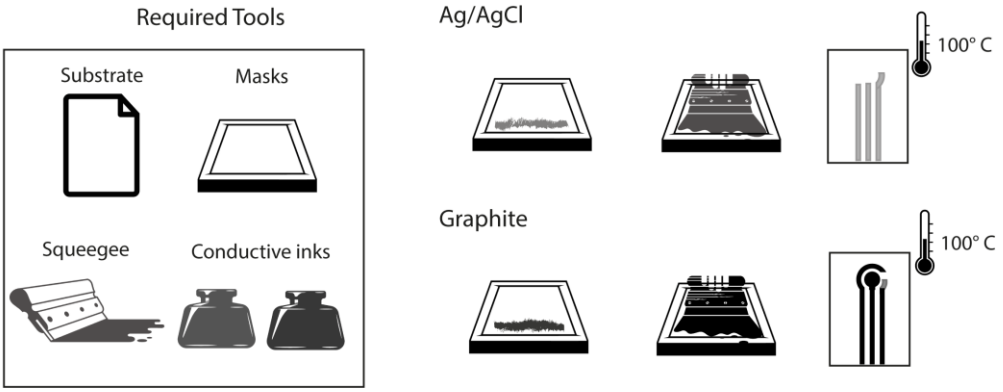

Figure II.2. Screen-printing procedure.

2nd step: using a modifier to enhance and/or tailor electrodes performance

Some materials were introduced into the construction of SPEs to improve their sensing performance, including printed polymer films, antimony films, and graphene-based nanomaterials. The use of biological/biomimetic molecules can also be considered as a modification of the electrode surface toward a specific target analyte (Figure II.3).

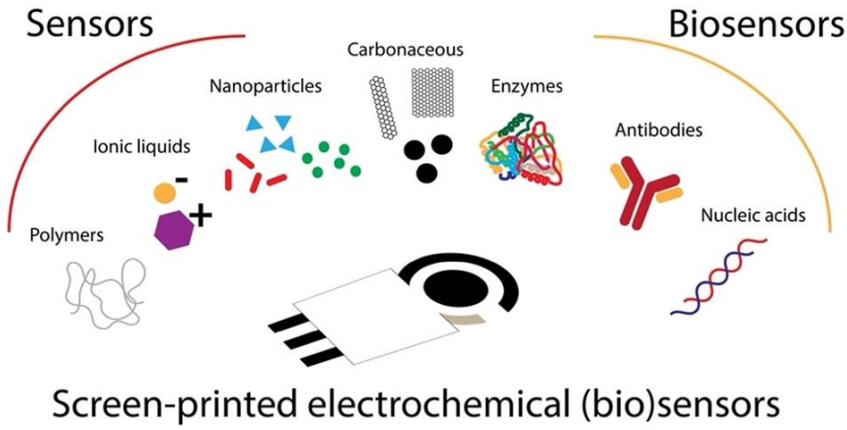

Figure II.3. Modifiers to improve electrode performance.[18]

One strategy to increase the performance and selectivity of electrochemical (bio)sensors is the use of nanostructured materials such as metal nanoparticles, carbon-based nanomaterials (carbon black and graphene), carbon coatings, membranes, and even some conductive polymers can be used to improve performance; it depends on the analytical needs.[23,24] Gold nanoparticles (AuNPs) possess distinctive characteristics, such as high surface area, excellent conductivity and biocompatibility, which make them very suitable for improving the sensitivity, selectivity and electrochemical performance of (bio)sensors. The large surface area of these nanoparticles provides abundant sites for the attachment of recognition elements such as specific ligands or biomolecules, while their catalytic properties contribute to the efficient electrochemical reduction of heavy metal ions, enabling detection at lower concentrations. The use of screen-printed electrodes in combination with gold nanoparticles creates a cost-effective and scalable platform for heavy metal detection, making

it ideal for various applications, including environmental monitoring, industrial safety, and water quality assessment.[25,26]

- *Synthesis of AuNPs.* Gold nanoparticles were synthesized using chloroauric acid ($HAuCl_4$) used as a precursor, sodium citrate as a capping agent and sodium boron hydride ($NaBH_4$) as a reducing agent. Before proceeding with the synthesis, all the glassware and magnetic anchor used were washed thoroughly, first with aqua regia (HCl/HNO_3 = 3 : 1, v/v) three times, rinsed in double-distilled water, washed with a piranha solution (H_2SO_4/H_2O_2 = 7 : 3, v/v) three times, and finally rinsed again with double-distilled water before use; both steps were repeated twice. The synthesis was conducted using 9 mL of double-distilled water to which 1 mL of the 10 mg mL^{-1} solution of chloroauric acid was added. Next, 2 mL of 10 mg mL^{-1} sodium citrate was added, and finally, 500 μL of 20 mM of the reducing agent was added dropwise. The reaction took place at room temperature overnight.[27–29] Once the synthesis was completed and the gold nanoparticles were obtained, they were analyzed by UV/Vis spectroscopy in order to evaluate the successful synthesis.

-*Modification with Carbon-based materials.* Carbon-based materials were extensively incorporated into enzyme-based biosensors to significantly improve the surface-to-volume ratio, which in turn facilitates the docking of more enzyme molecules, enhancing enzyme-substrate interaction. These materials also exhibit high electrical conductivity, excellent optical properties, and biocompatibility, all of which contribute to efficient signal transmission and detection. A prime example of such a material is carbon black, which features fine particles, typically in the nanometer range, and a large surface area. In

the context of enzyme-based biosensors, the combination of carbon black and Prussian blue provides a promising strategy for immobilizing enzymes on screen-printed electrodes. Prussian blue, a dark blue pigment also known as ferric hexacyanoferrate (II), is a coordination compound with the formula $Fe[Fe(CN)_6]^{3-} \cdot xH_2O$. Its characteristic blue color results from the interaction between iron (III) ions and cyanide ligands. Combined with carbon black in biosensors, Prussian blue promotes immobilization of enzymes by creating a stable and biocompatible environment for enzymes. Its distinct chemical structure and electrochemical properties also facilitate electron transfer during enzymatic reactions, improving biosensor performance.[30–32] Meanwhile, the carbon black helps improve the conductivity of the composite. This combination optimizes the overall efficiency of the electrode and enhances enzyme immobilization by taking advantage of the complementary properties of both materials.[33]

3rd step: electroanalysis

Depending on the architecture of the (bio)sensor, different electroanalysis techniques can be adopted to study and quantify various analytes. Commonly used techniques are summarized below:

- ***Cyclic voltammetry.*** Cyclic voltammetry (CV) is one of the most widely used techniques to study the properties and redox behavior of chemical species. CV is a technique that performs a linear scan of the potential applied to the working electrode. This means that the potential applied to the electrode is varied over time in a controlled manner, so that during the experiment the potential is gradually increased or decreased at a constant rate. The result of this scanning is represented graphically with

a cyclic voltammogram, which shows the current (measured in amperes) as a function of the potential (measured in volts). [34–36] In order to better understand a typical cyclic voltammogram, it is important to note that the applied potential takes on a characteristic triangular shape and passes through an electrode immersed in a solution containing electroactive species with oxidation-reduction capacity. In the CV experiment, the triangular potential is scanned back and forth between two extreme values, called "initial potential" and "reversal potential." [37,38] During the scanning of the potential, the system is subjected to cycles of oxidation and reduction of the electroactive compounds in the solution. In this case, the potential is linearly increased from an initial value to a maximum value and then linearly decreased to the same initial value. In conclusione, la CV emerge come una tecnica analitica versatile e fondamentale nel campo dell'elettrochimica, che offre una comprensione approfondita dei processi redox delle specie chimiche. Attraverso questa metodologia, è possibile determinare con precisione i potenziali di riduzione delle specie coinvolte e studiare la loro cinetica elettrochimica.

- ***Differential pulse voltammetry.*** In pulsed voltammetry, a step-in pulse is applied and the current is measured once the capacitive current (IC) has decayed. The pulse width is carefully selected to ensure that this condition is met, allowing accurate measurements. In pulse-based techniques, such as differential pulse voltammetry (DPV) and square-wave voltammetry, the capacitive current is effectively removed by

subtracting it from the total current signal. Differential pulse voltammetry (DPV) works specifically by comparing current values at two distinct points: just before the end of the pulse and just before the beginning of the pulse. The difference between these two currents is plotted against the scale potential, resulting in a characteristic peak-shaped waveform. DPV is widely used to improve the sensitivity of electrochemical measurements, making it particularly effective for detecting analytes at very low concentrations. This enhanced sensitivity is particularly advantageous in applications where precise detection of trace substances is required.[39]

- ***Square wave voltammetry.*** Square wave voltammetry (SWV) is an advanced electrochemical technique known for its exceptional sensitivity. SWV stands out because it combines the strengths of several electrochemical techniques.[40] It combines the high sensitivity of pulse methods, the detailed knowledge of electrochemical mechanisms provided by cyclic voltammetry, and the information on the kinetics of rapid electrochemical processes offered by impedance techniques. As a result, SWV is considered one of the most powerful methodologies in electrochemistry. This technique works by applying a modified potential scale, generating square-shaped pulses of potential. The potential is varied gradually at a high frequency, usually between 20 and 100 Hz.[41] Each potential step consists of two opposite pulses: a forward (direct) pulse and a reverse (reverse) pulse, each with identical duration and amplitude.[40,42] These alternating pulses create a potential cycle,

allowing cathodic and anodic scans of the electrode without the need to reverse the overall potential, as required by cyclic voltammetry. In SWV, a square wave is superimposed on the applied potential during the voltammetry process. The currents at the end of the forward and reverse pulses are recorded as a function of the scaled potential. This approach increases sensitivity and reduces background noise, making SWV particularly effective for detecting analytes at low concentrations and achieving a better signal-to-noise ratio.

- ***Chronoamperometry.*** In chronoamperometry (CA), a constant potential is applied to the electrochemical system, and the resulting current is recorded over time. The main mechanism driving mass transport in this technique is diffusion, and the current-time response provides valuable insights into concentration changes near the electrode surface. As the reaction progresses, the diffusion layer, responsible for transporting the reactants to the electrode, continues to expand. This results in depletion of the reactant at the surface, causing a gradual decrease in the concentration gradient. As a result, the current decreases with time, reflecting these changes. CA is widely used to study various electrode processes, particularly to study reaction kinetics and understand the rate at which reactions occur. It is also an effective method for monitoring changes in the concentration of specific analytes over time, making it useful in many applications, including electrochemical detection and analysis. This technique provides detailed information about the behaviour of

electrochemical reactions, helping researchers understand the dynamics of electron transfer, the rates of electrochemical reactions, and the overall transport processes at the electrode interface.

- ***Impedance Spectroscopy.*** Electrochemical impedance spectroscopy (EIS) is a powerful analytical technique used to study the electrical properties of electrochemical systems. It provides information about the various physical and chemical processes occurring within these systems, making it valuable for a wide range of applications. Impedance spectroscopy is a technique that measures the impedance of an electrochemical system over a range of frequencies, providing valuable information about the electrical properties of the system. By analyzing impedance, this method helps to characterize the electrode interface, including important parameters such as resistance and capacitance. The technique makes it possible to study the physical and chemical processes occurring within the system by observing its response at different frequencies.[43]
- ***Anodic stripping voltammetry.*** Anodic stripping voltammetry (ASV) is an advanced electrochemical technique widely used for the sensitive detection and quantification of trace metals in a variety of samples, with a focus on environmental analysis. The method involves two basic steps: initially, metal ions are reduced and deposited on the surface of an electrode under controlled potential conditions. After a defined deposition period, the potential is then scanned in a positive direction, causing the deposited metals to be oxidized and return to their

ionic form, generating distinct current peaks that correspond to the specific metals present. The ASV is particularly valued for its high sensitivity, which allows detection limits in the parts-per-billion range. This makes it very effective for monitoring pollutants such as heavy metals in environmental samples such as water and soil. In addition, the ASV's ability to analyze multiple metal ions simultaneously increases its versatility, making it an essential tool for both environmental monitoring and industrial applications. The process involves potential-controlled electrolysis to deposit the analyte on a working electrode, followed by oxidation to regenerate the ion species. Because of its ability to detect low concentrations of electroactive species, ASV is the preferred method for trace analysis of heavy metals and other similar substances.[44,45]

All electrochemical measurements will be made using a portable, battery-powered instrumentation, EmStat Blue (PalmSens), which is a small USB and battery-powered potentiostat connected to a laptop computer. The currents can be recorded and displayed on a laptop and/or smartphone using a dedicated application, e.g., PStouch, which can be used with all PalmSens and EmStat potentiostats.

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CHAPTER 3: INNOVATIVE GREEN-BIOBASED MATERIALS FOR ELECTROANALYTICAL SENSORS AND BIOSENSORS

The goal of the first part of this PhD was to develop new sustainable platforms consisting of electrochemical sensors that could be used for the detection of organophosphate pesticides whose use is an environmental hazard. The need for greener alternatives in sensor technology has grown in recent years, especially given the widespread environmental impact of traditional plastic-based materials. Here it is described in this chapter the optimization and characterization of bioplastic-based substrates applicable to sensor and biosensor platforms. Polymer-based devices were developed that can serve as environmentally friendly sensing solutions, as they can replace traditional non-degradable polyester-based substrates.[1] Bioplastics (using polyhydroxyalkanoates (PHAs) and modified cellulose derivatives) are proposed as an alternative to traditional materials and are intended to meet the growing demand for useful technologies in food safety and environmental monitoring.

Screen-printed conductive inks were used to construct electrochemical sensors on substrates for the determination of organophosphate pesticides. Pesticide identification, which is the focus of this study, will thus be in congruence with the world-dominating philosophy of sustainability. As the results presented so far indicate, PHA-based materials hold great promise for application as sensors, also contributing to the broader goal of minimizing the environmental impact associated with diagnostic technology. The successful

incorporation of these sustainable materials into electrochemical sensing platforms paves the way for green technology and innovation for environmental monitoring.

3.1 CHARACTERIZATION AND APPLICATION OF POROUS PHBV-BASED BACTERIAL POLYMERS TO REALIZE NOVEL BIO-BASED ELECTROANALYTICAL (BIO)SENSORS

3.1.1 Introduction

Since the publication of the United Nations 2030 Agenda for Sustainable Development in 2015,[2] sustainability has become the primary driver for the development of new technologies, approaches, and actions.[3] A clear example of this evolution is the improvement of cost-effective and sustainable diagnostic techniques during the Covid-19 pandemic, which contributed to the enhancement of social sustainability indices through the development of point-of-care tests.[4] Globally, research and development in the field of sensing devices and transducers is experiencing an interdisciplinary surge, spanning topics such as materials, procedures, optimization strategies, and applications.[5–9] The creation of disruptive technologies has become a common requirement for the sustainable improvement of sensor performance and functionality.

Among the various sensor architectures, electrochemical sensors stand out for their simplicity, making them a reliable alternative to other techniques due to their ability to measure coloured or turbid solutions, their suitability for continuous monitoring, and their potential for reagent-free settings.[10–12] The electrochemical glucose biosensor used to monitor blood sugar levels (glycemia) is the most widely sold device worldwide,[13] and the literature contains numerous examples of decentralized printed electrodes for fatigue/stress monitoring through

the skin, liquid biopsy on-chip, and tools for precision agriculture to be used on crops.[14–17]

However, the development of "green" sensing platforms plays a fundamental role in reducing the use of hazardous reagents and, consequently, in minimizing the formation of unwanted by-products, which are one of the main issues in waste management and disposal.[18,19] It is estimated that 86% of all plastic packaging is used only once before being discarded, generating a waste stream that persists in the environment and releases harmful pollutants.[20] E-waste also represents a significant environmental issue, and the development of electrochemical strips is increasingly focused on finding "greener" alternatives to plastic-based platforms, including biodegradable and sustainable materials.[21] Although paper-based platforms were demonstrated to be a valuable alternative to traditional plastic materials, it should be noted that the costs and procedures associated with their production (depending on purity and porosity) are not always advantageous. Indeed, although cellulose is an abundant, renewable, biodegradable, and non-toxic polymer, its purification from plant fibers involves chemical treatments (e.g., extraction, bleaching).[22] The choice of polymer-based materials is widespread due to their wide range of mechanical and functional properties, along with their lightweight nature, making them highly appreciated in several technological fields.[23–26] The use of fully bio-based and biodegradable materials, such as bacterial polymers, represents a promising alternative.[27] Among these, polyhydroxyalkanoates (PHA) are particularly attractive due to their well-known outstanding biodegradability in open environments, biocompatibility, and

bioresorbability.[28,29] Typically, food and agricultural wastes are used as feedstock in the bioconversion process,[30,31] but biomasses from the degradation of industrial textile dyes or post-consumer agricultural polyethylene have also been found to be suitable feedstocks for PHA production.[32,33] The nature of the substrate and the bacterial strain are key factors in obtaining different types of hydroxyalkanoic monomers, which can be utilized by microorganisms during the biosynthetic polymerization process.[34]

Today, various types of PHAs, both homopolymers and copolymers, can be produced.[35] In particular, the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) was extensively studied in various technological fields due to its malleability and processability. PHBV was primarily used in biomedical applications for its biocompatibility, but it has also been explored as a piezoelectric sensor, a thermo- and humidity-triggered actuator, and for producing green electronics.[36–41] Depending on specific needs, PHBV can be customized with other materials; for example, it can be made more hydrophilic by adding cellulose-based compounds.[42]

Regarding the use of bacterial substrates in sensing applications, some examples were reported in the literature, mostly focusing on bacterial cellulose due to its mechanical properties, biocompatibility, and porous structure, which can mimic the extracellular matrix of the skin. Morales-Narváez developed a plasmonic bacterial nanopaper-based device for the evaluation of uncontrolled UV exposure.[39] Similar platforms, based on luminescent macrofibers with quantum dots on bacterial cellulose nanofibers, were developed to measure pH and glucose in both serum and saliva.[40] Silva et al. demonstrated the

suitability of printing electrodes onto microbial nanocellulose produced by oxidative fermentation for detecting lead, cadmium, uric acid, and 17β-estradiol in sweat.[41] Sgobbi showcased the use of bacterial cellulose to develop sweat lactate biosensors by printing conductive inks modified with Prussian Blue and lactate oxidase enzyme.[42] Although the use of bacterial nano- and micro-cellulose was explored for both colorimetric and electrochemical sensing, the use of PHBV-based substrates has not been reported in this context. In this study, the aim was to thoroughly characterize both PHBV and a cellulose-modified PHBV as novel substrates for application in the (bio)sensor field. Specifically, these two alternative substrates were screen-printed with conductive inks to create electrochemical strips, and their performance was evaluated and compared with traditional polyester (the most commonly used substrate in screen-printed manufacturing). The substrates were analyzed through various approaches, including morphological (scanning electron microscopy (SEM), contact angle), mechanical (tensile tests), electrochemical (cyclic voltammetry, chronoamperometry, impedance spectroscopy), and (bio)analytical (detection of iron ions and organophosphate pesticides) techniques. In particular, the determination of organophosphate pesticides, is very useful given the growing importance of these compounds in food safety and environmental protection. The various characterization methodologies highlighted the potential of PHBV-based materials for future sustainable applications in the electroanalytical field, with a focus on their use for monitoring and detection of organophosphate pesticides.

3.1.2 Experimental section

Materials

Chloroform ($CHCl_3$) and methanol were purchased from Sigma Aldrich and used without further purification. PHBV (Sigma Aldrich, 8 mol% of hydro-valerate (HV) unit) was purified before its use in order to completely remove the fermentation residues. Specifically, as-received PHBV was solubilized in warm $CHCl_3$ for 2 h, filtered through Celite and reprecipitated in a large excess of cold methanol, as previously reported elsewhere.[43] Number average molecular weight (Mn) of 235,600 g mol^{-1} and weight average molecular weight (Mw) of 677,300 g mol^{-1} were determined after the purification step by gel permeation chromatography (GPC). Polyethylene glycol monomethylether 1900 (PEO) was purchased from Alfa Aesar. Micro-fibrillated cellulose (MFC) aqueous suspension (concentration of 3.2 wt%) was produced by previously established optimized method,[44] starting from bleached Eucalyptus wood pulp. Metals standard, butyrylcholinesterase, organophosphate inhibitors have been purchased from Merck. Polyester sheets for comparison experiments were purchased from MacDermid (UK) and the polyester was a Autostat HT5 hazy and heat stabilized.

Preparation of dispersible MFC

The MFC aqueous suspension (as prepared) cannot be added directly to the polymeric solutions for miscibility reason. Also, the simple drying of the MFC suspension is not a viable option since it leads to MFC aggregation, which is difficult to redisperse in the polymeric solution. To overcome this problem, an aqueous PEO solution (10 wt%) was added to the aqueous MFC suspension and the solution

was stirred at room temperature using an ULTRA-TURRAX® stirrer operating at 8500 rpm and sonicated for 1 h. The so-obtained PEO/MFC mixture was then freeze-dried by an Epsilon 2–4 LSCplus (Martin Christ GmbH). The very fast freezing and the water removed in supercritical conditions allows obtaining a composite material mainly constituted by MFC micro-fibrillated in solid PEO. The prepared MFC-based material was then finely cryo-milled (using liquid nitrogen) by an IKA A10 basic.

Substrate preparation

Purified PHBV was solubilized in $CHCl_3$, and casted in a petri dish (diameter 15 cm) then left to dry overnight at room temperature under aspiration hood. Two substrate thicknesses were prepared. Specifically, 0.8 g of PHBV were dissolved in 15 mL of $CHCl_3$ to obtain a film with a thickness of approximately 50 µm, whereas 2.5 g of PHBV in 25 mL of $CHCl_3$ were used to obtain a substrate 150 µm thick.

To improve the typical low hydrophilicity of the PHBV-based materials, 5 wt% (with respect to the polymer amount) of the prepared MFC was added to the aforementioned PHBV/$CHCl_3$ solutions. The dispersion of the MFC in the polymeric solutions was obtained by the combination of magnetic stirring and dip-sonication for about 5 h before casting the solution in the petri dish.

Fabrication of the electrochemical strips

To fabricate the electrochemical strips, serigraphy was adopted with an in-house experimental setup. Following the selection of the substrate (i.e. polyester, PHBV, PBHV/MFC) the three electrodes were manually screen-printed as described in the Chapter 2. After each step,

the ink was cured for 30 min in the oven at 70 °C. The diameter of the working electrode was 4 mm.

Scanning electron microscopy

SEM analyses were performed by an Ultra Plus FESEM scanning electron microscope (Zeiss, Germany). Cut surfaces of all samples were mounted on stub and gold-sputtered at 20 nm thickness with the HR208 Cressington sputter coater and analyzed at 5–10 kV with an SE2 detector as already reported.[45]

Tensile tests

Mechanical properties of the two PHBV-based substrates were characterized by a universal tensile tester (Instron 3365 A). In addition, a polyester sample, that is the gold-standard substrate, was also tested. The samples were prepared for the tests by scissor cutting a rectangular shape of dimensions 30 mm × 10 mm (L x W).

The tensile machine was equipped with a 500 N load cell and screw side action tensile grips. Load and extension data were recorded at a sampling frequency of 100 Hz. The mechanical failure tests were performed by straining the samples at a constant speed of 5 mm/min until failure occurred.

Contact angle characterization

Static water contact angle (WCA) was characterized by DSA30S (KRUSS) contact angle goniometer equipped with a digital camera. Water (Milli-Q) droplets of 4 μL were placed on the surface of the prepared substrate. Six static measurements were carried out on each sample to evaluate the average WCA value and corresponding standard deviation. Since the prepared substrates show a rougher side and a flat side due to the solvent casting method used for the preparation, the

WCA measurements were carried on both sides of the substrate to evaluate how the surface roughness affects the hydrophilicity of the surface.

Electrochemical measurements

All the experiments were carried out using screen-printed electrodes connected to a portable potentiostat (PalmSens 4) and all the measurements were performed by covering the three electrodes (working, counter, reference) with a 100 µL drop of the working solution.

3.1.3 Results and discussion

3.1.3.1 Morphological characterization

The first characterization of the two substrates was consistent with a morphological observation, to evaluate the porosity of different materials with regard to their composition. In fact, the two PHBV-based substrates were prepared with different hydrophilic properties. It should be noted that, in particular for printed electroanalytical application, the hydrophilicity might affect the deposition of conductive inks, with different performances of the printed strips, as reported in literature.[46] For this study the two different substrates were prepared using different thicknesses, namely 50 mm and 160 mm.

In addition to this, following the preparation procedure the two sides of the PHBV-based films displayed different roughness, depending on the fact that one side was in contact with the glass of the petri dish (it will be called the “bottom side”), and the other side was in contact with air (it will be called the “top side”). Due to this reason, the first

characterization involved the evaluation of the morphology through SEM experiments, as shown in Figure III.1.

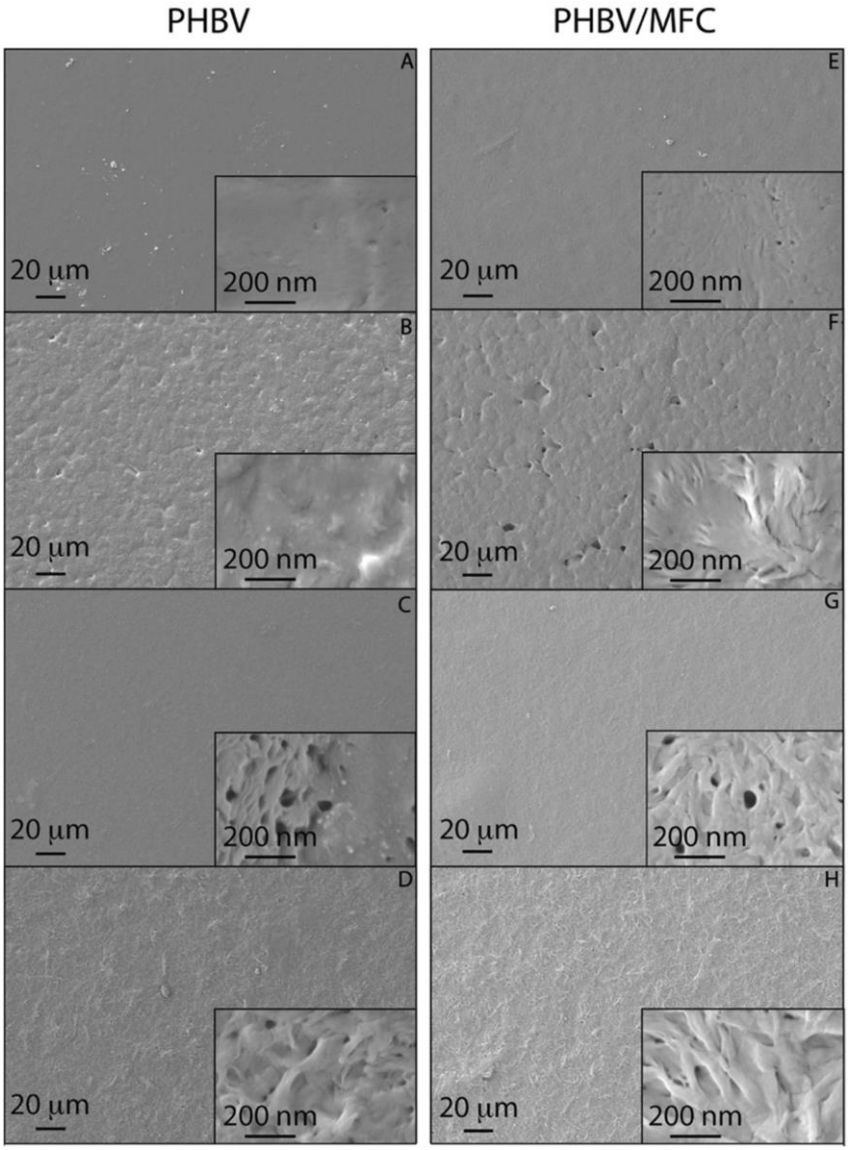

Figure III.1. SEM micrographs of PHBV and PHBV/MFC substrates. In particular A, B, E, F correspond to 50 µm substrates, and C, D, G,

H correspond to 160 μm substrates. A, E, C, G correspond to bottom side, while B, F, D, H correspond to top side.

The experiments were performed acquiring different magnifications and analyzing two different sides of each sample. In detail, Figure III.1A, B, E, F correspond to top side and bottom side of 50 μm PHBV and to bottom side and top side of 50 μm PHBV/MFC, respectively. It was evidenced that the bottom sides showed a homogeneous and smooth surface, while the top sides revealed a more marked roughness. Regarding the 160 μm samples, Figure III.1C, D, G, H displayed a greater porosity, and while the pure PHBV samples appeared wrinkled, the PHBV/MFC revealed a dense fibrotic network which was appreciated both on bottom and tops sides, ascribable to the presence of the micro-fibrillated cellulose during preparation.

Mechanical properties evaluation

The second characterization focused on evaluating the mechanical properties of the two PHBV-based substrates. To perform these studies, all the substrates were chosen with a thickness of 160 mm due to two main reasons: 1) the thicker substrate was easier for the handling and the screen-printing, and 2) this was the same thickness as the polyester sensors that is mainly used to realize flexible devices. Figure III.2 shows the results of the mechanical tests.

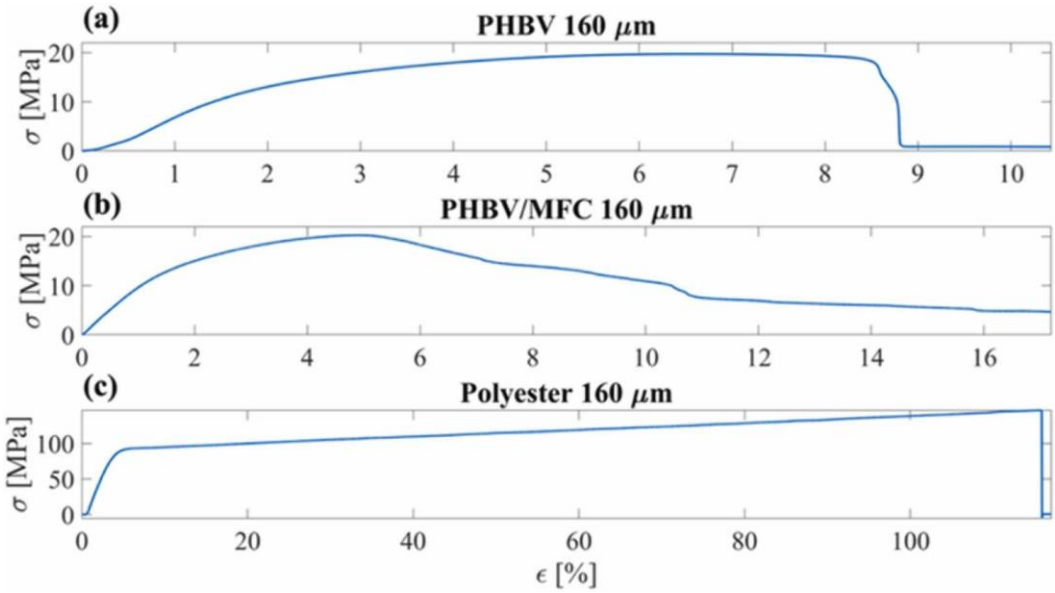

Figure III.2. Stress-strain curves of the two samples and the control. (a) Stress–strain curve of the PHBV sample; (b) Stress–strain curve of the PHBV/MFC sample; (c) Stress–strain curve of the control (i.e., Polyester sample) showing a much larger value of fracture than the two previous samples.

As reported, the two PHBV-based substrates showed mechanical failure under an e value of approximately 10%, while the control showed failure at approximately 115%. The two substrates showed a maximum stress before failure with slight differences: the PHBV maximum stress was 19.7 MPa, while the PHBV/MFC maximum stress was approximately 20.3 MPa. The polyester showed a value much higher than the two examined samples (i.e., 146.7 MPa).

Contact angle measurements

The solvent casting method leads to substrates characterized by different roughness on the two sides of the sample. In particular, the one that is in contact with the Petri dish during the drying step presents

a very flat and smooth surface, whereas the side exposed to the air is usually rougher. On the other hand, the addition of MFC does not significantly affect the typical whitish color of the PHBV, which is due to the known high crystallinity degree of the PHAs (Figure III.3A).[47]

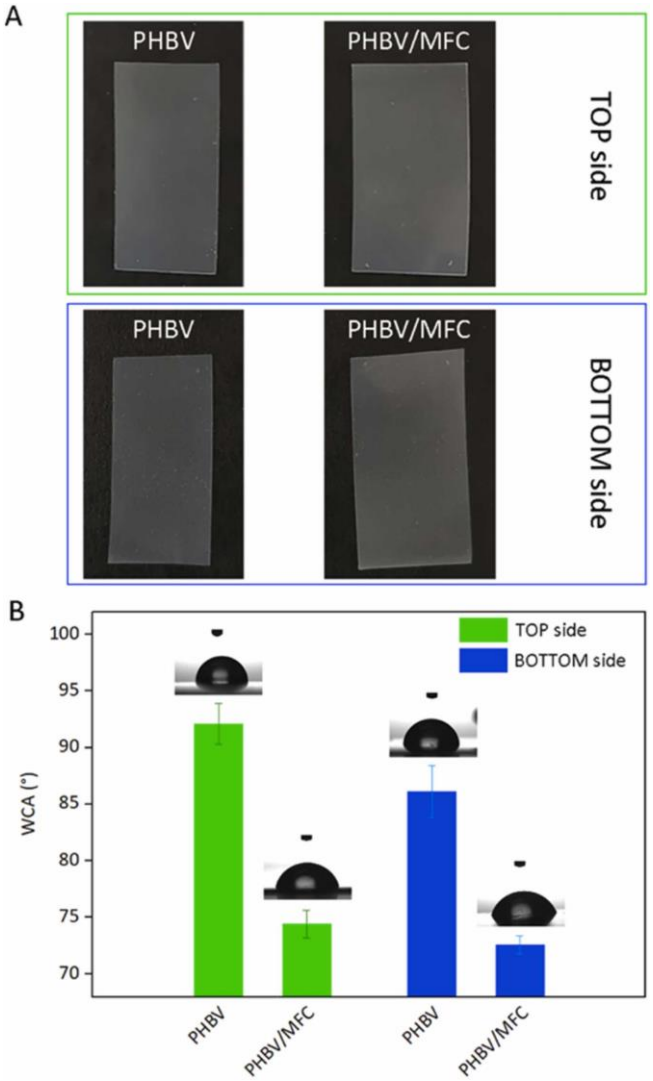

Figure III.3. (A) Photographs of both sides of the prepared specimens (160 µm thick). (B) Water contact angle (WCA) of the bottom and top sides of neat PHBV and PHBV/MFC substrates.

Macroscopically, the top side (rougher) appears opaquer than the bottom side (smoother), which instead is slightly bright, since it scatters less the light than the top side. As shown in Figure III.3B, the top side of the neat PHBV sample has a WCA above 90° as previously observed for this bio-polyester.[48] Interestingly, the incorporation of

only 5 wt% of MFC significantly drops the WCA of neat PHBV decreasing its value of 18° (Figure III.3B). This takes place thanks to the large number of hydroxyl groups of MFC, which increase the polymer surface free energy and leads to an increase of the wettability of the surface, similarly to what has been reported for traditional polyolefins.[42] With respect to top side, the WCA of the bottom side is slightly less affected by MFC (approximately 13°). However, also in this case the incorporated MFC decreases the WCA of the PHBV following the same trend observed for the top side (Figure III.3B). It is known that the roughness has an important effect on the final WCA of a surface,[49] thus explaining what is observed here, where the rougher surface (top side) shows a higher WCA compared to the smooth one regardless the addition of MFC.

3.1.3.2 Electrochemical characterization

Electrochemical impedance spectroscopy characterization

Electrochemical impedance spectroscopy was carried out at + 0.2 V in 0.1 M potassium chloride in the presence of 5 mM ferricyanide and the results are presented in Figure III.4. In the complex plane plots (Figure III.4a and inset) all the samples show a semicircle for high frequencies and a straight line at lower frequencies, with a slope below 45° for PHBV /MFC bottom side, PHBV /MFC top side and Polyester and above for PHBV bottom side and PHBV top side. The equivalent circuit used to fit the PHBV /MFC bottom side, PHBV /MFC top side and Polyester data is the typical Randles circuit (Figure III.4b, inset) composed by the cell resistance (R_W) in series with the electrical double layer capacitance (CPE_{dl}) and the charge transfer resistance (R_{ct}), respectively in parallel. In series with the latter there is an open

Warburg element (W), which describes the mass transfer of the system. For the hydrophobic samples (PHBV bottom/top side) the open Warburg element is replaced with the support capacity (CPE_s), since the hydrophobicity of the support slows down the mass transfer which is not observable.

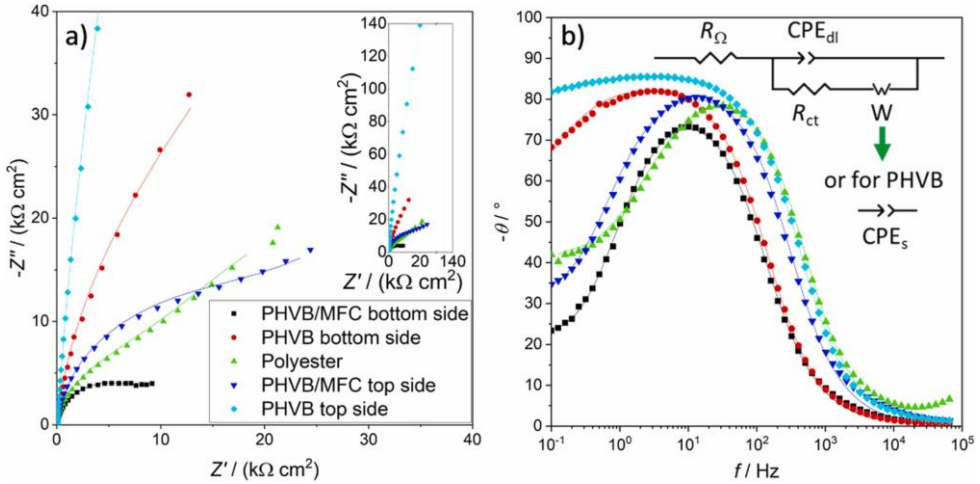

Figure III.4. EIS complex plane (a, magnification in inset) and Bode phase (b) plots registered at 0.2 V in 5 mM ferricyanide solution. Equivalent circuits used to fit impedance data are presented as inset.

Another element that demonstrates that hydrophobicity does not favor electrochemical performances is the detection of the maximum impedance value for these latter samples, in the order PHBV top side > PHBV bottom side > Polyester and PHBV/MFC top side > PHBV/MFC bottom side. In all cases, instead of a pure capacity, a constant phase element is used, which reflects the presence of surfaces that are not perfectly homogeneous, which deviate from ideality, measured by a parameter, between 0.5 and 1.

The Bode graph of the phase is in line with the previous results: at low frequencies PHBV/MFC bottom side, PHBV/MFC top side and

Polyester show points below 45°, while PHBV bottom side and PHBV top side above, confirming the presence of the open Warburg element or capacity, respectively. Furthermore, the trend is very similar for PHBV/MFC bottom side, PHBV/MFC top side and Polyester and for PHBV bottom side and PHBV top side. However, at higher frequencies the Polyester trend is more similar to PHBV bottom side and PHBV top side. The results previously obtained are confirmed by the parameters derived from the fitting (Table III.1).

Table III.1. Parameters values obtained from the fitting with equivalent circuits shown in Figure III.4, inset.

Sample	R_{Ω} / (Ω cm^2)	CPE_{dl} / (μF cm^{-2} s$^{\alpha-1}$)	α_{dl}	R_{ct} / (kΩ cm^2)	CPE_s / (μF cm^{-2} s$^{\alpha-1}$)	R_W / (kΩ cm^2)	α_W	τ / s
PHBV /MFC bottom side	73.08	27.51	0.92	8.28	-	0.039	0.10	0.0009
PHBV bottom side	43.74	38.41	0.94	84.62	41.62	-	0.77	-
Polyester	43.97	10.59	0.95	8.45	-	71.99	0.51	13.29
PHBV /MFC top side	51.07	14.68	0.95	18.28	-	5.80	0.26	0.23
PHBV top side	49.21	10.65	0.96	1008.24	1.75	-	0.64	-

The morphology of the support (bottom or top side) is fundamental in the formation of the electric double layer, as demonstrated by the similar CPE_{dl} values for bottom and top side surfaces, respectively. These values are higher for the bottom side ones because the capacitor that is formed is more uniform. The results are also confirmed by the CPE_s values. For the electrochemical performances, hydrophilicity plays a key role. In fact, the lowest R_{ct} is for PHBV /MFC bottom side, with a value comparable to Polyester taken as a reference, followed by PHBV /MFC top side. Hydrophobic samples (PHBV ones) perform less, with better results for smooth substrate. Furthermore, the PHBV /MFC bottom side and PHBV /MFC top side samples are more performing than Polyester due to the lower resistance to mass transfer (R_W), a phenomenon aided by the absorbency of the cellulose.

Cyclic voltammetry and chronoamperometry characterization

It should be considered that the choice of the substrates where the electrodes will be screen-printed may affect the electrochemical performance of the ultimate device. In fact, the adoption of porous materials, i.e. paper-based, could reduce the diffusion of the electrochemical species and thus making the device not appropriated for real application. To evaluate the effect of the different substrates on the effectiveness of the printed strips, cyclic voltammetry experiments have been carried out in presence of a 100-µL drop containing 5 mM ferricyanide dissolved in 0.1 M potassium chloride interrogating the scan rates, from 0.02 to 0.5 V/s (Figure III.5).

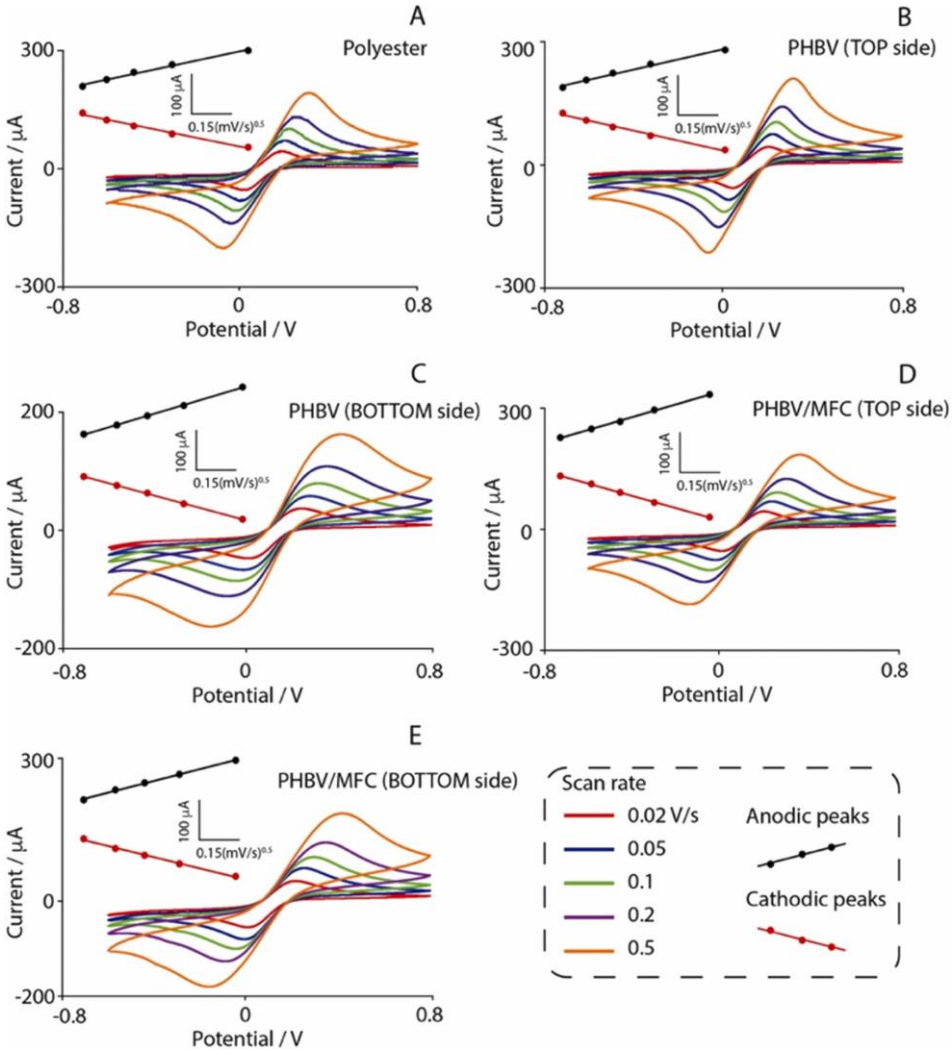

Figure III.5. Cyclic voltammetric experiments of electrodes screen-printed onto A) polyester, B) PHBV (TOP side), C) PHBV (BOTTOM side), D) PHBV/MFC (TOP side) and E) PHBV/MFC (BOTTOM side). All the experiments were carried out through the analysis of 100 mL drop of a solution containing 5 mM, using various scan rates, namely 0.02, 0.05, 0.1, 0.2 and 0.5 V/s. Inset: linearity among the intensity of the anodic (black curve) and cathodic (red curve) and the square root of the tested scan rates.

As is clearly visible, for all the substrates that were used to screen-print the strips, the cathodic and anodic voltammetric peaks resulted linear to the square root of the scan rate, and this behavior was consistent with a semi-infinite diffusion of the electroactive species at electrode surface, showing that reagents were able in diffusing and reaching the electrode area without any entrapment,[50–52] that might be observed in presence of porous substrates. The approximated slopes relative to the correlation existing between the anodic curves and the square root of the scan rate were calculated equal to ca. 238, 260, 200, 241 and 208 mA* $(s/mV)^{0.5}$ respectively to polyester, PHBV (TOP side), PHBV (BOTTOM side), PHBV/MFC (TOP side) and PHBV/MFC (BOTTOM side). Even if the PHBV/MFC displayed the presence of a cellulosic content, it was not relevant in affecting the semi-infinite diffusion of the model redox probe. No significant differences were observed within the experimental error, considering a 10–15% variation due to the manually scree-printing process. Moreover, for all the substrates tested, the ratio between the anodic current and the cathodic current was comprised in the 0.89–1.05 range. The substrates' performance were also tested after 50 bendings by carrying out a cyclic voltammetry in presence of 5 mM ferricyanide prior and after the bendings. As reported in Figure III.6 a slight difference in the signal was observed after 50 repetitions: polyester and PHBV/MFC were consistent with a decrease of the signal less than 7%, while PHBV showed a decrease of ca.13%. A possibility could be ascribable to the presence of cellulose which allowed a major resistance to these stresses.

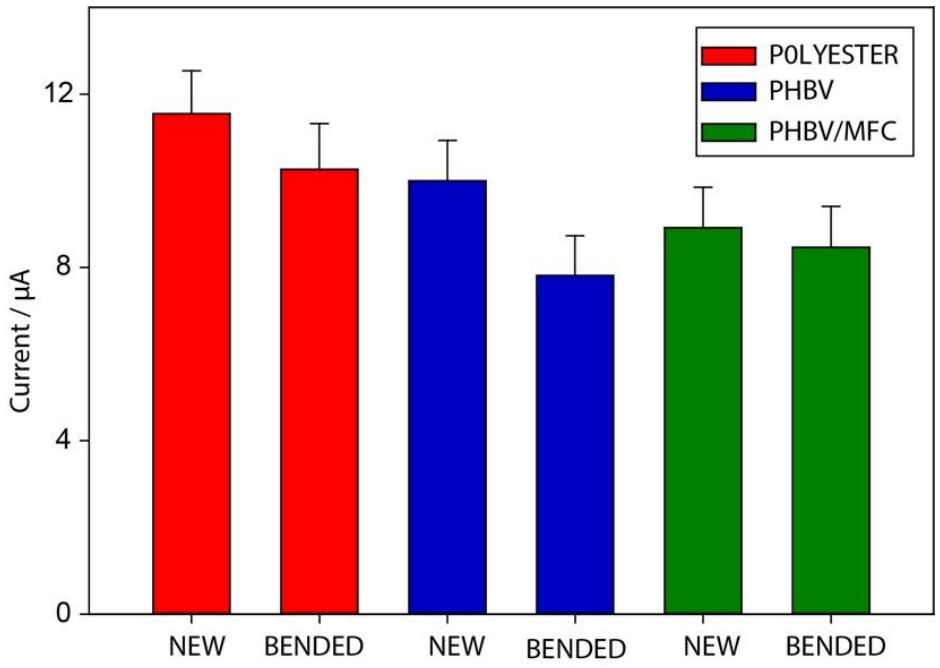

Figure III.6. Bending experiments measuring the anodic peak in presence of 10 mM ferricyanide prior and after 50 bendings.

In addition with these investigation, in order to evaluate the diffusion of the redox probe at the different substrates, the diffusion coefficient (D) for ferricyanide was calculated using chronoamperometry experiments in absence and in presence of

different concentrations of ferricyanide, namely 2, 4 and 8 mM, as reported in Figure III.7.

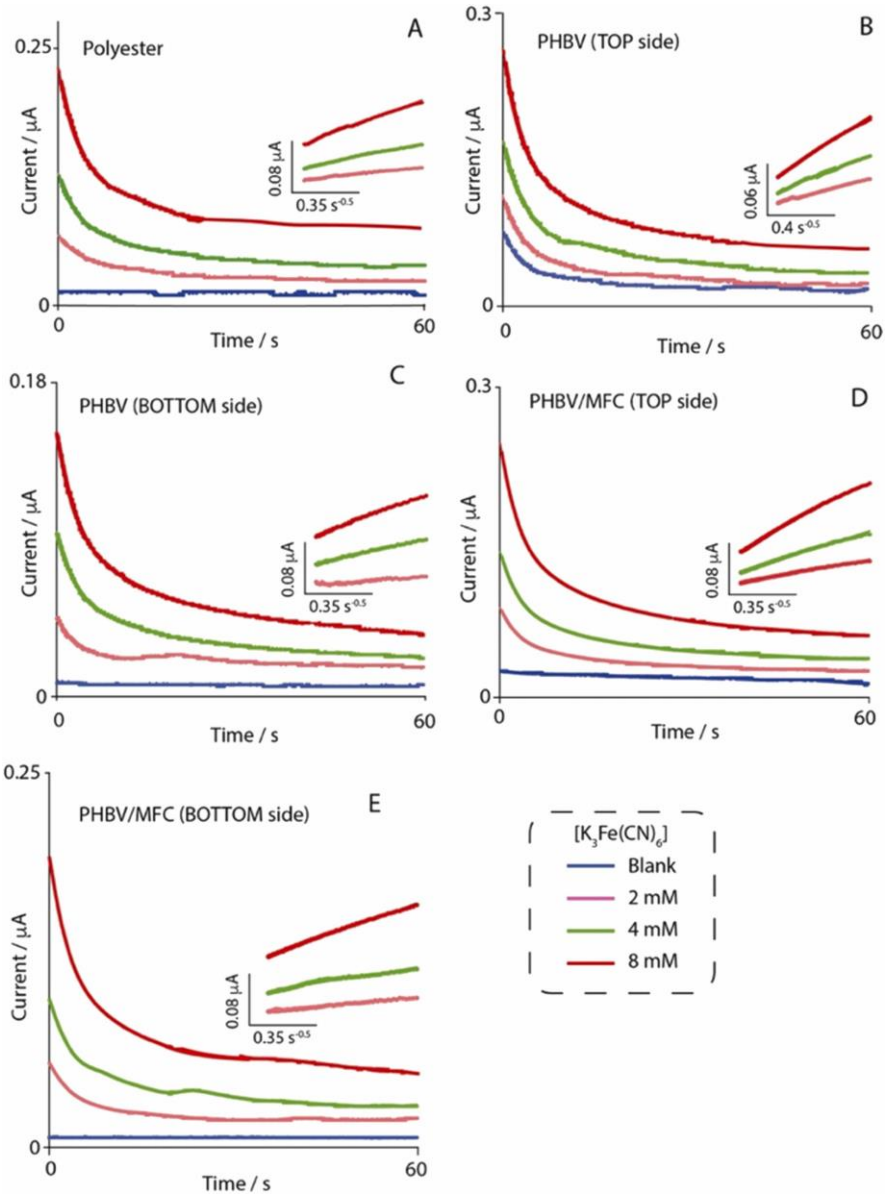

Figure III.7. Chronoamperometry experiments carried out in a 100-mL drop of 0.1 M KCl in absence (blue line) and in presence of 2 (pink line), 4 (green line) and 8 mM (red line) of ferricyanide. A, B, C, D and

E are relative to the use of, respectively, polyester, PHBV (TOP side), PHBV (BOTTOM side), PHBV/MFC (TOP side) and PHBV/MFC (BOTTOM side). Insets: show plots of I vs. $t^{0.5}$ obtained for the three different ferricyanide concentrations tested. All the measurements were carried out by applying 0.4 V (vs. Ag/AgCl) for 60 s.

To estimate the diffusion coefficient of the redox specie in combination with the selected substrates, Cottrell equation was taken into account.[53] All the experiments were performed by screen-printing the same amount and type of graphite-based ink, and according to the Cottrell approximation, the sampled current resulting from a potential step decay to zero inversely proportional to the square root of time. As reported in Figure III.7, the main images report chronoamperograms at three levels of ferricyanide concentration (plus the measurement carried out in 0.1 M KCl), while the insets are obtained by plotting the current vs. square root of time. If the slopes of these linear curves are plotted against the redox probe concentrations, the diffusion coefficient (D) for ferricyanide can be evaluated following the Cottrel equation. For all the tested substrates, the calculated diffusion coefficient for ferricyanide was ca. 10^5 cm^2/s and this appeared to be in good agreement with those reported in previous reports.[46,54] What it should be noted is that, in the present experimental setup, the presence of cellulosic portion does not play a crucial role in hindering the diffusion of ions considering the species contained in the working solution diffuses through the bottom (where the electrodes are screen-printed). However, the roughness of the substrates often represents the first drawback while printing electrochemical device, due to a lower

adhesion between the mask and the substrate; for this reason the following studies have taken into account the “BOTTOM side” of each of the two PHBV-based materials in comparison with polyester.

3.2.3 Iron sensing and organophosphates biosensing

The analysis of metal ions is of high importance in the field of sensors, in fact, their presence gives indications for many purposes, from diagnostics to agriculture, through environment and industry. Among the metals and their sensing importance, iron ions represent a versatile target that is related to various context: anemia affects a majority of individuals with advanced chronic kidney disease,[55] iron plays a fundamental role in the metabolism and growth of plants,[56] and the presence of iron in wastewater in industrial factories need to be carefully evaluated, such as the wastewater produced following manufacture of steel.[57] In this study, the three substrates were compared to the electrochemical detection of iron ions through the use of anodic stripping voltammetry. Briefly, the iron present in the working solution was initially electrodeposited on top of the working electrode and successively oxidized back in solution, and it produced a detectable signal (anodic peak) that was related to the amount of the ion present in the solution. The comparison among the three substrates was carried out by analysing iron ions in the ppm range, as showed in Figure III.8.

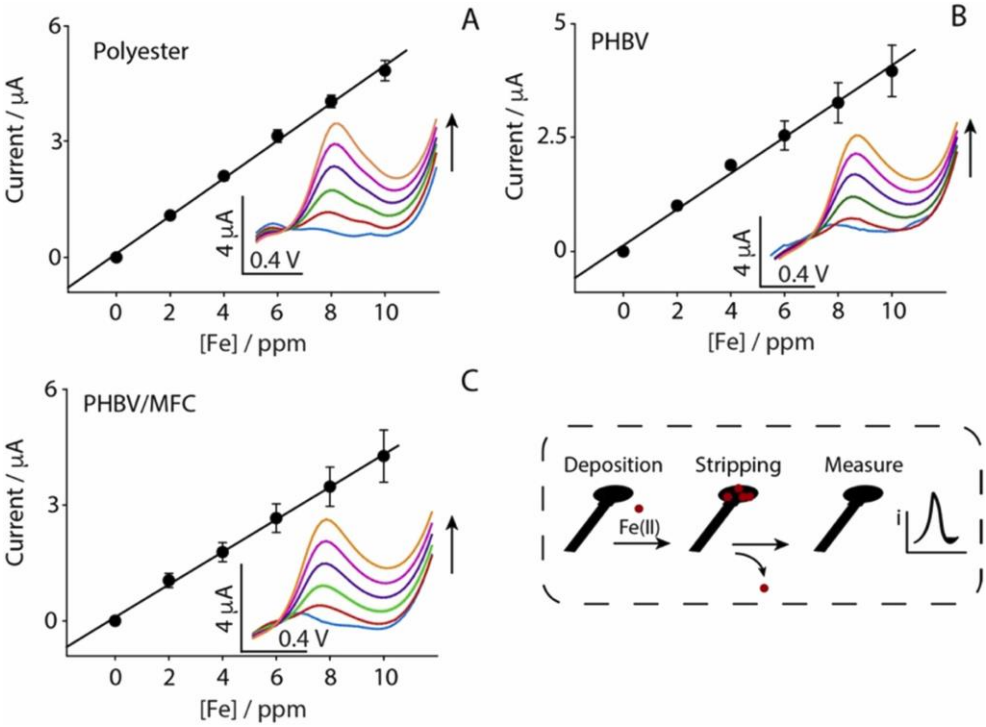

Figure III.8. Stripping voltammograms for increasing Fe levels up to 10 ppm obtained onto electrodes that were screen-printed on A) Polyester, B) PHBV and C) PHBV/MFC. Corresponding calibration plot using linear sweep anodic stripping voltammetry (LS-ASV). Experimental conditions: 0.1 M hydrochloric acid, deposition potential at −1 V for 100 s, scan rate of 0.2 V/s. Low inset: mechanism for stripping detection of iron ions is reported.

As shown in Figure III.8 all the substrates allowed a satisfactory detection of iron ions within the same range of concentrations. The equations describing the linear correlation among the current and the concentration were approximated as follows, $y = 0.49x + 0.10$ (R^2 of

0.994), $y = 0.40x + 0,14$ (R^2 of 0.964), and $y = 0.42\ x + 0,09$ (R^2 of 0.953), relatively to polyester, PHBV and PHBV/MFC, respectively, where y represents the current difference between metal ion and the blank, and x represents the metal level expressed in ppm. A detection limit (LOD) was calculated following the equation LOD= 3 * (standard deviation of the blank)/(slope of the curve), obtaining 90, 140 and 150 ppb, respectively to polyester, PHBV and PHBV/MFC. These results appear well-compared with other unmodified electrochemical architectures as the one reported by the Compton group with a LOD of ca. 2 mM iron (110 ppb), which also included 20 min to remove the dissolved oxygen in solution.[58] Another paper reporting the electrochemical detection of iron ions was recently reported using commercial graphite-based screen-printed electrodes (DropSens) through the anodic stripping voltammetry in presence of o-phenanthroline, ferrocyanide and 240 s of accumulation: even if this method allowed to reach a LOD of ca. 4 ppb, it should be noted that the experimental setup was more complex with respect to an unmodified SPE platform.[59] Another system to compare was also represented by a carbon-based platform modified with a coating of carbon nanotubes and Nafion, and the linear sweep stripping voltammetry allowed a LOD of ca. 35 ppb of iron ions (using an accumulation of 4.5 min).[60] It should be noted that various electroanalytical architectures based on modified systems have been reported for iron sensing, including gold nanoparticles, bismuth nanoparticles, graphene, carbon nanotubes, and Nafion.[61,62] In addition to this, the PHBV-based screen-printed electrodes may represent a starting point for the construction of

more sophisticated printed electroanalytical architectures. Even if the three tested substrates highlighted a similar sensitivity towards the detection of iron ions, it should be noted that the repeatability appeared slightly different. The repeatability was calculated as the relative standard deviation (RSD), and while the polyester-based strips (Figure III.8A) were characterized by a repeatability of ca. 7%, the RSD calculated for PHBV and PHBV/MFC was equal to 12% and 14%, respectively (RSD was calculated on three replicates, using three different electrochemical strips for each kind of substrates tested). This slight difference could be attributed to production strategy of the PHBV-based substrates with respect to polyester: the layer of the PHBV-based materials, as reported in the SEM images (Figure III.1), presented some morphological heterogeneity and this might lead to a slight source of variation in the final product. However, the results demonstrated the good performance of the novel substrates with detection limit in the ppb range.

In order to make the characterization of these substrates wider, an additional case of study was interrogated. The substrates were modified with Prussian Blue and butyrylcholinesterase enzyme to evaluate the presence of dichlorvos, as a model organophosphate inhibitor. The system was obtained by drop casting 8 μL Prussian Blue nanoparticles (1 mg/mL) and 2 μL of butyrylcholinesterase enzyme (1 U/mL). In presence of the enzymatic substrate (butyrylthiocolione), one of the by-product of the reaction is the thiocholine that can be easily oxidized at the electrode surface via Prussian Blue. Instead, the presence of the inhibitor (which de-activate the enzyme) has the effect of decreasing the enzymatic activity, thus

producing less thiocholine with the results of having a lower oxidation current.[63] As reported in Figure III.9, the organophosphate inhibitor was measured at the three substrates ranging from 1 ppb to 1 ppm.

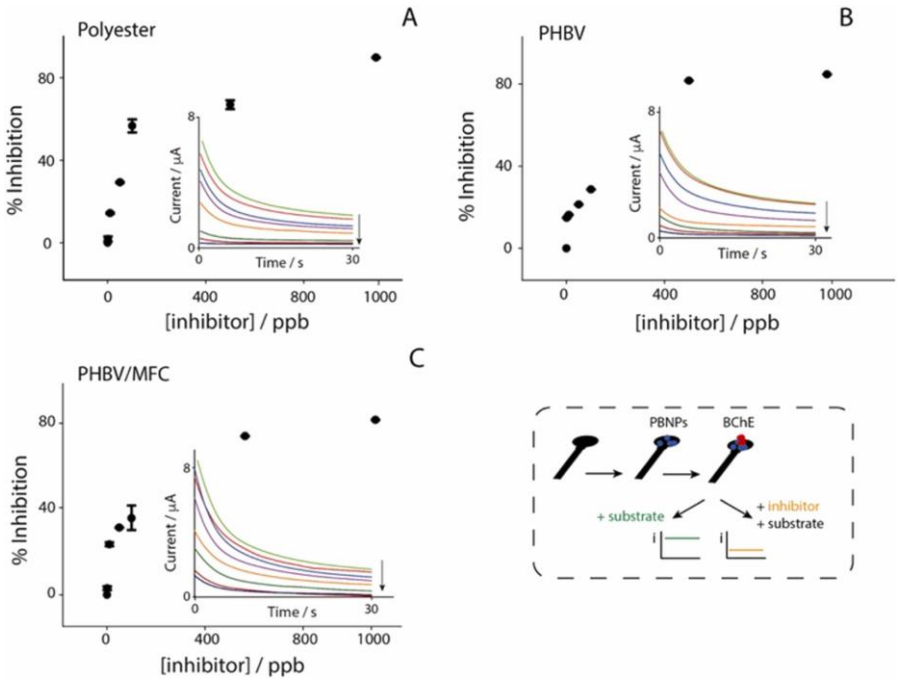

Figure III.9. Chronoamperometric recordings obtained in the range between 1 ppb and 1 ppm of organophosphate inhibitor using A) polyester, B) PHBV and C) PBHV/MFC. The inset shows both the composition of the device and the electrochemical scheme on which the detection is based.

As reported, the intensity of the chronoamperometric curves were consistent with a decrease as the inhibitor concentration increases. The three tested platforms were challenged in presence of inhibitor, i.e. dichlorvos, from 1 ppb to 1000 ppm. As the concentration of

substrate, a concentration of 10 mM was chosen to give a high current signal as the background. The calibration curves in the linear range, up to 100 ppb, were described by the following equations, $y = 0.54x + 3.1$, $y = 0.24x + 11$, $y = 0.36x + 10$, respectively to polyester, PHBV, PHBV/MFC, where y represents the percentage of inhibition and x is the concentration of the inhibitor expressed in ppb, and the detection limit for each device was calculated to be equal 0.8, 0.4 and 0.5 ppb, respectively. With respect to other electrochemical inhibition-based biosensors for the determination of organophosphate pesticides, the results reported in terms of LOD appear in agreement within the concentration range of a paper-based printed electroanalytical system based on Prussian Blue Nanoparticles that produced a 3 ppb LOD,[64] a flexible cobaltphthalocyanine-based screen-printed electrode with a ca. 5ppb LOD,[65] an amperometric AChE-based screen-printed electrode modified with a mixture of silver nanowire-graphene-TiO_2 sol-gel-chitosan characterized by a LOD of ca. 2 ppb[66] and also with the use of a different architecture based on the inhibition of choline oxidase based on poly(brilliant cresyl blue) films formed on multiwall-carbon nanotube, dichlorvos was detected down to ca. 0.5 ppb.[67] In addition, the repeatability was calculated on three measurements of 20 ppb standard solution, obtaining a value lower than 10% for all the substrates. However, the overall applicability of these new substrates looks promising, displaying satisfactory performance in presence of different species to be detected.

3.1.4 Conclusion

This work reported for the first time a complete characterization of novel materials based on PHBV to be used in electroanalytical sensing

application. These so-called bacterial polymers are fully bio-based and biodegradable sustainable materials, and they may potentially replace the use of plastic-based materials for realizing portable devices. PHBV and PHBV/MFC were deeply investigated through the use of morphological, mechanical and electrochemical approaches, and those were compared with the gold standard in developing screen-printed electrodes, namely polyester. All the characterization studies have demonstrated the suitability of these substrates to be combined with the screen-printing process, highlighting the satisfactory performance.

In particular, the novel materials were applied to the determination of iron ions and organophosphate inhibitors in aqueous solutions demonstrating the good preliminary findings. The replacement of "old" material with sustainable alternatives is of pivotal importance, especially in the field of sensors and biosensors. The use of bio-based and biodegradable substrates, such as the PHBV ones, opens to novel possibilities about the decentralization of portable devices. For instance, the growth of precision agriculture, where sensing devices are required to be in close contact with fruit, leaf, stem, soil etc. would be a perfect field of application for these bacterial polymers, which will be lowering the issue related to contamination and waste disposal, especially in remote areas.

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CHAPTER 4: WEARABLE ELECTROCHEMICAL SENSORS FOR PESTICIDE DETECTION: COPPER-BASED PESTICIDES IN VITICULTURE AND BROADER PESTICIDE APPLICATIONS

After successfully optimizing environmentally friendly bioplastic-based substrates for electrochemical sensors, the focus of this work shifted to the development of wearable sensing devices, particularly integrated into gloves, for in situ detection of agricultural pesticides. This chapter presents two distinct applications of wearable electrochemical sensors, both designed to meet the growing need for accurate, real-time monitoring of pesticide residues in agricultural environments.

The first application involved the creation of an electrochemical sensor to be worn on the glove, designed specifically to detect copper-based pesticides, commonly used in viticulture to control plant diseases such as downy mildew (*Plasmopara viticola*). Copper residues on grapevine leaves, a critical issue for both vineyard management and environmental health, were monitored using the developed wearable sensor. The sensor's ability to detect copper ions directly on the surface of vine leaves was validated through a collaboration with Quintodecimo, a winery located at Mirabella Eclano (AV), Italy. The development and optimization of the glove-based sensors were significantly advanced during a stage at the Department of Nanoengineering at the University of California, San Diego (UCSD), under the supervision of Professor Joseph Wang, whose expertise in

wearable electrochemical devices greatly contributed to refining the sensor designs and enhancing their performance for real-world applications. The glove sensor proved effective in detecting copper at concentrations relevant to agricultural practices, offering a simple and portable solution for vineyard management.[1]

Building on the success of the copper detection sensor, the second application focused on expanding on-glove technology to detect organophosphate insecticides directly on fruit surfaces. Organophosphates are widely used in agriculture, but their residues in food products pose significant health risks. In this case, an enzyme-based electrochemical biosensor was integrated into the glove to detect traces of organophosphates, such as dichlorvos, on the peels of fruits such as apples and oranges. This wearable sensor system, which works in conjunction with a smartphone-powered reader, provided a low-cost, portable solution for rapid detection of pesticide residues in the field, enabling real-time monitoring and informed decision making in agricultural and food safety settings.[2]

These two developments-first for detecting copper in vineyards and then for detecting organophosphate residues on fruit-highlight the versatility and potential of wearable electrochemical sensors for precision agriculture. This chapter delves into the design, optimization, and field testing of these sensors, showing how wearable technology can be leveraged to improve agricultural practices, ensure food safety, and minimize environmental impact.

4.1 SENSING AT YOUR FINGERTIP: ON-GLOVE ELECTROCHEMICAL SENSOR FOR COPPER DETECTION ON VINE LEAVES

4.1.1 Introduction

As discussed in Chapter 1, Cu is a widely used natural mineral pesticide, used to control bacteria and fungi, permitted for use in organic farming systems. Cu-based pesticides are recognized for thecontrol of downy mildew in vineyards, a disease caused by Plasmopara viticola,[3] but also used in the treatment of various plant diseases, such as apple scab, potato blight, coffee rust and cocoa broom disease.[4] Despite its long history of use since the 19th century, growing concerns about Cu accumulation in the soil and its potential toxic effects on both plants and the environment have led to stricter regulations. Specifically, European Commission Regulation 2018/1981 limits the use of plant protection products containing Cu compounds to a maximum of 28 kilograms per hectare over a seven-year period (about 4 kilograms per hectare per year), in an effort to minimize the accumulation of Cu in the soil and reduce the exposure of non-target organisms.[5,6] Excess Cu in soil can significantly disrupt microbial activity and interfere with plant growth, photosynthesis, root and shoot development and lead to chlorosis, necrosis and leaf discoloration.[7] In addition, long-term exposure to high levels of Cu is harmful to human health, potentially causing neurological disorders and liver and kidney damage.[8–12]

Given the need for more precise monitoring of Cu residues in agricultural settings, electrochemical detection techniques have gained significant attention due to their simplicity, affordability, and potential

for in situ applications. Among the various analytical methods, atomic absorption spectroscopy (AAS),[13,14] inductively coupled plasma mass spectrometry (ICP-MS),[15,16] and electrochemical stripping voltammetry are widely used to detect Cu.[17]

Electrochemical methods are particularly advantageous because they can be used for decentralized and on-site monitoring, offering the possibility of obtaining real-time data without the need for complex sample preparation or laboratory equipment.[18,19] Various electrochemical approaches, mainly focused on the anodic stripping voltammetry, have been noted in literature for agricultural and environmental applications, using diverse architectures and materials, including carbon nanotubes, pencil graphite-modified quantum graphene electrode, DNAzyme coupled to iron-based metal organic framework, bismuth films, gold nanoparticles coupled to alginate microspheres integrated into a 3D-printed case.[20–23]

Due to their features, electrochemical methods have shown to be implemented for applications that are always closer to humans and environment. A wide range of wearable devices for on-body monitoring of metals have been developed,[24–26] but also some innovative lab-on-a-tip [27] and on-glove applications.[28–30]

The development of wearable devices represents a paradigm shift in diagnostics for humans and the same concept could be applied for monitoring the health of plants and vegetables. Several progresses have been made in the field of precision agriculture,[31] also with the integration of artificial intelligence and machine learning to evaluate growth of the plant, nutrients in soil, etc. [32–34] The possibility to have analytical devices in direct contact with plants and soil is of

considerable interest for many reasons: it would permit to evaluate crops health, to monitor pollutants and also to reduce the uncontrolled use of fertilizers and pesticides. However, among the existing approaches for analysis decentralization, the use of gloves appears as a promising solution, especially in outdoor field settings.[35] A glove can be easily engineered with electrochemical strips, exchanged among end-users, made disposable, connected to portable hand-held reader and applied towards diverse targets, e.g., water, soil, fruits, leaf, etc.

In this context, the present work builds on previous efforts in the field of precision agriculture and wearable sensing devices, with a focus on the development of an electrochemical sensor for the detection of Cu ions directly on grapevine leaves. This research was conducted in collaboration with Quintodecimo, a winery located in Mirabella Eclano (AV), Italy, in collaboration with Professor Luigi Moio. The activity with Quintodecimo provided valuable insights in the field and allowed for concrete testing of the sensor in a real vineyard. The sensor was developed and optimized with the assistance of Professor Joseph Wang, during the doctoral stage at the Department of Nanoengineering, University of California, San Diego (UCSD), where the design and implementation of the electrochemical glove sensor was refined.

As a proof-of-concept, the integration of an electrochemical strip with a glove was evaluated for the first time towards the detection of Cu ions onto leaves. As case of study, the engineered electrochemical glove was applied towards the detection of Cupravit Bio Advanced (a copper-based pesticide commonly used in grapevine production). In particular, Cu ions were sampled and detected just by touching the

surface of the leaf, and a with a following addition of acidic solution. The use of gold nanoparticle-modified screen-printed electrode was effective in detecting Cu ions within the levels used in wine production, without any pre-treatment task, thus making the system suitable for real-world application. This works might open to new developments in the field of precision agriculture, representing a starting point easily generalizable to other species of interests, i.e., pesticides, nutrients, bacteria.

4.1.2 Experimental section

Materials

The Cu-based pesticide, Cupravit Bio Advanced, was supplied by Bayer (Crop Science, Milan, Italy) and consists of metallic copper in the form of tribasic copper sulphate. Hydrochloric acid solution was purchased from Sigma-Aldrich. All reagents used were of analytical grade, corresponding to the highest level of purity available, with a purity of over 98% as indicated by the producer. All solutions were prepared in double-distilled water. The garden leaves were collected at the garden of the Department of Pharmacy, University of Naples. The vine leaves were sampled in the Quinto Decimo company (Mirabella Eclano, Avellino). For the flexible polyester-based substrate on which the sensors were fabricated, Autostat HT5 with a thickness of 125 μm, manufactured by MacDermid, UK, was used. This substrate was chosen for its excellent strength and flexibility characteristics, making it ideal for electrochemical applications in harsh environments. The adhesive tape used to insulate the moulded electrodes, and the adhesive tape used to assemble the system onto the nitrile glove are commercially available products, manufactured locally in Italy.

All electrochemical measurements were performed using a portable, battery-powered instrumentation, namely a potentiostat, Sensit Smart (PalmSens, Utrecht, The Netherlands,), connected to a smartphone, where the recorded currents are displayed using a dedicated application, such as PStouch (PalmSens, Utrecht, The Netherlands).

Fabrication of the electrochemical strips

The three-electrode system was screen-printed manually, on a polyester substrate, as illustrated in the Chapter 2. The final diameter of the working electrode is 4 mm, resulting in an active electrode area of approximately 12.6 mm^2. A general adhesive tape was used to define the working area on the screen-printed electrodes (SPEs) and to avoid diffusion of the solution towards the connectors. Once the electrodes printed on polyester were obtained, they were modified with a volume of 2 µl of gold nanoparticles (AuNPs) (equivalent to approximately 2.5 nmol), by drop casting and assembled onto a nitrile glove. AuNPs were used to improve the sensitivity and accuracy of the electrochemical sensor through the deposition of Cu ions via Cu reduction onto gold. AuNPs increase the surface area of the electrode and facilitate electron transfer due to their excellent conductivity properties, thus improving the detection of Cu ions.

The three-electrode system, manually printed on a polyester substrate, was assembled onto nitrile gloves using adhesive tape. This type of tape was chosen to ensure a secure and stable adhesion of the electrodes to the glove surface. The adhesive tape ensures that the electrodes remain well positioned and functional during use, maintaining the accuracy of electrochemical measurements. The use of adhesive tape

was preferred over special glue because of its ease of application and its ability to adapt to flexible glove surfaces.

AuNPs synthesis

For the synthesis of gold nanoparticles, we used the protocol illustrated in the Chapter 2.[36]

Cu (II) ions measurements

The electroanalytical technique used to measure Cu ions was linear sweep-anodic stripping voltammetry (LS-ASV). Measurements were conducted using a 0.1 M hydrochloric acid solution as the working solution. In the anodic stripping voltammetry, the anodic peak potential for Cu ion redissolution was measured at about +0.3 V vs Ag/AgCl. This range was established after applying a deposition potential of −0.4 V, which facilitates the accumulation of Cu on the electrode surface. Subsequently, the redissolution process was monitored in a 0.1 M hydrochloric acid solution, which allowed efficient stripping of Cu from the electrode to the solution. This configuration ensures accurate and sensitive quantification of Cu ions, optimising the sensor's performance in electrochemical detection.

The experimental parameters used, optimised by Miglione et al., are as follows: a deposition potential of −0.4 V was applied for 300 s, scanning the anodic potential from 0.1 to 0.6 V, with an E step of 0.01 V and a scan rate of 0.5 V s^{-1}.[37] In addition, the AuNP-based electrochemical sensor was interrogated in the presence of various interfering species including zinc, nickel, cadmium, iron and lead, also highlighting a satisfactory selectivity.[37] The specificity of the sensor for Cu^{2+} ions is due to the combination of the chemical and physical properties of gold nanoparticles, which act as a platform for the

selective electrochemical reduction of Cu. The redox potential of Cu is significantly different from that of other interfering metals in solution, such as zinc, nickel, cadmium, iron and lead. This allows the sensor to selectively distinguish Cu^{2+} ions during voltammetric analysis. Furthermore, the high conductivity of gold nanoparticles facilitates efficient electron transfer for Cu, further enhancing the sensitivity and selectivity of the sensor towards Cu^{2+}, even in the presence of binary mixtures with other metals. These factors, combined with optimised experimental conditions, ensure a highly specific response for copper detection.

On-glove electrochemical detection

The on-glove detection of Cu ions was performed by following simple procedures as follows. As shown in Figure IV.1, a Cupravit-containing aqueous solution was sprayed on the leaves, which it was allowed to dry at room temperature. Successively, the analyte was sampled by simply rubbing the surface of the leaf with the gloves for 5 s. Finally, testing area of the engineered glove was drop cast with 100 microliters of 0.1 M HCl and the electrochemical measurement was carried out using a portable potentiostat connected to a smartphone. The sensors assembled on the glove were connected to the Sensi Smart device using copper wire connectors. Copper wires ensure a secure and stable electrical connection due to their excellent conductivity, which minimises interference and ensures reliable signals. These copper wires have been connected to the Sensit Smart via specially designed connectors. The connectors were chosen to perfectly fit the device's input ports, ensuring efficient transmission of electrochemical signals from the sensors. The electroanalytical technique used to measure

copper ions was linear sweep-anodic stripping voltammetry (LS-ASV).[38]

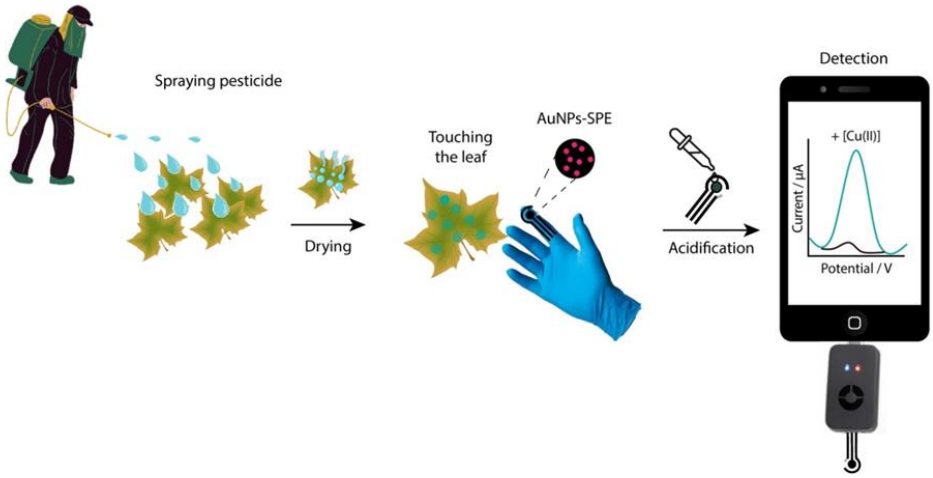

Figure IV.1. Schematic representation of the electrochemical on-glove sensing device for direct monitoring copper ions on leaf without pretreatment.

4.1.3 Results and discussion

As mentioned in the materials and methods section, the commercial Cupravit Bio Advanced was used to perform the study. This compound contains copper in the form of tribasic copper sulphate and, in accordance with agricultural practice, a standard solution of 1.5 g l^{-1} was prepared and applied to the surface of a model leaf, of the laurel tree, *Prunus laurocerasus*. The initial characterization study involved the Cu ions sampling directly from the leaf. Initially, a known amount of Cupravit was drop cast on the leaf and subsequently re-dissolved in double-distilled water. Briefly, a 300 ppm solution of Cupravit was drop cast on the leaf, using three different volumes, i.e., 10, 20 and 50 µl. After the water was evaporated, the dried Cu ions were re-dissolved

in the same amount of water, depending on the starting volume. Subsequently, the glove was put in contact with the aqueous drop containing the re-dissolved Cu ions, for ca. 5 s, and it was acidified with 100 microliters of hydrochloric acid towards the electrochemical stripping detection.

As observed in Figure IV.2A, no significant differences were observed, and it was chosen to continue the optimization with a 10 µl volume. Subsequently, this preliminary experimental setup was adopted towards the detection of increasing amounts of Cu ions, always taking into consideration a starting solution of 1.5 g l^{-1} Cupravit. As reported in Figure IV.2B, the detection at the glove was characterized in a range comprised between 75 and 750 ppm. A linear correlation was observed, and it was described by the following equation: $y = 0.09x + 0.68$, $R^2 = 0.988$, where y represents the stripping current expressed in microampere, and x represents the Cu ion concentration expressed in ppm. The limit of detection (LOD), calculated as $3\sigma B/slope$, where σB is the standard deviation calculated from blank measurements and the slope is equal to y/x in the calibration curve, was found to be about 4 ppm. The inter-electrode repeatability calculated on three replicates of 300 ppm Cupravit, expressed as the relative standard deviation (RSD), was lower than 10%. It should be noted that all the scatter points reported in all the figures are the results of single use sensors and different leaves: it highlights the precision of the method.

Considering the satisfactory results obtained with the preliminary application of the glove onto the wet leaf, the following step was about to evaluate the application towards dried sample. In order to provide

the readers a clearer representation of the two experimental approaches, a scheme of the two experimental setups has been included in Figure IV.2C. Accordingly, the results showed in Figure IV.2B have been obtained by touching a copper-containing redissolved drop on the surface of the leaf, while the final experimental setup will be characterized by touching the dried Cupravit without performing the re-dissolving through water drop. As reported in Figure IV.2D, a dynamic variation of the recorded current was observed for the range of concentrations tested, up to 750 ppm, even if a linear trend was observed up to 75 ppm. The linear part of the calibration curve was described by the following equation: $y = 0.70x—2.40$, $R^2 = 0.973$. With this experimental setup, a detection limit was obtained equal to ca. 1 ppm. By observing the two linear equations appear clear how the dried drop sampling provided a higher sensitivity within a lower concentration range of Cupravit, with respect the liquid sampling (Figure IV.2B). This result is attributed to the higher Cu sampling onto dried leaf compared with the sampling in presence of the water drop: in fact, the contact of the glove with the liquid drop onto the leaf only allows a small sampling of coppers, while the direct contact between leaf and glove is capable to boost the sampling. This also justifies the wider and less sensitive linear range observed in Figure IV.2B in comparison with the narrow and more sensitive observed in Figure IV.2D.

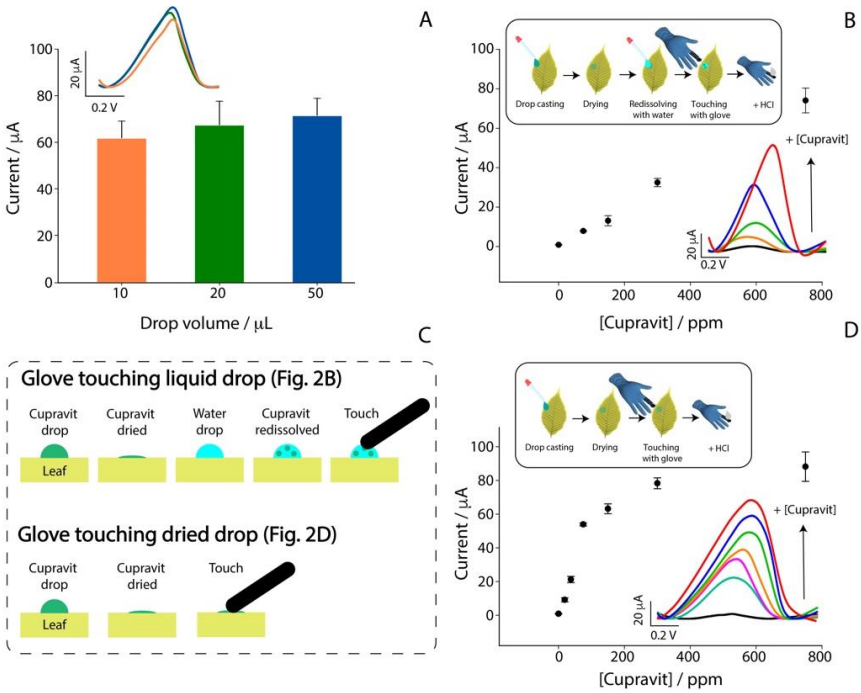

Figure IV.2. A) Optimization of the volume of Cupravit to be placed on the leaf by drop casting. The histogram shows the comparison in the detection of a 300 ppm Cupravit solution deposited on the leaf by drop casting three different volumes 10, 20 and 50 mL. Inset: Voltametric curves relating to the measurements of the three volumes; B) Calibration curve for Cupravit, up to 750 ppm, obtained by testing the device on garden leaves by redissolving copper ions in water. Upper inset: Schematic protocol. Lower inset: Voltametric curves relating to the detection of Cupravit. Experimental parameters: E dep = -0,4 V; t dep = 300s; E begin = 0,1 V; E end = 0,6 V; E step = 0,01 V; Scan rate = 0,5 V/s; C) Schematic representation of the two experimental approaches for both touching dried and liquid drop; D) Calibration curve for Cupravit up to 750 ppm, obtained by touching the garden

leaves using the dry drop. Upper inset: Schematic protocol. Lower inset: Voltametric curves relating to the detection of Cupravit.

Once the dried sampling was demonstrated to be effective with the control of the drop casting of Cupravit onto the leaf (10 microliters), the final step was about the application of the glove by adopting settings more similar to those applied in agricultural settings.
For this reason, in order to evaluate the performance of the glove sensor in detecting Cu at the working levels used by farmers, getting as close as possible to a real application, it was decided to treat several leaves by spraying known concentrations of Cupravit on each of them with a common sprayer and let them dry. This time, the glove was rubbed randomly on the surface of both garden leaves and vine leaves sampled by QuintoDecimo, a wine producer. As illustrated in Figure IV.3, for both the types of leaves that have been tested, Cu ions have been detected in the range comprised between 15 and 1500 ppm, demonstrating the system's ability to detect different amounts of Cu present, directly on the leaf without pre-treatment of the sample.

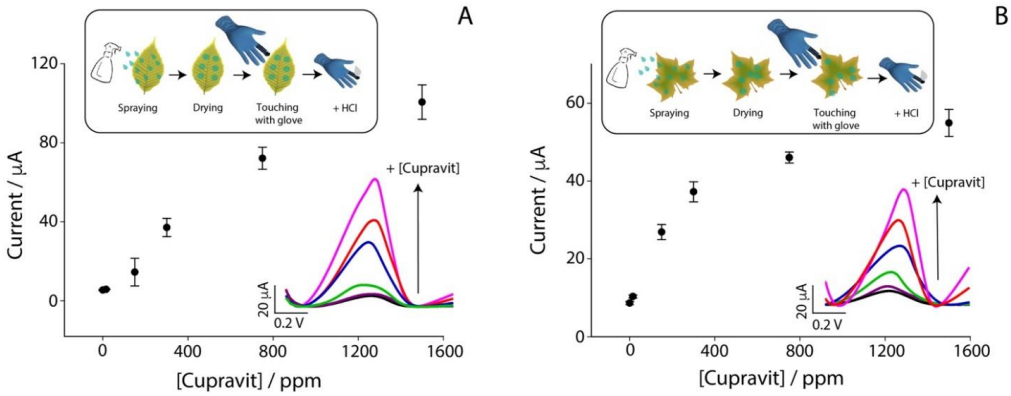

Figure IV.3. Application of the on-glove measurements on sprayed A) garden and B) vine leaves, touching the dried drops on the leaves and acidifying with 100 microliters of hydrochloric acid. Concentrations of Cupravit were tested up to 1500 ppm. Upper inset: Schematic protocol. Lower inset: Voltametric curves relating to the detection of different Cupravit leaves. Experimental conditions are reported in caption of Figure IV.2.

For both the leaves tested a satisfactory linearity was observed up to ca. 750 and ca. 400 ppm, respectively, for garden and vine leaves, with similar sensitivity within the linear range of ca. 0.1 mA ppm^{-1}. What is interesting to observe is both the increase of the RSD and the decrease of the sensitivity with respect the measures that have been performed in controlled settings (Fig. 2D). The use of spray does not allow to control the deposition of Cupravit, that is instead more random with respect to the previous drop casting during system optimization. The sprayed drops are variable in terms of volume and space distribution. However, the results displayed in Figure IV.3. are satisfactory, especially considering that for all the experiments unique leaf-glove couples were utilized.

4.1.4 Conclusion

For the first time, an on-glove electrochemical sensor was applied towards the detection of copper ions on leaves for empowering precision agriculture at the point-of-need for non-specialized users. The system was characterized by the integration of a gold nanoparticle-modified screen-printed electrode onto a glove, for the direct detection of copper ions onto leaves without any pretreatment steps. As a case of study, a commonly copper-based pesticide largely used in agriculture for wine production, namely Cupravit, was sprayed and detected onto leaves using a portable system. Both the characterization and real-samples application have demonstrated the satisfactory relevance of the results that allowed to detect Cupravit level in the high ppm range, in agreement with the current levels used in agriculture. With the proposed approach, the sampling was performed only by touching the surface of the leaf, thus opening to a future decentralization of relevant species in agriculture.

The on-glove system was conceived to provide a low-cost, sustainable and facile device to be applied in remote field settings, obviating the needs skilled personnel and complex analytical procedures. It represents a promising application for enhancing the analytical practice in both agriculture and environmental fields, with net implications for food industry, pollution surveillance and pest reduction.

4.2 AN ELECTROCHEMICAL BIOSENSOR FOR ON-GLOVE APPLICATION: ORGANOPHOSPHORUS PESTICIDE DETECTION DIRECTLY ON FRUIT PEELS

4.2.1 Introduction

In recent decades, environmental pollution has emerged as a critical issue globally, raising concerns in several fields, including eco-toxicology, environmental biology, eco-chemistry, and pathology.[39] A major challenge for the future is the conservation of global agricultural production, which is increasingly threatened by climate change and rapid global population growth, as highlighted in the United Nations 2030 Agenda for Sustainable Development.[40] To ensure food security and maintain ecosystems, sustainable agricultural practices are critical and must be approached from a holistic and integrated perspective.[41] Plants, as the primary link in the food chain, play a central role in this context. They convert solar energy into chemical energy, but emerging plant diseases, exacerbated by both biological and environmental stressors, increasingly limit crop productivity.[31]

Pesticides, particularly those used to combat plant pests and diseases, are essential for increasing agricultural yields. However, their widespread use also raises concerns about pesticide residues in food, which can have serious implications for human health and environmental safety.[42,43] Regulatory frameworks, such as Regulation (EC) No. 396/2005, set strict limits for pesticide residues in food, generally at levels below 0.01 ppm.[44] To comply with these regulations and ensure food safety, early detection of pesticide residues is critical. Recent advances in precision agriculture highlight the potential of new technologies to address these challenges. Precision agriculture

integrates sensors, data management and informed decision making to optimize agricultural production while minimizing environmental impact.[45–48] In particular, sensors and biosensors that can monitor crop health have proven to be useful tools for improving agricultural efficiency, especially when they can be deployed directly on plants or in the field, reducing the risk of contamination and improving the accessibility of monitoring. Traditional techniques, such as gas chromatography (GC-MS) and liquid chromatography-mass spectrometry (LC-MS/MS), offer high sensitivity and accuracy for pesticide detection, but are hampered by their complexity, high cost, and the need for specialized equipment and trained personnel.[49,50] This makes them unsuitable for on-site or rapid monitoring, especially in resource-limited settings.

To overcome these limitations, electrochemical (bio)sensors offer a promising alternative. These sensors are characterized by high sensitivity, portability, cost-effectiveness, and the ability to provide real-time results with minimal sample preparation.[51,52] Advances in electrochemical sensor technologies, particularly those based on SPEs, have made possible the development of portable and low-cost devices for on-site monitoring of pesticides and other contaminants.[53] In addition, wearable sensors, such as electrochemical strips integrated into gloves, are emerging as a revolutionary tool in precision agriculture. These sensors enable continuous monitoring of plant health in real time, directly in the field, thus facilitating immediate decision making and timely interventions.[48,54–60]

Based on these developments, this work focuses on the application of electrochemical sensing technologies previously demonstrated in

grapevine leaves,[1] in Chapter 4.1, to the detection of pesticide residues in agricultural products, such as fruit. Specifically, after successfully using a wearable electrochemical sensor to monitor copper residues on grapevine leaves, we have expanded this approach to detect organophosphate pesticides, which are commonly used in agriculture to protect crops from pests. Specifically, we designed a new enzyme-based electrochemical biosensor that uses inhibition of cholinesterase enzymes in the presence of organophosphate pesticides as a detection mechanism.[61]

This novel sensor system, based on a bio-hybrid composite of Prussian blue nanoparticles, carbon black, and butyrylcholinesterase enzyme, is integrated into a screen-printed electrode inserted into a glove. For the first time, we demonstrate the use of this device to detect pesticide residues, such as dichlorvos, directly on agricultural products such as apples and oranges. The device works together with a smartphone-powered reader, providing a low-cost, portable solution for rapid on-site detection. The detection limit for both fruits tested is in the nanomolar range, sufficient to detect organophosphate residues well below the maximum residue limits (MRLs) set by regulatory agencies, typically in the micrograms per kilogram range. This approach represents a significant step forward in the development of wearable sensors for precision agriculture, enabling farmers and food safety inspectors to monitor pesticide residues directly in the field and make informed decisions based on real-time data.

4.2.2 Experimental section

Materials

Butyrylcholinesterase (BChE) from equine serum, butyrylthiocholine chloride, Dichlorvos analytical standard, potassium nitrate and ammonium sulfate were obtained from Merck, USA. Metal standard solutions for interference studies (mercury, copper and cadmium) were bought from Fluka. Carbon black (CB) VXC72R powder and Prussian Blue were provided by Cabot Corporation, Italy. All the measurement were carried out in phosphate saline buffer (PBS) prepared dissolving a PBS tablet 140 mM NaCl at pH = 7.2 (Merck, USA) in 1L of bidistilled water.

Electro-chemical measurements have been carried out with the use of a portable potentiostat, EmStat 3 (PalmSens, Netherlands).

Screen printed platform

Electrodes were in-house produced onto a flexible polyester film as substrate (HT5, Autostat), kindly provided by McDermid Alpha (UK) and MacDermid Performance Solutions Italiana (Italy). The adoption of polyester-based substrates for manufacturing electrochemical strips compared to paper-based ones allow creating more robust platforms to be applied in decentralized context. The three-electrode design was manually screen printed as described in the Chapter 2.

Strip nanoengineering

The electrochemical printed strips were successively modified with 4 μL mixture composed of carbon black (CB), Prussian blue (PB), prepared dissolving 1 mg of the mixed powder in 1 mL of DMF/H_2O (1:1) and then drop casted onto the working electrode area and let dry;

then 2 µL of the 25 U mL^{-1} enzyme, BChE, was drop-cast at the optimized concentration and let dry (Figure IV.4).[62]

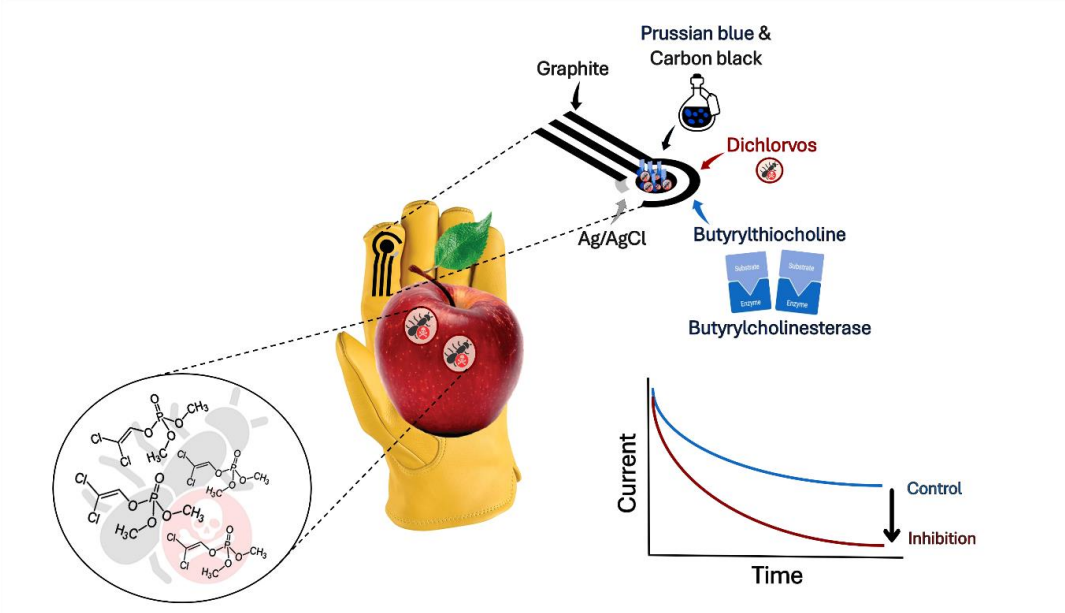

Figure IV.4. Schematic representation of the detection of dichlorvos directly on fruit peels by using on-glove electrochemical strips, nanoengineered with the electrocatalyst (Prussian blue), the conductivity enhancer (carbon black), the recognition bioelement (BChE), and the typical chronoamperometric inhibition-based response at increasing concentrations of inhibitor.

Pesticide quantification

The level of the pesticide is obtained by measuring the change of the BChE activity in the presence of the inhibitor. Briefly, the working area of the electrode, modified with the hybrid nanocomposite, was covered with the suitable volume of phosphate buffer containing 5 mM substrate (butyrylthiocholine) and the inhibitor (Dichlorvos) at increasing concentrations (0–100 ppb). After several minutes, the

enzymatic activity was calculated by measuring the amount of the electroactive byproduct of the BChE substrate, namely, thiocholine. The current was recorded in the presence and in the absence of the inhibitor, then used to calculate the inhibition level that was proportional to the inhibitor concentration. A chronoamperometric technique at 0.4 V (versus Ag/AgCl) was used to monitor the enzymatic activity, and the intensity of the chronoamperograms was recorded in the presence of the inhibitor, highlighting a decrease as the inhibitor concentration increased, as reported in Figure IV.4.

4.2.3 Results and discussion

Evaluation of the experimental setup

The detection method of the organophosphorus pesticide was based on the irreversible inhibition of the Butyrylcholinesterase (BChE) that hydrolyzes its substrate, butyrylcholine, giving byproducts butyric acid and choline. Unfortunately, both these products are not electroactive, and the enzymatic activity cannot be observed at the printed electrochemical device. For this reason, butyrylthiocholine was chosen as the enzymatic substrate, giving as the by-products butyric acid and thiocholine: while the former is not electroactive, the latter can be easily electrochemically revealed.[63] This was possible thanks to the modification of the working electrode with a nanohybrid composite made with the combination of BChE, PB, and CB: BChE produces thiocholine, PB is sensitive to its oxidation at less interfering potential, and CB has the dual function of hosting the components of the composite and improving conductivity.[62,64–67] Thus, the experimental setup of the biosensor was optimized in order to obtain satisfactory sensitivity and repeatability, as reported in Figure IV.5.

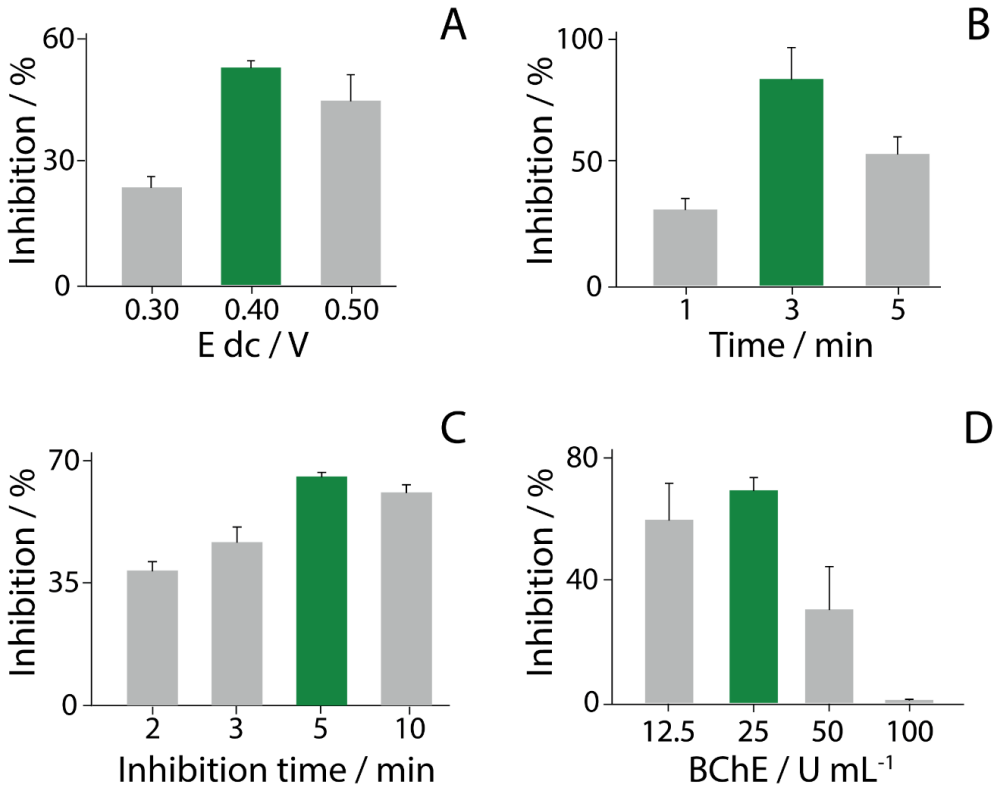

Figure IV.5. A) Optimization of the constant potential applied during the chronoamperometry; B) Optimization of the enzymatic reaction waiting time; C) Optimization of the inhibition time; D) Evaluation of the enzymatic activity between 12.5 and 100 U mL-1 BChE in the presence of 5 mM substrate and 100 ppb of dichlorvos, with the use of the optimized parameters, at an applied potential of 0.4 V (versus Ag/AgCl), reaction time of 3 min.

Several parameters were optimized: first, the constant potential applied during the chronoamperometric measurement, set at 0.4 V, then the waiting time that allows the enzymatic reaction in presence of the

substrate to occur, and the inhibition time required for the pesticide to inhibit BChE, set respectively at three and 5 min. Successively, the concentrations of both BChE (Figure IV.5 D) and the substrate (Figure IV.6) were considered. It is important to obtain the optimal condition to 1) have a good sensitivity, 2) evaluate future inhibition, and 3) reduce the cost of manufacturing.

With regard the substrate concentration optimization, the measurements were performed using increasing concentration of butyrylthiocholine up to 20 mM. A typical Michaelis–Menten behavior was observed. Considering that the difference between the responses due to 5, 10 and 20 mM of enzymatic substrate was negligible, 5 mM was chosen as the minimum concentration of substrate to reach the Vmax and it was selected for successive measurements in the presence of the inhibitor (Figure IV.6). Consequently, using 5 mM as the concentration of the enzymatic substrate, the nano-engineered platform was evaluated in the presence of different concentrations of BChE (Figure IV.5D) in a range comprised between 12.5 and 100 U mL^{-1} at the presence of a fixed amount of inhibitor (100 ppb), in order to obtain a good sensitivity and a high inhibition. The use of 100 U mL^{-1} of BChE produced the highest substrate conversion but the inhibition associated was low and the sensitivity of the method was not enough for inhibitor quantification, especially at lower concentration. The best compromise in terms of BChE amount and inhibition level was obtained by modifying the device with 25 U mL^{-1} of the enzyme with a 70 % inhibition in the presence of 100 ppb of dichlorvos.

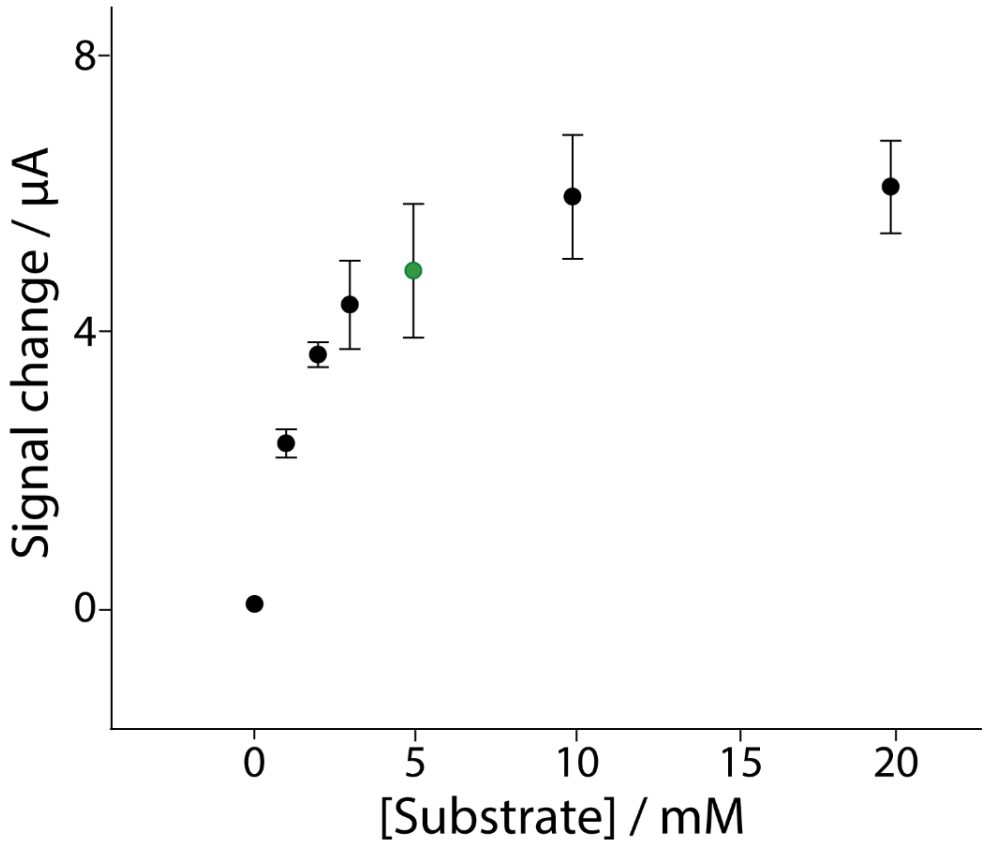

Figure IV.6. Calibration curve in the presence of various levels of enzymatic substrates, i.e., butyrylthiocholine, in the 0–20 mM range, at an applied potential of 0.4 V (versus Ag/AgCl), reaction time of 3 min, using 25 U mL^{-1} of BChE.

Analytical characterization of the device

After having optimized all the experimental parameters, a calibration curve was obtained in the range between 0 and 100 ppb of Dichlorvos, using PBS at pH = 7.2 as working solution, as reported in Figure IV.7.

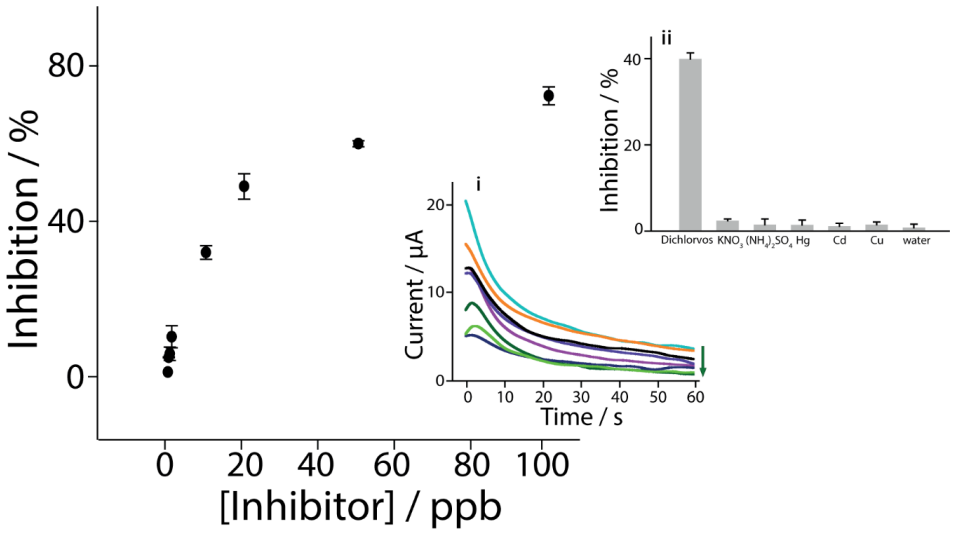

Figure IV.7. Calibration curve in the range between 0 and 100 ppb of inhibitor, using PBS at pH = 7.2 as working solution. Inset i) chronoamperometric recordings obtained in the range between 0 and 100 ppb of inhibitor. Inset ii) Selectivity studies conducted in presence of nitrates, ammonium, potassium, sulphates, mercury, copper, cadmium and water. Experimental conditions are the same as described in previous optimization studies.

The intensity of the chronoamperograms (inset i of Figure IV.7) was recorded in the presence of the inhibitor and it highlighted a decrease of the current as the inhibitor concentration increased. A linear trend was observed up to 20 ppb, while a dynamic interval was observed for higher levels of inhibitor: the observed exponential-like shape is consistent with the expected response of an enzyme inhibition assay in which at low concentrations of the inhibitor, the enzyme activity is only slightly affected, resulting in a linear relationship between the inhibitor concentration and the observed response. This initial linear range was

particularly useful for quantitative analysis, as it allowed for the direct correlation of the measured signal to the concentration of the analyte. However, as the inhibitor concentration increased, the enzyme activity was progressively inhibited, leading to a non-linear, exponential-like response. This behavior is attributed to the kinetics of enzyme inhibition, where the rate of inhibition was proportional to the concentration of the inhibitor. The exponential-like shape of the calibration curve at higher concentrations reflects the saturation of the enzyme active sites by the inhibitor, resulting in a diminishing response per unit increase in inhibitor concentration. The linear trend was described by the following equation: $y = 2.3x + 4.9$, where y represents the % of inhibition and x is the concentration of dichlorvos in ppb, with a calculated R^2 of 0.971. The detection limit, calculated as $3\sigma B/slope$ (σB represents the standard deviation calculated for blank measurements, slope is the slope of the calibration curve in the linear range), resulted equal to ca. 1 nM (0.3 ppb). The repeatability, as the relative standard deviation (RSD %) was calculated on four measurements of 20 ppb dichlorvos in PBS pH = 7.2, obtaining a value of 7 %. It should be noted that all the measurements were performed using one-shot platforms. Electrodes tested under the same experimental conditions but on different days and from different batches were also compared, obtaining an RSD% varying between 6 and 8 % while the Bias%, calculated on four measurements of 20 ppb of dichlorvos, as: (Average Actual value − Avarage Predicted Value)/Average Actual value) × 100, resulted equal to 2 %. Accuracy was calculated as 100 % − Error Rate, where the error rate was equal to the (Actual Value - Observed Value)/Actual Value × 100 and

resulted equal to 98 %, calculated on four measurements of 20 ppb.[68–71] The selectivity of the platform was evaluated in the presence of species, including toxic ones that could affect the enzymatic activity such as potassium nitrate (10 ppb), ammonium sulfate (10 ppb), mercury (10 ppb), copper (1 ppm) and cadmium (10 ppb).[6,72–74] As reported in the inset ii of Figure IV.7, the % of inhibition, calculated in presence of all these interfering species, was negligible when compared with the inhibition obtained with the same concentration of the pesticide, demonstrating the absence of the interference of these compounds at the level evaluated.

Application on fruit peels

In order to evaluate the applicability of the new platform and the ruggedness of the analytical method developed, the system was tested on real samples, considering the possibility of using this type of device directly on field, where the farmers can quickly evaluate the level of pesticides, just rubbing the fruit peel with the biosensor integrated onto a glove. The ultimate system was interrogated on two different fruit peels, namely apple and orange; the main problem associated with this architecture is that the rubbing can scratch the surface of the electrode and damage the hybrid nanocomposite, thus resulting in a less performing device. For this reason, we firstly tested the system "in solution," where the inhibitor was deposited onto the fruit, allowed to dry and subsequently re-suspended with bidistilled water prior to be sampled with the biosensor strip, as reported in Figure IV.8A and B.

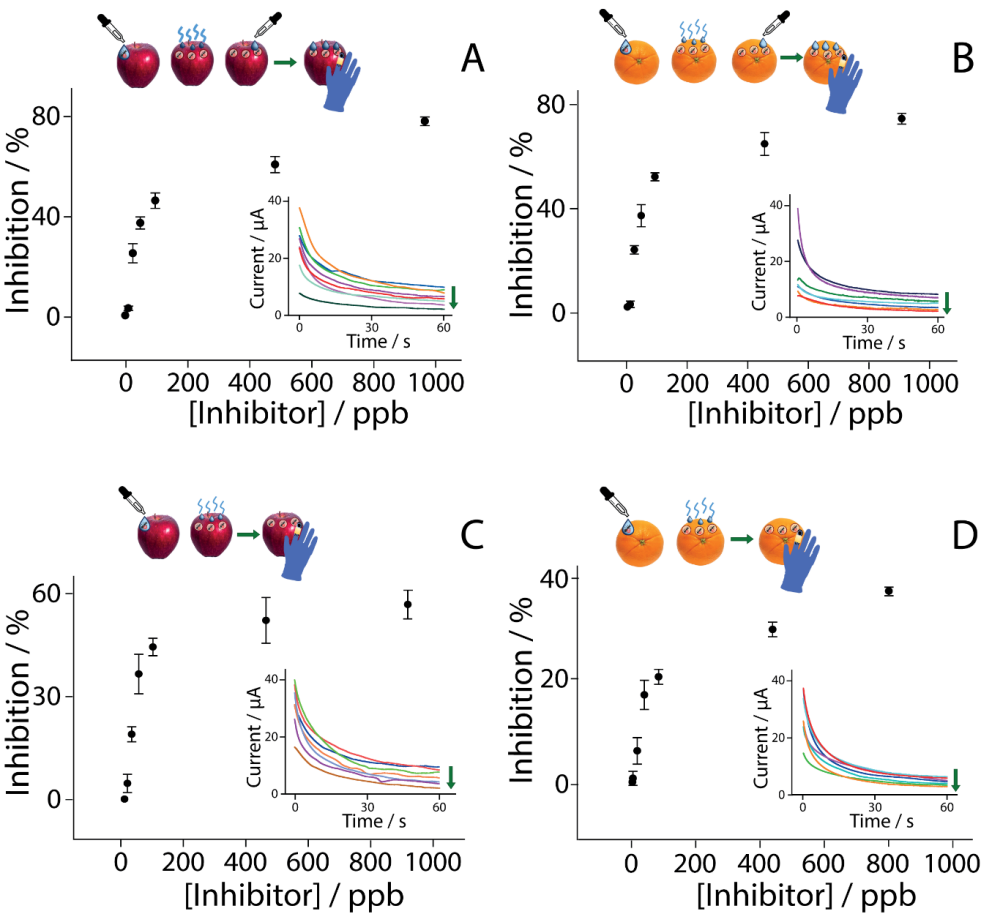

Figure IV.8. Calibration curves and chronoamperometric measurements obtained by testing the system on apple and orange peels. 4A) and 4B) biosensor rubbed "on drop" where the inhibitor was deposited and allowed to dry on apple and orange peels, and then wetting the area again with water and rubbing the sensor on the drop. 4C) and 4D) biosensor rubbed directly on the fruit peel where the inhibitor was deposited and allowed to dry. Experimental conditions are the same as described in previous optimization studies.

Additionally, the ultimate system was also tested in a "dry" mode, where the sensor was rubbed directly on the fruit peel where the inhibitor was deposited and allowed to dry (Figure IV.8C and D), without the re-suspension in water. As shown, the chronoamperometric measurements were then carried out by adding the solution containing the substrate as previously optimized. Meanwhile, the control experiments without pesticide were carried out under the same condition. As showed in Figure IV.8, the system gave satisfactory output for both cases, described by a similar exponential-like shape as the previous one, with an initial linear trend in the range between 0 and 50 ppb, described by the following equation: $y = 0.78x + 0.03$ and $y = 0.73x - 1.58$, with a detection limit of 8 and 40 nM when apple was analyzed, through solution and dry modes, respectively (Figure IV.8A and C). For the orange peel, tested in solution and dry, the linear range were described by the following equations: $y = 0.76x – 2.54$ and $y = 0.28x + 1.71$, with a detection limit of 12 and 24 nM, respectively (Figure IV.8B and D). The RSD %, calculated on four measurements, resulting equal to 8 and 9 % for the apple and orange tested both “in solution” and “dry”, respectively. The slight differences in terms of sensitivity might be attributable to the effect of strip's rubbing, which produces a lowering of the performance. The chemical composition of the peels of apples and oranges may differ significantly. Apples typically have a higher concentration of certain compounds, such as polyphenols and waxes, which can affect the extraction efficiency and subsequent detection of analytes. In contrast, orange peels may have a different composition that allows for more consistent extraction across samples. Moreover, apple peels are generally thicker and may contain

more fibrous material compared to orange peels, which could lead to variability in how well the analytes are extracted during sample preparation. This structural difference may result in lower sensitivity when analyzing dry apple samples. In solution, both fruits may present similar concentrations of analytes, leading to comparable results. However, when dried, the concentration of interfering substances or matrix effects may become more pronounced in apples than in oranges, resulting in a greater increase in LOD.[75] However, what it should be considered is that the sensitivity was capable to determine low concentration of dichlorvos in the range established by the regulating agencies. In addition, recoveries were calculated on two levels of Dichlorvos concentrations (10 and 50 ppb) and resulted equal to 81–104 % and 91–115 %, for orange tested through solution and dry modes, respectively and equal to 83–95 % and 106-97 % for apple tested through solution and dry modes, respectively.

On-glove biosensors, while promising for real-time monitoring, face several limitations that impact their durability and long-term stability. Mechanical durability is a concern due to the need for a balance between rigidity and flexibility to accommodate hand movements, which can lead to structural stress and degradation. In our system the use of a robust and flexible support as polyester try to address this limitation. Electrode stability is another issue, as exposure to sweat and environmental factors can cause electrode degradation, affecting sensor performance and accuracy. Signal consistency is also a challenge, with potential issues like hysteresis and nonlinearity exacerbated by dynamic hand movements. User comfort and wearability are essential to ensure continuous use, and environmental

factors such as temperature and humidity can further impact sensor reliability. Frequent calibration and maintenance are necessary to maintain accuracy, and interference from other signals, such as sweat and movement artifacts, as well as cross-reactivity with other substances, must be addressed to enhance the sensor's specificity and reliability.

4.2.4 Conclusion

In this work, we developed a screen-printed electrochemical biosensor for enzymatic detection of organophosphorus pesticides directly on fruit peels, just by rubbing their surface. Considering the results obtained, the use of common materials such as a simple polyester substrate, suitably modified, proved to be a suitable architecture for the realization of a portable device for the detection of organophosphate pesticides, which can be used for in situ analysis due to its strength and flexibility and easy integration into a glove. Dichlorvos was detected down to a nanomolar range in a few microliters of sample with a satisfactory repeatability and selectivity. The combination of the three-electrode configuration, printed on polyester with biomimetic PB and CB electrocatalysts, and the BChE enzyme allowed accurate quantification of the target analyte. Two different fruit peels were analyzed, and satisfactory detection limits were achieved. When comparing the on-glove biosensor to laboratory-based methods, its LOD of 1 nM positions it competitively among portable technologies while still being less sensitive than traditional laboratory techniques. However, the key advantage of the on-glove biosensor lies in its portability and user-friendliness, enabling real-time monitoring

directly in the field. This capability was crucial for immediate decision-making regarding pesticide application and food safety.

The results obtained pave the way for the rising of on-glove technology, with which the farmers could perform rapid and accurate pesticide measurement, without specific training and complex setup/equipment.

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CHAPTER 5: SMART REMEDIATION: FROM ELECTROCHEMICAL DETECTION WITH BIOCONTROL STRATEGIES FOR COPPER

The previous chapter discussed and addressed the detection of copper and other pesticide residues using the wearable glove sensor. This chapter now turns to another equally important aspect of environmental contaminant management, in this case copper in agriculture. At this stage of the research, the aim is to identify new and existing solutions for the remediation of copper pollution and propose the development of a remediate-and-sense system using the sensor technologies developed in this work. First existing remediation techniques will be examined, such as the use of alginate beads to remove metal ions, combining these natural, biocompatible beads with the electrochemical sensor technology previously developed. The beads, which act as a remediation agent, can be integrated into a customized chamber that can be 3D printed, enabling the simultaneous removal and detection of copper ions in environmental matrices such as water and soil. This combined remediation and real-time monitoring strategy could offer a new path to decentralized environmental monitoring, where each user not only measures pollution levels but actively remediates contaminants in situ.[1]

Subsequently, the scope will be expanded to remediation by studying biological control agents (BCAs) that can bioaccumulate copper. Specifically, studying the ability of beneficial bacteria, including *Bacillus amyloliquefaciens* and *Bacillus subtilis*, to reduce copper concentrations in contaminated culture media by natural processes.

Using an electrochemical sensor copper levels can be monitored to determine if these bacteria are able to significantly lower copper levels in the culture broth, avoiding the need for chemical detoxification, thus offering new avenues for sustainable, biologically-based remediation methods.

This chapter illustrates two different but complementary methods: 1) alginate bead remediation platform combined with an electrochemical sensor and 2) preliminary contribution of bioremediation with the beneficial intervention of microorganisms for copper reduction. Both approaches seek to address the growing environmental concerns about copper pollution in agriculture and the need for effective, cost-effective and scalable solutions. This chapter proposes a basis for sustainable environmental remediation and could have large-scale application in precision agriculture, wastewater treatment and environmental monitoring.

5.1 REMEDIATE AND SENSE: ALGINATE BEADS EMPOWERED BY PORTABLE ELECTROCHEMICAL STRIPS FOR COPPER ION REMOVAL AND DETECTION AT ENVIRONMENTAL SITES

5.1.1 Introduction

Cu is classified as an essential trace element because it is required for a number of metabolic and physiological processes in humans, animals, and plants.[2] It is associated with bone health, immune function, cardiovascular risk, and alterations in cholesterol metabolism.[3] It is required for human brain development and maturation and functions as a cofactor of key metabolic enzymes and as a signalling and regulatory molecule. The amount of Cu ions in organisms affects infant growth, host defense mechanisms and brain development.[4,5] As discussed in previous chapters, Cu is widely used in agriculture as an inexpensive bactericide and fungicide that is effective against a wide range of diseases, including grapevine blight, potato blight, and downy mildew, one of the most serious vine diseases worldwide.[6–8] However, the extensive use of Cu has led to the development of copper-tolerant microorganisms and negative environmental impacts due to its accumulation in soil and water bodies.[9] Exposure of crops and vegetables to high levels of Cu in soil can cause direct toxicity to plants, while chronic exposure to moderate levels of Cu can affect reproductive rates and vegetative growth (*e.g.*, biomass of aerial and underground structures).[10] With the aim to limit Cu intake, the World Health Organization recommends a maximum of 0.9 mg per day of Cu for adults.[11]

For these reasons continuous monitoring of Cu in agricultural soil and plants is essential to control its levels and improve environmental quality and human well-being. In this context, the availability of portable analytical devices that permit users, particularly those non-specialized, to detect pollutants and/or the presence of specific species in the environment represents an important opportunity to monitor both the ecosystem and consumer health. This approach differs from conventional techniques,[12,13] which, while offering high performance, require specialized laboratories with expensive instruments, and trained personnel for the analysis, thus limiting their applicability for in situ testing.[14]

The design and application of sensors and biosensors is of fundamental importance for this goal, and the use of electrochemical techniques is preferable to colorimetric/fluorimetric methods, due to the advantage of analysing coloured/turbid matrices; in fact, the electrochemical methods are not affected by measuring non-treated matrices, *i.e.* blood, soil, and wastewater. With a major focus on the detection of metals, various electrochemical approaches have been reported: Bobrowski *et al.* evaluated the functionality of a screen-printed carbon electrode coated with an antimony film to detect Cu (II) in treated soil samples, and by differential pulse anodic stripping voltammetry, a detection limit of 0.14 μg L^{-1} and a linearity up to 150 μg L^{-1} were obtained.[15] Adarakatti *et al.* designed a screen-printed platform using calixarene to detect lead, copper and mercury ions. The three metals were successfully detected in industrial effluents and wastewater samples, with detection limits of 38, 40 and 48 μg L^{-1}, respectively for lead, copper and mercury.[16] Electrodes were also screen-printed onto office

paper-based substrates to detect Cu in serum samples, down to 4 ppb using gold nanoparticles as the electrode modifier.[17] Recently Nguyen *et al.* have modified a silver-based electrode using MnO_2 as a modifier. Cu ions were detected in water sources down to 0.5 nM with a linearity comprised between 0.625–15 nM.[18] It is without any doubt that these portable approaches can be of high relevance in environmental and other fields.

However, the effectiveness of a decentralized detection system should be empowered by the possibility of remediating an environmental site of interest, and in this regard, several methods have been studied to remove metal pollutants from the environment. Remediation techniques include different approaches such as physical, chemical, and biological methods. Physical methods combine soil substitution and hot desorption processes including washing, soil replacement and isolation, surface capping, electrokinetic remediation and encapsulation. Chemical methods include vitrification, precipitation, ion exchange and adsorption. Biological methods are based on the use of microorganisms such as microbes and plant species, and depending on the site where the waste is removed, they are classified into *in situ* or *ex situ*.[19,20]

Although many previously listed physical, chemical and biological methods have been applied for the removal of heavy metals, chemical adsorption methods have been highlighted as efficient, inexpensive and easy to apply. [21] Adsorption is a process in which a solid or liquid substance accumulates molecules or particles of another substance on its surface.[22] Chemical adsorption is more popular for heavy metal removal because it has stronger interactions and higher adsorption

capacity towards heavy metals.[23] With regard to these approaches, the use of alginate, a natural anionic polysaccharide containing free hydroxyl and carboxyl groups, has been highlighted due to its abundance, non-toxicity and biocompatibility. [24] The green features of alginate and alginate-based composites have been reported to be applied towards metal removal up to the parts per million level: various applications towards the detection of copper, cadmium, lead, chromium and mercury ions, using alginate as hydrogel beads and also in the presence of chitosan, polyaniline, polypyrrole, graphene, conductive polymers, *etc*., were reported.[25–28]

However, even if alginate represents a promising and environment-friendly solution for remediation, the success of metal removal needs to be evaluated by external analytical methods. It should also be considered that an *in-situ* solution would be highly effective for the real-time surveillance of environmental sites. As per the theranostic application in clinical field, *i.e.* the possibility for diagnosing and providing a therapy for a certain disease using the same platform, a concept similar to remediate-and-sense, would have tremendous impacts, particularly to assure a prompt intervention in case of excessive pollution.

In light of this, the goal of the present work was to develop for the first time a combined tool for the removal and sensing of metal ions in environmental matrices, using Cu ions as the case of study. Briefly, an integrated device including alginate beads to remediate Cu ions and screen-printed electrochemical strips was developed and characterized for its application to liquid and solid matrices, *i.e.* water and soil. The removal ability of alginate beads was evaluated *via* the optimization of

the amount, size and time of contact with the polluted matrices. In particular, an experimental protocol was developed avoiding complex and invasive procedures, making the entire system a user-friendly platform for use in a decentralized context. The combination with printed-electrochemical strips for the rapid detection of metal ions is of particular importance if the on-site application is considered, and it represents a starting point towards further application in the environmental field, but not limited to this. It can be a starting point for converging towards precision and sustainable agriculture, reducing time and costs for on-site measurements and limiting the use of species that may be harmful to human health and the environment.[29,30]

5.1.2 Experimental section

Materials

Conductive inks (Ag/AgCl and graphite) were purchased from Acheson (Milan, Italy), hydrochloric acid and alginic acid sodium salt from brown algae (A0682, low viscosity 4–12 cP for a 1% aqueous solution at 25 °C) from Sigma-Aldrich (St Louis, MO, USA), standard copper solutions (1000 mg L^{-1}) from Fisher Scientific (Milan, Italy), and calcium chloride anhydrous from Merck Co. (Darmstadt, Germany). All reagents were characterized by a purity higher than 98%, as indicated by the producer. Soil was sampled from a garden pot. For the flexible polyester-based substrate, we used Autostat HT5 (125 μm), MacDermid, UK. This substrate was chosen for its robust and flexible characteristics, which are essential for our environmental applications. To insulate the printed electrodes, a clear adhesive tape as used in packaging was used. All measurements were done using a

portable potentiostat, EmStat3 (PalmSens, The Netherlands) connected to a laptop.

Fabrication of electrochemical strips

The three-electrode system was screen-printed manually, on a polyester substrate, as illustrated in the Chapter 2. Adhesive tape (commercially available) was used to insulate the printed electrodes. The diameter of the working electrode was 0.4 cm, and the electrochemical strips were 2.5 cm × 1 cm, respectively length *x* width.[31] The shelf life of the strips is around 1 year if they are stored in vacuum packs away from light and at room temperature.

Modification with AuNPs

To improve the analytical performance of the sensor for Cu measurement, gold nanoparticles, synthesized according to the procedure outlined in Chapter 2, were used.

Alginate bead synthesis

Calcium alginate beads were prepared by means of ionic cross-linking, starting from a sodium alginate solution (1% w/v) obtained by dissolution in distilled water, under continuous stirring, at room temperature, following a procedure adapted from previous studies.[32,33] The obtained solution was loaded in a plastic syringe (Terumo, 5 mL), supplied with a metallic needle (diameter size = 18G or 23G), and then extruded into an anhydrous calcium chloride gelling bath (1.0 M) through an infusion pump (KDScientific, MA, USA). The following parameters were selected: distance between the syringe and gelation media was 3 cm and the flow rate was 10 mL h^{-1}. The produced beads were cured in the gelation medium for around 15 min, under constant stirring, and then taken out and washed several times

with distilled water in order to remove possible residual calcium chloride. The average sizes of the obtained beads were measured from the micrographs acquired using an optical microscope, using the ImageJ (NIH) software. The surface of the beads was investigated by field emission scanning electron microscopy (FEG-SEM, Leo Supra 35, UK). The swelling test was carried out by dipping pre-weighed beads in distilled water (1 bead per mL distilled water), within a humidity-controlled incubator (temperature = 37 °C and humidity = 98%). The beads were extracted at regular intervals (5 to 360 minutes), cleaned superficially to remove surface water, accurately weighed, and observed using a digital microscope (Jusion 40) at selected time points. The swelling percentage (SP) was determined using the following expression: SP = (Mt–M0) *x* 100/M0, where M0 and Mt are the initial mass and mass at time *t*, respectively. The measurements were made in triplicate, and the average data were provided.

Cu(II) measurements

Linear sweep-anodic stripping voltammetry (LS-ASV) was performed for Cu detection, which first involves using a deposition potential to reduce Cu in the solution and then oxidizing it. Measurements were conducted using a 0.1 M hydrochloric acid solution as the working solution, which was also used as the supporting electrolyte for Cu detection. The use of an acid is necessary because the analysis of stripping is performed in the presence of acidic media, which are necessary to have metals in their oxidized forms.[34] In anodic stripping voltammetry, the anodic peak potential for the re-oxidation of Cu ions was observed around 0.3 V vs. Ag/AgCl. The experimental parameters were optimized as follows: a deposition potential of −0.4 V was

applied for 300 s, in the potential range from 0.1 to 0.6 V, with an E step of 0.01 V and a scan rate of 0.5 V s^{-1}.[17]

ICP-MS measurements

In order to evaluate the trueness of the platforms, all the samples were analyzed by ICP-MS. Briefly, samples were mineralized: 250 microliters were transferred into a 7 mL Pyrex. Hydrochloric acid and nitric acid were added to each sample (3 : 1 v/v). The reaction was conducted for 16 h at 90 °C, and after digestion the samples were diluted to 15 mL in ultrapure water. Measurements were performed using an Agilent 7700 ICP-MS instrument (Agilent Technologies) equipped with a frequency-matching radio frequency (RF) generator and a 3rd-generation Octopole Reaction System (ORS3) operating with helium gas in ORF. The following parameters were used: RF power = 1550 W; plasma gas flow = 14 L min^{-1}; carrier gas flow = 0.99 L min^{-1}; He gas flow = 4.3 mL min^{-1}. In this experiment, 103Rh was used as an internal standard. Lead and cadmium concentrations were measured with three replicates.

Remediate-and-sense

In order to realize a unique platform for both remediating and sensing Cu ions in environmental matrices, *i.e.*, water and soil, an easy and integrated approach was adopted. Briefly, the chosen environmental matrix was allowed to enter into contact with the alginate beads without any treatment, *i.e.* acid addition, heating, *etc.*, and consequently a printed strip was used to *in situ* monitor the presence of Cu ions, as displayed in Figure V.1.

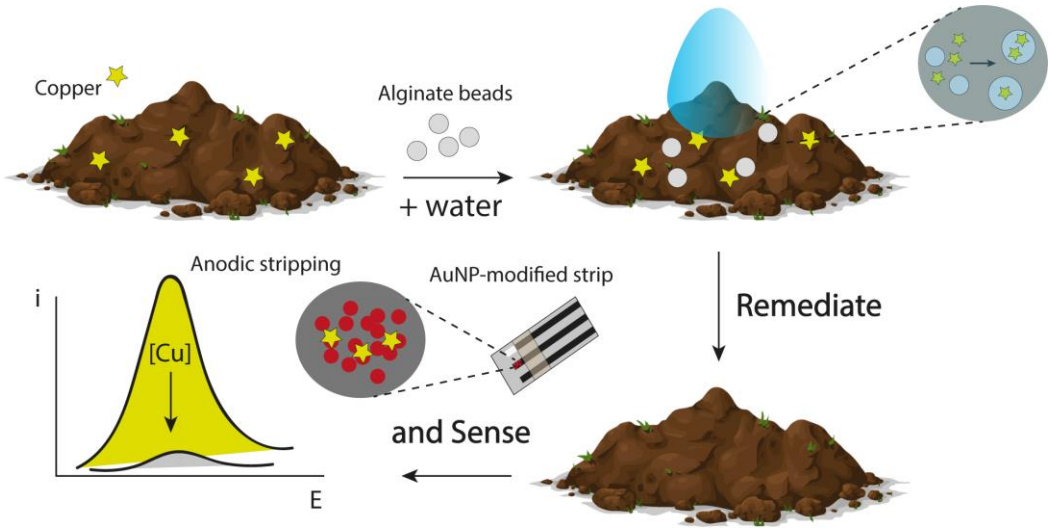

Figure V.1. Architecture mechanism of the remediate-and-sense concept carried out in soil samples.

5.1.3 Results and discussion

AuNP-modified printed strips for copper detection

As reported in our previous findings, Cu ions were successfully detected in biological matrices using paper-based electrochemical strips.[17] In this case, a flexible substrate made of polyester was used to realize the printed strips: the choice was due to the need for flexibility and mechanical robustness for *in situ* application in environmental settings. However, even if the role of AuNPs was demonstrated to be fundamental for the analysis of Cu ions, and other ionic species, *e.g.* mercury, arsenic, and iron, it should be noted that the deposition of AuNPs might change among different substrates, due to

different porosities of conductive inks that are printed. The first investigation was about the modification of the strip surface with various amounts of AuNPs. As observed in the inset i of Figure V.2, by carrying out cyclic voltammetry experiments in the presence of 5 mM potassium ferricyanide in a 0.1 M potassium chloride solution, it appeared obvious how the presence of AuNPs improved the performance of the system. The surface of the strip was modified with different volumes of gold nanoparticles, namely 2 and 8 μL, and it was clear how their presence was effective with the increase in the electrochemical performance, as demonstrated by the higher intensity of peaks and the lower potential difference between the oxidation and reduction peaks. In particular, the potential difference is a good indicator of the quality during the electrochemical process of electron exchange at the electrode surface: as a result, 2 μL was selected as the optimal compromise among electrochemical performance, time to modification and amount of modifiers. The next step involved the evaluation of the analytical performance in the presence of Cu ions in a wide range of concentrations, namely up to 400 ppb. The anodic stripping voltammetry (ASV) was adopted to obtain a calibration curve under standard conditions, using 0.1 M hydrochloric acid as the working solution.

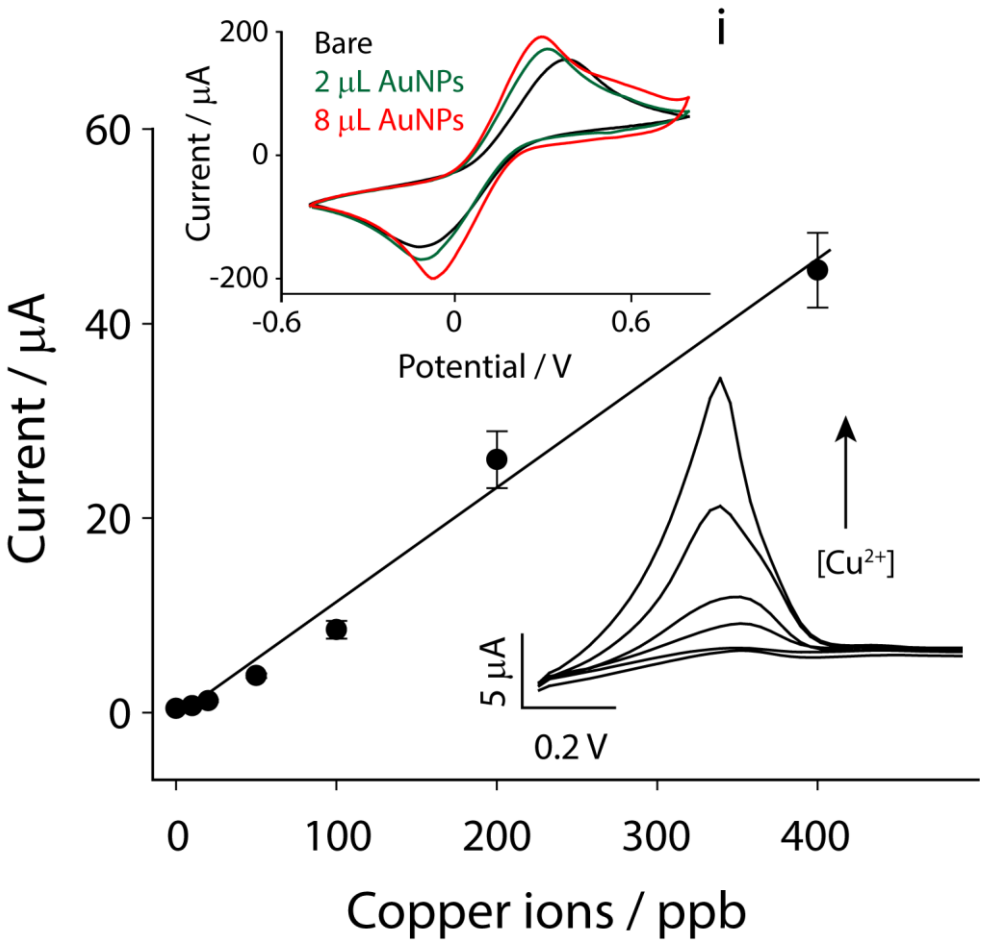

Figure V.2. Calibration curve for copper ions in a standard solution, *i.e.* 0.1 M HCl, obtained by modifying the electrochemical strips with 2 µL of AuNPs. Experimental parameters: *E* dep = −0.4 V; *t* dep = 300 s; *E* begin = 0.1 V; *E* end = 0.6 V; *E* step = 0.01 V; scan rate = 0.5 V s^{-1}. The voltammetric curves relating to the measured concentrations for copper are also reported. Inset i: Cyclic voltammograms for the evaluation of the optimal amount of AuNPs, between 2 and 8 µL. The measurements were conducted in triplicate in

the presence of 5 mM potassium ferricyanide in a 0.1 M potassium chloride solution.

As reported in Figure V.2, a linear correlation was obtained between the height of the current peaks and the concentration of Cu ions. The relationship is described using the following equation: $y = 0.12x - 0.88$, $R^2 = 0.983$, (where y represents the current difference between metal ions and the blank solution, and x represents the metal ion level expressed in ppb).

The limit of detection (LOD), calculated as $3\sigma B$/slope (σB is the standard deviation calculated for several blank measurements and the slope is relative to y/x in the calibration curve), was found to be *ca.* 1 ppb, while the limit of quantification (LOQ) was found to be *ca.* 4 ppb, with a relative standard deviation (RSD%) lower than 10%.

Alginate beads as remediating agents

In order to evaluate the capability of alginate beads to remediate Cu ions, two different types of alginate beads, obtained using two different needles (18G and 23G), were used, and named 18G alginate beads and 23G alginate beads, respectively. In particular, the size and amount of alginate beads, including the time of interaction between Cu ions and beads, were evaluated.

Namely, the remediation efficacy of alginate beads 18G and 23G was investigated in the presence of a 100 ppb solution of Cu ions dissolved in water. In detail, different numbers of beads (from 5 to 20) were used to identify the optimal density to be used for successive remediation studies, as shown in Figure V.3A and B. Before evaluation, the beads were allowed to dry for approximately 40 minutes at room temperature in contact with an adsorbent pad. Successively, various numbers of

beads, between 5 and 20, were put in contact with 200 microliter of a 100 ppb Cu ion solution.

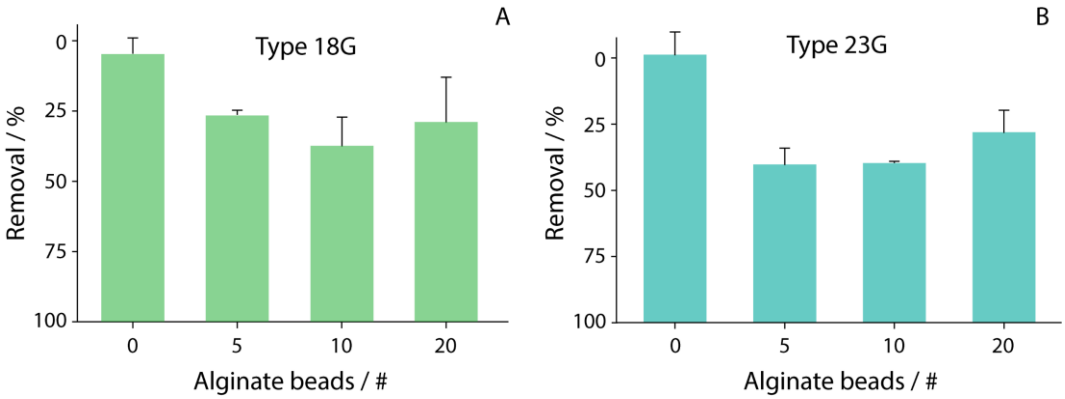

Figure V.3. Optimization of the size and quantity of alginate beads placed in contact with a 100 ppb copper ion solution. (A) Evaluation of the ability of 18G alginate beads to remediate copper ions, using different bead quantities, ranging from 5 to 20. (B) Evaluation of the ability of 23G alginate beads to remediate copper ions using different bead quantities, ranging from 5 to 20.

For the first evaluation, 180 min interaction among Cu ions and alginate beads was considered (the duration was chosen after assessing the removal capability within a wide range of times, data not shown). The efficacy of Cu removal was studied through the adoption of stripping voltammetry after the sampled solution was acidified to pH 1 with hydrochloric acid. The 23G alginate beads showed a slightly better removal efficiency than that of the 18G ones, perhaps due to the average size (1.75 ± 0.02 mm *vs.* 2.75 ± 0.02 mm, respectively), while

for both types of beads, 10 of them resulted with the highest removal, with percentages of *ca.* 40 and 50, respectively for 18G and 23G.

According to these findings, further studies were performed using the 23G alginate beads. As far as the formation of copper $[CuCl_4]^{2-}$ complexes is concerned, this is more likely at higher concentrations of hydrochloric acid. At concentrations of 0.1 M, the probability of forming $[CuCl_4]^{2-}$ complexes is low compared to solutions with much higher HCl concentrations. However, it is important to note that the formation of such complexes can still occur to some extent, particularly under certain conditions such as high temperatures or long reaction times. The predominance of $[CuCl_4]^{2-}$ complexes generally increases with the increase in HCl concentration.[35]

Alginate bead morphology

Following the identification of the optimal alginate beads, they were characterized. It should be noted that homogeneous beads were achieved, and in the performed swelling tests, the alginate beads showed slow water absorption in the initial phase, whereas, after 3 hours, there was a drastic increase in water absorption and in the next 2 hours the beads reached their maximum swelling. In detail, they were characterised by a maximum water absorption of 250%, reaching an equilibrium between 240 minutes and 360 minutes, with the increase in size as high as 60% of the initial diameter (Figure V.4A). In addition, the surface of the produced beads was homogenous and rough, promoting the metal adsorption, as evident from the acquired SEM micrograph displayed in Figure V.4B.

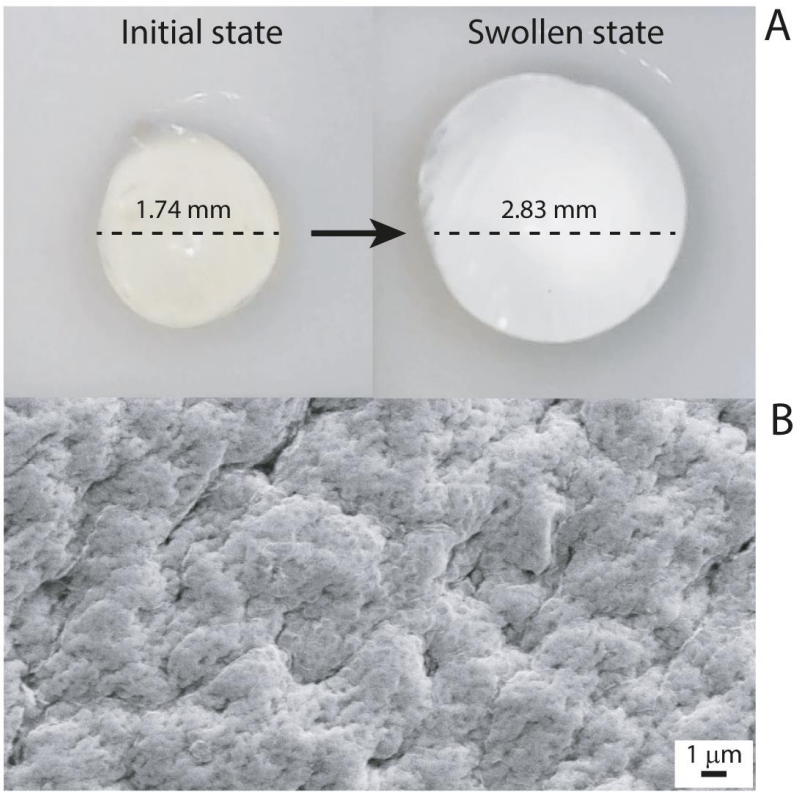

Figure V.4. (A) Micrographs of the alginate beads in the initial state (left) and the swollen state. (B) SEM images of the alginate bead surface.

Remediate-and-sense in soil samples

The developed system that combines the possibility of removing Cu ions and evaluating the performance of remediation in real time with an integrated strip was applied towards contaminated soil. However, before evaluating the performance for remediation, the electrochemical strip performance obtained in the standard solution was compared with those obtained in soil. In particular, a gram of soil was acidified with 5 mL of 0.1 M hydrochloric acid. Successively, a fraction was used for the direct measurement, while the remaining amount of acidified soil

was filtered using a syringe with a 0.45 µm membrane, to remove the gross impurities. As reported in Figure V.5, all the "blank" solutions were spiked with the increasing amount of Cu ions in the 0–80 ppb range, to evaluate the difference in sensitivity for future applications.

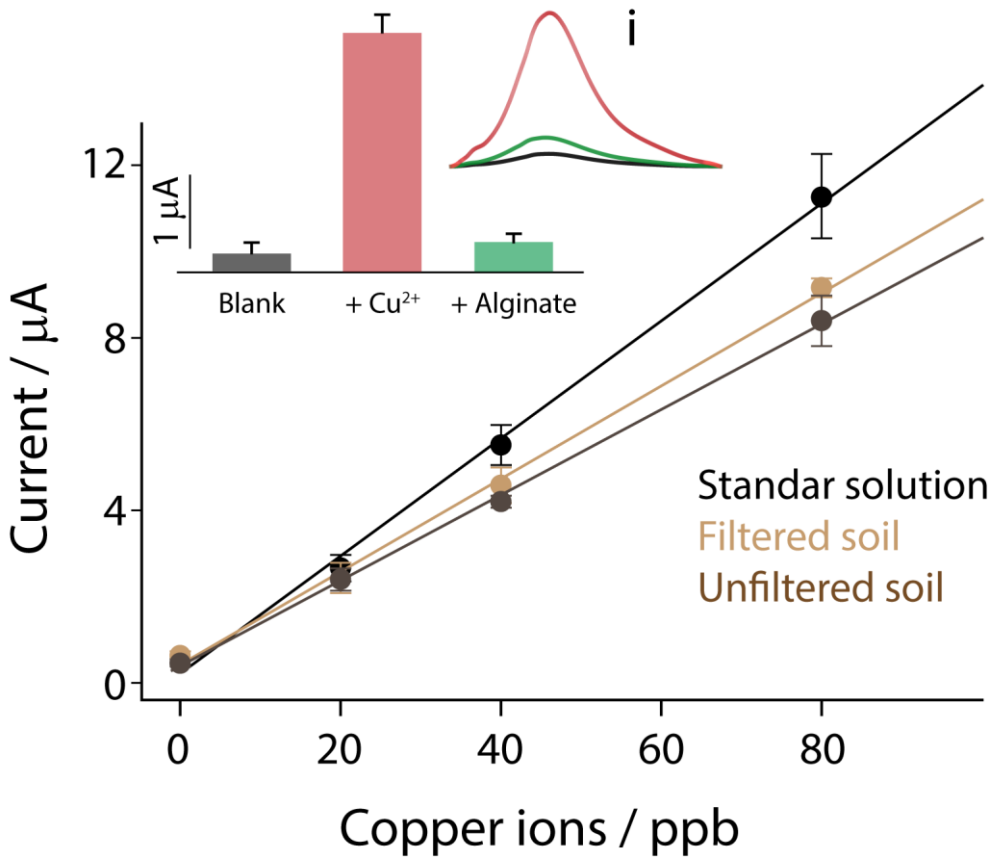

Figure V.5. Study of the effect of the soil matrix on the sensitivity of the electrochemical strip. Three different calibration curves were obtained for copper ions in the range of 0 to 80 ppb in the standard solution, *i.e.* 0.1 M HCl, filtered and unfiltered soil. All measurements were carried out in triplicates. Inset: Comparison of the detected copper ions in three different samples: blank soil, 50 ppb copper-spiked soil and 50 ppb copper-spiked soil with the addition of 10 alginate beads.

Inset i: Voltammetric curves related to the measured concentrations of copper.

By observing Figure V.5, it should be noted that a slight decrease in sensitivity was obtained by comparing the measurements carried out with and without soil, and this is ascribed to the presence of a complex matrix like soil. In particular, the sensitivity obtained in a standard solution of 0.1 M hydrochloric acid was equal to 0.14 μA ppb^{-1}, while the sensitivity obtained after soil contact were equal to 0.11 and 0.10 μA ppb^{-1}, respectively to filtered and unfiltered soils. All measurements were carried out in triplicate, and all the correlations were characterized by an R^2 value of 0.993. All the metals inside the soil were quantified by ICP-MS measurements, and the presence of Cu ions was not high enough to be detected by the present method. Successively the optimized type and amount of alginate beads were applied to copper-spiked soil as a proof of concept. Prior to start, the soil was spiked with 50 ppb of Cu ions. Successively 10 alginate beads were added to the soil together with distilled water. Only water and alginate beads were used to evaluate the removal activity in the presence of contaminated soil. As reported in the inset of Figure V.5, three samples were compared: blank soil, 50 ppb copper-spiked soil and 50 ppb copper-spiked soil with the addition of 10 alginate beads. After two hours of interaction, the solution was taken without filtration and the pH was adjusted to 1 using hydrochloric acid on the strip, avoiding any contact with the soil. What has been achieved was a quantitative removal, which reported the signals for spiked soil similar to those obtained for blank samples, and using 3D printing, it was possible to design and realize an *ad hoc* case for the complete

integration of the remediate-and-sense platform into a unique and compact device, as shown in Figure V.6.

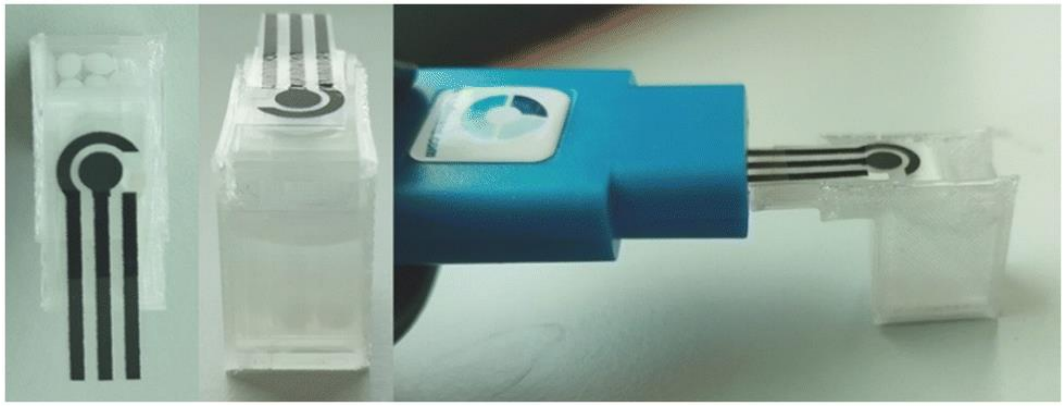

Figure V.6. Three-dimensional printed case for the integration of the printed electrode and alginate spheres.

In addition, diverse spiking of Cu ions was used in the soil, with levels between 50 and 200 ppb, and the efficiency of the approach of remediate-and-sense was evaluated by ICP-MS analyses of spiked soil, as shown in Table V.1, demonstrating the satisfactory trueness of the method.

Table V.1. Correlation among ICP-MS and electrochemical strips of various Cu-spiked soil concentrations and apparent recoveries of the two methods.

Spiked concentration (ppb)	**ICP-MS (ppb)**	**ICP-MS app. recovery (%)**	***E*-strip (ppb)**	***E*-strip app. recovery (ppb)**	**Correlation (%)[a]**
50	50 ± 1	100	55 ± 5	110	110
100	102 ± 2	102	97 ± 8	97	95
150	149 ± 3	99	140 ± 11	93	94
200	205 ± 3	102	220 ± 20	110	107

[a]Correlation % was calculated as (*E*-strip (ppb)/ICP-MS (ppb)) × 100.

5.1.4 Conclusions

The present work highlighted the opportunity to combine a remediation strategy with a portable analytical tool, to realize the so-called remediate-and-sense architecture. The approach was obtained by merging the efficacy of alginate microbeads and printed electrochemical strips to remediate copper ions and evaluate the efficacy with electrochemical measurements, respectively. The efficacy of alginate beads was evaluated by characterizing different-sized beads, using copper ions as the metal model to be remediated. In particular, the system was applied to a complex environmental matrix like soil. As a proof of concept, spiked soil was quantitatively

remediated using just 10 alginate microbeads, avoiding complex tasks and invasive procedures. In addition, the analytical performance of the strip was satisfactorily evaluated by a complementary analysis of ICP-MS, with the correlation between *ca.* 95 and 110%.

This study represents the first step towards the development of a fully printed analytical platform, where alginate might represent the substrate to screen-print electrochemical strips. A widely possible application can be made with this new architecture; in particular, it can represent a promising starting point for applications combined with pollutant removal methods, in order to converge towards precision and sustainable agriculture, limiting the use of species that may be harmful to human and environmental health.

To highlight the importance and contribution to the advancement of portable environmental remediation technologies, this work, published in the journal *"Analyst"*, was awarded a cover page in the same journal, as shown below.

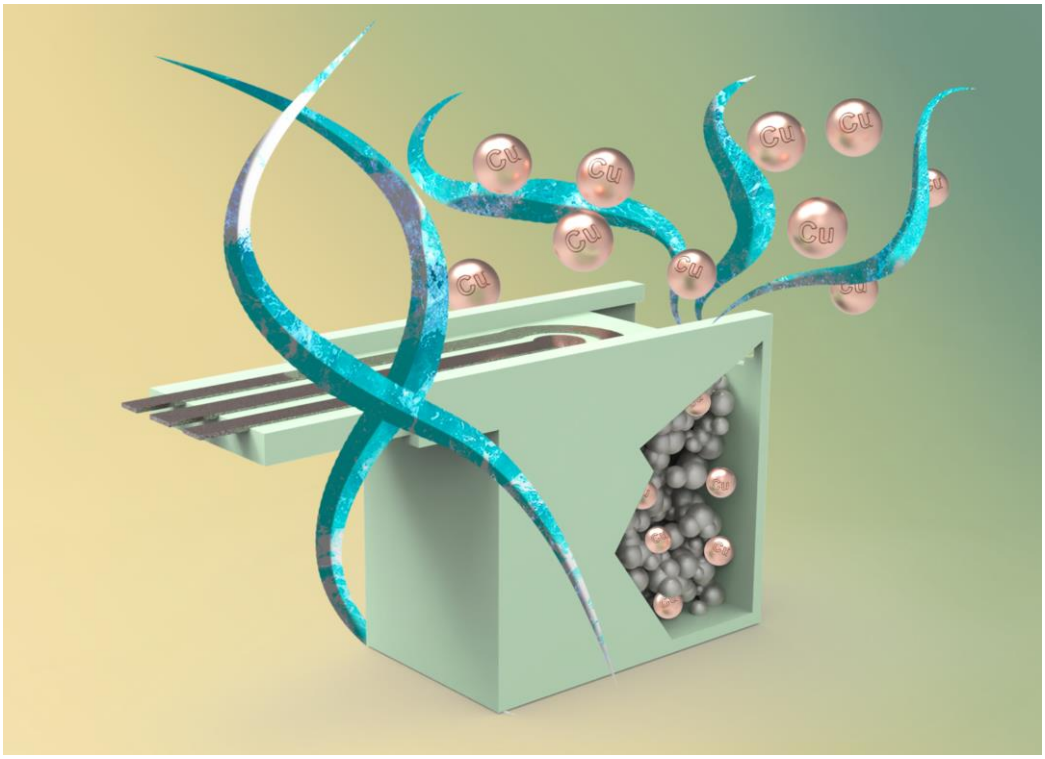

Figure V.7. Schematic presentation of advancement of portable environmental remediation technologies as a back cover page published in journal *Analyst*, 2024, **149**, 3302-3308.

5.2 INNOVATIONS IN COPPER BIOACCUMULATION: BIOLOGICAL CONTROL AGENTS AND ELECTROCHEMICAL SENSORS FOR EFFICIENT DETECTION

5.2.1 Introduction

As a traditional Cu remediation strategy, alginate beads have been widely described as a cost-effective and efficient metal removal technology with the potential to remediate metal contamination in the environment. The alginate-based systems used in this study mainly involved physical adsorption of Cu ions, with little or no active participation of the material with the contaminant. Despite the good Cu removal capacity, the use of alginate beads has some drawbacks. Although the adsorbent material is initially effective, the effectiveness decreases over time and more metal ions cannot be sequestered because it becomes saturated. Furthermore, this approach does not require any modification or detoxification of Cu and therefore does not alter its inherent toxicity. In addition, alginate beads are ineffective for long-term applications because they play only a temporary role as a Cu reservoir, having no regenerative or adaptive capacity to different external Cu concentrations. Therefore, although alginate-based methodologies may be effective in some applications, their passive nature is a serious drawback to achieving long-term sustainable remediation of heavy metals such as Cu. These drawbacks have stimulated the exploration of more efficient and effective remediation methods, particularly the application of biological control agents (BCAs) in the biodegradation/bioaccumulation of heavy metals, which

would represent a significant advance over traditional remediation methods.[36]

Bioremediation, or the use of living organisms, particularly microorganisms, to degrade, immobilize or transform pollutants, has emerged as a promising solution to the environmental challenges posed by heavy metals such as Cu.[37–41]

Although several types of BCAs have been studied, *Bacillus* species have received considerable attention because of their high tolerance to metal toxicity and their natural potential to bioaccumulate heavy metals such as Cu. *Bacillus* species such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* sequester and detoxify metals using a variety of mechanisms, including biosorption, bioaccumulation, and biotransformation. These not only reduce the bioavailability and toxicity of Cu ions, but are much more complex than simple physical adsorption.[42–45] Because these bacteria not only capture Cu ions but also internalize them, they can be stored in less toxic forms, either as intracellular or precipitated insoluble forms. *Bacillus* species are known to be refractory, surviving metalliferous biogeochemical cycles and under conditions of macronutrient deficiency; they also exhibit bacterial endotoxins and remove heavy metals from solutions due to accumulated stressors.[46]

Bacillus species are spore-forming Gram-positive bacteria that can survive conditions that are lethal to many common microorganisms; consequently, they are extremely effective in contaminated soils and environments where other bioremediation agents are unsuccessful. In addition, *Bacillus* species have been shown to precipitate Cu ions into less bioavailable forms, thereby reducing Cu toxicity and mobility in

the environment through bioprecipitation and remineralization of retained metals, and the combination of these biological processes makes Bacillus species a promising biologic for long-term remediation of heavy metals and a great improvement over passive methods such as alginate beads.[47–50] These biological processes make *Bacillus* species a powerful tool for long-term remediation of heavy metals and a significant advance over passive approaches such as alginate beads.

Here it is presented a new Cu bioremediation strategy using BCAs, specifically *Bacillus*, in an active Cu bioremediation effort in collaboration with the Department of Agricultural Sciences at Portici. These microorganisms provide a dynamic and sustainable means of removing environmental Cu contaminants through bioaccumulation. In addition, this study incorporates real-time monitoring to assess Cu removal performance by *Bacillus* species, providing a more dynamic and precise approach than conventional methods. Nano sensing, in the form of electrochemical sensors with gold nanoparticles, was used to continuously monitor Cu uptake by *Bacillus* species, which can serve as an easily obtainable, sensitive and non-invasive means of monitoring the remediation process. This real-time monitoring technology provides immediate information on the success of bioremediation processes, thus supporting dynamic adaptation of remediation approaches if necessary.

Preliminary results of this study revealed that the microorganisms *Bacillus amyloliquefaciens* and *Bacillus subtilis* can reduce Cu concentrations in contaminated culture media. Sensor technology was then integrated into the bioremediation process, allowing the treatment to be refined by providing real-time data on metal bioaccumulation,

resulting in a much more effective and efficient process for Cu removal.

Another vital benefit of *Bacillus* species in Cu bioremediation is the plant growth-promoting activity attributed to growth-promoting rhizobacteria (PGPRs). They can promote plant growth through functions such as production of plant hormones, improved nutrient uptake, and resistance to soil-borne diseases. This is particularly beneficial in the context of copper-contaminated agricultural soils, as it not only addresses the pollution problem but also increases plant health and agricultural productivity. The use of *Bacillus* species for bioremediation is not only effective but also environmentally friendly, as it promotes plant growth, restores soil fertility and creates a balanced ecosystem leading to sustainable agricultural practices.[46]

Therefore, *Bacillus* species in combination with real-time electrochemical monitoring can potentially suggest more ecological, on-demand, and more efficient solutions. This study introduces *Bacillus* species as Cu bioaccumulators with optimally controlled bioremediation monitored in real time. In addition to stimulating plant growth through PGPR activity, *Bacillus*-based bioremediation is an integrated, environmentally friendly, cost-effective, and scalable approach targeted at Cu pollution, with potential for agricultural and industrial applications.[51–53] The key role of detection technology innovates bioremediation methods-these combined strategies can pave the way for more sustainable and efficient management of heavy metals in the environment.

5.2.2 Experimental section

Microorganisms and culture conditions

This study identified *Bacillus amyloliquefaciens* and *Bacillus subtilis* as promising BCAs for Cu bioremediation. Isolation of these bacterial strains from agricultural soil samples was carried out using standard microbiological techniques. The isolates were grown on nutrient agar plates at 30°C and stored at 4°C for short-term maintenance. To prepare for experimental studies, bacterial strains were subcultured in nutrient broth (NB) from stock plates to obtain an active culture.

For bacterial inoculum preparation, a colony of Bacillus strains was transferred into 50 mL of NB and grown at 30°C under continuous agitation (150 rpm) for 24 h. The resulting bacterial suspension was standardized to a working concentration of ~10^8 CFU/mL by measuring optical density (OD) at 600 nm. The inoculum was stored at 4°C for about 1 week before being used in the copper bioremediation tests. Copper sulfate ($CuSO_4$-$5H_2O$) was used as a source of Cu to induce Cu contamination in liquid culture media. Copper sulfate stock solutions were prepared by dissolving a precise amount of $CuSO_4$ in deionized water to final concentrations of 100, 500 and 1000 ppb.

These stock solutions were then introduced into 100 mL of nutrient broth to obtain the final copper concentrations required in each treatment.

For the Cu bioremediation experiment, a test medium consisting of 100 mL of nutrient broth of known Cu concentration with the addition of 1 mL of standardized Bacillus inoculum (about 10^8 CFU/mL) was transferred. The bacterial cultures were incubated overnight in a continuous shaker at 30°C and 150 rpm. During this time interval,

samples were taken at defined times (3, 6, 12, and 24 h) to monitor Cu depletion and microbial growth.

Monitoring of copper reduction

The decrease in Cu concentrations was determined using a polyester electrochemical sensor modified with gold nanoparticles. To correlate the sensor response to Cu concentration, standard Cu solutions were used to calibrate the sensor. At each sampling, aliquots of the culture medium were extracted, and the Cu content was analyzed with the electrochemical sensor.

Cu (II) measurements

Electrochemical measurements of Cu were carried out using the same experimental parameters explained in Section 5.1.2.

5.2.3 Results and discussion

Copper depletion by Bacillus amyloliquefaciens in liquid culture medium

The first experiment consisted of testing the bacterium *Bacillus amyloliquefaciens* for Cu depletion in a liquid culture medium (nutrient broth-NB) supplemented with different concentrations of Cu (100, 500, 1000 ppb). Thus, the objective was to study the suitability of the bacterium for Cu bioremediation and to monitor the depletion process through an electrochemical sensor. The results are encouraging and demonstrate the need to consider bioremediation methods in agriculture in the future (Figure V.8).

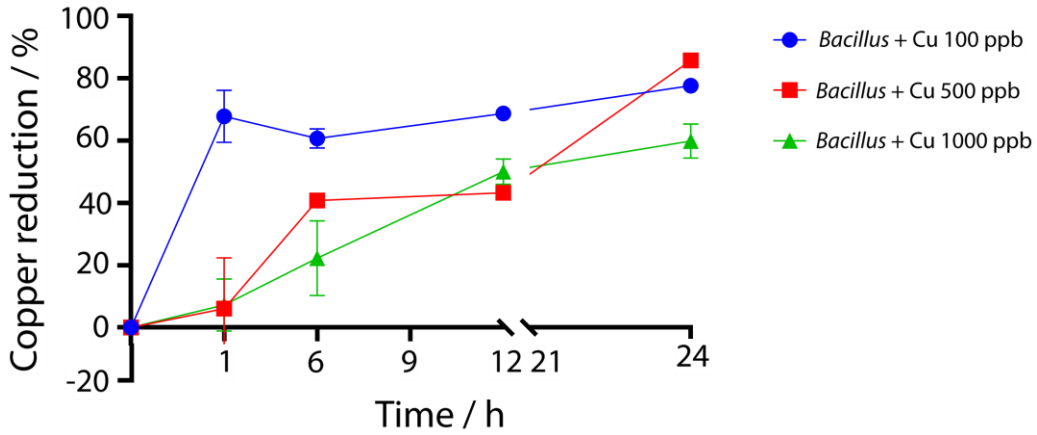

Figure V.8. Percentage of reduction of different copper concentrations as a function of time in the presence of *Bacillus amyloliquefaciens*.

Cu reduction by *B. amyloliquefaciens* was found to be dependent on metal concentration and time. At lower Cu concentrations (100 ppb), the bacterium achieved 67% removal within 3 hours, which increased to 77% after 24 hours. This suggests a great ability of this bacterium to bioaccumulate and biotransform Cu, including the processes of biosorption and bioprecipitation, in which Cu is bound and partially immobilized in less toxic species. These reductions are substantial, since Cu is a heavy metal and is extremely toxic to most organisms. The highest Cu concentrations (500 and 1000 ppb) had a reduced rate of reduction, with reductions at 6 hours of 22% and 40%, respectively. At 24 h, however, Cu reduction was 77% and 59% for the concentrations of 500 and 1000 ppb, respectively. This indicates a dose-dependent Cu removal capacity by *B. amyloliquefaciens*. At high concentrations, the reduction kinetics are slow, probably due to the

crowding of binding sites. A higher concentration of Cu can also compromise the efficiency of the process due to higher toxicity resulting in less bacterial growth and less metal transformation. In addition, the electrochemical sensor has enabled timely and accurate measurements, obviating the need for sophisticated laboratory methods to measure Cu in the medium. Emerging advanced monitoring technologies, such as this sensor, are therefore of great use in real-time bioremediation approaches, where speed and accuracy are critical to maximize the use of BCAs.

This behaviour suggests that the Cu removal capacity of *B. amyloliquefaciens* is dose dependent. At higher concentrations, the reduction kinetics are slower, probably due to saturation of the binding sites. The efficiency of the process may also be affected by the higher toxicity of Cu at higher concentrations, which could slow the growth of the bacterium and its ability to transform the metal.
In addition, the use of an electrochemical sensor has enabled rapid and accurate measurements, eliminating the need for complex laboratory techniques to quantify Cu in the medium. The integration of advanced monitoring technologies, such as this sensor, offers significant advantages for real-time bioremediation applications, where speed and accuracy are critical to optimize the use of BCAs.

Figure V.9 shows growth curves under various Cu concentrations in media containing *B. amyloliquefaciens* to assess the potential of the bacteria to continue growing under these Cu stress conditions. These experiments were conducted with the aim of assessing Cu tolerance in bacteria and how this may in turn influence a bacterium's ability to mediate bioremediation processes, e.g., Cu bioaccumulation.

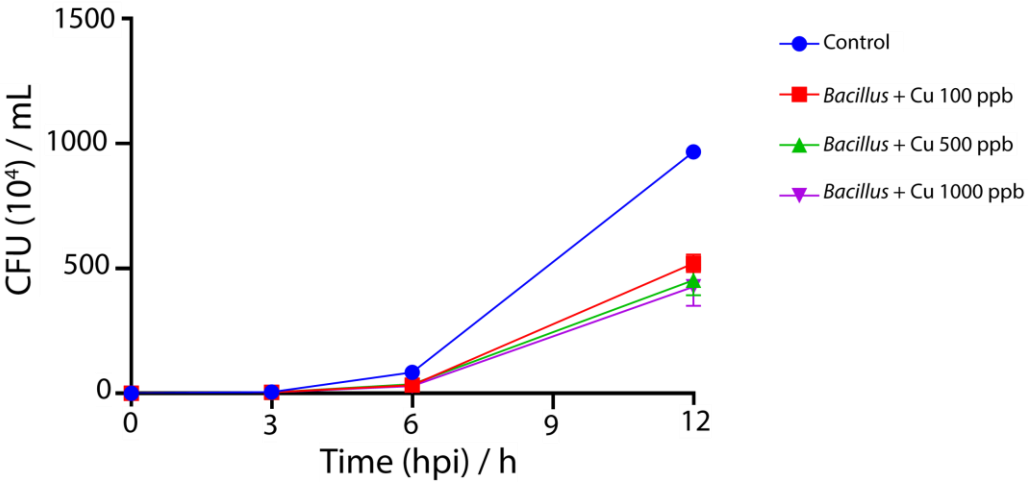

Figure V.9. Growth curves in media containing only *Bacillus amyloliquefaciens* (blue), *Bacillus amyloliquefaciens* together with copper 100 pmm (red), 500 ppm (green) and 1000 ppm (purple).

Bacterial growth was monitored at 12 h and 24 h post-inoculation (hpi) and expressed in CFU/ml by electrochemical determination of Cu concentration. In this regard, at 12 hpi all assayed Cu concentrations showed a small reduction (CFU/ml of ($5x10^6$) compared to the control experiment (*Bacillus* without Cu at $9x10^6$ CFU/ml). Indeed, at 24 h, no major growth differences were found between Cu treatments, as all conditions reached a CFU/ml concentration of $1x10^8$, indicating that

the behavior of the bacterium was adaptive to the presence of Cu in the culture medium.

Copper Depletion by Bacillus Amyloliquefaciens and Bacillus Subtilis in culture medium with higher copper concentration

In the second experiment, a similar approach was used to evaluate the copper depletion capacity of *Bacillus amyloliquefaciens* and *Bacillus subtilis* in a culture medium containing higher Cu concentrations, specifically 15 ppm and 30 ppm. Cu content was monitored continuously with the electrochemical sensor, and the effect of Cu depletion by the bacteria was analyzed after 24 hours.

Both bacterial strains were able to reduce a significant amount of Cu in the culture medium over 24 hours, as illustrated by the histograms in Figure V.10.

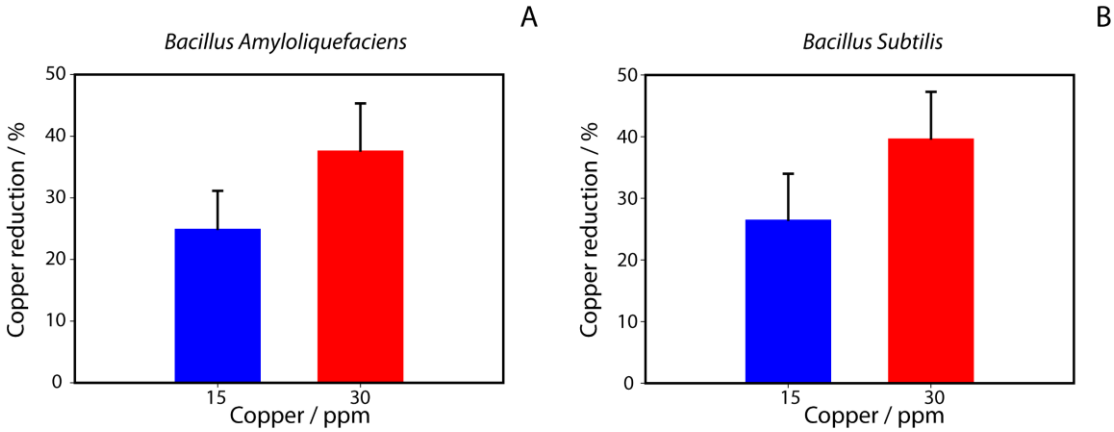

Figure V.10. Comparison of bioaccumulation ability by microorganisms A) *Bacillus Amyloliquefaciens* and B) *Bacillus Subtilis* in the presence of 15 ppm and 30 ppm copper.

At a Cu concentration of 15 ppm, *B. amyloliquefaciens* achieved a 25 % reduction in Cu content, while *B. subtilis* demonstrated a 26 % reduction. These reductions are in line with trends observed in the first experiment, in which bacterial activity was able to remove Cu, but at a slower rate due to the increase in initial metal concentration. As a final step, a Cu concentration of up to 30 ppm significantly increased Cu removal and recovery efficiency. The reduction achieved by *B. amyloliquefaciens* was 38 % at 30 ppm compared to *B. subtilis*, which achieved a 40 % reduction. This indicates that although higher Cu concentration provides more energy for the bacteria to work, resulting in more efficient reduction, there is a level up to which this increase is beneficial to the bacteria, beyond which factors such as stress or nutrient limitations in the culture medium may interfere.

At a concentration of 15 ppm, both strains behaved similarly, but at 30 ppm *B. subtilis* showed marginally better reduction. This could imply that *B. subtilis* has a predominant adaptation or more effective metal uptake mechanism at higher Cu concentrations. However, this variation is rather small and not necessarily important with regard to bioremediation applications. Both bacterial strains were capable of promising Cu detoxification but failed to completely remove the metal from the culture supernatant. These results demonstrate that these bacteria can be used in copper-contaminated soils, but more work needs to be done to improve their bioremediation efficiency, as in situ conditions typically include higher Cu concentrations and a more complex environmental context. Although these results are promising, further experiments are needed to optimize bioremediation. In future studies, Cu concentration can be run over a wide range to evaluate the

effectiveness of the bacteria at various levels of contamination. In particular, testing the bacteria at significantly higher Cu concentrations could give insight into the maximum tolerance to the metal and its ability to be reduced. Furthermore, to fully understand the practical applicability of these bacteria under field conditions, different soil types and environmental conditions (e.g., pH, temperature, and organic matter content) should be evaluated. Real field trials would allow their performance to be evaluated in the face of natural variations in the environment, including temperature, moisture and competing contaminants. Further research could also consider the synergistic relationship of *Bacillus amyloliquefaciens* in combination with *Bacillus subtilis*. Co-culture could also provide improved removal, as each strain may possess complementary capacities, resulting in greater metal tolerance, increased bacterial growth, and improved metal uptake and reduction.

5.2.4 Conclusion

Overall, BCAs used in unison with real-time electrochemical electronic receptors constitute a more dynamic and sustainable copper bioremediation technology than the passive alginate bead technology. Initial results of this study suggest that *Bacillus amyloliquefaciens* and *Bacillus subtilis* are highly efficient in decreasing the concentration of copper pollutants through biological processes such as biosorption, bioaccumulation and biotransformation, which significantly reduce the toxicity and mobility of copper in the environment. In addition, electrochemical sensors built into the plant allow real-time monitoring of copper uptake, facilitating dynamic optimization of bioremediation processes. All of these BCAs reduce copper toxicity and promote plant

growth as PGPR, which is potentially useful in copper-contaminated agricultural soils. The combined benefit of heavy metal detoxification and improved soil fertility makes bacillus-based bioremediation an economical, scalable and sustainable approach. Although further optimization is needed to improve process efficiency under harsh and complex environmental conditions, these promising results indicate that the use of *Bacillus* species with advanced in situ technologies holds great promise for improving heavy metal pollution control in agricultural crops and industrial applications.

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CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 OVERVIEW AND SUMMARY OF ACHIEVEMENTS

Pesticides are a major problem in agricultural and environmental systems that can adversely affect both human health and the equilibrium of ecosystems over time. Pesticides are used worldwide, and their continued use produces risks as the world's agricultural practices adapt to the increasing demands of food production. These chemicals, while useful for crop protection and agricultural cultivation, can leave long-term residues in the environment, which can become a problem for food quality, ecosystem health and biodiversity integrity. The development of advanced, sustainable and efficient tools for pesticide detection and decontamination is therefore essential to safeguard human health and the environment. The aforementioned innovative approaches motivated this Ph.D. thesis to study the design and application of biosensors, particularly wearable sensing devices, are proposed to provide highly sensitive pesticide detection technologies using environmentally friendly materials that are sustainable. Established methods for analysing the problem of pesticide identification are chromatographic methods (HPLC, etc.) and spectrophotometry. Although these methods can be extremely accurate and well validated, they are unusable in the field because they are complex, expensive and generally require specialized equipment and personnel. In addition, these methods require a lot of time and sample transportation, resulting in delays and expense. The present work underscores the importance of rapid, field-based detection (especially in underdeveloped and remote areas where laboratories are less

developed). Due to the lack of rapid and reliable biological tests to detect pesticides, the search for new solutions, developed mainly based on biosensor technology, was the focus of attention.

Nanomaterials offer remarkable properties and novel transduction mechanisms that could help solve many drawbacks of classical procedures. Inexpensive and portable, the sensors provide real-time data. Biosensor technologies geared toward portable devices can address the complexity of pesticide contamination and impact the practicality and sustainable life aspect of farmers, food safety inspectors, and environmental scientists. This description, provides much of the data for research on in situ biosensors for pesticide detection that can be synthesized quickly and inexpensively. This logic is integrated in a new application with the wearable sensor platform. This technology has made it possible to analyze pesticides in the field with glove-like systems, avoiding sample collection and laboratory analysis, for example. These sensors will help farmers and farm workers identify pesticide residues on plant, fruit or soil surfaces in real time, helping them to react immediately to pesticide application and improve food safety and protection for farm workers, and consequently that of consumers. The transfer of a versatile approach based on bioplastics, particularly polyhydroxyalkanoates (PHAs) in their tandem, PHBV (poly(3-hydroxybutyrate-co-3-hydroxyvalerate), as well as PHBV composite with microfibrillated cellulose (MFC), i.e., PHBV/MFC, for the production of electrochemical sensors is a key novelty explored in this thesis work. Since these polymers can be biodegradable and biobased, there is significant potential in their

application to replace synthetic industrial polymers such as polyester. This research achieves a twofold goal: while working toward the development of rapid pesticide sensors, the issue of the toxicity of these compounds was addressed by using PHAs and their derivatives as substrates for sensor construction. To this end, the results suggest that PHAs have potential as an alternative to conventional fossil-derived plastics, but also show promising mechanical and electrochemical properties for sensitive pesticide sensing. PHBV and PHBV/MFC composites can be used as good detectors of iron ions and organophosphate inhibitors in aqueous medium with reasonable results. Organophosphate pesticides are a growing concern for food supply security and environmental sustainability.

We note that new user-wearable technologies, with significant potential for precision agriculture products, are rapidly emerging and represent unique opportunities for real-time assessment of pesticide contamination in the field. For example, electrochemical sensors placed on a glove can enable farmers and farm workers to perform low-cost, real-time analysis for proper sampling in a timely manner. The main innovation described in this paper is the creation of an electrochemical sensor for detection of copper ions by touching the surface of grapevine leaves. The sensor was developed with the collaboration with the Quintodecimo winery was extremely helpful because provided real-life background experience and supported the idea to develop and test the sensor in a vineyard. At UCSD, in the Department of Nanoengineering, work was further refinde both the design and performance of the wearable sensor. As a result, it was possible to develop a glove system for the direct detection of copper

ions in grapevine leaves. This method is particularly relevant to agriculture, as it allows farmers to check directly for the presence of the copper-based pesticide in vineyards, directly from the surface of the leaves in a very short time, without having to go through any kind of preparation. The handheld sensor was able to detect the copper residues present both at a high concentration as well as at low ppm levels, and showed good correlation to pesticide levels commonly used in agricultural production. In addition, the detection process requires minimal contact with the leaf surface, thus enabling decentralization of pesticide monitoring, particularly in remote agricultural regions. The second application leveraged the previous success of the electrochemical glove-based sensor used to detect copper ions and extended the use of this wearable technology to the detection of a different class of pesticides, organophosphates. In this case, the glove-based technology was extended to the in-situ detection of organophosphate pesticides (dichlorvos) on fruit peels. Taking advantage of this same developed instrumentation, the new sensor enabled direct detection of pesticide residues in situ, in a single step, without additional peripherals or laboratory processes. The idea behind this method was to adapt our previous experience of a glove-mounted sensor system for detecting copper ions on vine leaves to a system for detecting organophosphate pesticides. This sensor, sensitive and selective enough to detect pesticide concentrations down to one nanomolar, was used to obtain in situ feedback on pesticide contamination by simply rubbing the surface of the fruit. Together with the biomimetic carbon black and prussian blue electrocatalysts and the enzyme, butyrylcholinesterase (BChE), the biosensor on the glove

successfully detected organophosphate residues. These new wearable biosensor technologies represent a major breakthrough and an important aspect of precision agriculture and field monitoring of pesticide residues directly on crops.

These new wearable biosensor technologies represent a major advance in precision agriculture and field monitoring of pesticide residues directly on crops. The research has not stopped at sensing, but also explored the potential of so-called remediate-and-sense systems, in which pollutant detection is integrated into active strategies for contaminant remediation. This combination of products also makes it possible to solve the generalization of environmental pollution, in addition to the problem of pesticide residue contamination. The goal of this study was to integrate previously studied electrochemical sensors with a classical remediation tool, such as alginate beads. Due to their biocompatibility and ability to remove metal ions, the use of biocompatible alginate beads coupled with sensor technology-capable of removing and detecting metal ions-can provide a useful combination for remediation and detection platforms. In this system, alginate beads were used for remediation, while printed electrochemical strips served as a real-time sensor to detect copper ions. In a test study, this system was shown to successfully clean copper-contaminated soil without much intervention and using only 10 alginate beads. The generated composite beads were incorporated into a 3D-printed chamber that enables the simultaneous removal and detection of copper ions from environmental matrices (e.g., soil and water). This innovative approach and the application of state-of-the-art technology that combines in situ monitoring of contaminant levels with remediation capabilities can

enable a distributed model of environmental monitoring in which the user is not only informed of the extent of contamination present but is also able to be directly responsible for ex-situ removal of contaminants to remediate them on site. The scope of remediation was further expanded with bioremediation of copper using biological control agents such as *Bacillus amyloliquefaciens* and *Bacillus subtilis* in which the bioaccumulation processes of the bacteria were able to extract copper from the substrate. This approach is not only more sustainable but could replace conventional remediation measures in some situations. Here, it was outlined a proof-of-concept with a microbial method that could be exploited for the removal of copper in in situ using these bacteria-based on electrochemical sensors that can allow monitoring of copper levels in real time during the bioremediation process over time. Thus, the re-sanification process combined with electrochemical monitoring allows for more accurate and adaptive management of heavy metal-contaminated agricultural soils. The pervasive nature of pesticide contamination makes systems known as remediate-and-sense, which integrate these two components into a single system, the perfect counterpart.

In this way, precision agriculture could be transformed no longer as a way to improve land use and yield, but rather as a handful of on-the-ground implementations that will go a long way toward achieving sustainable practices and environmental stewardship in a world where food production and ecosystem conservation are empowered.

6.2 FUTURE DIRECTIONS

Several potential future research directions can be drawn from the results of this study. Another important aspect is the development of multi-analyte sensors that can detect different types of pesticides or pollutants simultaneously. This would make the technology more robust, flexible and applicable in different agricultural and environmental conditions. Another important aspect is the improvement of bioplastics used in sensor development. More research on materials such as PHAs can improve sensors to make them more durable and efficient with minimal impact on the environment. Improvement of these materials could help sensors operate efficiently even in unfavorable conditions. Another potential area of research is the development of wearable sensors linked to real-time data processing systems. This could enable real-time monitoring of pesticide levels, thus helping farmers and inspectors prevent incidence and protect the environment from pests. In addition, new remediate-and-sense systems that combine detection with pollution remediation could be greatly improved. These systems could become even more effective at extracting pollutants and providing real-time data if more advanced bioremediation techniques (such as bacteria or engineered plants) were used.