Volume 6, Issue 6, 2016, 1659 - 1675 ISSN 2069-5837

Biointerface Research in Applied Chemistry

www.BiointerfaceResearch.com

Review Article Open Access Journal

Received: 15.06.2016 / Revised: 10.10.2016 / Accepted: 15.10.2016 / Published on-line: 05.11.2016

Pesticide Detection in Soil Using Biosensors and Nanobiosensors

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ABSTRACT

Soil is a complex and heterogeneous environment which present a great importance in food production and maintenance of socio-economic activities. The preservation depends on the contaminants monitoring in order to avoid drastic impact. The soil contamination by pesticides constitutes a threat to the quality, impacting the biodiversity and nutrient cycling, as well as the quality of water bodies and the atmosphere. By the soils diversity and complexity is required efficient techniques for sample extraction an analysis. Some convectional techniques are complex, time consuming and required trained operators. In this way, is important to develop simple, low cost and sensitivity methods, compatible with classical methods to detect pesticides in soil. Thus, using sensor and biosensor with a biological element of recognition it is possible to selectively detect and recognize one or more analytes. At the nanoscale the surface modification by sensitive coating gives to cantilevers nanosensors versatility for detect specific compounds. The cantilever nanobiosensors show a promise potential to be applied to determine with high sensitivity pollutants in soil.

Keywords: *soil; pesticides; nanobiosensors; cantilever*.

1. INTRODUCTION

Soil is a complex and heterogeneous environment, composed of minerals, organic matter and organisms with characteristics varying according to the climate and the source material. The soil is very important for food production and maintenance of socio-economic activities, and its preservation depends on the monitoring of contamination in order to avoid drastic impacts [1, 2, 3, 4].

According to Singh (2014) [5], the world population will increase to around 8 billion of people in 2025 and 9 billion in 2050, and is widely recognized that global agricultural productivity must also increase to keep up with this increase in population. Actions are required to ensure increased agricultural productivity without compromising soil.

The addition of inputs to the soil as fertilizer, nutrient content increases, but, on the other hand, can cause toxicity due to trace elements that remain present in the environment. The pollution in the soil may also be due to effluents and incorrect disposal of solid waste like as domestic, industrial and rural [6, 7]. In relation to industrial effluents, the major concern is the accumulation of metals in soil, which are toxic to living beings. The use of agrochemicals, such as pesticides, to combat various types of pests, such as insects, fungi and weeds, when used uncritically, causes soil, water and air contamination [5, 8, 9, 10].

For pesticides detection in soil, the traditional analytical methods are the chromatographic ones that, although efficient, safe, and with high detection limits, they are complex, time-consuming analysis, requiring highly trained operators and demanding costly reagents. In view of these adversities, we can still see a great field to be explored. Thus, this study aimed at reviewing pesticides contamination in the soil and the existing methods for these contaminants detection in this environmental matrix with the use of biosensors and nanobiosensors, two very promising alternatives.

2. PESTICIDES AS CONTAMINANTS IN SOIL

Due to the rapid population growth, the use of pesticides in agriculture has intensified in order to increase agricultural productivity to meet the growing demand for food.

The term pesticide includes a substance or a mixture of substances, natural or synthetic that controls, eliminate or modify the physiology of a living organism capable of damaging agricultural, domestic and commercial environments [11]. According to the International Union of Pure and Applied Chemistry (IUPAC), pesticides are substances capable to prevent, destroy or combat unwanted organisms that someway can affect the production, processing, storage and transportation of foods, agricultural products in general, wood and wood products or animal feed [12].

Pesticides may be classified according to the following criteria [13, 14, 15, 16]:

a) Target pests:

- Herbicides (weeds), for example: Alachlor, Atrazine, Simazine, Cybutryne, Terbutryn, Diuron, Isoproturon,

Trifluralin, Aclonifen, Bifenox, Cypermethrin and Glyphosate;

- Fungicides (fungi), for instance: Hexachlorobenzene, Pentachlorobenzene and Quinoxyfen;
- Insecticides (insects), for example: Chlorfenvinphos, Chlorpyrifos, Dichlorvos, Endosulfan, Hexachlorocyclohexane, Trichlorobenzenesa and Heptachlor;
- Acaricides (mites), for instance: Dicofol.

b) Chemical nature of the active ingredients: organochlorines, organophosphates, carbamates, pyrethroids / pyrethrins;

c) Action mode: systemic and non-systemic or contact;

d) Action Spectra: Selective and Non-Selective;

e) Timing of application: Before plantation, Pre-emergence and Post-emergence;

f) Toxicity:

- Class Ia (Extremely hazardous),
- Class Ib (Highly hazardous),
- Class II (Moderately hazardous),
- Class III (Slightly hazardous),
- Class U (Unlikely to present acute hazard), which are determined based on lethal dose, 50% (LD_{50}) for rats.

Pesticides have become essential in modern agriculture, since they control and eliminate pests that infest crops, reducing harvest losses [17]. On the other hand, pesticides can contaminate foods [18], soil [9], air [19] and water [20]. Because of this, the use of pesticides results in consequences on the soil [21] and aquatic organisms [22, 23]. Additionally, direct or indirect exposure to these products causes potentially adverse effects to humans. Cardio-vascular, respiratory, endocrine, neurological (Parkinson and Alzheimer), reproductive diseases and cancer are often associated with exposure to pesticides [24, 25, 26, 27]. The exposure of pregnant women to pesticides can induce early changes in glucose metabolism of the newborn [28]. Heu et al., (2012) [29] evaluated the effects of exposure of human skin cells to glyphosate using the atomic force microscopy (AFM) technique, and verified changes in the cells topography after exposure to glyphosate, demonstrating that glyphosate damages the cells.

There are two types of pesticides sources in the environment, punctual and diffuse. The punctual sources are direct application points, that is, industry waste, spillage during preparation, loading and transportation. The diffuse sources are related to the accidental release during storage or application and soil leaching [14].

The main entry ways for pesticides in the soil are either through plants pulverization or its direct application to the soil [30], where the persistence in this environment is influenced by several factors such as, pesticides physico-chemical properties: molecular size, water solubility, polarity, volatility, molecular structure, chemical function and acid-base nature; soil properties: soil type, moisture content, organic carbon content, pH, redox potential, microbial population; environmental conditions: climate, topography, air currents; variables related to the application of pesticides: concentration, frequency and mode of application; and also the use of other chemicals [31, 32, 33, 34]. Among the factors above mentioned, the organic matter in the soil is highlighted, since the higher the content increase pesticide adsorption in the soil, and therefore, longer be its permanence in this environment [35].

The ending of the pesticide in the environment is influenced by the following characteristics [36]:

a) Solubility: represents how easily a pesticide can be leached or drained by the soil;

b) Tendency to soil adsorption: measured by the partition coefficient, which represents the pesticide tendency to bind soil particles, where higher this coefficient, higher is the pesticide tendency to remain in the soil;

c) Persistence in the environment or half-life: time required to reduce the concentration by half of pesticide in relation to the initial value.

Thus, a pesticide that has low water solubility, long half-life period and a high partition coefficient has a greater tendency to persist in the soil. The pesticide behavior in the soil is governed by physical, chemical and biological processes, which control the transport to water, air or food, as its transformation through degradation processes [31] (Figure 1).

The transferring of pesticides from soil to waters can either reach the surface water or the underground water. Pesticides are adsorbed by soil particles in erosion process or in water flow that are responsible for the contamination of surface waters. The underground water contamination occurs by pesticide leaching, where the chemicals are carried to aquifer-feeding waters [37]. The pesticides transference from soil to air occurs by volatilization, that is the conversion process of solid or liquid to gas form [36].

The adsorption-desorption processes and pesticides degradation affect their ecotoxicological impact because most chemical products are sorbed by oil particles and chemicals (hydrolysis) or microbiologically (micro-organisms) degraded. Pesticides bind to the soil particles are less mobile and less accessible to degradation and, thus, more persistent in the environment. The degradation process, cannot lead to complete pesticide mineralization, turning it into intermediate metabolites with greater mobility, persistence and toxicity to a type of non-target pest [38, 39, 40]. Photodegradation is the pesticides break by sunlight and can occur in the plant leaves, soil surface and air [36].

Pesticides and their metabolites can be grouped based on the type of interaction with the soil particles. Those are strongly bind to the soil present hydrophobic characteristics, low volatilization, persistent and bio-cumulative, for example: dichlorodiphenyltrichloroethane, endosulfan, endrin, heptachlor, lindane and their metabolites. However, the pesticides weak or moderately bind to the soil, of polar character, can be runoff or

leached from the soil, promoting a serious problem for drinking water, as well volatilize quickly [35].

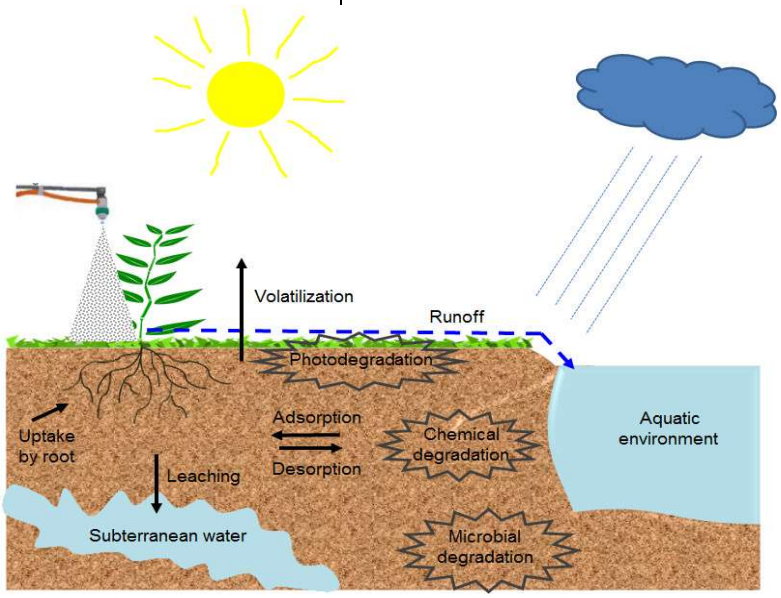

Figure 1. Transport and degradation processes of pesticides in soil.

One of the causes of high persistence and/or half-life is the accumulation of residues or metabolites in the soil, even those pesticides have been prohibited. At China, some organophosphate pesticides have been banned for more than 30 years, but as they were widely used in agriculture between the 1950s and 1980s, they can still be found in the environment. The area of agricultural soils of the Yangtze River delta is an example. The soil analysis of this region, one of the most populous and economically promising in China, indicated contamination by aphthalate esters used as plasticizers in polymer industry; organochlorine pesticides used in agriculture; and polybrominated diphenyl ethers, flame retardants used in furniture and textile industries and in electronics, which are endocrine disruptor compounds. Organochlorine pesticides were detected in 241 samples analyzed, with the following detection rates: α-hexachlorocyclohexane – 91%; β-hexachlorocyclohexane – 67%; γ- hexachlorocyclohexane – 99%, δ-hexachlorocyclohexane – 74%; p,p'-dichlorodiphenyltrichloroethane - 79%; o,p'-dichlorodiphenyltrichloroethane – 38%; p,p'-tetrachlorodiphenylethane – 71%; o,p'-tetrachlorodiphenylethane – 23%; p,p'-dichlorodiphenyldichloroethane – 99%; o,p'-dichlorodiphenyldichloroethane – 44%; aldrin – 54%; endrin – 6%; heptachlor – 7%; heptachlorepoxide – 28%; hexachlorobenzene – 59%. The concentrations of organochlorine pesticides ranged from 1.0 $ng.g^{-1}$ to 3520 $ng.g^{-1}$ with an average of 59.3 $ng.g^{-1}$[41].

Another inherent problem on the use of pesticides is that they are not present to the environment in their pure form. Their commercial formulation includes substances called inert ingredients such as adjuvants and carriers, responsible for performance improved and stability of the pesticide [42, 43]. However, because they are chemically active, may be toxic and cause contamination of the environment, including soil.

During the pesticide manufacturing process, wastes are generated containing many toxic compounds and non-biodegradable ones consisting of active ingredients, solvents, sub-products and even other liquids and solid wastes that due to their complexity may remain in the environment and thereby, cause soil contamination in areas close to these sites. Another form of contamination is the transporting or incorrect storage of pesticides. When analyzing the soil around a pesticide plant in China, the horizontal distribution profile showed that the local most polluted by chlorinated was not the closest to the production plant, since the highest concentration of dichlorodiphenyltrichloroethane and hexachlorocyclohexane was 100 m from the plant and the highest concentration of endosulfan was 200 m away. In terms of the vertical distribution profile, while organochlorine production has ceased for about 20 years, concentrations in $mg.kg^{-1}$ was measurable in all soil layers investigated (0-20; 20-40; 40-60 cm), showing a persistence of residues in soil [44].

According to Chen et al. (2012) [45], accidents on the industrial site, such as fires, can generate significant quantities of toxic products during combustion, and the flow of water to extinguish the fire, mixed with unburned pesticides and other toxic products, may cause soil and water reservoirs contamination.

Due to high persistence and bioaccumulation of some pesticides, they may be dispersed in the environment, carried by air currents to regions distant from the place of use, and even where there are not any agricultural practices. The study of soils samples from the King George Island, west Antarctica, is an example that showed the predominance of hexachlorobenzene in all analyzed soil samples (67.9 to 532 $pg.g^{-1}$ in dry weight), followed by dichlorodiphenyltrichloroethane (18.8 to 308 $pg.g^{-1}$ in dry weight), hexachlorocyclohexanes (6.25 to 232 $pg.g^{-1}$ in dry weight) and chlordane compounds (nd – 59.7 $pg.g^{-1}$ in dry weight) [46].

Soil contamination caused by pesticides, either through direct application or diffuse contamination urges the need to establish laws and regulations to set the maximum permissible concentrations in that environment. Every country should have a legislation dedicated to pesticides usage restrictions, but only just a few countries have such legislation.

Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for community action to achieve the sustainable use of pesticides. Among the actions are the sales of pesticides, equipment for the application of pesticides in soil and the most appropriate practices to be adopted for use, including the proper handling and storage and the appropriate destination packages. However, in this directive the maximum permitted values of pesticides in soils to member countries are not defined [47].

However, Brazilian [48] and Chinese [49] laws establish values for contamination of pesticides in soil that depend on the place (Table 1).

The application of pesticides is a threat to the quality of soil, directly affecting biodiversity and nutrient cycling [50] and, indirectly, the quality of water bodies and air [31]. Because of this, it is essential monitoring of pesticide concentrations in soil, continuously, with the development and improvement of analytical methods for their detection and quantification.

Table 1. Maximum limits allowed in the Brazilian and Chinese soil due to its place.

Pesticide	Agricultural			Residential		Industrial	
	China[a]		Brazil[b]	China[a]	Brazil[b]	China[a]	Brazil[b]
	Organic matter content ≤20 g.kg^{-1}	Organic matter content >20 g.kg^{-1}					
Aldrin	-	-	0.003	0.06	0.01	0.3	0.03
Dieldrin	-	-	0.20	0.06	0.6	0.3	1.3
Endrin	-	-	0.40	2.00	1.5	10	2.5
Total dichloro diphenyl trichloro ethane	0.10	0.10	0.55	1.0	2.0	4.0	5.0
Total polychlorinated biphenyl	0.10	0.20	0.01	0.50	0.03	1.5	0.12
Atrazine	0.10	0.10	-	2.0	-	6.0	-
2.4 diclorofenoxiacetic	0.10	0.10	-	50	-	500	-
Glyphosate	0.50	0.50	-	-	-	-	-

[a] Amounts for soil (mg/kg). [49].
[b] Amounts for soil (mg/kg dry weight).[48].

3. METHODS FOR DETECTION OF PESTICIDES IN SOIL

Due to the diversity and complexity of soil types and interactions of their constituents with pesticides, different physico-chemical properties of pesticides and low concentrations expected for those pollutants in the soil [51], there is a need for efficient sample extraction techniques before their determination by chromatography, which is the most used method [52].

Chromatography is one of the most important techniques in environmental analysis and includes methods for separation of small quantities of complex mixtures, with high resolution, in which the sample is balanced between a mobile and a stationary phase [53]. The detectors used in the chromatographic analysis must have a high sensitivity and a wide range of linear response. The detectors most commonly used for pesticide analysis in accordance with Table 2, are: a) flame ionization detector that is based on the ions generation during firing of the eluates in a flame of hydrogen and air; b) electron capture detector that can be applied to detect electron-absorbing components (high electronegativity), specially halogens, organometallic compounds, nitriles, or nitro compounds; c) mass spectrometer detector, used both in liquid as gas chromatography, consists of a ions source that fragment the analyte ions of different mass, which are classified according to their relation (mass/charge) [61]. Table 2 shows examples of pesticides determination in soil, in different places, with different sample preparation techniques and equipment chromatographic, obtaining different detection limits.

Table 2. Sample preparation techniques, equipment and detection limits for analysis of pesticides in soil in different places.

Place	Sample preparation technique	Equipment	Pesticide	Detection limit (ng.g^{-1})	Reference
Yangtze river (China)	Soxhlet extraction	GC-μECD	α-Hexachlorocyclohexane	0.002	[54]
			Hexachlorocyclohexane	0.002	
			β-Hexachlorocyclohexane	0.050	
			γ-Hexachlorocyclohexane	0.056	
			δ-Hexachlorocyclohexane	0.214	
			Heptachlor	0.032	
			Aldrin	0.025	

Place	Sample preparation technique	Equipment	Pesticide	Detection limit ($ng.g^{-1}$)	Reference
			Heptachlorepoxide	0.028	
			trans-Chlordane	0.024	
			o,p'-Dichlorodiphenyldichloroethane	0.023	
			α-Endosulfan	0.022	
			cis-Chlordane	0.022	
			Dieldrin	0.022	
			p,p'-Dichlorodiphenyldichloroethane	0.023	
			o,p'-Tetrachlorodiphenylethane	0.021	
			Endrin	0.020	
			β-Endosulfan	0.019	
			p,p'-Tetrachlorodiphenylethane	0.018	
			o,p'-Dichlorodiphenyltrichloroethane	0.018	
			p,p'-Dichlorodiphenyltrichloroethane	0.034	
			Methoxychlor	0.136	
Vegetable Farms in Isfahan and Rudsar (Iran)	USL-SPE + DSLLME	GC-MS	Parathion	0.075	[55]
			Methylparathion	0.20	
			Disulfoton	0.025	
			Sulfotep	0.012	
Iran	SFE + DLLME	GC-FID	o,o,o-Triethylphosphorothioate	3	[56]
			Thionazin	6	
			Sulfotepp	2	
			Disulfoton	1	
			MethylParathion	9	
			Parathion	5	
			Famphur	9	
Southwest zone of Oromia region (Ethiopia)	MAE	GC–MS	Metalaxyl	0.10	[57]
			Malathion	0.10	
			Chlorpyrifos	0.12	
			Kresoxim-methyl	0.11	
Gaillacvineyard (France)	PLE	GC-MS	Flazasulfuron	3.68	[58]
			Metalaxyl	3.75	
			Chlorpyrifos	4.64	
			Folpet	7.77	
			Myclobutanil	3.08	
			λ-Cyhalothrin	1.43	
			Flumioxazin	5.19	
Iran	MSPD	GC – μECD	α-Hexachlorobenzene	0.4	[59]
			β-Hexachlorobenzene	0.3	
			δ-Hexachlorobenzene	0.6	
			γ-Hexachlorobenzene	0.4	
			Heptachlor	0.2	
			Aldrin	1.0	
			Heptachlorepoxide	0.4	
			Endosulfan	0.9	
			4,4'-Dichlorodiphenyldichloroethane	0.5	
			Dieldrin	0.3	
			Endrin	0.9	
			4,4'-tetrachlorodiphenylethane	0.5	
			Endosulfan2	0.5	
			4,4'-Dichlorodiphenyltrichloroethane	0.1	
			Endrinaldehyde	1.1	
			Endosulfan sulfate	1.8	
Region in Beijing (China)	QuEChERS method	LC–MS/MS	(+)-Indoxacarband (-)-Indoxacarb	1.2	[60]
			(+)-Carfentrazone-ethyland (−)-Carfentrazone-ethyl	0.8	
			(+)-Quizalofop-ethyl and (−)-Quizalofop-ethyl	0.6	
			(+)-Benalaxyland (-)-Benalaxyl	0.6	
			(+)-Isocarbophosand (-)-Isocarbophos	1.8	
			(+)-Fenamiphosand (-)-Fenamiphos	0.6	
			(+)-(2R, 3R)-Paclobutrazoland (-)-(2S, 3S)-Paclobutrazol	0.6	
			(+)-Simeconazoleand (-)-Simeconazole	0.6	
			(+)-Napropamideand (-)-Napropamide	0.6	

GC-μECD = gas chromatograph equipped with micro-electron capture detector; USL-SPE + DSLLME = Ultrasound leaching-solid phase extraction followed by dispersive-solidification liquid–liquid microextraction; GC-MS = gas chromatograph with mass spectrometry detector; SFE + DLLME = Supercritical fluid extraction coupled with dispersive liquid–liquid microextraction; GC-FID = gas chromatography-flame ionization detector; MAE = microwave-assisted extraction; PLE = pressurized liquid extraction; MSPD = Matrix Solid Phase Dispersion; QuEChERS = Quick, Easy, Cheap, Effective, Rugged, Safe; LC–MS/MS = chiral liquid chromatography coupled with tandem mass spectrometry.

The excellent sensitivity of chromatographic methods is evidenced by the low detection limits shown in Table 2. However, these methods have some limitations, such as complex and lengthy analysis, highly skilled operators and high costs of reagents and materials [62]. Due to these limitations, it is important to develop simple methods and with lower cost, sensitivity and reliability level compatible with the classical analytical methods for detecting pesticides in soil. In this sense, the use of sensors is becoming more popular to complement the analysis, and as an alternative to replace the existing classical methods [63, 64]. Nanotechnology has contributed much to the development of sensors, by use of nanomaterials for building devices that can significantly improve analytical performance such as sensitivity, detection limit, among others [65].

3.1 Sensors for detection of pesticides

Sensor is defined as a device that identifies and transform an information into measurable signal to be used [66]. The sensor concept can be extended taking into account the type of sensing layer used. When an element of biological recognition (biological elements, organisms, tissues, cells, organelles, membranes, enzymes, enzyme components, receptors, antibodies, nucleic acids, organic molecules) is used as a sensing layer, the sensor is called biosensor [67]. Thus, a biosensor is a device capable of providing specific, quantitative or semi-quantitative analytical information using an element of biological recognition that is in direct contact with the transducing element [68].

A biosensor consists of three basic components (Figure 2): a) biological recognition element, that selectively recognize one or more analytes from a large number of other substances; b) physical transducer, which processes the signal produced by the interaction between the recognition element and the analyte of interest in a measurable signal; c) electronic system for signal amplification and data recording [69].

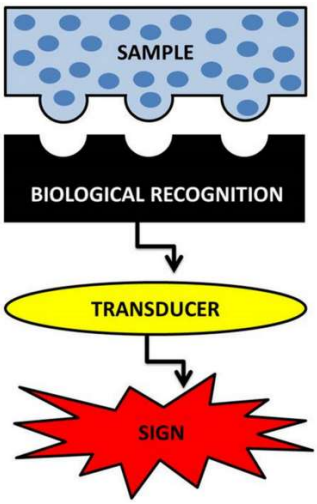

Figure 2. Schematic diagram of biosensor operating.

An ideal biosensor must have some characteristics as fast, accuracy, reproducible, sensitive and selective [70]. The sensitivity and performance of the biosensor can be enhanced with the miniaturization and/or with the use of nanoscale sensing layers, where these devices are now being called nanobiosensors. Nanotechnology has opened the possibility for using materials with small scale between 1 and 100 nanometers, of which properties are very interesting and different from the same materials on a larger scale [71, 72], and increased specific surface area of these devices [73], making them suitable and promising as sensing devices.

There are many nanomaterials investigated for their use as a sensing layer in nanobiosensors such as nanotubes, nanowires, nanorods, nanoparticles, and quantum dots [74]. The use of nanoparticles has been highlighted by amplifying the recognition signal of many molecules due to diversity in structure and size, manufacturing efficiently at atomic scale, it is highly sensitive, low cost, and of rapid response [73, 75].

3.1.1 Biosensor classification criteria

Biosensors can be classified according to the type of interaction between the sensing layer and the analyte, methods used to detect desired interactions, the nature of the recognition elements (sensitive layer), and transducing system [76].

In terms of the transducing system, biosensors can be classified as:

- Electrochemical biosensors: Based on the selective interaction between the analyte and the recognition element (sensor layer). An electrode is used as a transducing element. The interaction can produce an electrical signal related to the concentration of the substance to be studied. Such biosensors are subdivided in; potentiometric, voltammetric, amperometric and electrochemical impedance spectroscopy biosensors [62].
- Optical biosensors: Based on the changes of the substances optical properties with the purpose of monitoring the analyte concentration. Absorption, refractive index, fluorescence, polarization, and wavelength are among the optical properties that can be used either individually or

combined in order to generate the sensing signals [77, 78].

- Piezoelectric biosensors: Based on the measuring of changes in resonance frequency due to mass alterations and/or microviscosity [79]. The materials used in the design of such biosensors are piezoelectric materials that resonate by the application of an alternate external magnetic field [76].
- Thermometric biosensors: Based on changes in temperature in relation to analyte concentration [80].
- Biomechanical biosensors: The adsorption of molecules on the biosensor's surface causes a mechanical response, such as deflection [81].

The types of interaction between the sensing layer and the analyte are classified by bioaffinity and biocatalytic interaction. For bioaffinity, the interaction between the analyte of interest and the recognition element occurs without a catalytic transformation. A marker, such as an antigen-antibody at the receiver can be used to measure this interaction. Biocatalytic interaction already uses biocatalysts consisting of either enzymes or animal cells, and plant tissues. These recognition elements can be coupled to different types of transducers [82].

The recognition elements of biosensors can be classified into [62]:

- Enzymatic biosensors that use enzymes as recognition elements;
- Cell biosensors, using cells or micro-organisms for detection;
- Immunosensors are biosensors that monitor antigen-antibody interactions

In enzymatic biosensor, the enzyme used as a recognition element may be in direct contact with the transducer, and the detection of substances based on inhibition of the enzyme has an increasing advance. These biosensors can measure the enzyme activity in the presence or absence of inhibitor, are based on enzyme catalysis of the substrate by evaluating the disappearance or appearance of a reactant or reaction product [62, 83].

In order to maintain the enzyme activity is extremely important adequate immobilization of the enzyme on the device because various factors can influence the loss of enzyme activity, such as temperature, pH and inhibitors. Thus, the immobilization method should ensure access of biological material (enzyme) to the analyte to be determined and the transducer proximity [71, 84].

Generally, there are four types of immobilization (Figure 3):

- Adsorption: Based on low-energy interactions between functional groups of bioreceptor and substrate, considered a simple and fast way. Physical adsorption can occur based on weak van der Waals forces or chemical absorption by strong covalent bonds;
- Encapsulation or entrapment: It is based on polymerization of the matrix, which used to entrapment the biomaterial. Allows the control of thickness of the polymeric layer by electrical load measurements;
- Covalent bond: It is based on immobilization of the enzyme functional group on the support matrix;
- Cross covalent bond or crosslinking: Form a cross-linked system of enzyme molecules, which is considered a rigid network [71, 84, 85, 86].

There is an increasing use of enzymatic biosensors for pesticide detection, which is based on the degree of enzyme inhibition when in contact with the analyte, assuming that the enzyme activity is determined under controlled conditions (temperature, pH, volume, and substrate concentration) [14]. The Table 3 shows some enzymes used as recognition element in enzymatic biosensors for detecting pesticides in various matrices and their detection limits. When using enzymatic biosensors for pesticide detection, determination of the analytical parameter is performed using different known concentrations of the solutions element to be determined; to subsequently apply this device to detect the pesticide in real samples [103]. The analytical performance of the enzymatic biosensor must be validated by recognized analytical methods, addressing characteristics such as sensitivity, repeatability, selectivity and stability, and evaluating potential interferents when used for detection in real samples (environmental or biological) [104]. Surely, a preliminary sample preparation stage may be required when employing complex samples.

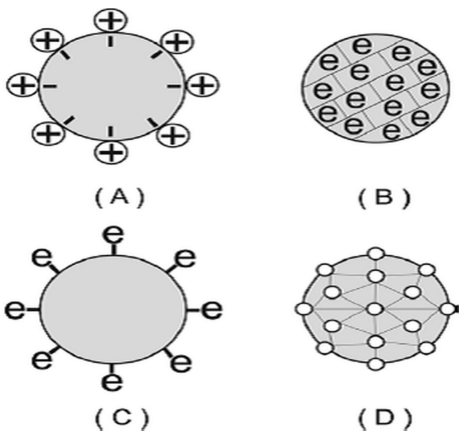

Figure 3. Scheme of the four basic types of enzyme immobilization methods: (a) adsorption; (B) encapsulation; (C) covalent bond; (D) cross-covalent bond [85] (Reprinted from Brazilian Society of Chemistry with permission).

Table 3. Enzymes used as recognition element in enzymatic biosensors for detecting pesticides in various matrices and their detection limits.

Pesticide	Enzyme	Sample	Detection Limit	Reference
2,4-dichlorophenoxyacetic acid	Alkaline-phosphatase	Calibration curve *	0.5 $\mu g.L^{-1}$	[87]
Acephate	Acetylcholinesterase	Grape, Apple, Mango, Orange, Banana, Tomato, Rice, Wheat	0.044 ppm	[88]
Carbaryl	Acetylcholinesterase	Tap water and lake water	5.45 10^{-13} µM	[89]
Carbofuran	Acetylcholinesterase	Cabbage	0.1 $\mu g.g^{-1}$	[90]
		Soil	0.1 $\mu g.g^{-1}$	
Chlorfenvinphos	Acetylcholinesterase	Tap water	4,90 $ng.L^{-1}$	[91]
Chlorpyrifos	Acetylcholinesterase	Tap water and lake water	5.3 x 10^{-14} µM	[89]
Chlortoluron	Tyrosinase	River water, well water and tap water	0.02 µM	[92]
Diazinon	Butyrylcholinesterase	Soil	35 ppb	[93]
Dichlorvos	Acetylcholinesterase	Apple	2.5 x 10^{-12} M	[94]
		Lettuce leaves	2.99 × 10^{-13} M	[95]
		Calibration curve *	0.68 $\mu g.L^{-1}$	[96]
		Water	4.0 $\mu g.L^{-1}$	[97]
Fenitrothion	Alkaline-phosphatase	Calibration curve *	45.5 µM	[98]
	Butyrylcholinesterase	Soil	21 ppm	[93]
Heptonophos	Butyrylcholinesterase	Soil	650 ppb	[93]
Malathion	Acetylcholinesterase	Grape, Apple, Mango, Orange, Banana, Tomato, Rice, Wheat	0.058 ppm	[88]
	Alkaline-phosphatase	Calibration curve *	0.1 $\mu g.L^{-1}$	[87]
Metham-sodium	Alkaline-phosphatase	Calibration curve *	36.5 µM	[98]
Methamidophos	Acetylcholinesterase	Apple juice	3.1 x 10^{-13} M	[99]
Mevinphos	Butyrylcholinesterase	Soil	1.4 ppm	[93]
Monocrotophos	Acetylcholinesterase	Apple juice	2.7 x 10^{-12} M	[99]
		Water	5.9 $\mu g.L^{-1}$	[97]
Paraoxon	Acetylcholinesterase	Tapwater	2.46 $ng.L^{-1}$	[91]
		Apple	6.0 x 10^{-14} M	[94]
	Organophosphorus hydrolase	Calibration curve *	10^{-7} M	[100]
Parathion	Acetylcholinesterase	Tap water	0.542 $ng.L^{-1}$	[91]
		Water	4.2 $\mu g.L^{-1}$	[97]
	Butyrylcholinesterase	Soil	3.9 ppm	[93]
Pirimicarb	Laccase	Vegetables	0.04 $mg.Kg^{-1}$	[101]
Sulcotrione	Hydroxyphenylpyruvatedioxygenase	Calibration curve*	1.4 x 10^{-10} M	[102]
Tetradifon	Alkaline-phosphatase	Calibration curve *	4.1 µM	[98]

* Not applied in real samples

Generally, in studies of enzyme inhibition by pesticides, the biosensor is initially immersed in a solution containing the substrate at a defined concentration and in the absence of inhibitor (solution 1), and the base signal (I_0) is measured. Then the biosensor is incubated in a working solution containing a specific inhibitor concentration, for a predetermined time and, thereafter, transferred to a solution containing the enzyme substrate (same concentration of the solution 1) and the base signal (I_i) is measured again (Figure 4). Thus, the amount of inhibitor can be related to the inhibition percentage (% IR) obtained from (Equation 1), where the increased amount of inhibitor in the reaction medium leads to a decrease in enzymatic activity [106].

$$\% IR = [(I_0 - I_i)/I_0] \times 100 \quad \text{(Eq. 1)}$$

This process can be monitored by many types of transducers, wherein the compounds capable of suffer some type of change in specific properties when the system suffers interference from an inhibitor can be used in the reaction medium or immobilized together with the enzyme [85].

In Figure 4 is shown an electrochemical biosensor such have a biological recognition element the acetylcholinesterase enzyme, which is one sensitive enzyme to inhibition by organophosphate and carbamate. When an inhibitor (eg: organophosphate) is not present in solution, the acetylthiocholine substrate is converted to acetic acid and thiocholine by the action of the enzyme present on the biosensor. The thiocholine, in turn, is oxidized by applying voltage. Since, in the presence of an inhibitor, the conversion of acetylthiocholine decreases or is zero. The anodic oxidation current is inversely proportional to the

concentration of the inhibitor in the sample and depends on the exposure time [105].

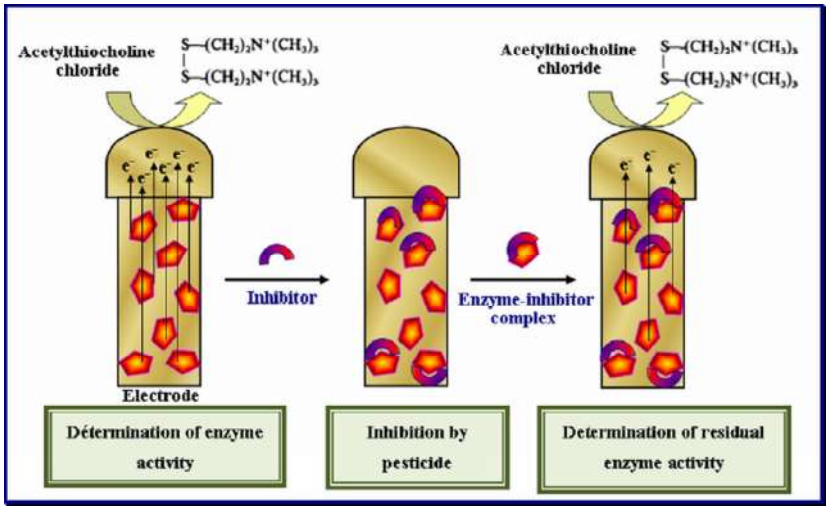

Figure 4. Operating of an electrochemical biosensor based on acetylcholinesterase inhibition for pesticides [105] (Reprinted from Elsevier with permission).

3.1.2 Application of sensors for detection pesticides

In the literature there are many studies about the application of sensors for pesticide detection in different samples of environment. An electrochemical sensor consisting of a carbon electrode modified with copper nanowires was developed by Mirabi-Semnakolaii and Daneshgar (2011) [107] for detection of trifluralin herbicide. The presence of copper nanowires improved the conductivity, resulting in increased of rate of electron transfer. This sensor showed a linear response in concentration range from 100 to 0.2 nmol.L^{-1}, with 0.008 nmol.L^{-1} of detection limit and quantitation limit of 0.15 nmol.L^{-1} for trifluralin, and the supporting electrolyte phosphate buffer solution of 0.05 mol.L^{-1} and pH 4.0. When applied to soil samples previously dried, sieved, extracted with acetone and diluted in phosphate buffer, showed a recovery of 99.3 to 101.5%.

Deep et al. (2014) [108] studied the development of a printed carbon electrode modified with styrene sulphonic acid doped with polyaniline for the manufacture of an immunosensor used in detection of atrazine pesticide. The mechanism used was the interaction of atrazine with the anti-atrazine antibody, immobilized on the sensor surface. The detection was specific and highly sensitive (0.01 ng.mL^{-1}atrazine) in concentrations from 0.01 to 50 ng.mL^{-1}.

For detection of atrazine in water samples Tortolini et al. (2016) [109] used biosensor amperometric based on mushroom tyrosinase. Atrazine could be detected due to inhibition of enzyme activity in the presence of the catechol substrate, where it catalyzes the oxidation of catechol o-quinone. The authors concluded that with 20 min incubation on the pesticide, the device showed a minimum detection limit of 0.03 ppm. Furthermore, they found that the interaction tyrosinase/atrazine is reversible.

Chen and Yang (2013) [110] evaluated the application of a biosensor based on liquid crystal using paraoxonase enzymes (PON1, PON2, and PON3 from the organophosphorus hydrolysis family) for the detection of organophosphorus in aqueous solution, more specifically paraoxon. The detection mechanism was based on the monitoring of changes in pH values per minute during the enzymatic hydrolysis of organophosphorus. Such type of biosensor demonstrated good specificity for detection, without using complex instrumentation, presenting low detection limit (1 µM of paraoxon) and response in real time.

A fiber optic biosensor using acetylcholinesterase enzyme in immobilized form was developed by Choi et al. (2001) [111], to detect organophosphate compounds in contaminated water, based on enzyme inhibition. These compounds are used in agriculture for crop protection purposes, and rapid detection in groundwater is very important. The detection was based on the reduction of product o-nitrophenol in the presence of the substrate-nitrophenylacetate, due to inhibition by organophosphorus compounds on acetylcholinesterase. The biosensor can successfully detect organophosphate compounds until 2 ppm with response time about 10 min.

Gupta et al. (2015) [112] developed a quartz crystal microbalance sensor for determining atrazine traces of residual water. The surface was coated with imprinted 2-hydroxyethyl methacrylate –phenol atrazine film on allylmercaptane. The sensor presented a linear response ranging from 0.08 to 1.5 nM with 0,028 nM of detection limit. The results indicated the efficiency of this sensor besides presenting, good sensitivity, fast response and low cost.

Vamvakaki and Chaniotakis (2007) [113], developed lipomose nanobiosensors for detection of two widely used organophosphorus pesticides: dichlorvos and paraoxon and the determination of total toxicity in drinking water samples. A detection design based on fluorescence has been chosen as a transducer, presenting high sensitivity, low detection limits and a wider detection range. The enzyme used was acetylcholinesterase encapsulated in the internal nano-environment of liposomes, which has been proven to greatly improve enzyme stabilization, where activity is measured by means of inhibition at the presence of pesticides. The authors concluded that the nano-sized liposomes provided the appropriate environment for acetylcholinesterase stabilization, enabling their use in fluorescent biosensors. Pesticides concentrations down to 10^{-10} M can be monitored using this inhibition.

Kesik et al. (2014) [91] used an amperometric biosensor

based on a conductive polymer, using carbon nanotubes and acetylcholinesterase for detection of organophosphorus pesticides. Inhibition responses of paraoxon, parathion and chlorfenvinphos on enzyme activity were detected. The response time was 6 s, linear range between 0.05 mM and 8.00 mM, detection limit of 0.09 mM, considered low and with high sensitivity (24.16 $\mu A.mM^{-1}cm^{-2}$).

Manisankar et al. (2008) [114] investigated the electrochemical behavior of three pesticides isoproturon, voltage and dicofol in soil samples. Used glassy carbon electrode modified multilayer carbon nanotubes (MWCNTs/GCE) and polyaniline (PANI) or polypyrrole (PPY) deposited on MWCNT/GCE. The electroativo behavior of pesticides was made from the cyclic voltammetry studies. The detection limit was 0.1 $\mu g.L^{-1}$ for isoproturon, 0.01 $\mu g.L^{-1}$ for voltage and 0.05 $\mu g.L^{-1}$ for dicofol on PANI/MWCNT/GCE modified system.

The novel silver/cupper alloy nanoparticles and graphene nanocomposite paste electrode was fabricated and its electrochemical activity investigated using cyclic voltammetry and electrochemical impedance studies and it was employed for non-enzymatic sensitive determination of chloropyrifos, an active member of organophosphate pesticides in soil and water samples. The cyclic voltammetry and electrochemical impedance spectroscopy showed a high catalytic activity of the nanocomposite, which was attributed to its increased active surface area, high electrical conductivity leading to fast rate of electron transfer. Nanoparticles of silver/cupper increased the electrical conductivity and the number of active sites of the nanocomposite, which showed high stability and sensitivity, good selectivity, wide linear range, low detection limits, good repeatability and reproducibility [115].

The surface of a glassy carbon electrode was modified with the introduction of graphene quantum dots, with subsequent addition of pralidoxime. The introduction of graphene quantum dots caused a significantly increased on the effective area of the electrode, increasing the amount of immobilized pralidoxime. The pralidoxime belongs to the family of oximes, which reverses the connection of the organophosphate with the acetylcholinesterase enzyme. Electrochemical measurements using soil samples were made, and in optimal conditions (0.1 M acetate buffer, pH 5.5), and the potential of 0.5 V and 200 s, wherein the pralidoxime oxidation current difference was proportional to the concentration of the fenthion in $1.0x10^{-11}$ to $5.0x10^{-7}$ M, with detection limit of $6.8x10^{-12}$. However, each modified electrode works only once, where after its use, the electrode was polished with alumina paste for cleaning before the next change [116].

Chauhan and Pundir (2012) [117] described a method for constructing an electrochemical biosensor for the detection of demalathion, chlorpyrifos, monocrotophos and endosulfan based on the covalent immobilization of acetylcholinesterase (purified from maize seedlings) on iron oxide nanoparticles decorated carboxylated multi-walled carbon nanotubes (MWCNTs-c) electrodeposited onto indium tin oxide - coated glass plate. The inhibiting effect of pesticides on the enzymatic activity of acetylcholinesterase was evaluated in incubation times ranging from 2 to 20 min in a pesticide solution (10 $nmol.L^{-1}$), where it was found that inhibition of the enzyme increased with the duration of the incubation period until reaching a constant level in 10 min (Figure 5). The immobilized enzyme could be reactivated by immersion in a solution of 2-pyridine aldoxime methiodide 4.0 $mmol.L^{-1}$, which is a reactivator of acetylcholinesterase. When soil samples (extract) were inoculated with 10, 20 and 30 ppb of malathion and reapplied in the same biosensor that had been used for the analysis of non-enriched extract, the inhibition percentage was between 32.0 and 63.2 %.

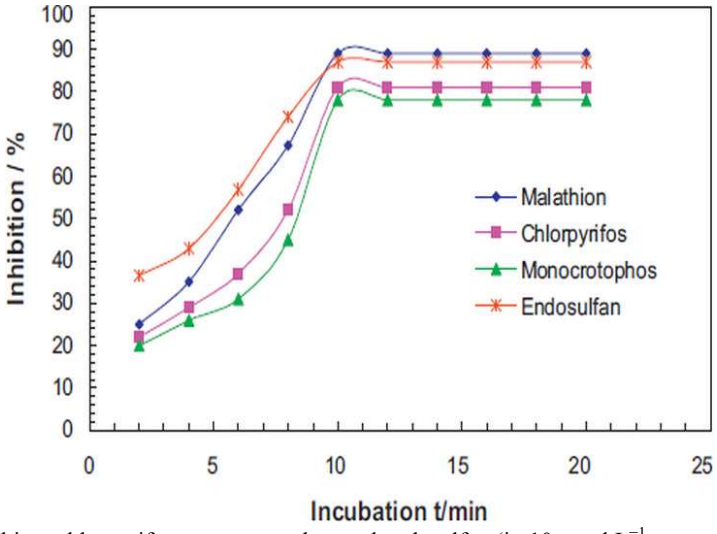

Figure 5. Effect of inhibition time on malathion, chlorpyrifos, monocrotophos and endosulfan (in 10 $nmol.L^{-1}$concentration). Applied potential + 0.4 V vs. silver/chloride silver [117] (Reprinted from Elsevier with permission).

A microbial optical biosensor for the detection of pesticide methylparathion was investigated by [118]. Whole cells of *Flavobacterium* sp. were immobilized by entrapment in glass fiber filter and used as biological component associated with optical transducer for detecting methylparathion. *Flavobacterium* sp. contains the enzyme organophosphorushydrolase, which hydrolyzes methylparathion into a detectable product, p-nitrophenol. The analysis was performed by measuring absorbance at a maximum wavelength of 410 nm based on the relationship between the amount of hydrolyzed methylparathion and the

amount of p-nitrophenol formed. Soil samples were used that have undergone a pre-treatment consisting in soil incubation in bicarbonate-carbonate buffer 0.2 M (pH 8.5) containing 10% methanol and, after decanting, the aqueous layer was used as the sample biosensor. The biosensor was stable for 1 month, stored at 4 °C, with a detection limit of 0.3 mM of methylparathion, which was estimated from the linear range (4-80 M) of the calibration curve of the enzyme test.

A glassy carbon electrode was modified with mercury film in the presence of thiocyanate and used in the determination of atrazine in soil and water samples by adsorption. Adsorption of atrazine on the electrode surface modified with mercury layer was made under open circuit conditions in the stirred solution. The linear range was 0.5-60 $\mu g.L^{-1}$ (R^2 = 0.9978) and the detection limit of 0.024 $\mu g.L^{-1}$ [119]. The authors emphasis that this procedure is applicable to the determination of atrazine in complex real samples such as soil samples.

Nanobiosensors are also used for quantification of trace amounts of pesticides in biological samples. An example is the use of a fluorescent biosensor composed of an immunochromatographic test strip with quantum dots for biomonitoring of 3,5,6-trichloropydinol, a metabolite of chlorpyrifos pesticide. The detection principle is based on a competitive immunoreaction, which 3,5,6-trichloropyridinol competes with 3,5,6-trichloropyridinol-quantum dots conjugate to bind with immobilized anti-3,5,6-trichloropyridinol antibodies in the test strip, where quantum dots captured help as signal vehicles for the fluorescent reading. This nanobiosensor detected 1.0 $ng.mL^{-1}$ of standard 3,5,6-trichloropyridinol substance in 15 min, and when tested in mouse plasma showed an average recovery of 102 %, demonstrating to be a fast and precise tool for detection of 3,5,6-trichloropyridinol [120].

4. NANOBIOSENSORS OF CANTILEVERS – AFM

The invention of the atomic force microscope (AFM) in 1986 [121] opened the possibility of creating a new detection tool of various analytes through the functionalization of cantilevers, which can also be used as sensors [122]. Thus, AFM is quite a versatile technique, since it can be used on either topographic characterization of the sample surface through its imaging or non-topographic sample surface analyzes related to the use of AFM as a nanoscale sensor, called nanomechanical systems.

Sensors based on cantilever are versatile and compact devices for specific detection of many substances, presenting real-time response, low detection limits and high sensitivity [123]. Cantilever consists of a movable beam on which one end is fixed to a support and may have "V" (triangular) or "T" (rectangular) shape [124, 125]. They generally have a tip at the free end [81].

For use as sensors, cantilevers should be coated with a sensing layer that is highly specific to the molecule that is desired to detect, where this process is called functionalization [125]. Both the AFM tip and the cantilever (needless of its tip) can be chemically modified. On the first case it originates the AFM tip nanobiosensors and on the latter case originates the cantilever based nanobiosensors.

Cantilevers surface functionalization is one of the most important steps in the construction of these devices since affect their characteristics such as sensitivity and detection limit. The cantilever functionalization, composed of silicon dioxide, can be normally done through the deposition of silanes (-SiOX) [81]. Another widely used type of functionalization is the addition of a gold layer [126] on the silicon dioxide surface of the cantilever and then alkaline chain molecules with thiol group (-SH) are deposited on this layer. Sensing layers with different properties and functions may be obtained with the use of thiols with different chain lengths and different functional groups at one end, making this type of functionalization very interesting [127]. This functionalization is carried out with the aim of obtaining functional groups that enable the bind of biological recognition element [128]. This procedure is performed that the biomolecules are not denatured and leached during the use of nanobiosensor, still remaining stable for long time of use [129]. The enzymes used as the sensors in the specific pesticides detection must have a specific binding to the target molecule, offering selectivity. Pesticide detection by enzymes in nanomechanical systems is normally based on a mechanism of inhibition [130]. For nanomechanical system, the operating principle is based on the analytes adsorption on its surface modified with a sensitive layer, causing a change in the vibrational frequency (dynamic mode) or in the deflection (static mode) (Figure 6). In the static mode, the sensitive elements are deposited on only one side of the cantilever and after contact with the analyte, there is a change in the surface tension on the functionalized side in relation to the other side, causing the deflection. In the dynamic mode, the sensitive elements are deposited on both sides of the surface, which causes a change in the resonant frequency [76].

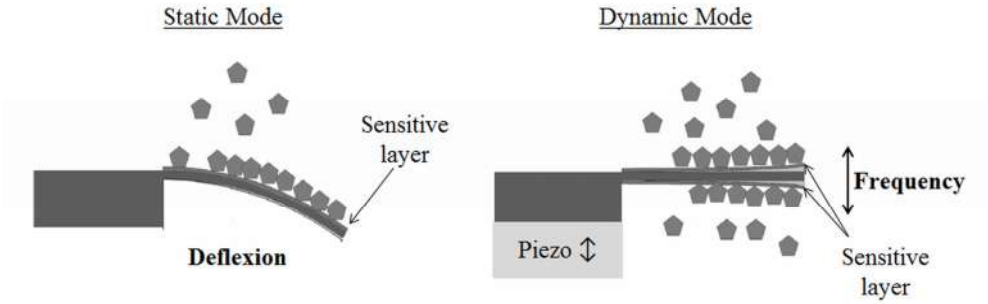

Figure 6. Some operation modes of nanomechanical cantilever sensors: static and dynamic mode (Adapted from [131] (Reprinted from Springer with permission).

The mechanical properties of cantilevers, such as the spring constant (k) and the resonance frequency, are responsible for the cantilever sensors performance [132]. The spring constant is a measure of the cantilever flexibility, determined by the geometric properties (length, width and thickness) and the Young's modulus, related to the cantilever material properties [125, 133]. The resonance frequency is the result of the cantilever vibratory motion, related to the cantilever spring constant, geometry and the mass change adsorbed onto the cantilever [133]. The AFM tip sensors are based on the Atomic Force Spectroscopy technique (AFS), an AFM technique module. It detects specific intermolecular interactions, allowing the functional groups mapping on a substrate at both micro and nanometric scales. Thus, the AFS based AFM tip nanossensors are highly sensitive and selective devices [134-136], since they allow the quantification of forces between the tip and sample. To date, there are no reports of using cantilever sensors for agrochemicals detection in soil, but they have been used for pesticides detection in both water and atmospheric contaminants, as described below.

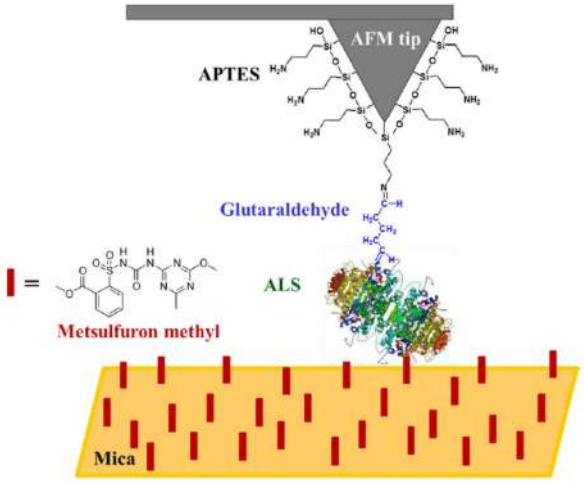

Figure 7. Illustration of the tip functionalization with 3-aminopropyltriethoxysilane (APTES), glutaraldehyde and acetolactate synthase (ALS) and the interaction with metsulfuron methyl herbicide [130] (Reprinted from MDPI with permission).

Silva et al. (2013) [130] detected the herbicide metsulfuron-methyl by force curves using AFM tip nanobiosensor, which was functionalized with the acetolactate synthase enzyme, obtained from bacteria and yeast. The AFM tip functionalization occurred by 3-aminopropyltriethoxysilane gaseous evaporation in the presence of triethylamine, glutaraldehyde solution, and acetolactate synthase enzyme-enriched extract. The herbicide metsulfuron-methyl (in methanol) was immobilized on the mica substrate (Figure 7). Comparing the non-functionalized cantilever with the functionalized one it was observed an increase of approximately 250 % adhesion force relative, showed a specific interaction enzyme-herbicide.

A computer simulation with the steered molecular dynamics (SMD) modeling software was used to evaluated the interaction force between active layer (acetyl-CoAcarboxylase enzyme) and herbicide diclofop. The Figure 8 show the scheme of decoupling process of herbicice diclofop from the active site of acetyl-CoAcarboxylase enzyme. The theoretical force of 1.6 ± 0.5 nN per enzyme is in agreement with the experimental one measured with AFM. With this study it was possible to determine both the coverage of the AFM tip with active layer and the average number of active sites attached [137].

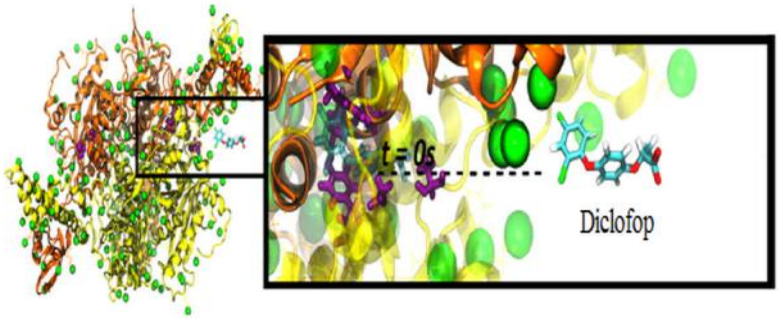

Figure 8. Ilustration of the herbicide diclofop decoupling process from the active site of the ACC enzyme [137] (Reprinted from Elsevier with permission).

Deda et al. (2013) [138] evaluated the use of acetolactate synthase enzyme for detection the herbicides imazaquin and metsulfuron-methyl using functionalized AFM tip. The functionalization consisted of the gaseous evaporation of 3-aminopropyltriethoxysilane in the presence of triethylamine, followed by immersion in glutaraldehyde and after in the solution of the enzyme. The substrate was coated with glutaraldehyde and herbicide solution (Figure 9). The authors observed the adhesion force between the AFM tip and the herbicide monitoring the laser beam position, which is focused at the endpoint of the cantilever and reflected to a photodetector. It was observed an increasing of the adhesion force of 132 % and 145 %, respectively for imazaquin and metsulfuron-methyl.

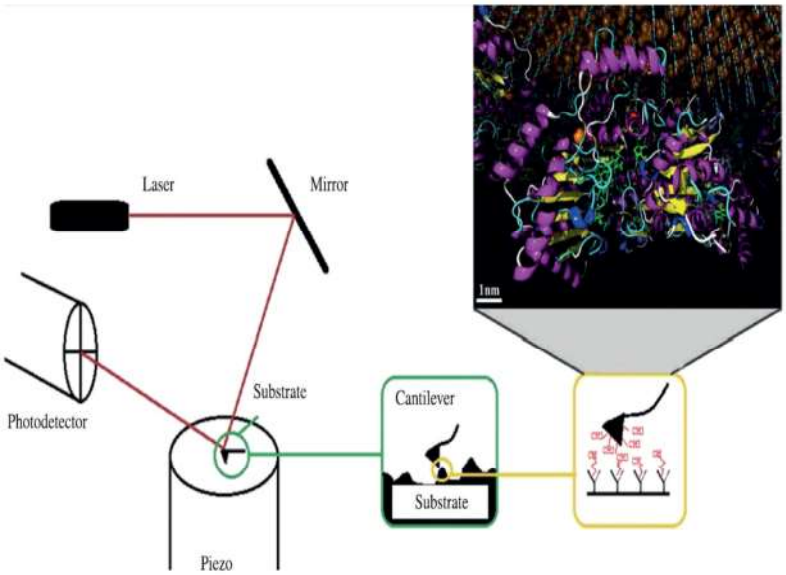

Figure 9. Illustration of chemical interaction between the functionalized tip and the herbicide [138] (Reprinted from Scielo with permission).

Plata et al. (2008) [139] used piezoelectric microcantilever sensor to determinate total carbonate in soil samples. The authors observed that microcantilever sensor responded to nitrogen pressure in an interval from 2 to 5 s, in a linear range of 35-1036 mbar and precision of 1.02 %. The cantilever sensor signal quickly returned to the baseline after the measurements.

Application of these nanomechanical systems for detection pollutants is emerging technology that will enable the creation of more efficient and selective techniques, where different contaminants can be detected from the correct choice of the biomolecule used as sensing layer. The development of science and nanotechnology will contribute to producing sensors useful as analytical tools.

5. CONCLUSION

The increase in world population with the consequent greater demand for food and raw materials led to a widespread of pesticides use, causing environmental contamination, mainly soil. Due to pesticides characteristics such as toxicity and their persistence in the environment and present in trace amounts in the soil, the development of analytical methods that require less analysis time compared to traditional chromatographic analysis methods to detect pesticides with high sensitivity is extremely important. Biosensors, and in particular nano-biosensors, have stood out, as the specific biological recognition combined with the device's great surface area, thus improving analytical performance through high sensitivity and a low detection limit. So, cantilever nano-biosensors derived from Atomic Force Microscopy technique, have showed versatile and compact devices with a wide

range of applications in pesticides detection with high sensitivity due to their small size and highly specific sensing layer (biomolecule).

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7. ACKNOWLEDGEMENTS

The authors would like to thank Cnpq, Capes, Fapergs and Finep responsible for the financial support.