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Review Article

Composting of Soils Polluted with Pesticides: A Microbial Approach and Methods for Monitoring

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Abstract

This review provides information about pesticide-polluted soils treated by composting, viewed from a microbial approach. It describes how pesticides pollute the environment when they are applied, the processes by which they can be transformed (chemically or biologically) in soil and explains the composting as a bioremediation technology to restore soil quality. It highlights the participation of main groups of microorganisms (bacteria, actinomycetes and fungi), which are involved on pesticide biodegradation during the composting processes. Also, there are cited the main soil enzymes (dehydrogenases, ureases, β-glucosidases), that must to be monitored in composting of soil polluted by organophosphates and organochlorines pesticides. It also contains principles and fundamentals of microbiological methods to measure the population dynamic in a composting process. Provides information about methods for a direct or indirect detection of microbial activity and the most innovative and comprehensive molecular methods for exploring biodiversity on composting soils.

INTRODUCTION

Pesticides are the most cost-effective means of pest and weed control. However, there is a high concern about the environmental impact that has caused the massive application of pesticides, as these chemicals can migrate to others sites [1]. Pesticides applied on to soils can bring environmental hazards, influence soil quality, and induce detectable changes in size, structure, and functionality of the microbial community, thereby altering life functions, dynamics, and biodiversity of soil organisms [2-4].

A technology for soil bioremediation is composting, as it utilizes microorganisms to degrade pollutants [5]. Composting is defined as the microbial degradation of organic matter under aerobic conditions to obtain a stable material that can be used as organic fertilizer [6]. Composting can help to stabilize and/or degrade pesticides in contaminated soils, thus contributing to their bioremediation [7,8]. When compost is spread onto soil, organic matter is mineralized and pesticides can undergo physicochemical and biological processes that may change their chemical forms and their bioavailability [9-11].

Therefore composting is a suitable process for stabilizing pesticides in soils through degradation by microbial communities, to finally enhance soil quality [3,12]. Soil quality is usually characterized by its abiotic factors (pH, water holding capacity, texture), but increasing recognition is now being given to biotic factors [13]. Microorganisms (biotic factors) are indicators of soil quality because of their roles in biogeochemical cycles (C, N, P, and S) and maintenance of soil structure [11].

In the soil composting, it is involved a high catabolic activity by microbial populations composed of a wide variety of mesophilic, thermotolerant and thermophilic aerobic microorganisms [6,10]. For purposes study, in this article, will be grouped soil microorganisms as follows: non actinomycetes bacteria (NAB), actinomycetes (although are also known as actinobacteria) and fungi. They are the main pollutant-degrading microbes in soils and composts, that have been widely considered to be the crucial governing factors in the remediation of contaminated soils [3,14,15]. The understanding of microbial interactions and their roles during the composting process of pesticide contaminated soils are still relevant [16,17]. Therefore, a deeper understanding in the dynamics of microbial communities found in soil/compost mixtures is necessary, in order to assess the effect of composting in remediation of contaminated soils [18,19].

The information about microbial populations on soil is obtained through culture media, however many microorganisms are non-cultivable, therefore, using molecular methods is important. This review presents a microbiological approach for composting pesticide contaminated soils, describing the methods and techniques used to explore the microbial biodiversity during composting of contaminated soils, from the use of culture media to select specific microorganisms, to the molecular methods for study soil microbial communities.

Persistence and Biodegradation of Pesticides onto Soil

The behavior of pesticides in soil is governed by a variety of physical, chemical and biological properties (Figure 1). According to Yavari *et al*., 2015 80 [20], the main effective properties are solubility in water, vapor pressure (VP), Henry's law constant (KH) or air-water partitioning coefficient (Hc), octanol/water partition coefficient (Kow), soil organic carbon/water partition coefficient (Koc), acid dissociation constant (pKa), and half-life (t1/2). Pesticides characteristics such as the nature and position of functional groups, substituent and unsaturated bonds and can be used to estimate the pesticides sorption behaviors and the risks of environmental pollutions [1,21]. Next are described briefly, the mechanisms physicochemical and biological, for persistence and biodegradation of pesticides in soils:

a) The mobility of pesticides in soil, their bioavailability and transfer to other environments, depends on the mechanisms and kinetics of adsorption and desorption from soil particles. These mechanisms are key constituents for modeling the mobility, availability, and bioactivity of pesticides applied in the environment and determining the fraction of chemicals susceptible to leaching, degradation, and uptake by target and non target organisms. Adsorption plays a fundamental role in the adjective – dispersive transport dynamics, persistence, transformation and accumulation of pesticides.

The molecular nature of soil organic matter has been proved to be key in adsorption of non-polar and polar pesticides (e.g. diuron, simazine, atrazine). Organic matter is highly reactive toward ionic and polar pesticides because ionizable functional groups within natural organic matter (e.g., carboxylate, phenolate, amino, and phosphate groups). In addition, aromatic moieties and hydrophobic micropores within organic matter promote the sorption of many hazardous organic compounds. Clays minerals have negative charge, because of that, also play a major role in the adsorption of ionic pesticides in soils [1,20].

b) Chemical degradation of pesticides is governed by abiotic factors, includes soil properties such as chemical composition, organic matter, clays percentage, water content, oxidation-reduction potential and pH [22]. Chemical degradation occurs through reactions such as photolysis, hydrolysis, oxidation and reduction, for example organophosphates can be chemically degraded by alkaline reactions in soils [1,23].

c) The biodegradation of pesticides in soil involves a variety of complex biochemical reactions that result from physical interactions between pollutants, soil matrix and biological interactions among different microorganisms [24]. Pesticides are transformed by metabolic reactions leading to changes in their chemical structure through diverse reactions. Their biodegradation involves a transformation by oxidation, reduction, or hydrolysis reactions. After that, pesticides or its metabolites could be conjugate to sugars and amino acids, resulting in an

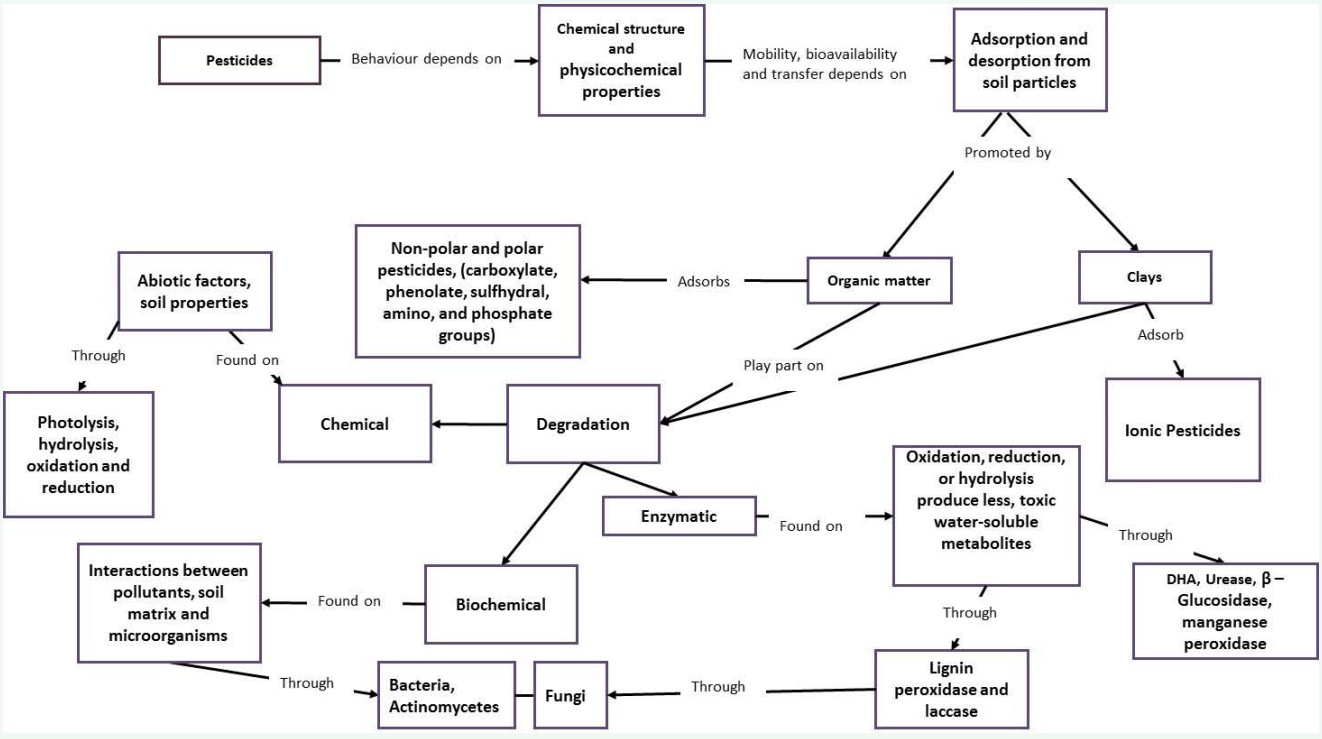

Figure 1 Interaction and degradation of pesticides in soils.

increased water solubility and a decreased toxicity. Finally the conversion of metabolites into inorganic compounds. In these processes, bacteria and fungi generate intra- or extracellular enzymes such as hydrolases, peroxidases, oxygenases, and others [25,15].

Composting: Bioremediation Technology for Clean-Up of Soil Polluted with Pesticides

According to Megharaj *et al.,* (2011) [26] the aim of composting is reduce volume and water content of green wastes, destroy pathogens, and remove odor-producing compounds. This process is applied as a bioremediation technology for handling polluted soil. On composting, polluted soil is piled up and is spread out in thin layers, to allow aeration conditions, also is essential to verify constantly, temperature, pH, water potential, organic matter and the amount of pesticide or metabolite [27-29]. The contaminated material is typically mixed with an organic bulking agent such as, manure, to improve airflow in the pile and increasing the porosity, also it is necessary to add water to control moisture content [30]. Composts of yard manure, cow dung, corn fermentation byproduct, corn stalks and sawdust have been used to improve the herbicide removal of atrazine, trifluralin and metolachlor in contaminated soils [31]. Add compost to soil has improved degradation of herbicides, benthiocarb(S-4-chlorobenzyl diethylthiocarbamate) and MCPA (4-chloro-2-methylphenoxyacetic acid). In addition, chlorophenols can be effectively degraded during composting of contaminated sawmill soil [26]. More than 90 % of the chlorophenols disappeared in a composting pile produced out of straw compost and chlorophenol-contaminated soil [3].

During the composting, pesticides can be degraded during the first phase of rapid decomposition. Heat which is generated by microbial metabolism is trapped in the compost matrix and most of the microbial decomposition and biomass formation occur during the thermophilic stage of composting. The mixing of remediated soil with contaminated soil can increase the effectiveness of composting because the remediated soil with acclimated microorganisms significantly influences pollutant degradation in the composting process [26].

A successful composting process requires efficient microbial consortia that can degrade pesticides to minimum level according to environmental normativity [9,27]. Therefore, it is required an exploration of total microbial population and their biochemical activities [32,15].

Pesticides-Degrade Microorganisms on the Composting of Contaminated Soils

Soil composting is a micro ecosystem typically inhabited by non actimomycetes bacteria (NAB), actinomycetes, an intricate web of fungal hyphae and protozoa [11]. The interactions between these microbial groups make it very difficult to establish the direct and/or indirect effects of pesticide additions on the microbial community composition [33]. A fraction of soil microbiota can quickly develop the ability of pesticide degradation, when continuously applied to soil [34].

During the composting process, microorganisms use the organic matter and/or pesticides as carbon and nitrogen sources and electron donors [35,36]. Normally in the first stage of composting, arise mesophilicNAB and fungi organic matter decomposers, because of they have different hydrolytic enzymes (cellulases, hemicellulases, proteases, lipases, phosphatases and arylsulphatases), which are involved in the depolymerization of different constituents of organic wastes and pesticides. In the next stage, thermophilic microorganisms appear, especially actinomycetes, parallely populations of coliforms pathogens decline. The final stage of composting is characterized by new mesophilic populations of NAB, actinomycetes and fungi, also formation of humic-like substances during compost maturation and complete stabilization, pesticides are transformed into simple or less toxic molecules [6,37,38]. Table (1) presents soil microbial groups reported for pesticide biodegradation. Below, microorganisms involved in composting pesticide-polluted soil are described:

Non actinomycetesbacteria (NAB): The NAB are heterotrophic bacteria, satisfy their need of energy by taking pesticides as only carbon source. A large group of Gram negative and positive NAB genera isolated from soil, have been reported to degrade organophosphates compounds. *Serratia* sp. SPL-2 can degrade methidathion, *Pseudomonas aeruginosa* Is-6 can degrade acephate, methamidophos, methyl parathion, dimethoate and malathion. Soil bacterial communities containing isolates of *Agrobacterium* sp., *Bacillus cereus*, *Bacillus subtilis*, *Brucellamelitensis*, *Klebsiella* species, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Serratiamarcescens* are capable of degrading chlorpyriphos as a sole carbon source after an incubation of 20 days. Diflubenzuron (100~500 µg/g) had a stimulatory effect on *Azotobactervinelandii* in soil [39,40].

Actinomycetes: Actinomycetes are either aerobes or anaerobes, motile or non-motile, and spore-/non-spore forming bacteria with a high G+C content. These soil microorganisms constitute a significant fraction of the microbial population in soils (commonly more than 1 million microorganisms per gram) [41]. Because of actinomycetes are organic matter decomposers, they have a potential use as agents for composting and biodegradation of certain pesticides organochlorines, s-triazines, carbamates, acetanilides, organophosphorus and sulfonylurea in polluted soils. [42-44]. Pesticide-degrading actinomycetes belonging to *Arthrobacter*, *Brevibacterium*, *Clavibacter*, *Corynebacterium*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Rhodococcus* and *Streptomyces* genera have been previously described [45,46].

The biodegradation of organochlorine pesticides by several actinomycetes was reported by Briceño et al. [41] in which two isolates named as *Streptomyces* sp. strain AC5 and *Streptomyces* sp. strain AC7, were capable to grow in a medium with chlorpyriphos and biodegrade them at concentrations of 25 mg/L and 50 mg/L for 72 h. The actinomycetes of genus *Streptomyces* and *Micromonospora* have the ability to degrade alachlor, diuron [44], lindane, chlordane, methoxychlor and cypermethin. Moreover degradation of s-triazines, carbamates, acetanilides, organophosphorus and sulfonylurea is predominantly attributed to actinomycetes [47].

Fungi: Soil fungi produce extracellular ligninolytic enzymes like manganese peroxidase, lignin peroxidase and laccase. By

Table 1: Soil microorganisms pesticide degraders [6, 35,36].

Group	Microorganism	Pesticide
Actinomycetes	*Arthrobacter*sp.	Endosulfan
	*Micromonospora*sp. And Streptomycessp.	Alachlor, chlordane,chlorpyriphos, cypermethin,diuron, lindane andmethoxychlor
Bacteria	*Burkholderia*sp.	Fenitrothion
	*Bacillus*sp.	Mesotrione
	Bacilluspumilus	Chlorpyrifos
	*Enterobacter*spp.	Chlorpyrifos
	*Ochrobactrum*sp.	Methylparathion
	Providencia stuartii	Chlorpyrifos
	Pseudomonasfrederiksbergensis	Dimetoate and malathion
	Pseudomonassp.	Dianizon
	Serratialiquefaciens	Dianizon
	Serratiamarcescens	Dianizon
	*Sphingomonas*spp.	Isoproturon
	*Stenotrophomonas*sp.	DDT
Fungi	*Aspergillus niger*	Endosulfan
	*Chlorophyceae*sp., *Chlorella*sp., *Scenedesmus* spp.and *Stichococcu s*sp.	Fenamiphos
	Trametesversicolor (R26)	Atrazine
	*Verticillium*sp. DSP	Chlorpyriphos

example laccase and peroxidase, can degrade completely bentazon (a very recalcitrant herbicide), by the co-presence of a variety of humic materials. Fungi enzymes have a greater ability to resist the application of pesticides, except for fungicides, cyazofamid, pyrazofos and captan can suppress specifically their electrons transfer in mitochondrial activity [33]. White rot fungi have been reported to have a high capacity of pesticide removal such as monocrotophos, methamidophos, dimethoate, fenpropathrin, acetamide, profenophos, chlorpyriphos, carbosulfan during composting [3,48]. A consortium of *Phanerochaetechrysosporium, Trametesversicolor, Bjerkanderaadusta* and *Bjerkanderafumosa* was assessed for pentachlorophenol removal [49].

Enzyme Activity of Soil and Compost on Pesticides Degradation

Soil enzyme activity is the direct expression of the soil biological community to presence of available pollutants. Soil enzymes are present in two forms, intracellular, within bacteria and fungi, or extracellular, enzymes immobilized onto soil particles. These enzymes, carrying out reactions that transform pesticides by oxidation, reduction, or hydrolysis, producing metabolites more water-soluble and less toxic than the original compounds [9,50,51].

Hydrolysis is the first reaction who drives for the complete degradation of organophosphates pesticides. Methyl parathion hydrolase is an important organophosphorus hydrolase, also known as phosphotriesterase, is capable of hydrolyzing organophosphate pesticides such as methidathion, acephate, methamidophos, methyl parathion, paraoxon, tetrachlorvinphos, dimethoate,malathion,chlorpyrifos.Thishydrolasehasbeenfound in methyl parathion-degrading *Pseudaminobactersalicylatoxidans* mp-1, *Achromobacterxylosoxidans* mp-2, *Ochrobactrumtritici* mp-3, *B. melitensis* mp-7, *Plesiomonas* sp. M6, *Sphingopyxis* sp. DLP-2, *Pseudomonas stutzeri* HS-D36, *Flavobacterium* sp. and *Pseudomomasaeruginosa* [24,4,52]. By other side, a high activity of hydrolytic enzymes in a compost could be related to the potential ability of its resident microbiota to degrade pesticides.

Others soil enzymes such as phosphatases, β-glucosidases, dehydrogenases, ureases, are sensitive indicators of soil quality and its ability to stabilize organic matter on composting, because they are involved in biogeochemical cycles and can respond to changes in soil organic matter or presence of persistent organic compounds by natural or anthropogenic factors [12,53,54,55].

Phosphatases, β-glucosidases and cellulases are very important enzymes involved in the transformation/ decomposition of organic matter in soil. Acid and alkaline phosphatase hydrolyze organic esters into inorganic phosphate and arylsulphatase is associated with fungal and bacterial hydrolysis of ester sulphate to produce SO_4. β -glucosidase catalyzes the hydrolysis of disaccharides in soil to form β – glucose, the hydrolysis products are important energy sources for microorganisms [56,57].

Another important enzyme during composting is dehydrogenase (DHA). DHA is an indicator of organic matter stabilization because it is involved in the respiratory metabolism of an overall microbial activity in soils [36]. This enzyme is measured by the reduction of the triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF) at 37 ºC for 24 h in darkness and expressed as mg of TPF released per gram of dry matter (DM) [32,58]. DHA activity is considered one of the most important enzyme activities used as an indicator of overall

microbial activity, because it is intracellular in all living microbial cells and is linked to the microbial respiratory process. Several pesticides have been reported that affect DHA in soil, by example hexaconazole, chlorpyrifos and quinalphos had an overall DHA inhibition [57].

Urease catalyzes the hydrolysis of urea into CO_2 and NH_3 and is a key component in the nitrogen cycle of soils [57,18]. This enzyme is quantified by the ammonium released in soil samples, incubated with urea during 2 h at 37 ºC with toluene, to inhibit microorganism growth [60,61]. It has been reported that in pentachlorophenol polluted soils, urease activity decrease, but when soil is treated with compost, urease activity is improved [59]. In diuron contaminated soils, a high urease activity was found, this fact could be related to the hydrolysis of this nitrogen-containing herbicide, because of urease catalyses the cleavage of N–C bonds in ureic compounds.

Methods for Monitoring Microbiota, During Composting of Pesticide-Contaminated Soils

For monitoring the behavior of microbial growth or their catalytic activity, in soils polluted or composting, is important to have an accurate and rapid method to measure it. Figure 2 present the relationship between the different methods most commonly used, for phylogeny, biodiversity, abundance or activity studies. Next are described briefly their purpose and application.

Enumeration of Total Aerobic Heterotrophs

Also known as plate count technique, in this method, previously it must to prepare the sample in order to release microorganisms from the matrix of soil, then disperse them in a suitable diluents so that individual cells can be enumerated by cultivation in a solid medium. It quantifies the viable microorganisms in a sample by counting the number of colonies that form on a solid growth medium inoculated with dilutions of a soil sample. Units are expressed as colony forming units per gram of soil dry weight (CFU/g). It is especially important to determine the type of microorganisms to be cultured on agar, considering that selective media based on their nutritional requirements has to be selected, in addition to specific compounds like antibiotics that inhibit growth of non-desired microorganisms [10,62,63].

In a composting process of post-harvest tomato plants over six months, populations of mesophilic and thermophilicNAB and actinomycetes were measured with enumeration of total aerobic heterotrophs method. The microbial populations were increased at the mesophilic and thermophilic stages of composting in a magnitude order $1x10^8$ CFU/g [10].

Soil Microbial Biomass by Fumigation-Extraction

Soil microorganisms represent only 5% of the organic matter but play a critical role in soil fertility by mineralization of organic compounds. In the fumigation-extraction method,

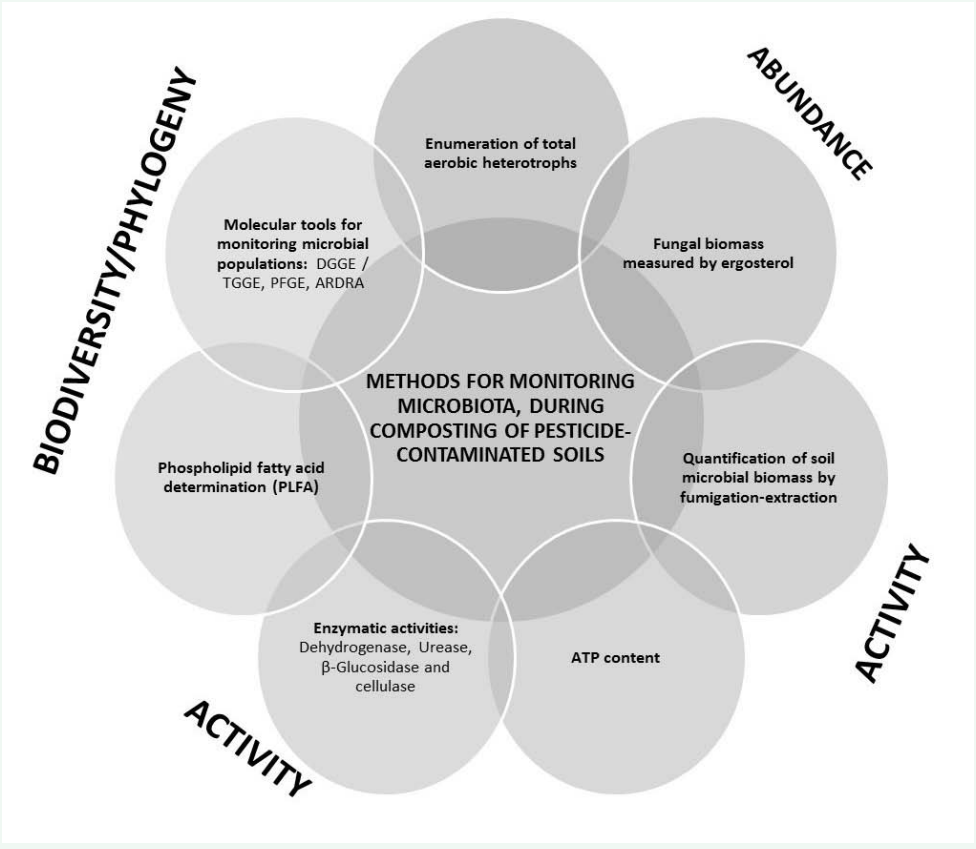

Figure 2 Relationship between the methods commonly used, for phylogeny, biodiversity, abundance or activity microbial studies, in order to explore microbiota of soils polluted with pesticides, treated by composting.

soils are exposed to chloroform vapour for 24 h in order to lyse the microbial cells. The difference between fumigated and non-fumigated carbon (C) is a measure of the chloroform labile C which is then multiplied by a factor to give microbial biomass C (Kec factor of 0.45) is often used to calculate the microbial biomass C value. Microbial biomass is an important indicator of microbial activities and provides direct assessment of the linkage between microbial activities and the pesticides transformations. If microbial biomass value is high around 229 kg-C/ha, indicates microbial community is capable to remove it or use it as carbon source. A drawback for this method is: fumigation and extraction cannot be dispersedin very compressed soils [18,64,65].

ATP Content

ATP content provides valuable information on transformation trends of pesticides in soils. The substrate luciferin, reacts with ATP and luciferase in the presence of Mg^{2+} to yield an enzyme–luciferin–adenosine monophosphate intermediate. This, in the presence of O_2, breaks down to produce free adenosine monophosphate, inorganic P, and light. The light emitted is measured by a photometer and plotted against ATP content to form a standard curve [65].

Fungal Biomass Measured by Ergosterol

The Ergosterol content of soils indicates the extent of fungal membranes as well as fungal and ectomycorrhizal biomass. Ergosterol is extracted by methanol and detected using high performance liquid chromatography with an UV detector. Shifts in microbial community structure due to soil contamination or changes in vegetation can be detected using ergosterol to microbial biomass C ratio [66].

Phospholipid Fatty Acid Determination (PLFA)

Determination of the composition of soil or compost microflora, can be determined by the profile of phospholipid fatty acids. The PLFA technique is based on the extraction, fractionation, methylation, and chromatography of the phospholipid fraction of soil lipids [64]. This method is an excellent tool for following the overall microbial succession during composting. The PLFA´s are assigned to microbial groups, by example 10Me18:0 is related to actinomycetes, all monounsaturates containing from 14 to 19 carbon are related to Gram-negative bacteria, polyunsaturated (18:3 and 18:2) to be fungal markers, and iso- and anteiso-branched with 14-19 carbon + 15:0 and 17:0 as representing Gram-positive bacteria non actinomycetales [67]. The PLFA was used to assess the dynamic of the microbial community during the composting of poultry manure (PM) and cow manure (CM). At the beginning of the process, the fungal biomass was significantly greater in PM and CM than in the respective co-composted materials with biochar (PMB and CMB); this difference declined gradually during the process. In contrast, the Gram+ to Gram– ratio was increased by the presence of biochar. After 12 weeks of composting, factor analysis based on the relative abundances of single PLFA´s revealed changes in the microbial community structure which depended on the original organic wastes (CM vs PM). The ratio monounsaturated/saturated PLFA´s can be used as an indicator of physiological or nutritional stress in microbial communities, normally is lower in microbial communities that inhabit environments polluted or limited in organic carbon [38].

DNA Based Methods for Monitoring Microbial Populations in Soil Composting

Recent advances in nucleic acid extraction from soil, have gained great interest because of their potential to describe soil microorganisms that are not accessible by cultivation-dependent techniques and have allowed the identification of unknown sequences potentially ascribable to new taxa [10,68,69]. Microorganisms biodiversity of contaminated environments can be monitored in order to assess the presence of pesticide degrading bacteria and fungi for biostimulation strategies. The most used molecular marker for this kind of studies is the ribosomal gene RNA 16S for prokaryotes or 18S for eukaryotes. Sequence analysis of this molecule allows phylogenetic reconstructions that describe the structure and composition of the microbial population in a determined habitat, thus identifying potential microbial species for bioremediation, especially for uncultivable bacteria [70].

Molecular methods such as real time PCR allows the amplification and quantification of 16S rRNA gene; Amplified DNA Ribosomal Restriction Analysis (ADRA) allows identification of genetic fingerprints of the microbial community [71,72].Gradient denaturing / temperature gradient gel electrophoresis (DGGE / TGGE) is a type of electrophoresis that allows the separation of DNA fragments of the same size but different nucleotide sequence for identification of the polymorphism of 16S rRNA. Using a gradient denaturing / gradient temperature bands can be purified for sequencing, and therefore identify microorganism of the microbial community [73], the DGGE is a highly sensitive, relatively reproducible method it allows simultaneous analysis of numerous samples to evaluate the differences and similarities that may exist between them [74].

Denaturing gradient gel electrophoresis (DGGE) is a very versatile method for screening the total microbial community DNA from a soil sample [75]. DGGE analysis of microbial communities produces a banding pattern, which can be quite sensitive to spatial and temporal sampling variations [61,76,77]. By example, through this method, could be identified actinomycete strains from a pesticide contaminated soil, capable to remove and dechlorinatelindane, chlordane and methoxychlor [43]. In addition, DGGE has been used to demonstrate changes in the bacterial community profiles over time in a green wastes compost, it could be possible to reveal a wide diversity of sequence types in the clone libraries. Indigenous bacterial functional and community diversity were increased in pentachlorophenol polluted soils treated with vermicompost, this behavior was revealed by 16S rRNA phylogenic trees, the dominant DGGE bands belonged to the five families Flavobacteriaceae, Pseudomonadaceae, Comamonadaceae, Sphingobacteriaceae and Moraxellaceae [75].

Pulsed Field Gel Electrophoresis (PFGE) is the standard reference technique to establish most bacteria and fungi in soil samples, because it has a high discriminatory power and excellent reproducibility [78,79]. A final method for studying bacterial diversity in microbial ecosystems is by building a metagenomic DNA bank of the microbial habitat. Phylogenetic characterization of microbial diversity; characterization of new genomes; elucidation of new metabolic pathways for

the synthesis of primary and secondary metabolite could be obtained from a genomic library in order to identify biological contaminants, systems resistance compounds and the discovery of new enzymes and biopolymers [80,81].

CONCLUSIONS

The application of composting technology as a remediation strategy for contaminated soils requires an understanding of the microbes involved in pollutant biodegradation and biotransformation. The knowledge about the effect of more types of pollutant-degrading microorganisms as amendments during composting of contaminated soil is still important despite the availability of various microorganisms and their microbiological properties to provide useful information on bioremediation of pesticide polluted soils by composting. It is essential to understand the possible roles of soil enzymes in order to maintain soil health and its fertility management in ecosystems.

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