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Biodegradation of Pesticides

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1. Introduction

The rapidly growing industrialization along with an increasing population has resulted in the accumulation of a wide variety of chemicals. Thus, the frequency and widespread use of man-made "xenobiotic" chemicals has led to a remarkable effort to implement new technologies to reduce or eliminate these contaminants from the environment. Commonly-used pollution treatment methods (e.g. land-filling, recycling, pyrolysis and incineration) for the remediation of contaminated sites have also had adverse effects on the environment, which can lead to the formation of toxic intermediates (Debarati et al., 2005). Furthermore, these methods are more expensive and sometimes difficult to execute, especially in extensive agricultural areas, as for instance pesticides (Jain et al., 2005). One promising treatment method is to exploit the ability of microorganisms to remove pollutants from contaminated sites, an alternative treatment strategy that is effective, minimally hazardous, economical, versatile and environment-friendly, is the process known as bioremediation (Finley et al., 2010).

Thereafter, it was discovered that microbes have the ability to transform and/or degrade xenobiotics, scientists have been exploring the microbial diversity, particularly of contaminated areas in search of organisms that can degrade a wide range of pollutants.

Hence, biotransformation of organic contaminants in the natural environment has been extensively studied to understand microbial ecology, physiology and evolution due to their bioremediation potential (Mishra et al., 2001). The biochemical and genetic basis of microbial degradation has received considerable attention. Several genes/enzymes, which provide microorganisms with the ability to degrade organopesticides, have been identified and characterized.

Thus, microorganisms provide a potential wealth in biodegradation. The ability of these organisms to reduce the concentration of xenobiotics is directly linked to their long-term adaptation to environments where these compounds exist. Moreover, genetic engineering may be used to enhance the performance of such microorganisms that have the preferred properties, essential for biodegradation (Schroll et al., 2004).

About 30% of agricultural produce is lost due to pests. Hence, the use of pesticides has become indispensable in agriculture.

The abusive use of pesticides for pest control has been widely used in agriculture. However, the indiscriminate use of pesticides has inflicted serious harm and problems to humans as

well as to the biodiversity (Gavrilescu, 2005; Hussain et al., 2009). The problem of environmental contamination by pesticides goes beyond the locality where it is used. The agricultural pesticides that are exhaustively applied to the land surface travel long distances and can move downward until reaching the water table at detectable concentrations, reaching aquatic environments at significantly longer distances. Therefore, the fate of pesticides is often uncertain; they can contaminate other areas that are distant from where they were originally used. Thus, decontaminating pesticide-polluted areas is a very complex task (Gavrilescu, 2005).

Organochloride pesticides are synthetic and were widely used in the 1970s, mainly in the United States (<http://www.epa.gov/history/topics/ddt/02.htm>, accessed in May 2011). Although their use has been banished in many countries, they are still used in developing countries. Organochloride pesticides are cumulative in the organisms and pose chronic health effects, such as cancer and neurological and teratogenic effects (Vaccari et al, 2006). Many xenobiotic compounds are recalcitrant and resistant to biodegradation, especially the organochloride pesticides (Diaz, 2004; Dua et al., 2002; Chaudhry & Chapalamadugu, 1991). In general, these highly toxic and carcinogenic compounds persist in the environment for many years.

Organophosphorus pesticides are actually more widely used in the United States (http://www.chemicalbodyburden.org/cs_organophos.htm, accessed in May 2011). These pesticides affect the nervous system of insects and humans, in addition to influencing the reproductive system (Colosio et al., 2009; Jokanovic & Prostran, 2009). These chemical agents block the prolonged inhibition of the cholinesterase enzyme activity. These chemical agents block prolonged inhibition the activity of the enzyme cholinesterase (ChE), responsible for the nervous impulse in organisms (Yair et al., 2008). The excessive use of organophosphorus in agriculture has originated serious problems in the environment (Singh & Walker, 2006). Although, these pesticides degrade quickly in water, there is always the possibility that residues and byproducts will remain, in relatively harmful levels in the organisms (Silva et al., 1999; Ragnarsdottir, 2000).

Carbamate pesticides are important in the agriculture due to their broad activity spectrum. In addition to a wide range of compounds, they are relatively degraded and generally have a low degree of toxicity to humans (Wolfe et al., 1978). However, they inhibit the enzyme acetylcholinesterase, therefore they are considered toxic to humans. The inhibition of the hydrolysis reaction of acetylcholine (ACh) results in the accumulation of ACh, causing various symptoms, such as sweating, lacrimation, hypersalivation and convulsion of extremities (Suzuki & Watanabe, 2005).

Decontamination of pesticide-infested environments is a difficult matter and can be very costly. In fact, the damages from pesticides in the environment are practically irreparable. Any measure used to decrease the effects of pesticides in the environment will always be a palliative solution and never definitive for the problems caused. Regrettably, there is always irreparable damage to the organisms and the environment, as for instance, the extinction of bird species and microorganisms in the world.

The biological methods are advantageous to decontaminate areas that have been polluted by pesticides. These methods consider the thousands of microorganisms in the environment that in order to survive seek for alternatives to eliminate the pesticides that were sprayed. Many native microorganisms develop complex and effective metabolic pathways that permit the biodegradation of toxic substances that are released into the environment. Although the

metabolic process is lengthy, it is a more viable alternative for removing the sources of xenobiotic compounds and pollution (Diaz, 2004; Schoefs et al., 2004; Finley et al., 2010).

On account of the grave risks synthetic pesticides pose to the organisms, there is an incessant search for pesticide safety and for the development of sustainable agriculture. The biological pesticides are based on natural compounds that effectively control the infestation of pests in agriculture. The advantage is that, contrary to synthetic pesticides, they are efficient and do not cause collateral damage (Fravel, 2005; Gerhardson, 2002; Raaijmakers et al., 2002).

The scope of this work demonstrates the use of the degradation of pesticides using microorganisms. This topic is inexhaustible and we are going to underscore the most recent points, including studies on the biodegradation of organochloride, organophosphorus and carbamate pesticides by microbiological process. Afterwards, in perspective, this chapter will show the use of natural pesticides in the biological control of pests.

2. Biodegradation

According to the definition by the International Union of Pure and Applied Chemistry, the term biodegradation is "Breakdown of a substance catalyzed by enzymes *in vitro* or *in vivo*. This may be characterized for the purpose of hazard assessment such as:

1. Primary. Alteration of the chemical structure of a substance resulting in loss of a specific property of that substance.
2. Environmentally acceptable. Biodegradation to such an extent as to remove undesirable properties of the compound. This often corresponds to primary biodegradation but it depends on the circumstances under which the products are discharged into the environment.
3. Ultimate. Complete breakdown of a compound to either fully oxidized or reduced simple molecules (such as carbon dioxide/methane, nitrate/ammonium and water). It should be noted that the biodegradation products can be more harmful than the substance degraded." (<http://sis.nlm.nih.gov/enviro/glossaryb.html>, accessed in May 2011; <http://www.epa.gov/OCEPATERMS/bterms.html>, accessed in May 2011).

Microbial degradation of chemical compounds in the environment is an important route for the removal of these compounds. The biodegradation of these compounds, i.e., pesticides, is often complex and involves a series of biochemical reactions. Although many enzymes efficiently catalyze the biodegradation of pesticides, the full understanding of the biodegradation pathway often requires new investigations. Several pesticide biodegradation studies have shown only the total of degraded pesticide, but have not investigated in depth the new biotransformed products and their fate in the environment.

2.1 Organochlorine pesticides

2.1.1 Introduction

The organochlorine pesticides are known to be highly persistent in the environment. This class of pesticides includes the chlorinated derivatives of diphenyl ethane (dichlorodiphenyltrichloroethane - DDT, its metabolites dichlorodiphenyldichloroethylene - DDE, dichlorodiphenyldichloroethane - DDD, and methoxychlor), hexachlorobenzene (HCB), the group of hexachlorocyclohexane (α -HCH, β -HCH, γ -HCH, δ -HCH, or lindane), the group of cyclodiene (aldrin, dieldrin, endrin, chlordane, nonachlor, heptachlor and

heptachlor-epoxide), and chlorinated hydrocarbons (dodecachlorine, toxaphene, and chlordecone), (Menone et al., 2001; Patnaik, 2003). Figure 1 shows some structures of organochlorine pesticides.

Unlike the organophosphate and the carbamate pesticides, the toxic properties of the organochlorine pesticides are not very similar (Matolcsy et al., 1988). Although the toxicological properties are analogous to organochlorines with similar structures, like heptachlor and chlordane, the toxicological degree can vary by substituting a chlorine in the molecule. For instance, the substitution of chlorine atoms in the DDT ring for a methoxide group decreases the toxicity (Patnaik, 2003).

DDT is the most well known pesticide from the organochlorine group. The use of organochlorine pesticides started in 1939, when Paul Hermann Müller realized that the DDT, first synthesized by Othmar Zeidler in 1874, was an efficient insecticide (Matolcsy et al., 1988). The DDT's high efficiency, its low water solubility, its high persistence in the environment and its mode of action, unknown until that moment, contributed to the increasing use of DDT (Konradsen et al., 2004).

The industrial manufacture of DDT is based on the synthesis described by Zeidler. Chloral, chloral alcoholate or chloral hydrate is reacted with chloro-benzene in the presence of sulfuric acid, oleum or chlorosulfonic acid. The products obtained from the synthesis reaction contain several impurities, including the *ortho-para* [1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane] and *ortho-ortho* [1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*o*-chlorophenyl)ethane] isomers of DDT, and 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane (*p,p'*-DDD), its *ortho-para* isomer, 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o,p'*-DDD). The 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (*p,p'*-DDT), is about 70% of the product mixtures (Matolcsy et al., 1988).

The *p,p'*-DDT is resistant to light, atmospheric oxygen and weak inorganic acids, but is rapidly decomposed to the biologically inactive *p,p'*-DDE (Matolcsy et al., 1988; Ahrens & Weber, 2009). During the World War II, powder DDT was pulverized on the population's skin to prevent epidemics of typhus transmitted by lice. The insecticide was also used in other countries to control the malaria-bearing mosquitoes (Konradsen et al., 2004). The use of DDT to control malaria bearing mosquitoes earned Müller the 1948 Nobel Prize in Medicine. After the war, the use of DDT was adopted as an agricultural pesticides (Benn & McAuliffe, 1975; Ottaway, 1982; Mariconi, 1985), the results were so impressive, that its use continued for 25 to 30 years in most countries. The problem occurred when DDT, like most organochlorine, reduced its efficiency, forcing the use of higher dosages. Consequently, large specialized laboratories sought to develop formulas which were characterized by greater efficiency and biodegradability (Turk, 1989).

At the end of the 1950s, the biologist Rachel Carson began to gather examples of environmental damages attributed to DDT (D'Amato et al., 2002). Between 1970 and 1980, DDT agricultural use was banned in most developed countries. In 2004, the Stockholm Convention, outlawed several persistent organic pollutants, such as aldrin, dieldrin, endrin, toxaphene, mirex and heptachlor, and restricted DDT use to vector control (Ahrens & Weber, 2009; Arisoy & Kolankaya, 1998). In 2009, lindane and chlordecone were added to the outlawed list by the Fourth Conference of the Parties (Ahrens & Weber, 2009).

Although most organochlorine were banned from some countries, organochlorine pesticides are still widely studied due to their recalcitrant nature, that is, even after years since the use has been banned, organochlorine contaminated sites are not rare. Not to mention, that the DDT use is still allowed to control malaria bearing mosquitoes, even though, narrowly.

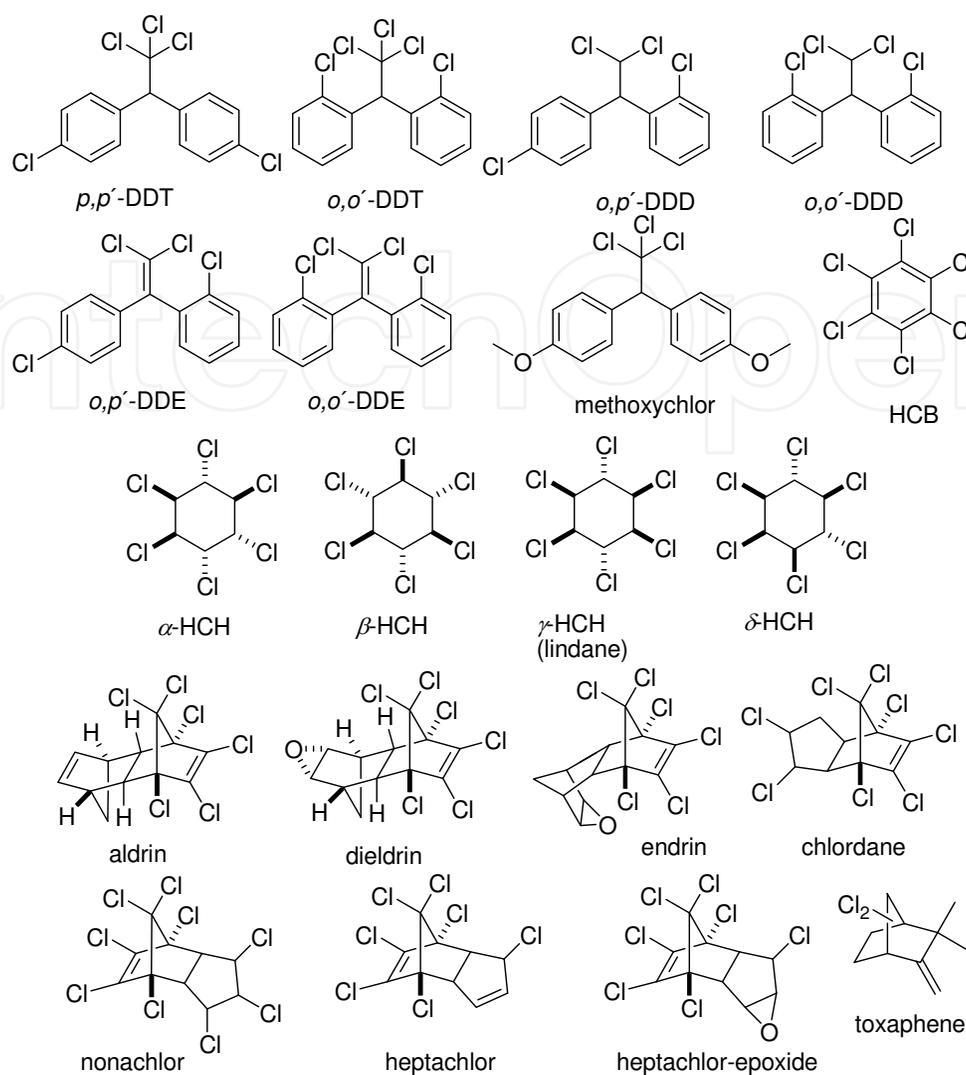


Fig. 1. Structures of organochlorine pesticides

2.1.2 Microbial degradation of organochloride pesticides

The fate of pesticides in the environment is determined by both biotic and abiotic factors. The rate at which different pesticides are biodegraded varies widely. Some pesticides such as DDT and dieldrin have proven to be recalcitrant. Consequently, they remain in the environment for a long time and accumulate into food chains for decades after their application to the soil (Kannan et al., 1994).

Most of the studies involving the biodegradation of organochlorine pesticides are done in pure cultures. The culture is usually isolated from a soil sample, generally contaminated with organochlorine pesticides. The strains are characterized and tested with different concentrations of the pesticide studied. DDT-metabolising microbes have been isolated from a range of habitats, including animal feces, soil, sewage, activated sludge, and marine and freshwater sediments (Johnsen, 1976; Lal & Saxena, 1982; Rochkind-Dubinsky et al., 1987).

The degradation of organochlorine pesticides by pure cultures has been proven to occur *in situ*. Nature magazine published one of the pioneer works. Matsumura et al. (1968) were able to evidence the breakdown of dieldrin in the soil by a *Pseudomonas* sp. The bacteria strain was isolated from a soil sample from the dieldrin factory yards of Shell Chemical

Company near Denver, Colorado. Later, in 1970, authors showed the biodegradation of aldrin, endrin and DDT with bacteria that were shown to be able to degrade dieldrin (Patil et al., 1970).

Biodegradation of DDT residues largely involves co-metabolism, that is, it requires the presence of an alternative carbon source, in which microorganisms growing at the expense of a substrate are able to transform DDT residues without deriving any nutrient or energy for growth from the process (Bollag & Liu, 1990).

Under reducing conditions, reductive dechlorination is the major mechanism for the microbial conversion of both the *o,p'*-DDT and *p,p'*-DDT isomers of DDT to DDD (Fries et al., 1969). The reaction involves the substitution of an aliphatic chlorine for a hydrogen atom. Using metabolic inhibitors together with changes in pH and temperature, Wedemeyer (1967) found that discrete enzymes were involved in the metabolism of DDT by *Aerobacter aerogenes*. The suggested pathway for the anaerobic transformation of DDT by bacteria is shown in Figure 2. Degradation proceeds by successive reductive dechlorination reactions of DDT to yield 2,2-bis(*p*-chlorophenyl)ethylene (DDNU), which is then oxidised to 2,2-bis(*p*-chlorophenyl)ethanol (DDOH). Further oxidation of DDOH yields bis(*p*-chlorophenyl)acetic acid (DDA) which is decarboxylated to bis(*p*-chlorophenyl)methane (DDM). DDM is metabolized to 4,4'-dichlorobenzophenone (DBP) or, alternatively, may undergo cleavage of one of the aromatic rings to form *p*-chlorophenylacetic acid (PCPA). Under anaerobic conditions DBP was not further metabolized (Pfaender & Alexander, 1972). Through an investigation of the co-metabolism of DDT metabolites by a number of fungi (Subba-Rao & Alexander, 1985) were able to substantiate the pathway proposed by Wedemeyer (1967). There has been one report describing the conversion of DDE to 1-chloro-2,2-bis(*p*-chlorophenyl)ethylene - DDMU by bacteria (Masse et al., 1989).

Some studies have presented notable results on the biodegradation of organochlorine pesticides. Table 1 presents some of the microorganisms that were able to degrade organochlorine pesticides. Among microorganisms, bacteria comprise the major group involved in organochlorine degradation, especially soil inhabitants belonging to genera *Bacillus*, *Pseudomonas*, *Arthrobacter* and *Micrococcus* (Langlois et al., 1970). In order to predict some of the factors that influence the capacity of biodegradation of DDT by a *Sphingobacterium* sp., Fang et al. (2010), studied the biodegradation at different temperatures, pHs, concentrations of DDT and, with an additional source of carbon. Results of the experience showed that the degradation rates were proportional to the concentrations of *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE ranging from 1 to 50 mg.L⁻¹. The ability of *Sphingobacterium* sp. to degrade DDTs was somewhat inhibited by DDTs at the level as high as 50 mg.L⁻¹. According to the authors, this may be due to the fact that DDTs at high concentration are toxic to *Sphingobacterium* sp. and inhibit degradation. The experiment was also tested for different pHs, it was tested for pH 5, 7 and 9. The results indicated that a neutral condition is favorable for the degradation of DDT by *Sphingobacterium* sp., whereas higher or lower pH inhibits degradation. The influence of the temperature on the biodegradation was investigated by performing the experiments at temperatures of 20, 30 and 40 °C. The results indicated that the optimum temperature for the biodegradation of DDTs by a *Sphingobacterium* sp. in pure culture was at 30 °C. Ultimately, the biodegradation was available with an additional carbon source and results showed that the degradation half-lives of DDTs in the presence of glucose, yeast extract, sucrose, and fructose were significantly shorter than those in the treatment without an additional carbon source; and that the presence of glucose generates the fastest degradation of DDTs (Fang et al., 2010).

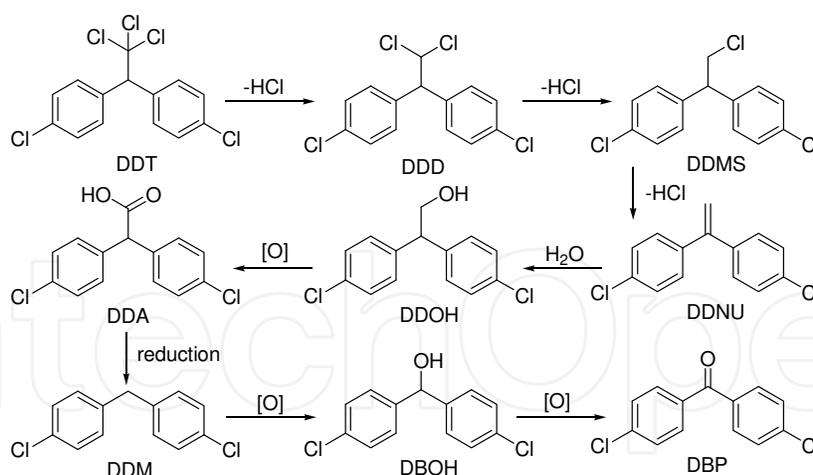


Fig. 2. Proposed pathway for bacterial metabolism of DDT (Adapted from Aislable et al., 1997).

Results obtained *in vitro* can be applied to contaminated sites for further investigation on the capacity of a microorganism to degrade an organochlorine pesticide. As a continuation of the study proposed by Fang et al. (2010), the bacteria that was evidenced to degrade DDT was applied to field soils after different treatments. The soil known to be contaminated with DDT was studied in four different conditions, the control, which did not receive any treatment; PV, the same soil, only with pumpkin vegetation; DI received inoculation of the *Sphingobacterium* sp. and; PVDI which was the contaminated soil with the pumpkin vegetation and inoculation with *Sphingobacterium* sp. The concentration of *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE was measured from each soil sample after 90 days, and was then compared to the initial concentration. Analysis indicated that the removal percentages of *o,p'*-DDT and *p,p'*-DDE in the PVDI treatment were statistically significantly higher (Fang et al., 2010).

According to Aislable & Jones (1995) and Aislable et al. (1997), the microbial degradation of DDT in soil apparently proceeds by a pathway analogous to that proposed by Wedemeyer (1967), (Figure 2). Under anaerobic conditions the first and major biotransformation product of DDT is DDD, with minor levels of DDA, DDM, DDOH, DBP, and DDE being detected (Guenzi & Beard, 1967; Mitra & Raghu, 1988; Xu et al., 1994; Boul et al., 1994). Reports of biodegradation of DDE in soil are rare, although, Agarwal et al. (1994) described the isolation of DDMU as a biotransformed product of DDE.

Studies with fungi have also evidenced the biodegradation of organochlorine pesticides. Ortega et al. (2011) evaluated marine fungi collected off the coast of São Sebastião, North of São Paulo State, Brazil. The fungi strains were obtained from marine sponges. The fungi *Penicillium miczynskii*, *Aspergillus sydowii*, *Trichoderma* sp., *Penicillium raistrickii*, *Aspergillus sydowii* and *Bionectria* sp. were previously tested in solid culture medium containing 5, 10 and 15 mg of DDD. The tests were also carried out with liquid medium in a rotary shaker, with the same amount of DDD per 100 mL liquid medium. The results showed that the fungi *P. miczynskii*, *A. sydowii* and *Trichoderma* sp. presented good growth in the presence of the pesticide. For further experiments *Trichoderma* sp. was selected as the standard microorganism, as it showed the best resistance to DDD in both solid and liquid medium (Ortega et al., 2011). In the experiments where DDD pesticide was concomitantly added into the growth of *Trichoderma* sp., 21% of the pesticide was degraded. The addition of H₂O₂ in the experiment promoted a degradation increase (75%). In the experiments where DDD was added after 5 days of *Trichoderma* sp. growth, and with the addition of H₂O₂, the total biodegradation occurred (Ortega et al., 2011). Many factors can affect the biodegradation, as described earlier, as for instance the presence of H₂O₂ increases the efficiency of the DDD degradation by *Trichoderma* sp.

| Pesticides | Toxicity ¹ | Microorganisms | References |
|---------------------|-----------------------|--|--|
| PCP | Class Ib | <i>Arthrobacter</i> sp. <i>Flavobacterium</i> sp. | (Stanlake & Finn , 1982) (Crawford & Mohn, 1985) |
| 1,4-Dichlorobenzene | Class II | <i>Pseudomonas</i> sp. | (Spain & Nishino, 1987) |
| DDT | Class II | <i>Aerobacter aerogenes</i> <i>Trichoderma viridae</i> <i>Pseudomonas</i> sp. <i>Micrococcus</i> sp. <i>Arthrobacter</i> sp. <i>Bacillus</i> sp. <i>Pseudomonas</i> sp. <i>Sphingobacterium</i> sp. | (Wedemeyer, 1966) (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) (Kamanavalli & Ninnekar, 2005) (Fang et al., 2010) |
| Lindane | Class II | <i>Basa thiooxidans</i> <i>Sphingomonas paucimobilis</i> <i>Streptomyces</i> sp. <i>Pleurotus ostreatus</i> <i>Phanerochaete chrysosporium</i> | (Pesce & Wunderlin, 2004) (Pesce & Wunderlin, 2004) (Benimeli et al., 2008) (Rigas et al., 2005) (Bumpus et al., 1993) |
| DDE | n.i. | <i>Trichoderma</i> sp. | (Ortega et al., 2011) |
| DDD | n.i. | <i>Phlebia</i> sp. | (Xiao et al., 2011) |
| Heptachlor epoxide | n.i. | <i>Phanerochaete chrysosporium</i> <i>Phlebia</i> sp. | (Arisoy & Kolankaya, 1998) (Xiao et al., 2011) |
| Heptachlor | O | <i>Bjerkandera</i> sp. <i>Trichoderma viridae</i> | (Lacayo et al., 2006) (Patil et al., 1970) |
| Toxaphene | O | <i>Pseudomonas</i> sp. <i>Micrococcus</i> sp. <i>Bacillus</i> sp. <i>Trichoderma viridae</i> | (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) |
| Aldrin | O | <i>Pseudomonas</i> sp. <i>Micrococcus</i> sp. <i>Bacillus</i> sp. <i>Trichoderma viridae</i> | (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) |
| Endrin | O | <i>Pseudomonas</i> sp. <i>Micrococcus</i> sp. <i>Arthrobacter</i> sp. <i>Bacillus</i> sp. | (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) |
| Dieldrin | O | <i>Pseudomonas</i> sp. | (Matsumura et al., 1968) |

Table 1. Microorganisms involved in degradation of organochlorine pesticides.

¹WHO Recommended classification of pesticides by Hazard. Class I is subdivided into two other classifications: Class Ia (extremely hazardous) and Class Ib (highly hazardous), Class II are the moderately hazardous, Class III are slightly hazardous and Class u, are unlikely to present acute hazard. The use of some pesticides have been discontinued and are classified as obsolete (O), (WHO, 2009).

n.i. – not informed

Studies involving the biodegradation of polychlorinated biphenyls (PCBs), used as a pesticide extender, have also been conducted, several isolated microorganisms have been proven to be capable of aerobically degrade PCBs, preferentially degrading the more lightly chlorinated congeners. These organisms attack PCBs via 2,3-dioxygenase pathway, converting PCBs to the corresponding chlorobenzoic acids. These chlorobenzoic acids can then be degraded by indigenous bacteria, resulting in the production of carbon dioxide, water, chloride, and biomass (Abramowicz, 1995). Anaerobic bacteria attack more highly chlorinated PCB congeners through reductive dechlorination. In general, this microbial process removes preferentially the *meta* and *para* chlorines, resulting in a depletion of highly chlorinated PCB congeners with corresponding increases in lower chlorinated, *ortho*-substituted PCB congeners (Abramowicz, 1995).

Despite the evidence that microorganisms with the ability to degrade DDT are resident in soil, its residues persist. The studies here presented showed that anaerobic conditions are beneficial to dechlorination of DDT, and additional carbon and hydrogen peroxide favors the biodegradation of some organochlorines. The decomposition rate depends on conditions in the soil and the bonding of the pesticide to soil surfaces. For most pesticides, aerobic decomposition proceeds much faster than anaerobic decomposition; however, there are classic exceptions to this, for instance DDT, whose decomposition proceeds ten times faster under anaerobic conditions (Scott, 2000).

Farm management practices also affect the rate at which pesticides are degraded. Irrigation of soils has been shown to enhance degradation of DDT to DDD, thought to be due to the creation of anaerobic microsites (Aislabie & Jones, 1995).

Due to the recalcitrant nature of most organochlorines, many such pesticides are still widely studied in order to find mechanisms that enhance their biodegradation in the environment. Genetic techniques can contribute to elucidate biochemical pathways involved in the microbial degradation of organochlorines, which represent promising alternatives towards developing highly efficient strains as well as the isolation and application of enzymes potentially involved in biodegradation.

2.2 Organophosphate pesticides

2.2.1 Introduction

Currently, among the various groups of pesticides that are used worldwide, organophosphorus pesticides form the major and most widely used group that accounts for more than 36% of the total world market. The most used among these is methyl parathion. Its accumulation has many health hazards associated to it, hence, its degradation is very important (Ghosh et al., 2010).

The organophosphorus pesticides (OP) are all esters of phosphoric acid and are also called organophosphates, which include aliphatic, phenyl and heterocyclic derivatives (Figure 3). Owing to large-scale use of OP compounds, contaminations of soil and water systems have been reported from all parts of the world. In light of this, bioremediation provides a suitable way to remove contaminants from the environment as, in most cases, OP compounds are totally mineralized by the microorganisms. Most OP compounds are degraded by microorganisms in the environment as a source of phosphorus and /or carbon. Classification of Pesticides. Thus, the OP pesticides can be hydrolyzed and detoxified by carboxylesterase and phosphotriesterase enzymes.

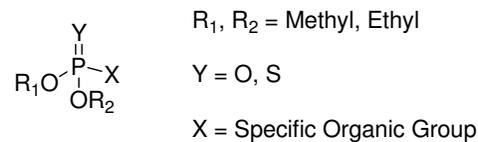


Fig. 3. General structure of organophosphate pesticides

Organophosphates are used to control a variety of sucking, chewing and boring insects, spider mites, aphids, and pests that attack crops like cotton, sugarcane, peanuts, tobacco, vegetables, fruits and ornamentals. OP pesticides are marketed by many of the world's major agrochemical companies. Some of the main agricultural products are parathion, methyl parathion, chlorpyrifos, malathion, monochrotophos, diazinon, fenitrothion and dimethoate (Figure 4).

The organophosphorates possess an efficient insecticide activity, due to its characteristic of irreversibly inhibiting the enzyme acetylcholinesterase in the nervous system, which acts in both insects and in mammal. In man, the organophosphates are absorbed through all routes, reaching high concentrations in fatty tissues, liver, kidneys, salivary glands, thyroid, pancreas, lungs, stomach, intestines and, at smaller proportions, in the central nervous system (SNC) and muscles. However, the organophosphates do not accumulate in the human organism, as it is readily biotransformed in the liver. The excretion of these compounds and of their metabolites is quite fast, taking place mostly in the urine and, at small proportions, in the feces, usually within 48 h. The largest excretion levels occur within 24 h after absorption (Oga, 2003; Griza et al., 2008).

Due to the above mentioned health hazards and other problems associated with the use organophosphorus pesticides, early detection and subsequent decontamination and detoxification of the polluted environment is essential. The present subject examines applications and future use of OP-degrading microorganism cultures from agricultural fields and enzymes for bioremediation (Karpouzas & Singh, 2006).

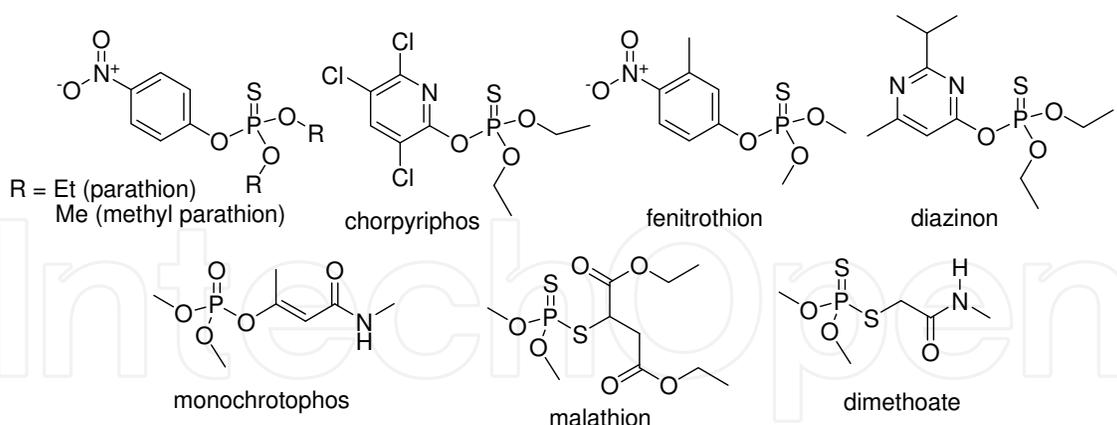


Fig. 4. Structures of organophosphate pesticides

2.2.2 Microbial degradation of organophosphate pesticides

Methyl parathion (*O,O*-dimethyl-*O*-(*p*-nitro-phenylphosphorothioate) is one of the most used organophosphorus pesticides. This product is widely used throughout the world and its residues are regularly detected in a range of fruits and vegetables. Investigation of microbial degradation is useful for developing insecticide degradation strategies using microorganisms. Bacteria with the ability to degrade methyl parathion have been isolated worldwide (Liu et al., 2003; Hong et al., 2005).

Multiplex tendencies characterize pesticide applications in farming. A number of pesticide mixtures, especially pyrethroid and organophosphorus pesticide mixtures have been formulated as an improvement over individual pesticides (Moreby et al., 2001). Construction of a genetically engineered microorganism (GEM), which can simultaneously degrade these two kinds of pesticides, could benefit the study and application of bioremediation in multiple pesticide-contaminated environments (Yuanfan et al., 2010). Methyl parathion hydrolase gene, *mpd*, which is responsible for hydrolyzing methyl parathion to *p*-nitrophenol and dimethyl phosphorothioate, has also been cloned from these strains. Sequences are effectively conserved in these strains (Yuanfan et al., 2010).

A fenpropathrin-degrading bacterium, *Sphingobium* sp. JQL4-5, was isolated and characterized. A stable, genetically engineered strain, JQL4-5-*mpd*, capable of simultaneously degrading fenpropathrin and methyl parathion was constructed by random insertion of the methyl parathion hydrolase gene (*mpd*) into the chromosome of strain JQL4-5. Soil treatment results indicated that JQL4-5-*mpd* is a promising GEM in the bioremediation of multiple pesticide contaminated environments (Yuanfan et al., 2010).

Organophosphorus hydrolase (OPH), isolated from both *Flavobacterium* sp. ATCC 27551 (Mulbry & Karns, 1989) and *Pseudomonas diminuta* MG (Serdar et al., 1989), is capable of hydrolyzing a wide range of oxon and thion OPs. However, OPH has already been shown to lack any hydrolytic activity toward numerous dimethyl OPs (Horne et al., 2002). The *mpd* gene encoding an organophosphate degrading protein was isolated from a methyl parathion (MP) degrading *Plesiomonas* sp.

The methyl parathion hydrolase gene (*mpd*) and enhanced green fluorescent protein gene (*egfp*) was successfully coexpressed using pETDuet vector in *Escherichia coli* BL21 (DE3). The coexpression of methyl parathion hydrolase (MPH) and enhanced green fluorescent protein (EGFP) were confirmed by determining MPH activity and fluorescence intensity. The recombinant protein MPH showed high enzymatic degradative activity of several widely used OP residues on vegetables. Subsequently, a dual-species consortium comprising engineered *E. coli* and a natural *p*-nitrophenol (PNP) degrader *Ochrobactrum* sp. strain LL-1 for complete mineralization of dimethyl OPs was studied. The dual-species consortium possesses the enormous potential to be utilized for complete mineralization of PNP-substituted OPs in a laboratory-scale bioreactor. These studies demonstrated that MP could be degraded via the MP → PNP → hydroquinone → Krebs cycle (Figure 5) by the dual-species consortium. The data confirm that the mineralization process of MP is initiated by hydrolysis leading to the generation of PNP and dimethylthiophosphoric acid, and PNP degradation, then, proceeds through the formation of hydroquinone. The accumulation of PNP in suspended culture was prevented (Zhang et al., 2008).

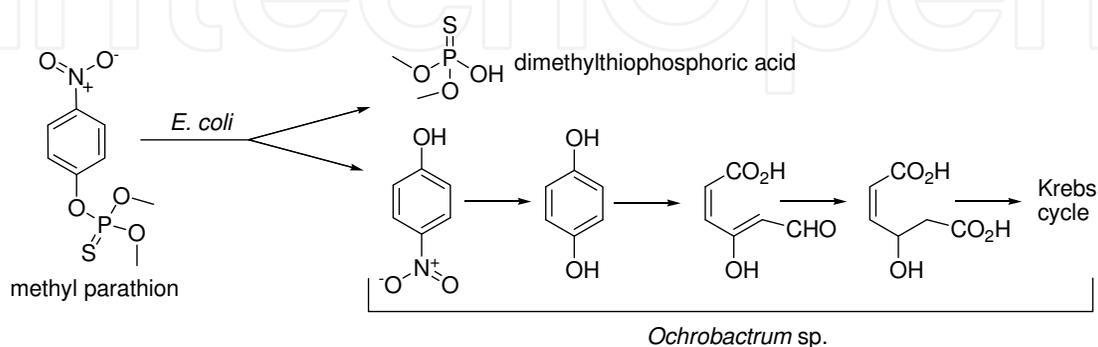


Fig. 5. Proposed pathway for the biodegradation of MP by microbial consortium (Zhang et al., 2008)

Thus, there is an increasing need to develop new methods to detect, isolate, and characterize the strains/enzymes playing a part in these degradation processes (Vallaey et al., 1996). Successful detoxification of recalcitrant organic chemicals may require the concerted effort of multispecies consortia.

Fenamiphos (FEN), ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate, is an organophosphate nematocidal used in protected horticultural crops. In soil, FEN is gradually oxidized to its sulfoxide (FSO) and sulfone (FSO₂), which also possess high nematocidal activity and is equally toxic to non-target vertebrates (Figure 6). Degradation studies of FEN in a range of soils showed half-life values ranging from 12 to 87 days. FEN and its oxidation products FSO and FSO₂ showed low to moderate affinity for soil adsorption and their soil accumulation may result in their eventual downward movement into groundwater. Indeed, previous studies have suggested that under favorable environmental conditions FEN could leach to groundwater where it could persist (Franzmann et al., 2000). Therefore, tools are needed for the decontamination of natural resources by the residues of chemicals such as FEN and its oxidation derivatives.

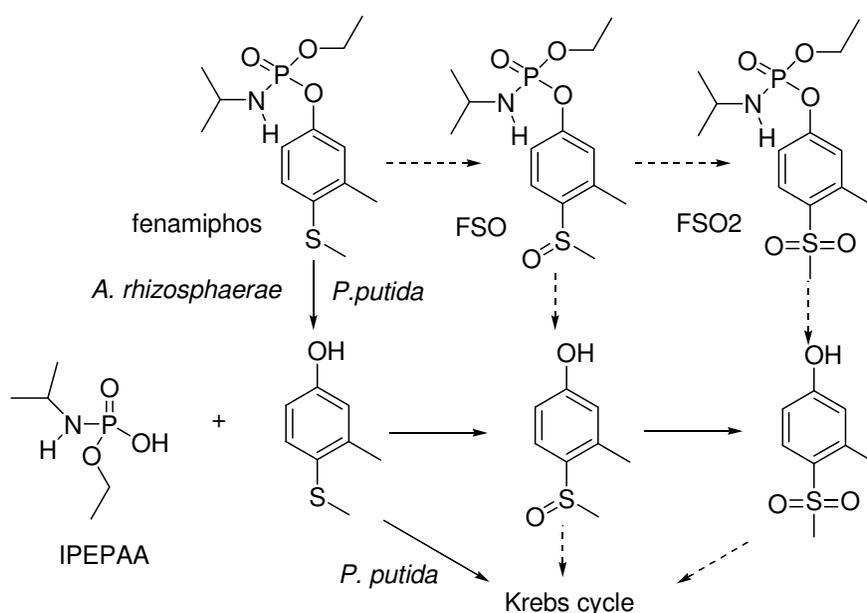


Fig. 6. Metabolic pathway of FEN by the isolated bacteria (Chanika et al., 2011)

Two bacteria identified as *Pseudomonas putida* and *Acinetobacter rhizosphaerae*, able to rapidly degrade the organophosphate fenamiphos, were isolated. Denaturing gradient gel electrophoresis analysis revealed that the two isolates were dominant members of the enrichment culture. Clone libraries further showed that bacteria belonging to α -, β -, γ -Proteobacteria and Bacteroidetes were also present in the final enrichment, but were not isolated. Both strains hydrolyzed FEN to fenamiphos phenol and ethyl hydrogen isopropylphosphoramidate (IPEPAA), which was further transformed, only by *P. putida*. The two strains were using FEN as C and N source. Cross-feeding studies with other pesticides showed that *P. putida* degraded OPs with a P-O-C linkage (Chanika et al., 2011). Thus, both bacteria were able to hydrolyze FEN, without prior formation of FSO or FSO₂, to FEN-OH which was further transformed only by *P. putida* (Figure 6), suggesting elimination of environmentally relevant metabolites. In addition, *P. putida* was the first wild-type bacterial isolate able to degrade OPs. All the above characteristics of *P. putida* and its

demonstrated ability to remove aged residues of FEN highlight its high bioremediation potential (Chanika et al., 2011).

Herein, it was shown that the construction of genetically engineered microorganism (GEM) and the dual-species consortium has the potential to be used in the degradations of different kinds of pesticides. These studies show the benefits of bioremediation in multiple pesticide-contaminated environments and mineralization of toxic intermediates in the environment, which can lead to complete bioremediation of contaminated sites that have an adverse effect.

2.3 Carbamate pesticides

2.3.1 Introduction

Carbamates were introduced as pesticides in the early 1950s and are still used extensively in pest control due to their effectiveness and broad spectrum of biological activity (insecticides, fungicides, herbicides). High polarity and solubility in water and thermal instability are typical characteristics of carbamate pesticides, as well as high acute toxicity. The carbamates are transformed into various products in consequence of several processes such as hydrolysis, biodegradation, oxidation, photolysis, biotransformation and metabolic reactions in living organisms (Soriano et al., 2001).

Chemically, the carbamate pesticides are esters of carbamates and organic compounds derived from carbamic acid (Figure 7). This group of pesticides can be divided into benzimidazole-, *N*-methyl-, *N*-phenyl-, and thiocarbamates. The compounds derived from carbamic acid are probably the insecticides with the widest range of biocide activities (Sogorb & Vilanova, 2002).



Fig. 7. General structures of carbamate pesticides

Highly toxic acetylcholinesterase (AChE)-inhibiting pesticides, organophosphates and carbamates are intensively used throughout the world and continue to be responsible for poisoning epidemics in various countries (De Bleecker, 2008). The carbamates are inhibitors of AChE and are responsible for the greatest number of poisonings in the rural environment. The use of pesticides in the Brazilian rural environment has brought a series of dire consequences to the environment as well as to the health of rural workers (Oliveira-Silva et al., 2001). The clinical effects of carbamate pesticides depend on the dose, route of exposure, type of carbamate involved, use of protective gear, and the premorbid state of the victim (Rosman et al., 2009).

The study of pesticide degradation is usually beneficial, since the reactions that destroy pesticides convert most pesticide residues in the environment to inactive, less toxic, harmless compounds (Lan et al., 2006).

The enzymatic hydrolysis of carboxyl esters by carboxyl esterases (CbEs) is based on the reversible acylation of a serine residue within the active centre of the protein (Gupta, 2006). Firstly, the substrate must gain access to the active site and this acylation causes a nucleophilic attack by the serine on the carboxyl carbamate producing a transition state

formation, in addition to forming a stable acylated enzyme (Figure 8). This acyl-enzyme intermediate is hydrolysed by nucleophilic attack of water that releases the corresponding carbamate acid, plus the free active enzyme again ready to initiate a new catalytic cycle (Reed & Fukuto, 1973; Sogorb & Vilanova, 2002; Hemmert & Redinbo, 2010).

Moreover, the investigation of biodegradation pathways are quite complex. In addition to the complexity of the (bio)degradation of the pesticides there are also other factors, such as pesticide nonextractable residues in soils. The definition of bound residues was described as “bound residues represent compounds in soils, plants, or animals which persist in the matrix in the form of the parent substance or its metabolite(s) after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix” (Barriuso et al., 2008).

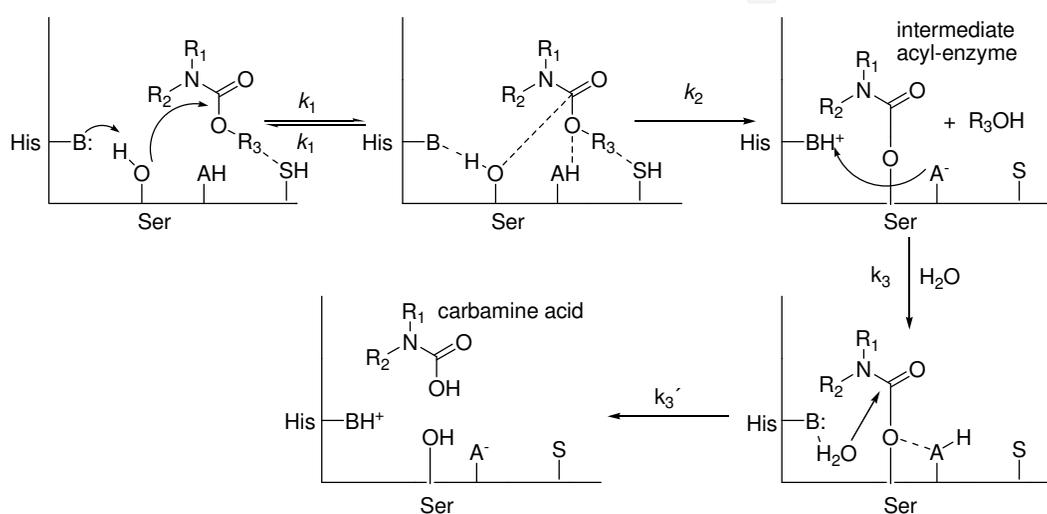


Fig. 8. Catalytic mechanism for carbamate hydrolysis by carboxyl esterases, k_1 defines the affinity of the enzyme for a given substrate and k_2 describes how quickly the acyl-enzyme intermediate is formed (Reed & Fukuto, 1973).

2.3.2 Microbial degradation of carbamate pesticides

The biodegradation of carbamates has been investigated by different microorganisms that metabolize carbamate pesticides. In most cases, the studies did not eliminate the possibility that abiotic processes are involved in the degradation.

A number of bacteria capable of degrading carbofuran (*Pseudomonas*, *Flavobacterium*, *Achromobacterium*, *Sphingomonas*, *Arthrobacter*) have been isolated and characterized in an effort to better understand the bacterial role to remove carbofuran from the environment. Carbofuran is one of the pesticides belonging to the *N*-methylcarbamate class used extensively in agriculture. It exhibits high mammalian toxicity and has been classified as highly hazardous. Carbofuran was degraded first to carbofuran phenol and the result was degraded to 2-hydroxy-3-(3-methylpropan-2-ol) phenol by *Sphingomonas* sp. (Kim et al., 2004) and *Arthrobacter* sp. (De Schrijver & De Mot, 1999), (Figure 9).

Carbendazim is a widely used broad-spectrum benzimidazole fungicide to control a wide range of fungal pathogens on cereals and fruits, it is also used in soil treatment and foliar application on the appearance of disease. The fungicide carbendazim was degraded by a microbial consortium obtained from several soil samples in Japanese paddy fields with continuous culture enrichment. Biodegradation using immobilized bacterial consortium was

investigated in various parameters, as temperature, pH, and nutrient concentration. The degradation ability of the consortium was increased by immobilization on loofa (*Luffa cylindrica*) sponge, in comparison with that of free-living consortium. This immobilized consortium on loofa sponge is a promising material for bioremediation of polluted water with these pesticides in paddy fields (Pattanasupong et al., 2004).

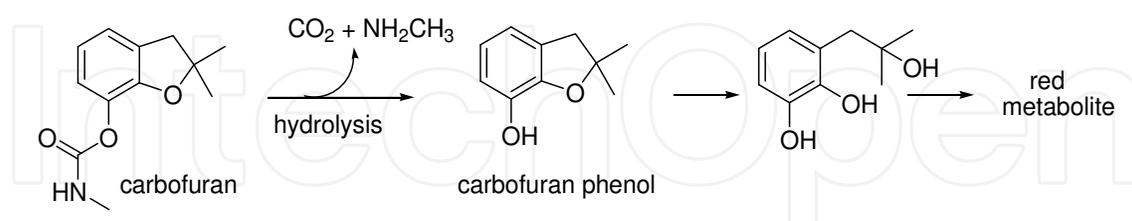


Fig. 9. Biodegradation of carbofuran by *Sphingomonas* sp.

Afterwards, the carbamate carbendazim was converted to 2-aminobenzimidazole by *Pseudomonas* isolates (Figure 10). In general, a limited number of xenobiotic pesticides are metabolized by single strain, but usually consortia of microorganisms are catalyzed for complete degradation. Several Actinomycetes that metabolize carbamate pesticides were isolated. In most cases, this is initiated by hydrolysis of the carbamate at the ester linkage. (De Schrijver & De Mot, 1999).

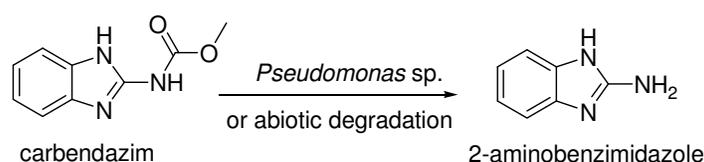


Fig. 10. Biodegradation of carbendazim by *Pseudomonas* sp.

Juvenoids are efficient pesticides with relatively low toxicity to humans (Figure 11). However, few studies have evaluated the effect of degradation by soil microorganisms on their toxicity. The effects of bacterial, fungal and yeast isolates on aerobic decomposition of ethyl *N*-{2-[4-(2,2-ethylenedioxy-1-cyclohexylmethyl)phenoxy]ethyl} carbamate during eight weeks were determined. Higher degradation activity was observed during the first week of the experiment and a substantial decrease in the rate of degradation occurred during the following seven weeks. This can be described both to the accumulation of degradation products and to impaired physiological state of microbial cultures during the long-term experiments (Novák et al., 2003).

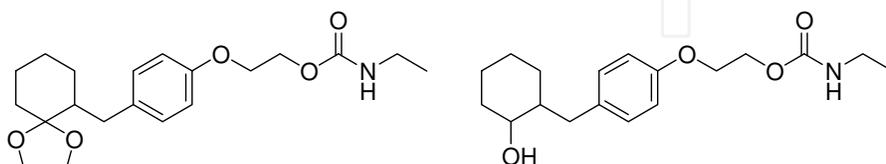


Fig. 11. Juvenoid pesticides

Ethylenethiourea is an important degradation product of ethylenebisdithiocarbamate fungicides (maneb, zineb, mancozeb), which are widely used in different kinds of crops (Figure 12). The ethylenebisdithiocarbamates are not highly toxic and degrade rapidly in the presence of moisture and oxygen, forming different types of compounds such as the polar

ethylenethiourea, which is relatively stable and is a potential contaminant for groundwater. Experiments conducted under biotic and abiotic conditions, showed complete degradation of ethylenethiourea in the presence of microbial nitrate reduction with pyrite, which occurs in deeper parts of the aquifers (Jacobsen & Bossi, 1997).

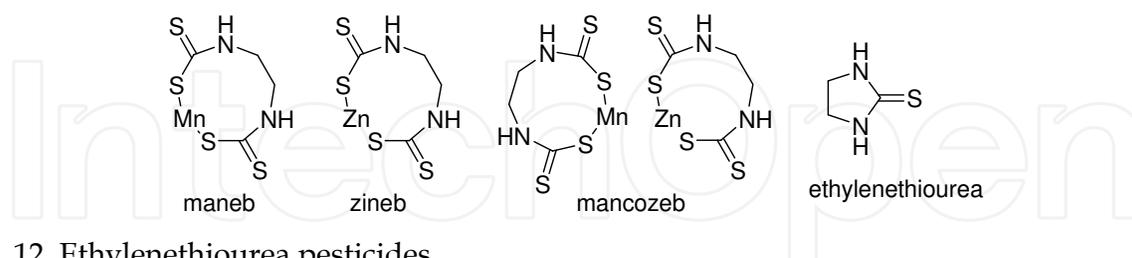


Fig. 12. Ethylenethiourea pesticides

In general, pesticide-degrading microorganisms are isolated via enrichment cultures. A novel strategy has been reported using a coexpression vector for the purpose of developing bacteria that can detoxify different pesticides. The organophosphate hydrolase gene from *Flavobacterium* sp. and carboxylesterase B1 gene (b1) from *Culex pipiens* were cloned in the coexpression vector. A single microorganism was capable of producing both enzymes for degradation of organophosphate (parathion), carbamate (pirimicarb) and pyrethroid pesticides (deltamethrin), (Figure 13). The technical capability of genetically engineering bacteria with more enzymes should open up new opportunities for extending the wide range of pesticides that can be biodegraded in the future (Lan et al., 2006).

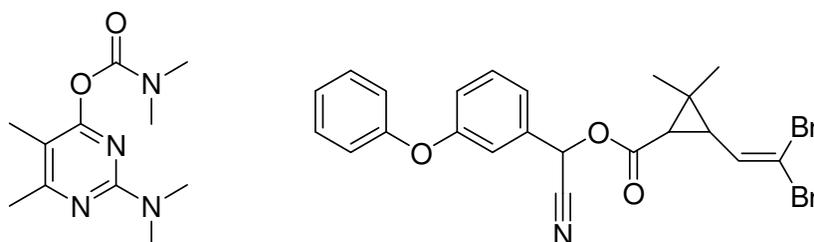


Fig. 13. Pirimicarb (left) and deltamethrin (right) pesticides

Recently, the isolation of a soil bacteria able to hydrolyze organophosphate and carbamate pesticides was performed. Cross-feeding studies with other pesticides showed that *Pseudomonas putida* degraded organophosphates with a P-O-C linkage (fenamiphos), and oxamyl (Figures 6 and 14) and carbofuran carbamates (Chanika et al., 2011). In addition, the biodegradation of insecticidal organophosphates and carbamates has been described by human brain esterases, which actively degraded 1-naphthyl acetate and other substrates (Sakai & Matsumura, 1971).

Contamination of surface water by organophosphate and carbamate compounds is of concern because of the potential toxicity to aquatic organisms, especially those at lower trophic levels. Many organophosphate and carbamate compounds have acute and chronic toxicity to fish and aquatic invertebrates. Bondarenko et al. (2004) showed that the persistence of diazinon and chlorpyrifos was much longer than for malathion and carbaryl in freshwater, and was further prolonged in seawater. Afterwards, microbial degradation contributed significantly to the dissipation of diazinon and chlorpyrifos in freshwater, but was inhibited in seawater. In contrast, degradation of malathion and carbaryl was rapid and primarily abiotic. The interactions of pesticide persistence with water location, temperature,

and type of pesticides suggest that site, and compound-specific, information is needed when evaluating the overall ecotoxicological risks of pesticide pollution in a watershed.

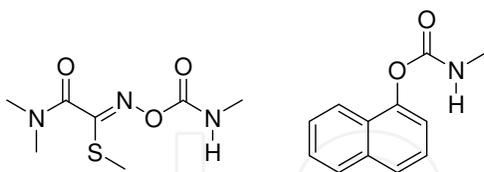


Fig. 14. Oxamyl (left) and carbaryl (right) pesticides

Kaufman and Blake (1973) have selected soil microorganisms capable of degrading isopropyl carbanilate (propham), 3',4'-dichloropropionanilide (propanil), 3'-chloro-2-methyl-*p*-valerotoluidide (solan), and methyl 3,4-dichlorocarbanilate (swep), (Figure 15). Degradation of the pesticides in enrichment solutions, and by pure cultures of effective microbial isolates (*Pseudomonas striata*, *Achromobacter* sp., *Aspergillus ustus*, *Aspergillus versicolor*, *Fusarium oxysporum*, *Fusarium solani*, *Penicillium chrysogenu*, *Penicillium janthinellu*, *Penicillium rugulosum* and *Trichoderma viride*) were demonstrated by the production of the corresponding aniline, chloride ion liberation and disappearance of the original compounds. Each organism demonstrated unique substrate specificity and was capable of degrading other aniline-based pesticides of the acetamide, acylanilide, carbamate, toluidine and urea classes.

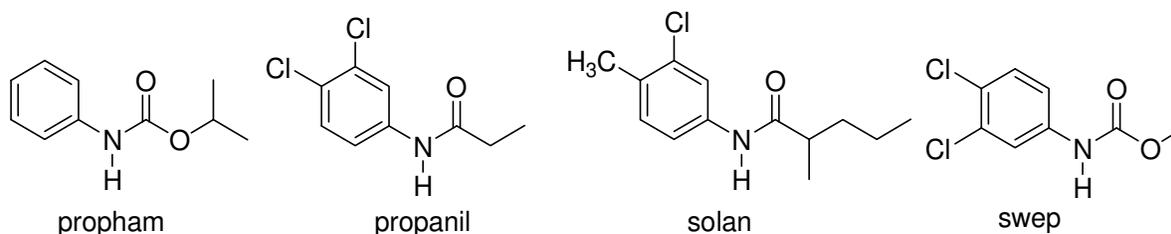


Fig. 15. Carbamate pesticides degraded by soil microorganisms

As described here, the carbamate pesticides are easily degraded by different types of microorganisms (fungi and bacteria). The degradation of these pesticides by enzymatic systems of microorganisms has contributed to the total removal of xenobiotics from soils, hence avoiding the contamination of waters and the environment.

2.4 Biological pesticides

Synthetic chemical pesticides provide many benefits to agriculture and food production, however, as previously discussed, they also present toxicity to non-target organisms and cause environmental pollution, therefore efforts to find new pest control alternatives have been studied, essentially due to the increasing concern about the effects of these compounds on human health and on the environment. Biodegradation and bioremediation of synthetic pesticides have been used as alternative green technologies to solve the problems related to the accumulation of these contaminants in soil and water. Another proposal to reduce the environmental impact of pesticides is the use of biological-derived products also known as biopesticides.

According to the Environmental Protection Agency (EPA), biopesticides are defined as naturally occurring pest control substances. They are classified into three groups (Joshi, 2006):

- a. Microbial pesticides: in which a microbial living organisms (bacteria, fungi, viruses, protozoans) is the active control agent;
- b. Plant pesticides: pesticidal substances produced by plants from introduced genetic material (plant incorporated protectants);
- c. Biochemical pesticides: naturally occurring substances that control pests by non-toxic mechanisms. These include substances that interfere with growth or mating such as pheromones.

The main advantage of biopesticides is their safety to non-target organism, biodegradability and their specificity, which permits the use of small dosages and power exposure, hence avoiding pollution caused by conventional pesticides (Rosell et al., 2008). In addition to being less harmful than chemicals, biopesticides have been of great value in integrated pest management (IPM) strategies where the use of biopesticides greatly decreases the use of chemicals, maintaining crop yields. The specificity of biopesticides contrasts with the broad spectrum of chemical counterparts. In contrast, biopesticides are also slow acting, have relatively critical application times, most suppress rather than eliminate the target population, have limited field persistence and short-shelf life.

Despite the range of biopesticides that have been described, our discussion focuses on microbial pesticides.

2.4.1 Microbial pesticides

Microbiological control is sustained by beneficial interactions resulting from competition, antagonism and parasitism of microorganisms against plant pathogens, insects and weeds (Montesinos, 2003). In general, microorganisms are able to suppress pests by producing a toxin, causing a disease or preventing the establishment of other organisms. Currently, several microorganisms involved in such processes are the active ingredient of microbial pesticides.

2.4.1.1 Bacteria

Most biopesticides available in the market are bacterial-based products. The well-known and widely used bacterial biopesticide comprises the gram-positive, spore-forming bacteria belonging to the genus *Bacillus* that are commonly found in soil. The majority of commercial microbial insecticides are preparations based on strains of *Bacillus thuringiensis* (Bt) that produces a crystalline inclusion body during sporulation (Frankenhuyzen, 2009). The crystal proteins (Cry proteins) are toxic to many insects and are defined as endotoxins (Bt toxin) that are generally encoded by bacterial plasmids (Gonzales & Carlton, 1980). Both spores and inclusion bodies are released upon lysis of the parent bacterium at the end of the sporulation cycle and if ingested, the spores and crystals act as poisons in certain insects. The protein is activated by alkaline conditions and enzyme activity of insect's gut hence, Bt is referred as a stomach poison (Chattopadhyay et al., 2004). The toxicity of the activate protein is dependent on the presence of receptor sites on the insects gut wall. This match between toxin and receptor sites determines the range of insect species killed by each Bt subspecies and isolates (Frankenhuyzen, 2009).

Cry proteins are produced as protoxins that are proteolytically converted into a combination of up to four smaller toxins upon ingestion. These proteins bind to specific receptors in the larval midgut epithelium causing the formation of large cation-selective pores that increase the water permeability of the cell membrane. A large uptake of water then causes cell

swelling and rupture of the midgut. Poisoned insects can die quickly from the toxin activity or may die within 2-3 days from septicemia due to the entering of gut contents into the bloodstream. Bt strains containing mixtures of up to 6-8 Cry proteins have been used as microbial pesticides since Bt var. *kurstaki* have been commercially available since 1961 (Montesinos, 2003). Formulations are active against insect order Lepidoptera (moths and butterflies); Diptera (flies and mosquitoes); Coleoptera (beetles and weevils) and Hymenoptera (bee and wasps) larvae (Frankenhuyzen, 2009). Of the recognized subspecies of Bt, var. *kurstaki* is toxic to gypsymoth, cabbage looper, and caterpillars (order Lepidoptera), var. *israelensis* is toxic to fungus gnat larvae, mosquitoes (species of *Aedes* and *Psorophora*), coffee berry borer (Mendez-Lopez et al., 2003), back fly, and some midges (order Diptera), var. *san diego* is effective against potato beetle, elm leaf beetle, and boll weevils (Whalon & McGaughey, 1998), var. *aizawai* is effective against wax moth larvae and diamondback moth caterpillar and var. *morrisoni* is toxic against moth and butterfly caterpillars (order Lepidoptera) (Chattopadhyay et al., 2004). A number of Bt-derived products were used in Europe to control Lepidoptera pests in vegetables, tomatoes, top fruit, vines, olives and forestry (Butt et al., 1999).

Besides Cry proteins (crystal delta endotoxins), Cyt proteins (cytolysins) have been described as another class of insecticidal protein produced by Bt (Yokoyama et al., 1998). Cytolysins interact with phospholipid receptors on the cell membrane in a detergent-like manner (Gill et al., 1987). The hydrophobic portion of the cytolysins bind the amphipathic phospholipids; transmembrane pores are formed and cells are lysed by osmotic lysis (Knowles & Ellar, 1987). The spore inclusions contain many proteins, which sometimes possess distinct activities and may act in a synergistic manner (Yokoyama et al., 1998).

With regard to toxicity, Cry proteins are non toxic to vertebrate species even at doses higher than $1 \times 10^6 \mu\text{g} / \text{kg}$ body weight, while dosages acutely toxic to susceptible insects are about $\mu\text{g}/\text{kg}$ body weight (Rosell et al., 2008), however Bt formulations can cause skin and eye irritation (Siegel & Shaddock, 1990). The acidic environment of the mammalian stomach does not favor solubilization and activation of the Cry proteins. These proteins are degraded very fast (often in some seconds), from 60-130 kDa to polypeptides less than 2 kDa that corresponds to peptides with 10 amino acids in length. The rapid degradation of these proteins by proteases in the mammalian gastrointestinal tract precludes their toxicity in mammals. Several studies in vertebrates have failed to find high affinity Cry protein binding sites on gut epithelial cell membranes (Rosell et al., 2008). Bt has thus become a bioinsecticide of great agronomical importance and is classified as toxicity class III pesticide (slightly toxic).

The commercial Bt products are powders comprised of a mixture of dried spores and toxin crystal proteins and these are applied to areas like leaves and roots where insects feed. The commercial Bt product contains about 2.5×10^{11} viable spores per gram. Bt products are marketed worldwide and they account for about 1% of the total agrochemical market. Bt products are known to lose their effectiveness to some extent when stored for longer than six months (Joshi, 2006).

Other species of *Bacillus*, including *B. sphaericus*, *B. popilliae*, *B. subtilis*, *B. lentimorbus*, *B. pumilus* and *B. firmus* have been applied as biopesticides (Schisler et al., 2004).

Bacteria belonging to other genera such as *Pseudomonas fluorescens*, *P. syringae*, *P. putida*, *P. chlororaphis*, *Burkholderia cepacia* and *Streptomyces griseoviridis* have also been used as biopesticides (Montesinos, 2003). However, these bacteria generally lose viability when

stored for several weeks, a disadvantage when compared with spore-forming *Bacillus* that demonstrates better shelf-life and facilitates the development of commercial products.

Insect resistance to Bt toxins has led to pursue suitable alternatives. Two more bacteria that are also known to produce insecticidal toxins are *Xenorhabdus* and *Photorhabdus* (both of these belong to the family *Enterobacteriaceae*). Both bacteria are entomopathogens, *Xenorhabdus luminescens* is found to occur in a specialized intestinal vesicle of the nematode *Steinernema carpocapsae* (Akhurst & Dunphy, 1993) with which it maintains a symbiotic relationship. *Photorhabdus luminescens* maintains a symbiotic relationship with nematodes of the family Heterorhabditidae (Poinar, 1990) and is present throughout the intestinal tract of these nematodes. In both mutualistic associations, the nematodes and the bacteria complement each other: the nematode acts as a vector and transports the bacteria into the target insect larva where it bores holes in the intestinal walls of the insect and releases the bacteria in the hemolymph. In the absence of the nematode, the bacteria cannot penetrate into the hemocoel (Tanada & Kaya, 1993). Both the nematode and the bacteria release insecticidal toxins, which eventually kill the insect (Poinar et al., 1977). The bacteria causes septicemia in the insect, the insect is killed and its tissues are used as nutrients (Kaya & Gaugler, 1993). Moreover, bacteria are required by the nematodes for their development into the infective juvenile stage and thus are required for efficient completion of the nematode life cycle. In the absence of the bacterium the nematode cannot reproduce (Tanada & Kaya, 1993). With emerging resistance to Bt among insects, *Xenorhabdus* and *Photorhabdus* are considered the next generation of microbial insecticides.

2.4.1.2 Fungi

Fungi often act as important natural control agents against insects, pathogenic fungi, nematodes and as herbicide. Many fungi utilized as biopesticides are pathogenic to insect hosts, therefore they are referred as entomopathogenic fungi; among them, members of Entomophthorales (Zygomycota) and Hyphomycetes are currently under research (Srivastava et al., 2009). Fungal strains are considered suitable for biopesticide development because, unlike other microorganisms, the infectious propagules (conidia) do not need to be ingested and contact with cuticle permits the fungi to penetrate the insect body (Thomas & Read, 2007).

Fungi can act as insecticide by two ways:

- a. Infection: most of the fungi species cause death to the insect through asexual spores called conidia. The conidium is the infective unit of entomopathogenic fungi and binds to the host cuticle by nonspecific interaction mediated by cuticle degrading enzymes present on the conidia or by fungal lectins. These conidia enter through the body wall of the host pest by dissolving the body wall by the combined action of enzymes, i.e., chitinase and protease, secreted by the fungi. Fungal penetration is further enhanced by mechanical force. The site of invasion is often between the mouth parts, at intersegmental folds or through spiracles, where locally high humidity promotes germination and the cuticle is nonsclerotized and more easily penetrated. Under favorable environmental conditions (>95% humidity) the fungus will break out through the cuticle and sporulate; it may grow profusely in the blood and give the carcass a characteristic mummified appearance. Therefore, insect death is probably the result of obstruction of blood circulation, starvation or physiological/biochemical disruption brought about by the fungus. The whole procedure takes 3–14 days for insect death (Roy et al., 2006).

- b. Mycotoxins: another fungi mode can cause death of the host by the production of mycotoxins, which can interfere in the nervous system of insects. Mycotoxins such as aflatoxin B, trichothecenes, patuline and ochratoxin are reported to be toxic to insects, (Figure 16), (Srivastava et al., 2009).

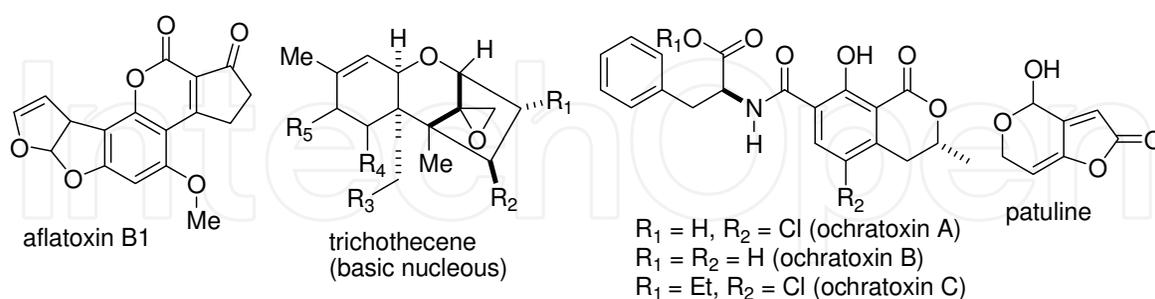


Fig. 16. Examples of mycotoxins produced by fungi

Fungi are known to infect a broader range of insects belonging to orders Lepidoptera, Homoptera, Hymenoptera, Coleoptera and Diptera. *Beauveria bassiana*, *Beauveria brongniari*, *Metarhizium anisopliae*, *Metarhizium flavoviride* and *Lagenidium giganteum* are examples of commercially available mycoinsecticides (Rosell et al., 2008).

Trichoderma harzianum, *T. viride*, *Talaromyces flavus*, *Glucocladium virens*, *Phytium oligandrum* shows fungicide activity against soil-borne pathogenic fungi (Montesinos, 2003). The application of a biopesticide containing the fungus *Verticillium lecanii* was reported to suppress the growth of plant pathogens as well as insect pests (Koike et al., 2005).

A formulation containing the unicellular fungi *Candida oleophila* O is used as a post-harvest biofungicide to control the pathogens *Botrytis cinerea* (gray mold) and *Penicillium expansum* (blue mold) which cause deterioration of apples and pears (Environmental Protection Agency-EPA, 2009).

The main difficulties to be overcome for applying entomophagous fungi in pest control are: scant production of mycotoxins; (ii) carcinogenic mycotoxicosis in non-target organisms; and (iii) slow effectiveness of entomophagous conidia. The combination of fungi formulations with plant extracts exploring their synergistic action is an alternative strategy to overcome these problems (Srivastava et al., 2009).

2.4.1.3 Viruses

Virus-based biopesticides have been used as insect control agents. The larvae of many insect species are vulnerable to viral diseases. Baculoviruses are a large virus group belonging to the family *Baculoviridae* and can infect different insect orders, particularly Lepidoptera and Diptera (Theilmann et al., 2005; Moscardi, 1999). Theilmann et al., 2005; Moscardi, 1999). Baculoviruses are classified into two genera: nuclear polyhedrovirus (NPV) and granulovirus (GV), (Cory & Hails, 1997; McCutchen & Flexner, 1999). Two morphologically distinct forms of infectious particles are generated in the baculovirus cycle, the occlusion derived virus (ODVs), comprising enveloped virions embedded within a crystalline matrix of protein (polyhedrin for NPVs and granulins for GVs), and budded virus (BVs), consisting of a single virion enveloped by a plasma membrane. Due to their specificity and high virulence to a number of insect pest species, they have been used worldwide to control lepidopteran pests in many crops (Moscardi, 1999). BVs are responsible for the systemic or cell-to-cell spread of the virus within an infected insect. ODVs, in turn, are responsible for the larva-to-larva transmission of the virus (Inceoglu et al., 2006).

Viruses, like bacteria, must be ingested to infect the insect hosts. During infection the host larvae is debilitated, resulting in reduced movement and increased exposure to predators. Post larval effects include the reduction in reproductive capacity and longevity. Disease and death insects serve as inoculums for virus transmission which may occur by rain and movement of insects on plants (Rosell et al., 2008). The commercial formulations that have been used include Granulosis virus to control *Byctiscus betulae*, Pine sawfly NPV to control *Diprion similis*, *Heliothis* NPV to control *Helicoverpa zea*, Gypsy moth NPV to control *Lymantria dispar*, *Mamestria brassicae* NPV to control *Heliothis* (Montesinos, 2003).

Insect viruses are safe to vertebrates, plants and non-target organisms. Limitations on the use of virus formulations include narrow spectrum of biological activity, slow mode of action (5–7 days after ingestion of NPVs and 7–14 days in the case of GV infections), and photolability (solar radiation), (Rosell et al., 2008). The major success of microbial control with viruses takes place in forestry. Forest pests are good targets for viral pesticides because the permanence in forest environment contributes to the pathogen cycle and the forest canopy also helps to protect viral particles from radiation. There have been different approaches directed to enhance the role of baculovirus as effective biopesticides. For instance, the effect of baculovirus may be enhanced by the synergistic action of specific chemical insecticides, such as the pyrethroids deltamethrin and permethrin (McCutchen & Flexner, 1999). To improve the potency and rapid action, recombinant baculovirus have been developed (Bonning & Hammock, 1996).

2.4.1.4 Protozoa

Some protozoan pathogens can kill insect hosts; however, many of them cause chronic infections with debilitating effects (Lacey & Goettel, 1995). One important consequence of protozoan infection is the reduction in the number of offsprings by the infected insects. Species of the genera *Nosema* sp. and *Vairimorpha necatrix* offer the greatest biopesticide potential. *Nosema locustae* is a specie of Microsporidium commercially available to control grasshoppers and crickets. It is most effective when ingested by immature grasshoppers (early nymphal stages). The spore formed by the protozoan is the infection stage in susceptible insects; it germinates in the midgut and causes a slow progress infection where the pathogen causes death three to six weeks after initial infection (Rosell et al., 2008). *Ostrinia nubilalis* that causes important damages to corn was controlled by *Nosema pyrausta* infection, which reduced the egg production per female 53 and 11% at the 16 and 27°C temperature, respectively (Bruck et al., 2001). *Nosema locustae* has been used to reduce grasshopper population in rangeland areas; although not all insects are killed, the infected grasshoppers consume less forage and the females produce fewer eggs. However, the utility of *N. locustae* as biopesticide remains questionable because of the difficulty to determine the treatment efficacy in this highly mobile insect.

2.4.2 Challenges of microbial pesticides

The main problems that should be solved regarding the widespread use of microbial pesticides include their specificity, once they are not effective against a wide range of pests. Although specificity is considered an advantage, it also limits the potential market and increases costs when compared to synthetics. Another important aspect is that biopesticide preparations are sensitive to heat, desiccation and ultraviolet radiation, reducing their effectiveness. Special formulations and storage conditions are necessary; this in turn can complicate the distribution and application of products. Molecular genetics of

microorganisms and genetic engineering technology will help in the development of new strategies for biopesticide improvement and its use. More work should be done to enhance shelf-life, to increase the speed of kill, the biological spectrum and the field efficacy of biopesticides.

3. Conclusion

The pollution of the environment by pesticides is a consequence of the continuous agricultural expansion, combined with the population increase. Pesticides are used in sizeable areas and applied to soil surfaces and accumulate beneath the ground surface, reaching rivers and seas. The natural microbiota is continuously exposed to pesticides therefore, it is no surprise that these microorganisms, that inhabit in polluted environments, are armed with resistance by catabolic processes to remove the toxic compounds. Biological degradation by organisms (fungi, bacteria, viruses, protozoa) can efficiently remove pesticides from the environment, especially organochlorines, organophosphates and carbamates used in agriculture. The enzymatic degradation of synthetic pesticides with microorganisms represents the most important strategy for the pollutant removal, in comparison with non-enzymatic processes. Regarding the use of biopesticides, their main advantage is their environmental-friendly nature when compared to chemicals.

To improve the use of microbe-based processes some questions still have to be answered, such as the long term impact of introducing microorganisms into the environment, as well as the narrow range of applications (particularly in the case of biopesticides).

The degradation of persistent chemical substances by microorganisms in the natural environment has revealed a larger number of enzymatic reactions with high bioremediation potential. These biocatalysts can be obtained in quantities by recombinant DNA technology, expression of enzymes, or indigenous organisms, which are employed in the field for removing pesticides from polluted areas.

The microorganisms contribute significantly for the removal of toxic pesticides used in agriculture and in the absence of enzymatic reactions many cultivable areas would be impracticable for agriculture.

4. References

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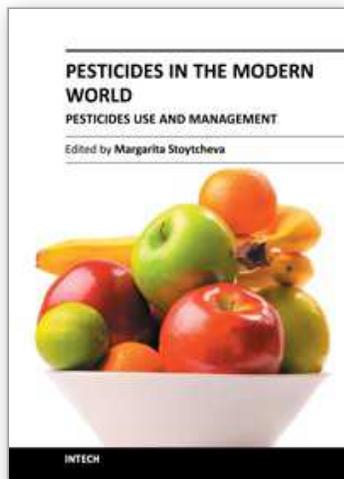
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