Chapter 7

Biodegradation of the Organophosphate Pesticide Profenofos by Marine Fungi

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Additional information is available at the end of the chapter

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1. Introduction

Pesticides play an important role in modern agriculture. Synthetic pesticides are recognized as a cost-effective method of controlling pests, improving productivity and food quality. However, while pesticides may have a beneficial effect on agricultural productivity, their indiscriminate use causes many serious problems to the environment and human health, since these compounds are toxic to non-target species (Diez, 2010; Coutinho *et al*., 2005).

The fate of pesticides in the environment is influenced by many processes (biological, chemical and physical) that determine their persistence and mobility (Gravilescu, 2005). Millions of tons of pesticides are applied annually, but it is believed that only a small fraction of these products effectively reaches the target organisms, and the remainder are deposited on the soil, contaminating non-target organisms and moving into the atmosphere and water (Eerd *et al*., 2003). Since many pesticide types are recalcitrant, they remain for a long time in soils and sediments, where they can enter the food chain directly or percolate into the groundwater (Rissato *et al*., 2004; Gravilescu, 2005).

Detoxification of pesticides *in situ* has been achieved by treatment of the contaminated soil with certain microorganisms or plants, a technology known as bioremediation or more specifically, phytoremediation in the case of plants (Sutherland *et al*., 2002). These microorganisms are the main biological agents capable of removing and degrading waste materials, to enable their recycling in the environment (Chowdhury *et al*., 2008). Since the conventional treatment options for the pesticide residues clean-up in the environment include removal of the contaminated material to be incinerated or disposed in landfills, *in situ* biological reme-

diation is seen as a safer, less disruptive and more cost-effective alternative treatment (Sutherland *et al.*, 2004).

Effective techniques for soil bioremediation are bioaugmentation, biostimulation, phytoremediation and enzymatic bioremediation. However, the three first techniques are limited by their dependence upon the growth rate of the remediating plants and microbes, which will vary with nutrients, aeration, pH and other factors relating to the contaminated soil (Scott *et al.*, 2008; Sutherland *et al.*, 2004). A successful bioremediation technique requires efficient organisms that can degrade pollutant to a minimum level. In the case of pesticides, an adequate rate of biodegradation is required to attain the acceptable level of a pesticide residue or its metabolites at the contaminated site in a limited period of time (Singh, 2008).

Organophosphate pesticides (OPs) are used worldwide in agriculture, municipal hygiene, disease vector control and against household pests; they were also a group of compounds used historically as chemical warfare agents (Yang *et al.*, 2008; Zheng *et al.*, 2007; Edwards and Tchounwou, 2005). OPs are phosphorus-containing pesticides whose insecticidal qualities were first observed in Germany during World War II (Edwards and Tchounwou, 2005). The principal types are phosphotriesters, thiophosphotriesters, and phosphorothiolesters. Phosphotriesters contain a phosphate center with three *O*-linked groups, thiophosphotriesters have the phosphoryl oxygens replaced by sulfur and in phosphorothiolesters, one or more of the ester oxygen are replaced by sulfur (Figure 1) (Bigley and Raushel, 2013).

This chemical class of pesticides has been used to replace the organochlorine pesticides, banned in the United States since the 1970s (Jauregui *et al.*, 2003). However, the OPs are also highly toxic pesticides, since they are potent irreversible acetylcholinesterase (AChE) inhibitors that have a profound effect on the nervous system of exposed organisms, including human beings (Edwards and Tchounwou, 2005).

The hydrolysis mechanism normally catalyzed by AChE depends on the attack of a serine residue at the active site on the carbonyl group in ACh, but in the presence of organophosphates, this residue is readily phosphorylated, as follows: a histidine residue at the active site captures a proton from the serine residue, increasing its nucleophilic character, so that it readily attacks the electrophilic phosphorus atom, releasing the leaving group (X) (Figure 2). Unlike the acetylated enzyme, the phosphorylated enzyme reacts slowly with water, allowing the dealkylation of the alkoxy substituent (R_2) attached to the phosphorus atom. The organophosphate compounds thus inactivate acetylcholinesterase by phosphorylation of the serine at the enzyme active site. The result is the formation of a strong hydrogen bond between a protonated histidine residue of the catalytic site and the negatively charged oxygen atom of the inhibitor. Therefore, the protonated histidine cannot function as a general base catalyst for the hydrolysis of the phosphorylated enzyme, which is a necessary step for the reactivation of AChE (Figure 2) (Mileson *et al.*, 1998; Santos *et al.*, 2007)

According the Brazillian Food, Drug and Sanitary Surveillance Agency (ANVISA), analysis of pesticide waste in food showed that OPs are those with the greatest number of occurrences in unsatisfactory samples. Among then, chlorpyrifos, methamidophos and acephate are the main active ingredients responsible for food contamination. Profenofos appeared to be the 12th

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APPENDIX

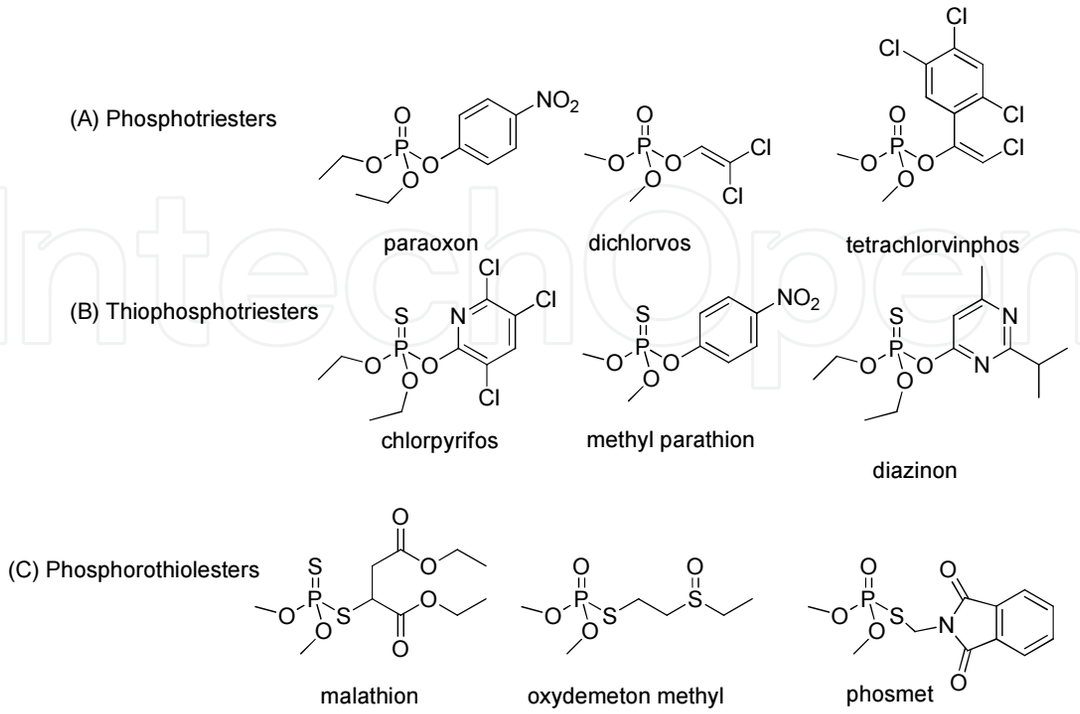

Figure 1. Chemical structures of main class of OPs. (A) Phosphotriesters; (B) Thiophosphotriesters; (C) Phosphorothiolesters

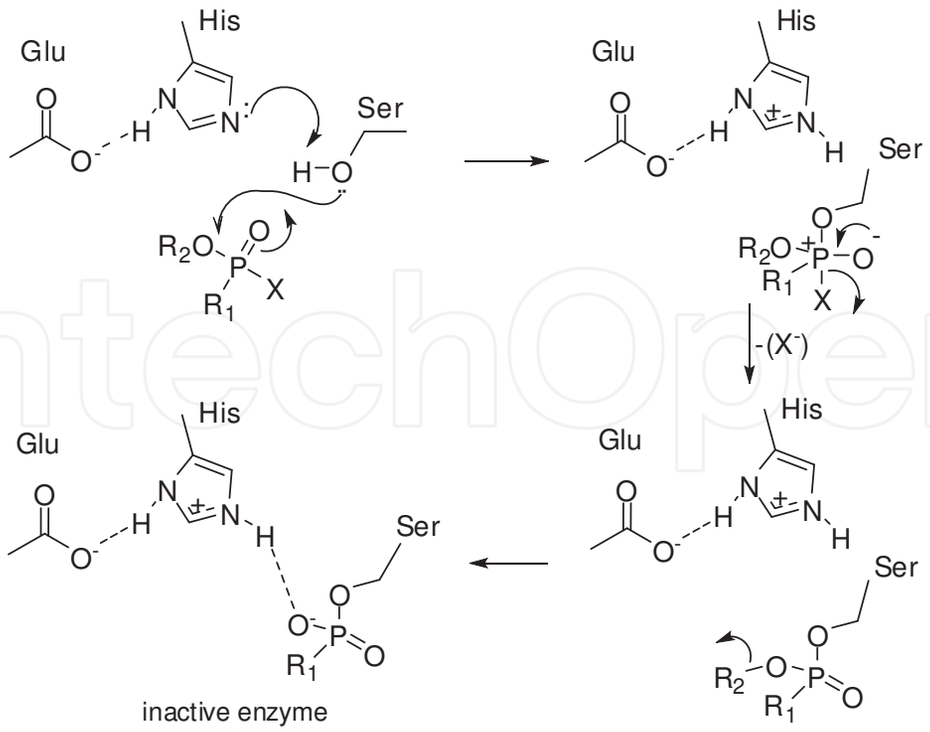

Figure 2. Mechanism of inhibition of acetylcholinesterase by organophosphate pesticides (Santos *et al.*, 2007)

commonest active ingredient in irregular samples of food, being found in samples of orange, strawberry and pepper (ANVISA, 2012).

Profenofos, *O*-(4-bromo-2-chlorophenyl) *O*-ethyl *S*-propyl phosphorothioate, is a broad spectrum, non-systemic foliar insecticide and acaricide. It is effective against a wide range of chewing and sucking insects and mites on various crops (Reddy and Rao, 2008). In the United States, profenofos is a "restricted use" pesticide sprayed only on cotton crops (McDaniel and Moser, 2004; EPA, 2012). However, in Brazil, this pesticide can also be used for foliar application on cotton, peanuts, potatoes, coffee, onions, peas, beans, green beans, watermelon, corn, cucumber, cabbage, soybean, tomato and wheat (ANVISA, 2011).

Classified as a moderately hazardous (Toxicity class II) pesticide by the World Health Organization (WHO) (Abass *et al.*, 2007; Malghani *et al.*, 2009), profenofos has a moderate order of acute toxicity following oral and dermal administration (McDaniel and Moser, 2004; Abass *et al.*, 2007). According to US Environmental Protection Agency (EPA), profenofos was first registered in the United States in 1982 and about 775,000 pounds (lbs.) of active ingredient are applied to cotton each year (EPA, 2012).

Chemical decontamination of organophosphates relies on bleach treatment, alkaline hydrolysis or incineration, but these conditions are harsh and the byproducts can be toxic (Ghanem and Raushel, 2005). Specific bioremediation of OPs requires highly specialized enzymes, so genetic engineering has been used to improve the properties of enzymes from various sources to enhance catalytic rates, stability and substrate range (Sutherland *et al.*, 2004).

A number of enzymes capable of detoxifying OPs have been discovered and the majority of them belong to the class of phosphotriesterases (PTE). Various PTEs have been identified: organophosphate hydrolase (OPH), methyl parathion hydrolase (MPH), organophosphorus acid anhydrolase (OPAA), diisopropylfluorophosphatase (DFP), and paraoxonase 1 (PON1) (Bigley and Raushel, 2013). All of these enzymes are found to promote the hydrolysis of organophosphate compounds. The most frequently cited enzyme in the literature, OPH, isolated from the bacteria *Pseudomonas diminuta* or *Flavobacterium* ATCC 27551, catalyzes the hydrolysis of a wide range of OP pesticides (Rogers, 1999; Chen and Mulchandani, 1988). Another enzyme reported involving the hydrolysis of OPs are carboxylesterases (CbEs), although the hydrolysis of OPs by PTEs is more efficient in the detoxification than the CbEs (Sogorb and Vilanova, 2002).

Zheng *et al.* showed that when OPH was coexpressed with CbE, the mixed enzymes degraded a variety of P-O bond containing OPs (chlorpyrifos, methyl parathion, dichlorvos and phoxim), whereas OPH had a very low catalytic activity for P-S bond containing OPs (malathion) (Figure 3). Thus, the hydrolase activities usually vary among structurally different OPs, ranging from the nearly diffusion-controlled limit for paraoxon to several orders of magnitude lower for phosphothiolesters, such as malathion (Zheng *et al.*, 2007).

Some degradation pathways are described in the literature for profenofos. The metabolic pathway of profenofos in cotton plants involves the cleavage of the phosphorothioate ester bond to yield 4-bromo-2-chlorophenol, followed by conjugation with glucose (Capps *et al.*, 1996). In the literature there are some cases of (bio)degradation of organophosphate pesticides

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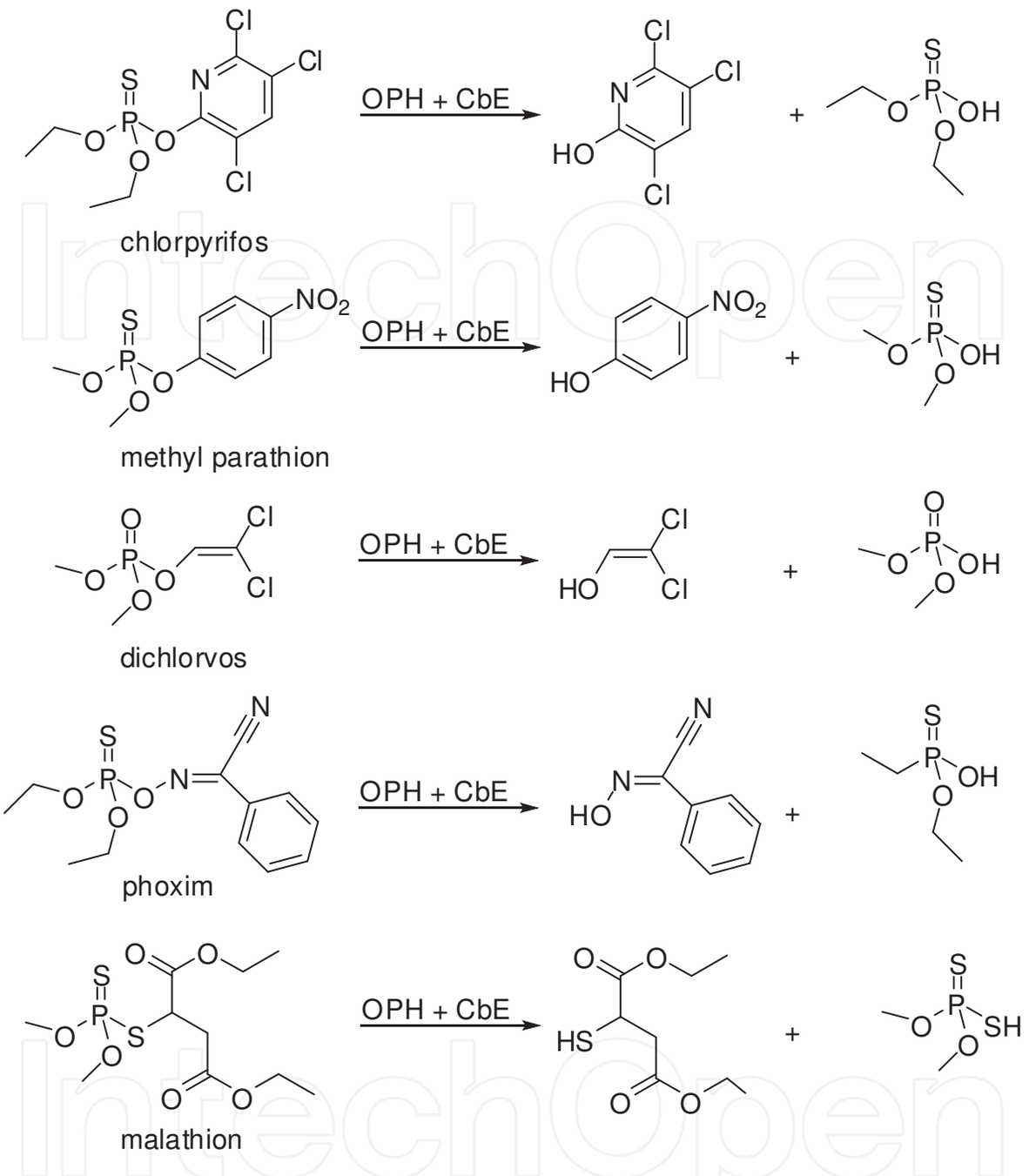

Figure 3. Hydrolysis of different OPs pesticides by OPH + CbE enzymes

that occur by subsequent biotransformations, yielding novel polar metabolites, such as glycosylated and sulfated derivatives. According to the Food and Agriculture Organization of the United Nations (FAO), in aerobic soil conditions, profenofos degraded rapidly, with mineralization and formation of unextracted residues. In sterilized soil, cleavage of the phenol-phosphorus ester bond in profenofos proceeded via chemical hydrolysis, with accumulation of 4-bromo-2-chlorophenol and formation of unextracted residues. The metabolic biotransformations of profenofos in plants and animals are similar and occur via hydrolysis to 4-

bromo-2-chlorophenol which is then conjugated by several enzymatic reactions (Figure 4) (FAO, 2012).

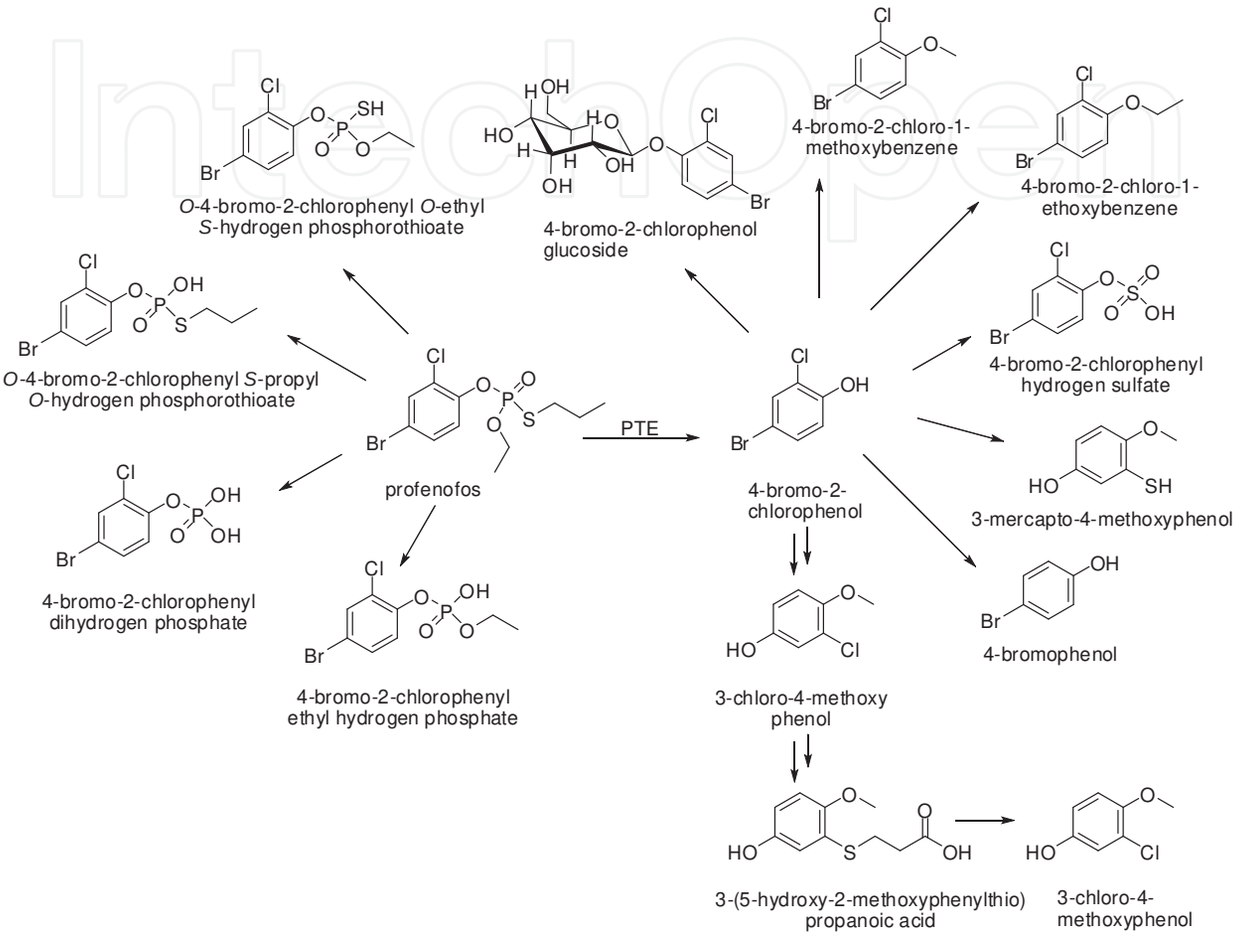

Figure 4. Proposed metabolic pathway of profenofos in soil, plant and enzymatic reactions

Fungi degrade a wide variety of compounds, a process known as mycodegradation. This process involves degradation to smaller molecules which may be toxic or non-toxic, as well as the removal of the pesticide molecule through a simple absorption or adsorption mechanism (Ramadevi *et al*., 2012).

The ability of bacterial species to degrade organophosphates is well established and researches have even proposed possible degradation mechanisms for the OPs (Van Eerd *et al*., 2003). However, the mechanisms of fungal degradation of these compounds are less established than those used by bacteria, since there are few studies on fungal degradation of OPs.

There are few studies on the biodegradation of profenofos by microorganisms. Malghani *et al.* reported the successful biodegradation of this compound by bacteria, and at the time of writing, the author stated that no studies on bacterial degradation of profenofos had been reported earlier

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(Malghani *et al.*, 2009). Filamentous fungi of the genus *Aspergillus* have been used in the biodegradation of OPs. For instance, *Aspergillus niger* showed high biodegradation of malathion pesticide (Ramadevi *et al.*, 2012), *Aspergillus flavus* and *Aspergillus sydowii* were capable of degrading pirimiphos-methyl, pyrazophos and malathion, even at high concentrations (1,000 ppm), utilizing these compounds as sole phosphorus and carbon sources, releasing the phosphorus moiety from these pesticides by means of their phosphatases (Hasan, 1999).

Marine enzymes have a great potential for use in biocatalytic reactions, as in biodegradation of pesticides, due to the peculiar characteristics of the marine environment. As the sea covers more than three quarters of the Earth's surface and provide abundant resources for biotechnological research and development (Rush *et al.*, 2007), marine organisms offer a dramatically different environment for the biosynthesis of molecules than terrestrial organisms, and are a vast untapped source of enzymes (Venter *et al.*, 2004; Venter *et al.*, 2010). In recent years, a variety of new enzymes with specific activities have been isolated from bacteria, fungi and other marine organisms; moreover, some can produce a considerable number of molecules with potential to be transformed into commercial drugs (Ghosh *et al.*, 2005; Haefner, 2003). In fact, the marine environment is a very rich source of extremely potent compounds exhibiting significant activities in anti-tumor, anti-inflammatory, analgesic, immunomodulatory, allergic and anti-viral assays (Newman and Cragg, 2004; San-Martín *et al.*, 2008).

Marine organisms in general (fungi, bacteria, algae, sponges, fish, prawns and other crustaceans) can be rich sources of novel enzymes, but most of the current bioprospecting activity focuses on microbial ones. A marine enzyme is a protein molecule with unique properties as it is derived from an organism whose natural habitat is saline or brackish water (Trincone, 2010; Sarkar *et al.*, 2010). These enzymes can be biocatalysts with properties such as high salt tolerance, hyperthermostability, barophilicity and cold adaptability. Microorganisms isolated from ocean sediment and seawater are the most widely studied sources of marine enzymes, especially proteases, carbohydrases and peroxidases (Ghosh *et al.*, 2005).

Enzymatic reactions catalyzed by marine fungi can be used when the fungi are cultured in media based on artificial seawater. The filamentous marine fungi *Aspergillus sydowii* CBMAI 933, *Penicillium raistrickii* CBMAI 931, *Penicillium miczynskii* CBMAI 930 and *Trichoderma* sp. CBMAI 932, grown in artificial seawater were able to catalyze the hydrolysis of benzyl glycidyl ether (Martins *et al.*, 2011). Similar results were observed in a study of ligninolytic enzyme production by the marine fungi *Aspergillus sclerotiorum* CBMAI 849, *Cladosporium cladosporioides* CBMAI 857 and *Mucor racemosus* CBMAI 847 (Bonugli-Santos *et al.*, 2010). Other studies have shown that marine bacteria and fungi cultured in the laboratory have specific requirements for salts, especially sodium, potassium, magnesium and chloride ions (Martins *et al.*, 2011; MacLeod, 1965; Kogure, 1998; Rocha *et al.*, 2009).

In this chapter, the first results obtained in the biodegradation of profenofos by whole cells of marine fungi are presented. Marine fungi were selected by us, with high potential to biodegrade profenofos and its main metabolite. The results presented in this chapter explore the potential of marine fungi in biotransformation and biodegradation of a xenobiotic (pesticide profenofos). The fungal biodegradation of OPs is still underexplored by researches, especially with regard to the biodegradation of profenofos, making this work extremely relevant. The

main objective of this study was the screening of Brazilian marine fungi with the enzymes required for detoxification of organophosphate pesticides (phosphotriesterases-PTEs and /or carboxylesterases-CBEs). The biodegradation of profenofos in the presence of these selected fungi was evaluated, assessing the degradation of the pesticide, as well as the formation of the metabolite, 4-bromo-2-chlorophenol. This results are environmentally important, because the pesticides applied to crops can be leached into rivers, lakes and seas under these different conditions, where they may suffer different biodegradation processes.

2. Materials and methods

2.1. General

Ethyl acetate (PA), used to extract the reaction mixtures and the salts used to prepare artificial sea water were purchased from a commercial source (Synth, Vetec, Brazil). Ethyl acetate (HPLC grade) for the analytical curve was purchased from a commercial source (Tedia, Rio de Janeiro, Brazil). The malt extract and agar used in solid and liquid culture media were purchased from commercial sources (Acumedia and Himedia, Brazil).

2.2. Pesticides

The analytical standards of chlorpyrifos and profenofos were purchased from Sigma-Aldrich, Brazil. Commercial pesticide containing profenofos was purchased from Syngenta® under the name Polytrin 400/40 CE. The commercial profenofos used in the marine fungi biodegradation test was donated by Professor Marcos R. de V. Lanza (IQSC-USP). The 4-bromo-2-chlorophenol was purchased from Sigma-Aldrich, Brazil.

2.3. Marine fungi

The Brazilian marine-derived fungal strains *Aspergillus sydowii*-CBMAI 934, *Aspergillus sydowii*-CBMAI 935 and *Penicillium raistrickii* CBMAI 931 were isolated from the sponge *Chelonaplysilla erecta; Aspergillus sydowii* CBMAI 1241, *Penicillium decaturense* CBMAI 1234 and *Penicillium raistrickii* CBMAI 1235 were isolated from the sponge *Dragmacidon reticulata; Trichoderma* sp. CBMAI 932 was isolated from the sponge *Geodia corticostylifera*. The sponges were collected in the South Atlantic Ocean at São Sebastião in São Paulo state, Brazil, by Professor Roberto G. S. Berlinck (Chemistry Institute of Sao Carlos, University of São Paulo, IQSC-USP). The marine fungi were isolated and purified in the microbiology laboratory of the Department of Ecology and Evolutionary Biology supervised by Professor Mirna H. R. Seleghim (UFSCar-Brazil). The fungi were identified by both conventional and molecular methods at the Chemical, Biological and Agricultural Multidisciplinary Research Center at UNICAMP, São Paulo, Brazil (http://www.cpqba.unicamp.br/). The isolated and identified marine fungi were deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI).

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2.4. Composition of marine fungi growth media

Composition of Artificial Sea Water (ASW) (1L): $CaCl_2.2H_2O$ (1.36g), $MgCl_2.6H_2O$ (9.68g), KCl (0.61g), NaCl (30.0g), Na_2HPO_4 (0.014 mg), Na_2SO_4 (3.47g), $NaHCO_3$ (0.17g), KBr (0.1g), $SrCl_2.6H_2O$ (0.040g), H_3BO_3 (0.030g).

Solid medium for stock cultures: agar (20 g. L^{-1}) and malt extract (20 g.L^{-1}) in ASW (1L) and adjusted to pH 8 by addition of 3M KOH.

Solid medium for fungal screening: agar (20 g. L^{-1}) and malt extract (20 g.L^{-1}) dissolved in ASW (1L) and adjusted to pH 5 by addition of 3M KOH or 1M HCl.

Liquid medium: malt extract (20 g.L^{-1}) in ASW (1L), adjusted to pH 7 by addition of 3M KOH or 1M HCl.

Liquid mineral medium supplemented with KNO_3(12.5 ppm): KNO_3 (12.5 mg.L^{-1}) dissolved in ASW (1L), adjusted to pH 7 by addition of 3M KOH or 1M HCl.

The culture media were sterilized in autoclave for 20 minutes (at 121 °C, 1.5 kPa). All manipulations involving marine fungi were carried out under sterile conditions in a Veco laminar flow cabinet. The stock cultures of the marine microorganisms were stored on solid culture medium (25 mL), in Petri dishes, maintained at 4°C in the refrigerator.

2.5. Cultivation of marine fungi on solid medium in the presence of profenofos

Marine fungi were screened by culturing on Petri dishes containing 25 mL of solid culture medium (2.0 g of malt extract, 2.0 g of agar and 100 mL of ASW) with the addition of profenofos and without (control culture). After the medium sterilization in the autoclave, the agar was cooled to 40-45°C and the profenofos was added at three different concentrations: 5.0, 10.0 and 15.0 μL per plate, solubilized in 100.0, 200.0 and 300.0 μL of dimethyl sulfoxide (DMSO), respectively. At room temperature, fungal mycelia from recent cultures were transferred to the surfaces of the agar plates with an inoculating loop. The fungi were incubated for 10 days at 35°C. Tolerance of profenofos was estimated by the size of the colony formed on the surface of the plates, relative to the control culture.

2.6. Analytical curve

Stock solutions of 500.0 ppm of profenofos, 4-bromo-2-chlorophenol (main metabolite) and chlorpyrifos (used as internal standard) were prepared.

Profenofos 500.0 ppm: 3.4 μL (1.3 mmol) of profenofos analytical standard and ethyl acetate (10.0 mL).

4-bromo-2-chlorophenol 500.0 ppm: 5.0 mg (2.4 mol) of 4-bromo-2-chlorophenol and ethyl acetate (10.0 mL).

Chlorpyrifos 500.0 ppm: 5.0 mg (1.4 mol) of chlorpyrifos and ethyl acetate (10.0 mL).

All standard solutions were prepared in a volumetric flask and made up to the containing 10.0 mL mark with ethyl acetate (HPLC grade). From these stock solutions were prepared the

working solutions, at concentrations of 5.0, 10.0, 20.0, 30.0 and 50.0 ppm of profenofos and 4-bromo-2-chlorophenol, in ethyl acetate (HPLC grade). The concentration of internal standard, chlorpyrifos, was maintained at 30.0 ppm in all assays. Next, 1.0 mL aliquots from the stock solutions were transferred into 1.5 mL vials. Triplicates samples, for each concentration of analyte, were analyzed by GC-MS-SIM.

2.7. Determination of profenofos concentration in commercial sample

The sample was prepared with 20.0 μL of commercial pesticide profenofos and 60.0 μL (2.4 mmol, 30.0 ppm internal standard) of stock solution of chlorpyrifos 500.0 ppm in ethyl acetate (HPLC grade), in a 100.0 mL volumetric flask. Duplicate samples of 1.0 mL were prepared and analyzed by GC-MS-SIM.

2.8. Biodegradation of profenofos by *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931 in liquid medium

The fungi *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931 were inoculated in solid culture medium (2.0 g of malt extract, 2.0 g of agar) in the presence of profenofos (50.0 ppm) and incubated for 7 days at 35°C. Two small slices of solid medium (1.2 cm x 1.2 cm) bearing the marine fungal mycelia were transferred to 250 mL Erlenmeyer flasks containing liquid medium (2.0 g of malt extract and 100.0 mL of ASW, pH 7). The liquid medium containing the inocula were incubated in an orbital shaker (Technal TE-421 or Superohm G-25) for 4 days (130 rpm, 32°C). After that, 50.0 ppm (18.6 μL, Section 2.6) of commercial profenofos was added. The reaction was incubated in the orbital shaker for 30 days (130 rpm, 32°C).

Extractions were performed at 10, 20 and 30 days. The reaction culture was filtered on a Buchner funnel to separate the mycelia from liquid medium. The mycelial mass obtained was rinsed and suspended in water and ethyl acetate (1:1). The mixture was stirred magnetically for 30 minutes and filtered again using Buchner funnel.

For the 10-days reaction, the extractions were then analyzed separately on the first mycelial extract and filtered medium. The liquid medium and mycelial extract were acidified to pH 6 and extracted separately, three times with ethyl acetate (3 x 25 mL) (Table 3).

For reactions at 20 and 30 days, the liquid medium and mycelial extract (after the Buchner filtration and extraction of mycelia with water and ethyl acetate, 1:1) were put together in an Erlenmeyer flask, acidified to pH 6 and extracted three times with ethyl acetate (3 x 25 mL). The filtered mycelial cells were dried in an oven (35 °C, 24 h) and then weighed (Tables 4-5).

After extractions, the organic phase was dried over anhydrous Na_2SO_4, followed by solvent filtration and evaporation, resulting in a final volume of 100.0 mL. The residual (no degraded) profenofos and the 4-bromo-2-chlorophenol released were analyzed by a gas cromatography coupled to a mass spectrometer in single-ion monitoring mode (GC-MS-SIM). Under these conditions, the concentration of pesticide and metabolite were determined by comparing the peak area of the samples with an analytical curve. The biodegradation results are summarized in Tables 6-10. Further degradation and growth experiments were performed, to test some parameters (Sections 2.8.1-2.8.4).

2.8.1. Biodegradation of 4-bromo-2-chlorophenol by A. sydowii CBMAI 935 and P. raistrickii CBMAI 931 in liquid medium

These reactions were prepared in 250-mL Erlenmeyer flasks containing 100.0 mL of liquid medium at pH 7 (Section 2.8) in which the 4-bromo-2-chlorophenol (50.0 ppm, 2.4 mmol) was added. The reaction was incubated in an orbital shaker for 30 days (130 rpm, 32°C). The inoculations, extractions and analyses proceeded as described in Section 2.8. The results are summarized in Table 6.

2.8.2. Biodegradation of profenofos by A. sydowii CBMAI 935 and P. raistrickii CBMAI 931 in liquid minimal medium supplemented with KNO_3

Two small slices of solid medium (1.2 cm x 1.2 cm) bearing the marine fungal mycelia were transferred to 250 mL Erlenmeyer flasks containing 100 mL of liquid mineral medium (1.25 g of KNO_3 and 100.0 mL of ASW, pH 7), previously sterilized in autoclave for 20 minutes at 121°C. Next, 100.0 ppm (37.2 μL) of commercial profenofos was added to the medium. The reaction was incubated in an orbital shaker for 30 days (130 rpm, 32°C). The extractions and analyses proceeded as described in Section 2.8. The results are summarized in Table 7.

2.8.3. Degradation of profenofos in the absence of marine fungi

To a 250 mL Erlenmeyer flask containing liquid medium (2.0 g of malt extract and 100.0 mL of ASW, pH 7), previously sterilized in the autoclave for 20 minutes at 121°C, 50.0 ppm (18.6 μL, Section 2.5) of commercial profenofos was added. The reaction was incubated in an orbital shaker for 30 days (130 rpm, 32°C). The extractions and analyses proceeded as described in Section 2.8. The results are summarized in Table 8.

2.8.4. Growth of marine fungi A. sydowii CBMAI 935 and P. raistrickii CBMAI 931 in the absence of profenofos

In a 250 mL Erlenmeyer flasks containing liquid medium (2.0 g of malt extract and 100.0 mL of ASW, pH 7), previously sterilized in the autoclave for 20 minutes at 121°C, two small slices of solid medium (1.2 cm x 1.2 cm) bearing the marine fungi was added, without any profenofos. The culture medium was incubated in an orbital shaker for 30 days (130 rpm, 32°C). The extractions and analyses proceeded as described in Section 2.8. The results are summarized in Table 9.

2.9. Biodegradation of the pesticide profenofos at various concentrations by *P. raistrickii* CBMAI 931

In four 250 mL Erlenmeyer flasks containing liquid medium (2.0 g of malt extract and 100.0 mL of ASW, pH 7), previously sterilized in the autoclave for 20 minutes at 121°C, commercial profenofos was added separately at 15.0 ppm (18.6 μL), 30 ppm (11.2 μL), 50.0 ppm (18.6 μL) and 60.0 ppm (22.3 μL). In the flasks were inoculated two small slices of solid medium (1.2 cm x 1.2 cm) bearing mycelium of *P. raistrickii* CBMAI 931. These reactions were incubated in an

orbital shaker for 30 days (130 rpm, 32°C). The extractions and analyses proceeded as in Section 2.8. The results are summarized in Table 10.

2.10. GC-MS analyses

The GC-MS system was a Shimadzu GC2010plus gas chromatograph coupled to a mass-selective detector (ShimadzuMS2010plus) in electron ionization (EI, 70 eV) mode. The GC-MS oven was fitted with a DB5 fused silica column (J&W Scientific 30m x 0.25mm x 0.25 μm). The chromatographic conditions were: initial oven temperature 100 °C (for 5 min), increased to 250 °C (for 10 min) at 5 °C/min; run time 45.0 min; injector temperature 200 °C; detector temperature 200 °C; injector split ratio 1:1; helium carrier gas at a pressure of 60 kPa. The analytes were first analyzed in SCAN mode in order to select the ion and the retention time for each compound. The selected-ion mode (SIM) analyses were performed to measure the biodegradation of profenofos. Table 1 shows the retention time and selected ion for each compound, used in the SIM-mode analyses.

Compounds	Retention time (min)	Selected ion (*m/z*)
4-Bromo-2-chlorophenol	10.605	207.85
Chlorpyrifos	27.730	313.90
Profenofos	31.190	138.95

Table 1. Method data of the SIM-mode analyses.

3. Results and discussion

3.1. Screening marine fungi on solid medium

The strains studied were the filamentous marine fungi *Aspergillus sydowii* CBMAI 934, *Aspergillus sydowii* CBMAI 935, *Aspergillus sydowii* CBMAI 1241, *Penicillium decaturense* CBMAI 1234, *Penicillium raistrickii* CBMAI 931, *Penicillium raistrickii* CBMAI 1235 and *Trichoderma* sp. CBMAI 932. These are multicellular microorganisms, which grow as mycelia, composed by branching microscopic filament named hyphae. Fungi were grown on solid medium at pH 5, which is a good pH for the cultivation of most fungi, while the optimum may vary from 3.8 to 5.6. These pH values favor fungi growth and inhibit growth of most bacteria, which optimal culture condition is at higher pH (Pelczar *et al.*, 1997).

Initially, the biotransformation of profenofos by marine fungi was conducted on solid culture media. The microorganisms were grown on Petri dishes containing 2% malt extract and artificial seawater (ASW). All the strains investigated were analyzed in the presence and absence of the profenofos pesticide, in duplicate tests. Fungi with biocatalytic potential to degrade profenofos were screened by comparing the growth of fungal colonies on Petri dishes at several concentrations of the pesticide and in its absence (control). Volumes of profenofos

added to the solid cultures were 5.0, 10.0 and 15.0 μL per Petri dish, corresponding to concentrations of 80.0, 160.0 and 240.0 ppm, respectively (Table 2).

After 10 days of growth at 35 °C, the colony diameters were measured and the average diameter (cm) of the colonies formed on each Petri dish was recorded. Since most of the colonies showed non-circular radial growth (Figure 5), they were measured between the furthest points. Figure 5 summarizes the qualitative results of the marine fungi growth on solid culture media in the absence and presence of profenofos, for the strains which growth-better.

When several colonies grow in a Petri dish, one colony can compete and/or inhibit the growth of another. In this experiment on solid medium, it was important to assess fungal growth on the plate surface to detect the presence or absence of microbial growth. However, the measurement of colonies had no quantitative purpose, and the test was done only to estimate the fungal growth.

Marine fungi	Colony diameter (cm)			
	Control culture	**80.0 ppm[a]**	**160.0 ppm[b]**	**240.0 ppm[c]**
Aspergillus sydowii CBMAI 934	4.0 x 3.0	1.0 x 1.5	1.0 x 1.0	1.0 x 1.0
Aspergillus sydowii CBMAI 935	Whole plate*	3.0 x 2.5	3.0 x 2.5	3.0 x 2.5
Aspergillus sydowii CBMAI 1241	3.5 x 2.5	3.0 x 2.5	3.0 x 2.5	2.0 x 2.0
Penicillium decaturense CBMAI 1234	2.5 x 2.5	1.0 x 1.5*	3.0 x 2.0	1.0 x 1.0*
Penicillium raistrickii CBMAI 931	3.5 x 3.0	3.0 x 3.0	3.0 x 2.5	2.5 x 2.5
Penicillium raistrickii CBMAI 1235	4.0 x 3.0	2.0 x 2.0	1.0 x 1.0	1.5 x 1.0
Trichoderma sp. CBMAI 932	Whole plate*	3.5 x 2.5	3.0 x 2.0	2.0 x 2.0

*Estimated measure, because the number of spores did not allow observation of the set of colonies
[a]5.0 μL profenofos and 100.0 μL DMSO
[b]10.0 μL profenofos and 200.0 μL DMSO
[c]15.0 μL profenofos and 300.0 μL DMSO

Table 2. Growth of marine fungi on solid agar medium of malt extract 2% with absence and addition of profenofos (35 °C, 10 days, pH 5).

Fungal development and growth requires a variety of inorganic and organic nutrients in the medium. Carbon is one of the most important elements for microbial growth, as carbon compounds provide energy for cell growth and serve as the basic units to build the cell materials. Nitrogen is also essential to the organisms, as well as other elements (hydrogen, oxygen and phosphorus) (Pelczar *et al.*, 1997). Thus, fungal growth in the presence of pesticides may indicate fungal tolerance to the pesticide toxicity; pesticide metabolism as a mechanism of defense of the microorganism to eliminate the xenobiotic compound; or even pesticide use as a source of nutrient for fungal growth, since the organophosphate pesticide profenofos has carbon, oxygen, sulfur and phosphorus in its structure.

In the screening of fungal strains on solid medium, in the presence of profenofos, excepting by the marine fungi *A. sydowii* CBMAI 934 and *P. raistrickii* CBMAI 1235, all other microorganisms (*P. raistrickii* CBMAI 931, *A. sydowii* CBMAI 935, *A. sydowii* CBMAI 1241 and *Tricho-*

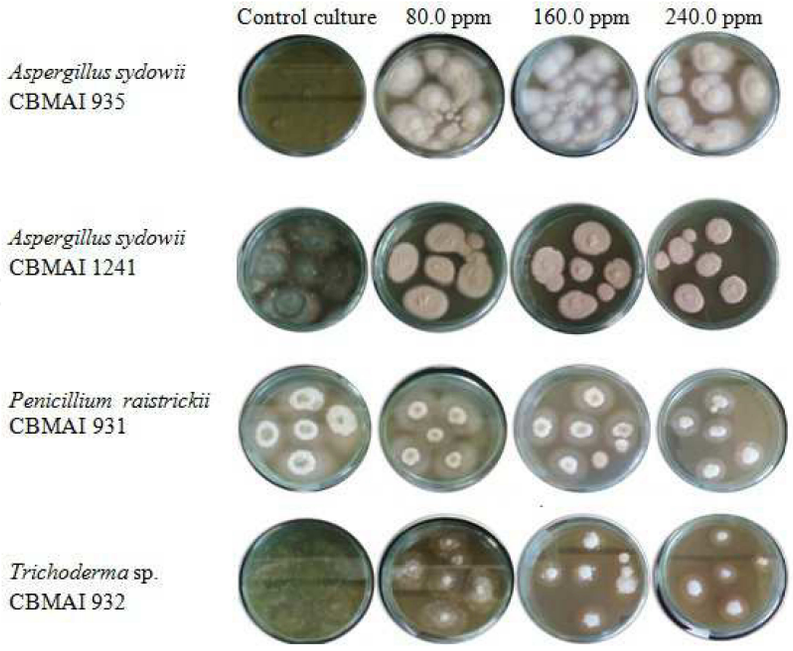

Figure 5. Marine fungi growing on solid culture medium containing various concentrations of profenofos pesticide (10 days at 35°C)

derma sp. CBMAI 932) showed excellent growth in the presence of the pesticide at all concentrations after 10 days, as shown in Table 2. Compared to the control culture, there was a slight inhibition of the cultures by the largest amount of pesticide (15.0 μL profenofos) (Figure 5). Since fungal growth was satisfactory in the highest concentration of the pesticide, it was possible to suggest that these strains showed good potential for biocatalytic degradation of profenofos.

There was a difference between the growth on the plate with 10.0 μL of pesticide and the other amounts, for the fungus *P. decaturense* CBMAI 1234. In this Petri dish, there was no sporulation and colonies were well defined, with no significant inhibition of growth in comparison with the control plate. Strains of *A. sydowii* CBMAI 934 and *P. raistrickii* CBMAI 1235 showed only a slight growth, compared to the other fungi. However, these last three fungi were able to grow even at higher concentrations of the pesticide (Table 3).

Finally, after this screening on solid culture medium, the strains of *P. raistrickii* CBMAI 931 and *A. sydowii* CBMAI 935 were selected to investigate and quantify the biodegradation of profenofos in liquid culture medium.

3.2. Analytical curves to determine the concentration of profenofos and 4-bromo-2-chlorophenol by GC-MS-SIM analysis

The OPs are particularly amenable to biodegradation because they are susceptible to hydrolysis by enzymes (Chen and Mulchandani, 1998). The best known enzymes that promote hydrolysis of OPs are phosphotriesterases (Ghanem and Raushel, 2005). The expected metabolite from hydrolysis of profenofos is 4-bromo-2-chlorophenol. Therefore, if this

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metabolite is a product of the mycelial reaction, enzymes were possibly active in the mycelial mass of the marine fungi *P. raistrickii* CBMAI 931 and *A. sydowii* CBMAI 935. Analytical curves by the internal standard method of GC-MS-SIM analysis were constructed in order to determine the concentration of the active ingredient profenofos in the commercial pesticide, the expected degradation product (4-bromo-2-chlorophenol) and the residual profenofos during the reaction. By analyzing, in the SCAN mode, a sample containing 4-bromo-2-chlorophenol (metabolite of profenofos), chlorpyrifos (internal standard) , the ion selected and retention time for each compound was determined (Figure 6, Table 1). For 4-bromo-2-chlorophenol (*m/z* 207.85) and chlorpyrifos (*m/z* 313.90), the molecular ions of each analyte was selected, while the base peak ion (*m/z* 138.95) was selected for profenofos. All samples were analyzed in SIM mode for quantification measurements and SCAN mode in the mass range of 50-550 u.m.a. for confirmation of the molecules identities.

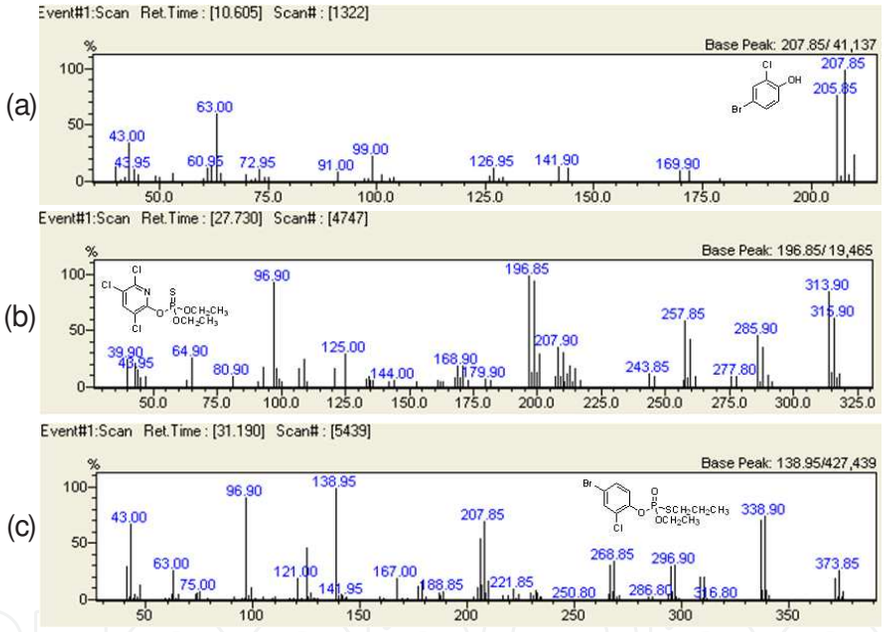

Figure 6. Mass spectra for analyses of the fragmentation patterns to select ions in the SCAN mode. (a) 4-Bromo-2-chlorophenol, (b) Chlorpyrifos (c) Profenofos

The internal standard technique is a useful method for minimizing errors due to variations in the used equipments. A substance used as an internal standard should be similar to the analyte, with a similar retention time, not react with another substance or matrix component, not be a part of the test sample and have a retention time different from those of the other substances in the sample (Ribani *et al.*, 2004). The pesticide chlorpyrifos (analytical grade) was used as the internal standard for the determination of profenofos and its metabolite. A graph was produced, the area ratio (area of the substance / area of the internal standard) versus the

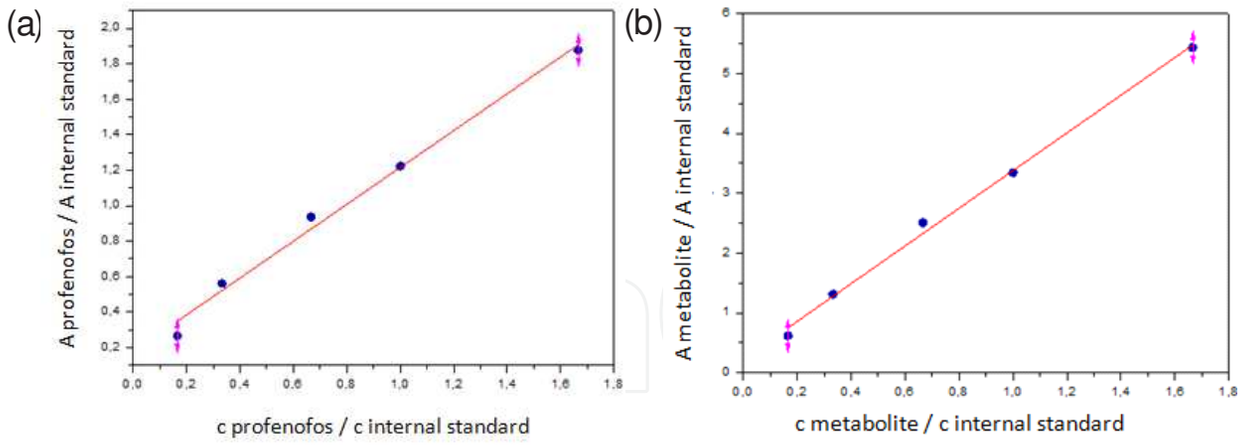

Figure 7. Analytical curves for (a) profenofos, (b) 4-bromo-2-chlorophenol

concentration ratio (variable concentration of substance / constant concentration of the internal standard) (Ribani *et al.*, 2004). This analytical curve was constructed for profenofos and 4-bromo-2-chlorophenol (metabolite of profenofos) at concentrations of 5.0, 10.0, 15.0, 20.0, 30.0 and 50.0 ppm (Figure 7).

The analytical curve for profenofos fitted by the linear equation y = 1.03965 x + 0.17522, with correlation coefficient r = 0.9955, and the one for the metabolite was fitted by the line y = 3.15088x + 0.2272, with correlation coefficient r = 0.99811.

The Brazil´s regulatory agency ANVISA recommends a correlation coefficient of 0.99; thus, the correlation coefficients obtained for the two analytical curves are within the parameters established in the literature (Ribani *et al.*, 2004).

3.3. Determination of the active ingredients concentration in the profenofos commercial sample

According to information from Syngenta® (Syngenta, 2012), the composition of the pesticide Polytrin, used in this study, was:

Inert ingredients: 560.00 g. L^{-1} (56.0% w/v)

Profenofos: 400.00 g. L^{-1} (40% w/v)

Cypermethrin: 40.00 g. L^{-1} (4% w/v)

The amount of active ingredient present in the working sample was measured, in order to develop reactions of biodegradation with the commercial sample of profenofos. To determine the volume of pesticide profenofos required to give a concentration of 50.0 ppm in the reaction, analyses were performed in duplicate with an arbitrary amount of pesticide (20.0 μL). The analytical data yielded 54.0 ppm for the concentration of active ingredient in 100 mL of medium.

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The results were in good agreement and showed that, to obtain a final concentration of 50.0 ppm in 100.0 mL of liquid culture medium, 18.6 μL of commercial profenofos must be added. According to these data, the total concentration of active ingredient (profenofos) in the working sample was approximately 320.0 $g.L^{-1}$.

3.4. Biodegradation of profenofos by marine fungi *P. raistrickii* CBMAI 931 and *A. sydowii* CBMAI 935

The inocula used for the profenofos biodegradation reactions were activated in Petri dishes containing 2% of malt extract solid medium and 50.0 ppm of the pesticide, in order to induce the production of phosphotriesterases or other enzyme classes (e.g., CbE = carboxylesterase) capable of degrading the OP. Enzyme induction occurs at the gene transcription level. Gene transcription is the first step in the flow of genetic information and, for this reason, gene expression is relatively easily affected at this point (Madigan *et al.,* 2010; Tortora *et al.,* 1995). An inducible enzyme is synthesized only when its substrate is present in the sample; hence, the inoculum was grown in the presence of profenofos, so that the enzymes of interest were already being expressed when the fungi were transferred to the liquid medium, to catalyze the biodegradation reactions.

The pH of the liquid medium was adjusted to 7, bearing to reports in the literature indicating that phosphotriesterases exhibit enhanced catalytic activity at neutral to basic pH. According to Eivazi and Tabatabal, hydrolysis of the pesticide paraoxon with animal enzymes showed good catalytic activity at pH 7.3. Assays of activity by the release of *p*-nitrophenol showed optimal activity at pH 7-11 (Eivazi and Tabatabai, 1977). The hydrolysis of organophosphates in the environment (in the absence of enzymes) is also affected by pH: the more alkaline the medium, the faster is the hydrolysis. According to Zamy *et al.* the half-life of profenofos in phosphate buffer at pH 8 is fifteen days, the pesticide being hydrolyzed in this buffer. Thus, the period in which the pesticide should be completely biodegraded by hydrolysis is considerable; however, the presence of enzymes would accelerate the process of degradation (Zamy *et al.*, 2004).

The step of mycelium extraction was important because the fungi, as well as bacteria, can absorb compounds with the aid of enzymes secreted into medium, which break or carry the complex organic molecules into the cells (Pelczar *et al.*, 1997). Thus, the extraction with magnetic stirring was used to extract both the pesticide that may be inside the mycelium (since this extraction causes the cell disruption) and adhered to surface of the cell membrane.

3.4.1. Biodegradation of profenofos by marine fungi P. raistrickii CBMAI 931 and A. sydowii CBMAI 935 after 10 days of reaction

At 10 days of reaction, the extracts of the mycelium and liquid medium were subjected to separate analysis by GC-MS-SIM. In Figures 8 and 9 chromatograms of each extraction are shown, with the analyses of the superimposed duplicates. The data concerning biodegradation of profenofos by fungal strains of *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931 are summarized in Table 3.

The duplicate reactions in the experiments with the fungus *A. sydowii* CBMAI 935 agreed well with each other: 53.0% and 45.0% of profenofos degraded. However, the duplicate reactions with *P. raistrickii* CBMAI 931, showed a significant discordance between for each reaction (81.4% and 48.0% degraded). As the growth behavior of the fungus varies for each experiment, other factors may have interfered and caused the difference between the results. It should also be noted that although the reactions are performed in duplicate, they occur independently and are, therefore, unique reactions.

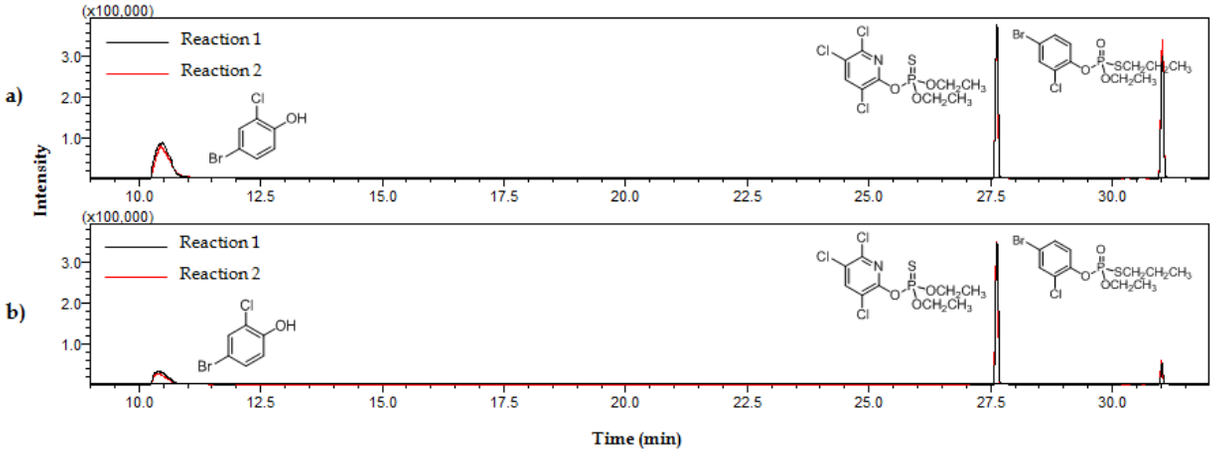

Figure 8. GC-MS-SIM analyses: Chromatogram of biodegradation of 50.0 ppm profenofos by *A. sydowii* CBMAI 935, at 10 days. a) Extract of the mycelium. b) Extract of the liquid medium

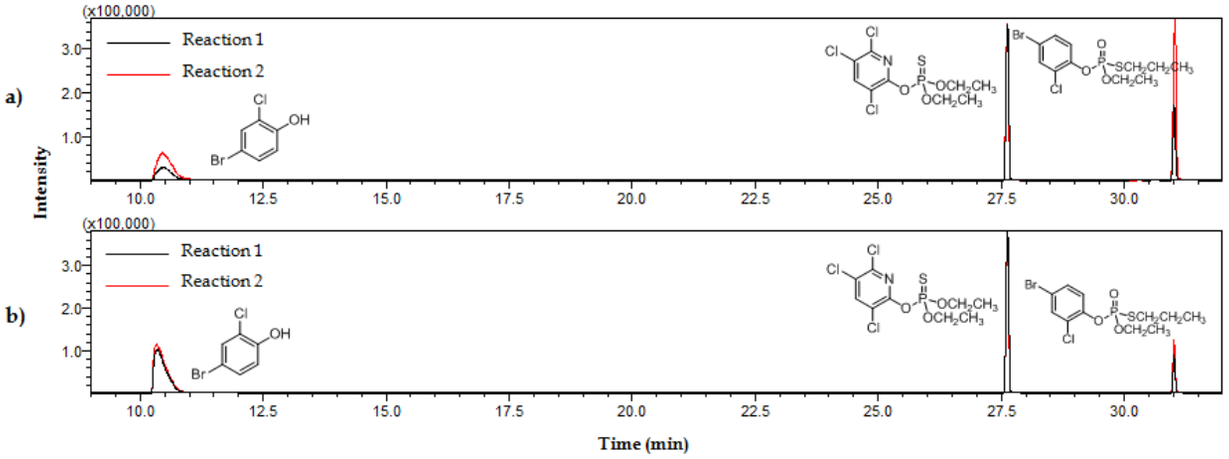

Figure 9. GC-MS-SIM analyses: Chromatogram of biodegradation of 50.0 ppm profenofos by *P.raistrickii* CBMAI 931, at 10 days. a) Extract of the mycelium. b) Extract of the liquid medium

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A. sydowii CBMAI 935 (50.0 ppm of profenofos)				
Duplicate reaction	**Fungal dry mass (g)**	**c[a] metabolite[b]**	**c[a] profenofos**	**% of profenofos degraded***
Reaction 1 (Extraction of liquid medium)	-	2.1	2.7[c]	53.0
Reaction 1 (Extraction of mycelium)	0.96	8.5	20.8	
Reaction 2 (Extraction of liquid medium)	-	1.4	2.4[c]	45.0
Reaction 2 (Extraction of mycelium)	0.64	7.7	25.1	
P. raistrickii CBMAI 931 (50.0 ppm of profenofos)				
Reaction 1 (Extraction of liquid medium)	-	8.4	1.1	81.4
Reaction 1 (Extraction of mycelium)	0.29	1.7	8.2	
Reaction 2 (Extraction of liquid medium)	-	9.3	3.0	48.0
Reaction 2 (Extraction of mycelium)	0.36	1.6	23.0	

[a]c (ppm) = concentration (data duplicates) determined by GC-MS-SIM

[b]4-bromo-2-chlorophenol

[c]estimated concentration

*total of profenofos degraded (mycelium + liquid medium)

Table 3. Quantitative biodegradation of profenofos by *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931, at 10 days, in liquid medium and mycelium (32 °C, 130 rpm, pH 7).

The concentrations of profenofos in the extracts of the liquid culture medium from the *A. sydowii* CBMAI 935 reaction were estimated by straight line extrapolation, since, owing to the low concentrations in the extracts, the peak areas for profenofos were not sufficient to be applied to the analytical curve; thus these values are only estimates.

In the GC-MS-SIM analyses, superimposing the mycelium and liquid culture medium extract profiles for each fungus, it was noted a higher concentration of the pesticide in the mycelium extract than in the liquid medium one. In GC-MS-SIM analyses of *P. raistrickii* CBMAI 931 it was also clear that the highest concentration of the metabolite (4-bromo-2-chlorophenol) was in the liquid medium. These data may suggest that the fungal mycelium are absorbing profenofos molecules and, after metabolization, excreting a part of the metabolite into the liquid medium. Rather than being absorbed it is also possible that these molecules are adsorbed to fungal cells membranes. A previous study conducted by us showed that the pesticide DDD was accumulated in the mycelium of the marine fungus *Trichoderma* sp. CBMAI 932 (Ortega *et al., 2011*). The higher concentration of profenofos in the mycelium could be explained by an intracelullar enzyme degrading profenofos. According to Chen and Mulchandani, the

organophosphate hydrolase enzyme is found within cells and, for biodegradation to occur, the pesticide should be transported into the interior of the cell. However, this kind of enzyme may limit biodegradation, because for microorganisms containing a high intracellular activity of degradative enzymes, total detoxification may be limited by the transport mechanism (Chen and Mulchandani, 1998).

3.4.2. Biodegradation of profenofos by marine fungi P. raistrickii CBMAI 931 and A. sydowii CBMAI 935 after 20 days of reaction

The reactions that were performed for 20 days were analyzed by making a single extract from the liquid medium along with the mycelia extract that was subjected to GC-MS-SIM analyses. Table 4 shows data regarding the biodegradation of profenofos for 20 days by strains *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931 in liquid medium.

The results for both marine fungi showed a good percentage of biodegradation of the pesticide profenofos at 20 days of reaction. However, *P. raistrickii* CBMAI 931 was more efficient than *A. sydowii* CBMAI 935, reaching an approximately average of 82% profenofos degradation, whereas *A. sydowii* CBMAI 935 showed degradation of approximately 71%.

A. sydowii CBMAI 935 (50.0 ppm of profenofos)				
Duplicate reaction	**Fungal dry mass (g)**	**c[a] metabolite[b]**	**c[a] profenofos**	**% of profenofos degraded**
Reaction 1 (Extraction of liquid medium and mycelium)	0.36	5.5	13.0	74.0
Reaction 2 (Extraction of liquid medium and mycelium)	0.33	4.9	16.3	67.4
P. raistrickii CBMAI 931 (50.0 ppm of profenofos)				
Reaction 1 (Extraction of liquid medium and mycelium)	0.19	13.8	11.0	78.0
Reaction 2 (Extraction of liquid medium and mycelium)	0.21	11.0	7.4	85.2

[a]c (ppm) = concentration (data duplicates) determined by GC-MS-SIM

[b]4-bromo-2-chlorophenol

Table 4. Quantitative biodegradation of profenofos by *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931, at 20 days in liquid medium (32 °C, 130 rpm, pH 7).

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3.4.3. Biodegradation of profenofos by marine fungi P. raistrickii CBMAI 931 and A. sydowii CBMAI 935 after 30 days of reaction

Finally, profenofos biodegradation reactions using marine fungi *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931 were performed for 30 days (Table 5, Figure 10). In this study, the following experiments were also carried out:

a. 30-day reaction containing only 4-bromo-2-chlorophenol (main metabolite) as the substrate, with the objective of assessing the biocatalytic potential of these marine fungi for complete degradation of the pesticide into non-toxic metabolites (Table 6, Figure 11);

b. 30-day reaction in mineral medium supplemented with potassium nitrate in order to assess whether the fungi are able to grow on medium with the pesticide as the sole carbon source, and nitrate as the nitrogen source (Table 7);

c. 30-day reaction for profenofos, in the absence of fungal mycelium, control experiment in order to determine the spontaneous rate of hydrolysis of the pesticide in the liquid medium (Table 8);

d. 30-day reaction with the fungi, in the absence of pesticide, control experiment in order to determine the growth of the marine fungi by measuring the mycelial mass produced without the pesticide influence (Table 9).

A. sydowii CBMAI 935 (50.0 ppm of profenofos)				
Duplicate reaction	**Fungal dry mass (g)**	**c[a] metabolite[b]**	**c[a] profenofos**	**% of profenofos degraded**
Reaction 1 (Extraction of liquid medium and mycelium)	0.38	12.0	12.5	75.0
Reaction 2 (Extraction of liquid medium and mycelium)	0.39	11.0	16.0	68.0
P. raistrickii CBMAI 931 (50.0 ppm of profenofos)				
Reaction 1 (Extraction of liquid medium and mycelium)	0.22	17.8	2.3*	95.4
Reaction 2 (Extraction of liquid medium and mycelium)	0.20	21.4	0.6	98.8

[a]c (ppm) = concentration (data duplicates) determined by GC-MS-SIM

[b]4-bromo-2-chlorophenol

*estimated concentration

Table 5. Quantitative biodegradation of profenofos by *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931, at 30 days in liquid medium (32 °C, 130 rpm, pH 7).

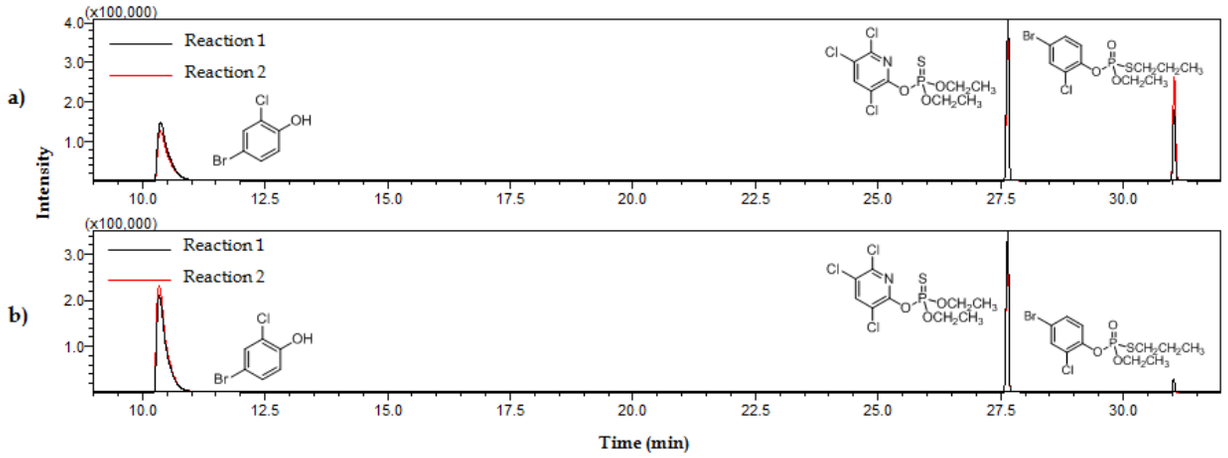

Figure 10. GC-MS-SIM analyses: Chromatogram of biodegradation of profenofos (50.0 ppm) at 30 days. a) *A. sydowii* CBMAI 935. b) *P. raistrickii* CBMAI 931.

A. sydowii CBMAI 935 (50.0 ppm of 4-bromo-2-chlorophenol)			
Duplicate reaction	**Fungal dry mass (g)**	**c[a] metabolite[b]**	**% of metabolite degraded**
Reaction 1 (Extraction of liquid medium and mycelium)	0.39	0.8	98.4
Reaction 2 (Extraction of liquid medium and mycelium)	0.37	0.9	98.2
P. raistrickii CBMAI 931 (50.0 ppm of 4-bromo-2-chlorophenol)			
Reaction 1 (Extraction of liquid medium and mycelium)	0.30	1.8	96.4
Reaction 2 (Extraction of liquid medium and mycelium)	0.35	1.2	97.6

[a]c (ppm) = concentration (data duplicates) determined by GC-MS-SIM

[b]4-bromo-2-chlorophenol

Table 6. Quantitative biodegradation of 4-bromo-2-chlorophenol (main metabolite) by *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931, at 30 days in liquid medium (32 °C, 130 rpm, pH 7).

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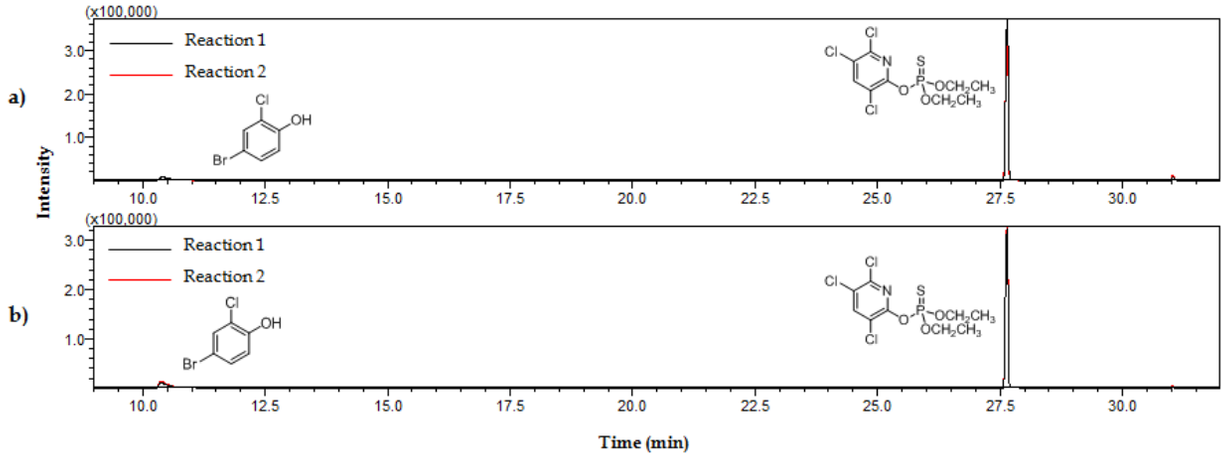

Figure 11. GC-MS-SIM analyses: Chromatogram of biodegradation of 4-bromo-2-chlorophenol (50.0 ppm) at 30 days. a) *A. sydowii* CBMAI 935. b) *P. raistrickii* CBMAI 931

Profenofos concentrations for reaction in liquid medium with 2% malt extract and reaction in mineral medium, in the presence of *P. raistrickii* CBMAI 931, were estimated by straight line extrapolation of the analytical curve for profenofos. The same was done for all concentrations of the metabolite, in the reaction with the fungi *P. raistrickii* CBMAI 931 and *A. sydowii* CBMAI 935.

A. sydowii CBMAI 935 (100.0 ppm of profenofos)				
Duplicate reaction	**Fungal dry mass (g)**	**c[a] metabolite[b]**	**c[a] profenofos**	**% of profenofos degraded**
Reaction 1 (Extraction of liquid medium and mycelium)	*	39.5	3.7	92.6
Reaction 2 (Extraction of liquid medium and mycelium)	*	38.0	5.7	88.6
P. raistrickii CBMAI 931 (100.0 ppm of profenofos)				
Reaction 1 (Extraction of liquid medium and mycelium)	*	46.3	2.2[c]	95.6
Reaction 2 (Extraction of liquid medium and mycelium)	*	44.8	1.8[c]	96.4

[a]c (ppm) = concentration (data duplicates) determined by GC-MS-SIM

[b]4-bromo-2-chlorophenol

[c]estimated concentration

*mycelial mass not obtained

Table 7. Quantitative biodegradation of profenofos by *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931, at 30 days, in liquid mineral medium supplemented with KNO_3 (32 °C, 130 rpm, pH 7).

Duplicate reaction	c[a] metabolite[b]	c[a] profenofos	% of profenofos degraded
Reaction 1 (Extraction of liquid medium and mycelium)	3.3	30.2	39.6
Reaction 2 (Extraction of liquid medium and mycelium)	4.2	31.0	38.0

[a]c (ppm) = concentration (data duplicates) determined by GC-MS-SIM

[b]4-bromo-2-chlorophenol

Table 8. Quantitative degradation of profenofos by spontaneous hydrolysis, at 30 days in liquid medium (32 °C, 130 rpm, pH 7).

A. sydowii CBMAI 935	
Duplicate reaction	**Fungal dry mass (g)**
Reaction 1	1.50
Reaction 2	1.49
P. raistrickii CBMAI 931	
Reaction 1	1.40
Reaction 2	1.23

Table 9. Quantitative mycelia mass produced by *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931, in absence of profenofos, at 30 days in liquid medium (32 °C, 130 rpm, pH 7).

It was observed that the biodegradation of profenofos by *A. sydowii* CBMAI 935 exhibited stagnation between 20 and 30 days of reaction, as can be seen by comparing Tables 4 and 5 data. However, between 20 and 30 days, there was a significant increase in the metabolite concentration. According to literature data, the organophosphates hydrolysis proceeds by breaking the bond between the phosphorus atom and the leaving group (Sogorb and Vilanova, 2002; Bigley and Raushel, 2012). Thus, the concentration of profenofos degraded should be about the same as the concentration of metabolite formed, where there is total degradation of the pesticide to the metabolite. However, according the United Nations Food and Agriculture Organization (FAO), after the hydrolysis and formation of 4-bromo-2-chlorophenol, the latter can be conjugated with another molecule, can react with a molecule from the fungal metabolism or even be metabolized (Figure 4) (FAO, 2012).

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So, a possible explanation for the incomplete conversion of reactants to products may be the 4-bromo-2-chlorophenol further degradation or the conversion to other metabolites by *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931. However, at 30 days of reaction, the biodegradation of the pesticide was stagnant (compared to 20 days), so that stagnation in the degradation or conversion of the metabolite may also have occurred, causing a slight accumulation at 30 days of reaction. This stagnation may be caused either by fungal death or reaching its stationary growth phase, after one of the nutrients in the liquid medium became scarce.

The final concentrations of profenofos in 30 days biodegradation reactions with *P. raistrickii* CBMAI 931 were estimated because of the very low concentration obtained, as previously mentioned. The biodegradation of the pesticide in the presence of this fungus was almost complete, suggesting that this has a greater biocatalytic potential than *A. sydowii* CBMAI 935 for profenofos degradation.

The results for the biodegradation of the metabolite were satisfactory, since there was an almost complete degradation (or conversion) of 4-bromo-2-chlorophenol by both fungi (Table 6). However, it was not possible to identify the other metabolites formed in this degradation.

Complementing the biodegradation studies, an experiment was conducted in liquid mineral medium supplemented with potassium nitrate that demonstrated, through the high percentage of degradation of profenofos, that the fungi could be using the pesticide as a source of carbon, since this was the sole carbon source present in the reaction medium. The concentration of pesticide in the mineral medium was 100.0 ppm, higher than in the earlier tests, since the pesticide was the sole source of carbon, it was needed a high concentration for the fungi growth. There was a greater final concentration of the metabolite, which could be partially degraded / converted in other molecules, since only 50% of the pesticide was converted to this product.

Through the control reaction of profenofos, in the absence of the fungus (and hence without enzymes), the spontaneous hydrolysis of the pesticide in the medium was assessed. This experiment revealed degradation of about 40%, indicating that this pesticide is not persistent in the environment, since a half-life of about a month is relatively low compared to other pesticides, such as organochlorines. However, approximately 60% of the pesticide was not degraded, showing that the enzymatic process is highly effective for promoting the biodegradation of profenofos. It should be noted, also, that the spontaneous hydrolysis does not promote the degradation of the metabolite, as does the enzymatic system (Table 8).

Aly and Badway discussed the hydrolysis of profenofos at 20°C with buffered solutions at pH 5, 7 and 9. A loss of 50%occurred in 106 days at pH5, 43 days at pH7 and 0.7 days at pH 9. Rate constants and half-lives (t1/2) revealed that this insecticide was relatively stable in acid medium and its stability decreased in higher pHs. The studies showed that the mode of decomposition of profenofos in acidic and neutral media is dealkylation, but in an alkaline medium it undergoes hydrolysis, resulting in substituted phenol and dialkyphosphoric acid compounds (Figure 12) (Ali and Badawy, 1982; Ahmed, 2012).

In the control reaction of *A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931, in the absence of the pesticide, there was a much greater mycelia growth indicating that the pesticide can partially inhibit the growth of fungi, as noted previously in the screenings carried out on solid medium (Table 9).

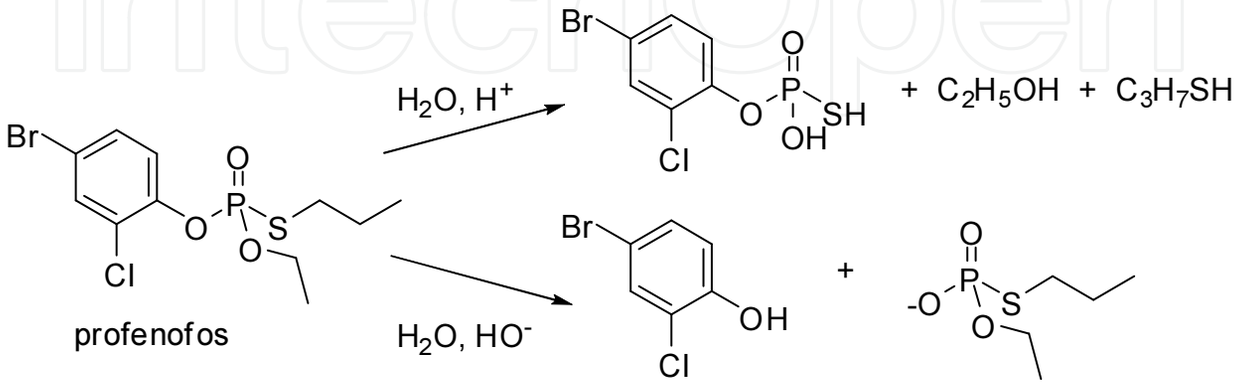

Figure 12. Degradation of profenofos in aqueous medium

3.4.4. Biodegradation of the pesticide profenofos by P. raistrickii CBMAI 931 at several concentrations

Aiming to evaluate the biodegradation of the pesticide profenofos at various concentrations, this test was carried out with variations of the initial concentration under standard biodegradation reaction (liquid culture medium with malt + profenofos + fungal inoculum for 30 days). The reactions were performed in duplicate, at concentrations of 15.0, 30.0, 50.0 and 65.0 ppm, and also a pesticide control (liquid culture medium + profenofos) was carried out and average results are shown in Table 10.

$c_{initial}$ [a] profenofos	c_{final} [a] profenofos*	c[a] metabolite[b]	%of profenofos degraded
15.0	0.5	4,90	98
30.0	0.6	20,40	98
50.0	2.5	24,1	95
65.0	3.2	27,9	95
30.0 (control)	14.9	10,8	50

[a]c (ppm) = concentration (data in duplicates) determined by GC-MS-SIM

[b]4-bromo-2-chlorophenol

Table 10. Quantitative biodegradation of profenofos by *P. raistrickii* CBMAI 931 in different concentrations, at 30 days in liquid medium (32 °C, 130 rpm, pH 7).

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Almost complete biodegradation was observed at lower initial concentrations of the pesticide (15.0 and 30.0 ppm). At higher concentrations (50.0 and 65.0 ppm), the results were also satisfactory, with 95% degradation of profenofos. This test proved that in liquid medium, as well as on solid media, the fungus *P. raistrickii* CBMAI 931 was resistant to higher concentrations of pesticide and showed an excellent potential for biodegradation of profenofos.

In the profenofos control, in the absence of the fungus, at a concentration of 30.0 ppm was observed a degradation of only 50% in a period of 30 days, confirming that the presence of fungi accelerates degradation reaction, possibly through the action of phosphotriesterases enzymes. The fungus also promoted the degradation or conversion of the part of the metabolite.

4. Conclusion

The growth of fungal strains on profenofos was promising, even at the highest tested concentration. The fungi *P. raistrickii* CBMAI 931, *A. sydowii* CBMAI 935, *A. sydowii* CBMAI 1241 and *Trichoderma* sp. CBMAI 932 may have a good biocatalytic potential in the presence of profenofos, according to the results of screening. Marine fungi should be further explored as sources of enzymes capable of degrading OPs, since studies in fungal bioremediation of pesticides has shown great potential, albeit much less explored than bacterial bioremediation.

The fungi *P. raistrickii* CBMAI 931 and *A. sydowii* CBMAI 935 were efficient in profenofos biodegrading in liquid medium, as well as promoting the transformation or degradation of the metabolite, 4-bromo-2-chlorophenol, and showing that the enzymatic system of these fungi effectively expressed the necessary enzymes for a complete degradation process. Further research is under way to assess the biodegradation of methyl parathion and chlorpyrifos.

Liquid medium reactions using *P. raistrickii* CBMAI 931 with increasing concentration of the pesticide leaded to the almost complete biodegradation (99.0 to 95.0%) of the pesticide profenofos at all concentrations (15.0, 30.0, 50.0 and 65.0 ppm), showing that this fungus is resistant to high concentrations of this pesticide. The fungus *P. raistrickii*-CBMAI 931 may be a good source of phosphotriesterases, which could be isolated and purified for applications in biotechnology, biodegradation reactions in soil and water contaminated with pesticides.

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