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Chapter 9

Screening of Marine-derived Fungi Isolated from the sponge *Didemnun ligulum* for Biodegradation of Pentachlorophenol

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60777

Abstract

Contamination by pesticides employed in agriculture has caused serious environmental harm. Pentachlorophenol (PCP) is a phenolic organochlorine compound and a dangerous pollutant which was banned from Brazil since 1985; however, there are still many contaminated areas. This pesticide is a serious problem because it has high toxicity and persistence at the environment due to its resistance to biotic and abiotic degradation. The use of microorganisms as degrading agents is considered an efficient method to reduce the adverse effects of environmental contaminants. It is noteworthy that fungi from marine environment are adapted to extreme conditions, including high chlorine concentrations, and can produce unique enzymes with interesting properties. Therefore, marine-derived fungi have an excellent enzymatic potential for the biotransformation of xenobiotics such as organochlorine pesticides. In this work, fifteen fungi strains isolated from a marine invertebrate, the ascidian *Didemnun ligulum*, were evaluated according to their ability to grow in solid culture media (3% malt extract agar) in the presence of different concentrations (10, 25, 30, 40, and 50 mg L^{-1}) of PCP. Among the tested strains, nine could grow in at least one concentration, and *Trichoderma harzianum* CBMAI 1677 showed optimal growth at the higher evaluated concentration (50 mg L^{-1}), showing toxicity resistance and suggesting its potential for biodegradation of PCP. In a later work, it was observed that *T. harzia-*

num CBMAI 1677 was able to degrade PCP. These results confirmed the efficiency of marine-derived fungi to biodegrade persistent compounds and could enable the development of bioremediation methodologies using these microorganism.

Keywords: Organochlorine pesticide, Agrochemicals, Marine Microorganisms, Biotransformation

1. Introduction

1.1. Pesticides

Pesticides are pure substances or mixtures of chemicals used to control undesired organisms during production, harvest, and food storage. These compounds can be organic or inorganic molecules classified according to their chemical structure or type of the target organism [1]. They can be introduced into the environment during their manufacturing, application, or subsequent leaching affecting target and nontarget organisms [2]. The term pesticide, used in this chapter, is a synonym of biocide, agrotoxic, and agrochemical, though, there are more specific definitions that include and exclude different chemicals groups [3]. Regardless of the term used, these compounds act by blocking a vital metabolic process of the target organism [4].

The use of different toxic substances against pests and diseases is dated from antiquity. Different natural products such as nicotine, pyrethrum, tobacco plants extracts (*Nicotiana tabacum* L.) [5] and inorganic compounds such as mercury and sulfur were employed in ancient times [6]. The modern use of pesticides is dated from the twentieth century with the intensive use of inorganic substances like sodium aceto arsenite, calcium fluoride, white arsenic, and others [7]. Since the 1930s, the increased agricultural production demanded the formulation and use of substances with best biocide action [7]. Intensive development of the chemical industry occurred with the Industrial Revolution, which led to an increase in the research, and consequently, the production of new pesticides, which was expanded on a global scale after 1940s [8].

The cultivated area increasing and need for higher agricultural productivity stimulated the use of pesticides, mainly in Brazil. In this sense, the use of pesticides in Brazilian agriculture began in the 1970s encouraged by the National Development Plan (in Portuguese, Plano Nacional de Desenvolvimento) [9]. In 2011, the pesticide market in this country was considered the largest in the world, representing 16% of the global market according to the National Health Surveillance Agency (in Portuguese, Agência Nacional de Vigilância Sanitária, ANVISA) [10].

Over the past 50 years, pesticides had been used to increase the food quantity and quality for a growing world population. While worries about their adverse effects in nontarget organisms, including humans, had been also increased [11]. These chemicals, while having a beneficial effect toward agricultural production, are alien to nature and can produce changes and imbalances [12]. Many of them are toxic not only to insects and harmful pests but also to other

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living beings that are essential to several environmental processes [6]. Different reactions may act in these chemicals affecting their fate and behavior during natural processes [13]. Therefore, pesticides may be one of the most dangerous contaminants to the environment, since they are very toxic, can bioaccumulate, and be part of chemical, physical, and biological processes in nature.

Pesticides used in agriculture remain in the soil at the application site, or are transported to different parts of the environment, such as sediments, plants, surface and ground waters, marine environments and even volatilized into the atmosphere, depending on their physical-chemical properties [14-16]. The metabolic fate of the pesticides also depends on the abiotic environmental conditions (temperature, pH, soil moisture), the microbial community, the pesticide characteristics (hydrophilicity, degree of solubility, molecular weight), and the chemical and biological reactions [18]. Once they entered in the soil, pesticides are transferred or degraded by evaporation, leching, infiltration, adsorption, absorption in inorganic matter and biotic and abiotic degradation [17]. The abiotic degradation occurs through physical and chemical transformations in reactions of hydrolysis, oxidation, reduction, photolysis, and rearrangement [18]. However, the enzymatic transformations performed by microorganisms and plants are the major detoxification pathways [11].

Pesticides are used in several products involving herbicides, fungicides, nematicides, insecticides, fumigants, and substances used as desiccants, defoliants, and growth regulators [19]. Based on the chemical functional groups of the active ingredients, pesticides may be classified as organochlorines, organophosphates, carbamates, and pyrethroids [20]. Organochlorines, which shows high toxicity and persistence because of their resistance to biotic and abiotic degradations, are especially worrisome [21].

1.2. Organochlorine pesticides

The age of the organochlorine compounds was started in 1948 with the Nobel Prize in Physiology or Medicine delivered to Paul Müller, who condensed chlorobenzene to synthesize *p*-dichlorodiphenyltrichloroethane (DDT), a high effective insecticide [22]. Since then, new types of organochlorines compounds had been developed and extensively used (Figure 1). However, the harmful effects of those compounds, such as persistence, toxicity, and bioaccumulation had been also reported [23].

In 1962, the American biologist Rachel Carson published the book "Silent Spring" alerting for the damage that insecticides, especially the DDT, could cause. Despite having been the target of much criticism, the publication was fundamental for the prohibition of organochlorine pesticides in the United States in the early 1970s [17]. Although the use of organochlorine pesticides in agriculture was banned, elimination methods are still studied since these compounds had been widely used from 1960 to 1980, and thus, a toxic waste accumulation occurred in various ecosystems around the world [24].

The organochlorine pesticides are highly thermostable compounds with cyclic structures [26] mainly formed by hydrogen, carbon, and chlorine [27] and recognized as the most toxic and persistent pollutants among organic compounds [28-29]. These compounds dissolve well in

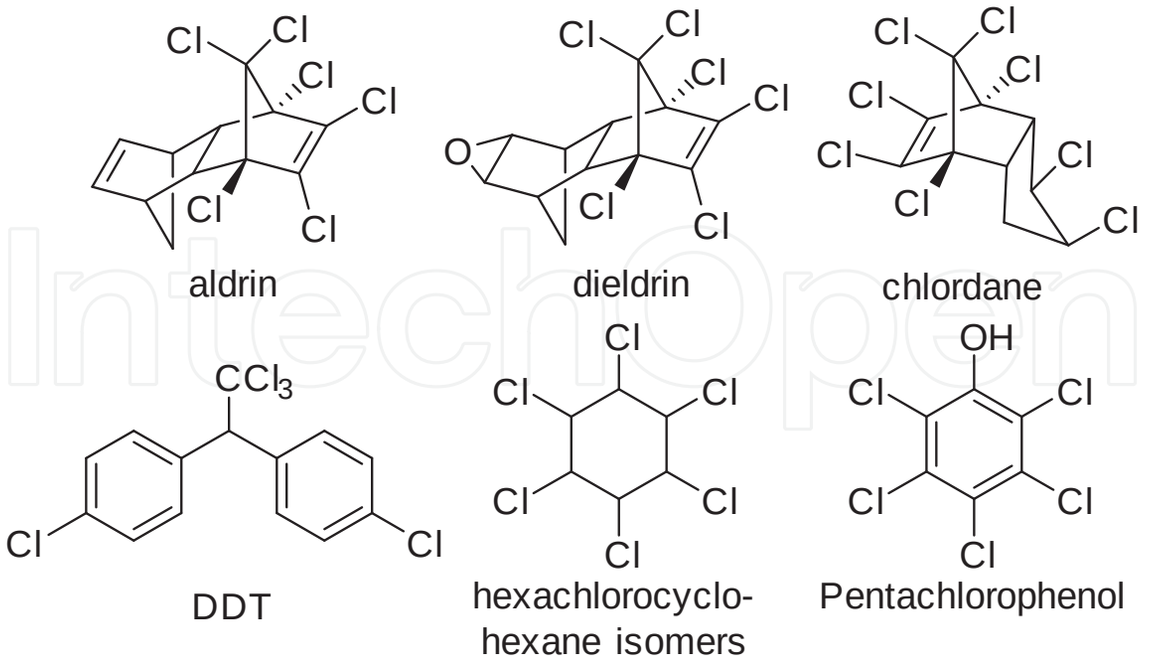
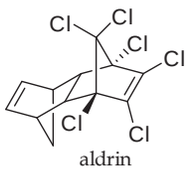
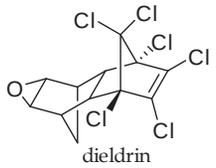


Figure 1. Synthetic organochlorines used as insecticides in the early days (Adapted from Santos et al. [25]).

lipids (fat-soluble), and favors its accumulation in adipose tissues of animals [23]. Thus, they are biomagnified through the biological chain [30], affecting the health of the top predators, including humans [31]. Additionally, organochlorine compounds may interfere in the normal functions of the endocrine system and disturb the reproduction in animals, since they show estrogenic and carcinogenic activity [32-33].

Organochlorine pesticides and some of their physical and chemical characteristics are described in Table 1. Among them, pentachlorophenol (PCP) is one of the most studied organochlorine compounds, because it slightly dissolves in water and has strong solubility, toxicity [34], volatility, ability to release dioxin (and its derivatives), and resistance to biodegradation [35].

Compound	CAS number	Solubility in water	Steam pressure
 aldrin	**309-00-2**	**27 μg L^{-1} at 25°C**	**2.31 × 10 mm Hg at 20°C**
 dieldrin	**60-57-1**	**140 μg L^{-1} at 20°C**	**1.78 × 10 mm Hg at 20°C**

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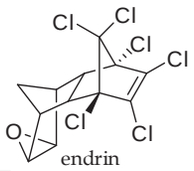
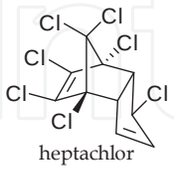
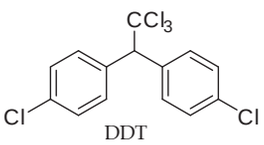
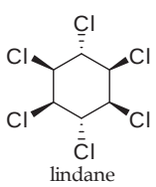
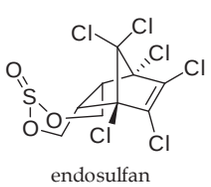
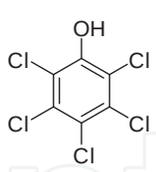
Compound	CAS number	Solubility in water	Steam pressure
 endrin	**72-20-8**	**220-260 μg L^{-1} at 25°C**	**7 × 10 mm Hg at 25°C**
 heptachlor	**76-44-8**	**180 μg L^{-1} at 25°C**	**0.3 × 10 mm Hg at 20°C**
 DDT	**50-29-2**	**1.25.5 μg L^{-1} at 25° C**	**0.02 × 10 mm Hg at 20° C**
 lindane	**58-89-9**	**7 mg L^{-1} at 20° C**	**3.3 × 10 mm Hg at 20° C**
 endosulfan	**115-29-7**	**320 μg L^{-1} at 25° C**	**0.17 × 10 mm Hg at 25° C**
	**87-86-5**	**14 mg L^{-1} at 25° C**	**16 × 10 mm Hg at 20° C**

Table 1. Physical and chemical characteristics of the main organochlorine pesticides (Adapted from Almeida *et al*. [36]).

1.3. Pentachlorophenol

PCP is used as an insecticide, fungicide, herbicide, and wood preservative [37]. Moreover, PCP is a by-product of the paper bleaching, disinfection of water containing phenols with chlorine or sodium hypochlorite, incineration of municipal solid waste and other processes [38-39]. PCP can be found in the air in the form of steam, adsorbed in soil and sediments, in surfaces and groundwater in its ionized salt form [40]. Table 2 shows some physical and chemical properties of PCP.

CAS number	87-86-5
Molecular formula	$C_6H\ Cl_5O$
Molar mass	266.34 g mol^{-1}
Melting point	190–191° C
Boiling Point	309–310° C (dec.)
Appearence	White crystalline solid
Density	1.978 g cm^{-3}
Vapor density	9.20 (air = 10)
Solubility in water	0.020 $g.L^{-1}$ at 30° C
Henry's Law constant	2.45×10^{-8} atm-m^3 mol^{-1}

Table 2. Physical and chemical properties of the PCP (Source: adapted from EPA [41]).

The PCP is produced by two different routes, i.e., the gradual chlorination of phenols in the presence of catalysts (ferric chloride or anhydrous aluminum chloride) and by dechlorination of hexachlorobenzene [42]. According to the Environmental Sanitation Technology Company of São Paulo State (in Portuguese, Companhia de Tecnologia e Saneamento Ambiental do Estado de São Paulo, Cetesb), PCP is a white solid insoluble in water, but highly soluble in oils and fat compounds. The commercial reagent of PCP contains about 85% of active ingredient, 6% of tetrachlorophenol, 6% of other chlorinated phenolic compounds and inert materials [43]. Other impurities are dioxins (tetra-, hexa-, and octachlorodibenzene-*p*-dioxin) and hexachlorobenzene as by-products of manufacture, which can be easily released to the environment. PCP is no longer marketed in Brazil, but pentachlorophenate, which is a water-soluble persistent product formed by the neutralization with sodium hydroxide, can be easily obtained because it is still used as wood preservative [44-45]. The high solubility of the sodium salt in water enables the persistence for long periods in water bodies, increasing the intoxication level [46]. Fish absorb PCP thorough their gills and alimentation, and then contaminate humans through the food chain [47]. According to Ondarza et al. [48], this accumulation in fish reflects the environment contamination degree.

According to the United States Environmental Protection Agency [41], several studies have provided data on PCP levels in human blood and urine (samples from general population or those with known PCP exposure), indicating that the main route of PCP absorption is inhalation during production and handling [49]. It can be easily absorbed by skin and gastrointestinal tract, and then dissipated throughout the body. Consequently, PCP is concentrated in heart, brain, adrenal glands, adipose tissue, liver, and kidneys [50], in which they cause serious damage and cancer [51].

Even with the prohibition of the PCP use in Brazil since 1985 (Ministry of Agriculture in Portuguese: Ministério da Agricultura), many areas remain contaminated. The main reason of the pollution is the indiscriminate use of PCP for several decades [38]. Studies show that PCP residues are still measured at high level in several environmental matrices, such as

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soil, water, sediment, organic matter suspension, atmosphere, and even in many organisms [52-53]. Thus, the use of biological degradation techniques is very important because these methodologies promote the complete mineralization of this compound or conversion to harmless products [54].

1.4. Microbial biodegradation of pesticides

The microorganisms are adaptable to adverse conditions and find ways to grow even in challenging environments [55]. Its potential for biotechnological applications are justified by their tolerance to extreme environmental conditions, rapid growth, low cultivation cost [56], and mainly by their enzymes, which can transform a wide variety of nonnatural chemical compounds [57].

Microorganisms can degrade xenobiotics contained in dyes, cosmetics, detergents, medicines, agricultural chemicals and can mineralize and degrade pesticides to nontoxic compounds [58, 59]. Therefore, microbial biodegradation is an effective method to reduce the harmful effects of pesticides. Biodegradation is considered the main process of pesticides elimination in soil [60] since microorganisms are capable of use these compounds as nutrients source for its enzyme-catalyzed transformations, which lead to changes of structure and toxicological properties and consequently, its polluting potential [61].

Organochlorine compounds are known to undergo dehydrochlorination, oxidation, dechlorination, rearrangement, hydrolysis, and photochemical reactions [65]. Among the pathways observed in microorganisms, the dechlorination under anaerobic condition and dehydrogenation under aerobic condition are the most important [18].

The selection of an appropriate microorganism is an essential step to perform a microbial biotransformation. If a microorganism can proliferate efficiently in environments with high concentrations of certain pollutants, such strain might be more adapted for the remediation of these contaminants [62]. Different bacterial and fungi genera had been used as efficient pesticides metabolizing organisms such as *Rhodococcus*, *Pseudomonas* and *Flavobacterium* [61], *Lentinula edodes*, *Phlebia radiata*, *Phanerochaete chrysosporium* [63], *Trametes hirsutus*, *Phanerochaete sordia*, and *Cyathus bulleri* [64].

In the biodegradation of organochlorine pesticides, some bacterial genera have been proven to be good biocatalysts, i.e., *Klebsiella* [66], *Staphylococcus* [67], and *Pseudomonas* [68]. Some fungi are also effective, i.e., basidiomycetes [69, 70] and white-rot-fungi, such as *Trametes villosa* [71], *Phaneroachaete chrysosporium*, *P. sordida* [72], *Phlebia radiata* [73], which are commonly used to biodegrade organochlorine compounds. But there are also reports of other fungal species involved in biodegradation of these compounds, i.e., *Trichoderma harzianum* [74], *Aspergillus niger* [75], and *Fusarium verticillioides* [76] with excellent results.

1.5. Biodegradation of PCP

The degradation of PCP in the environment can occur through chemical, microbiological, photochemical, electrochemical, and thermal processes [77, 78]. Microbial decomposition is an

important removal mechanism of this compound [78]; however, PCP causes oxidative phosphorylation and membrane cell disruption. Therefore, its toxicity slows biodegradation because of the growth inhibition effects on microorganisms [79].

Despite having these biodegradation unfavorable attributes, some microorganisms have the ability to use PCP and its metabolites as carbon and energy sources [80, 81]. Among the reported species, *Pseudomonas fluorescens* (TE3) [82], *Pseudomonas aeruginosa* (PCP2) [83], *Serratia marcescens* [84], *Pseudomonas stutzeri* (CL7) [81], and *Comamonas testosteroni* (CCM7350) are important examples [85].

Figure 2 shows the biodegradation pathway of PCP by *Sphingobium chlorophenoculium* ATCC 39723 [86]. This strain can degrade PCP to carbon dioxide and water (Figure 2).

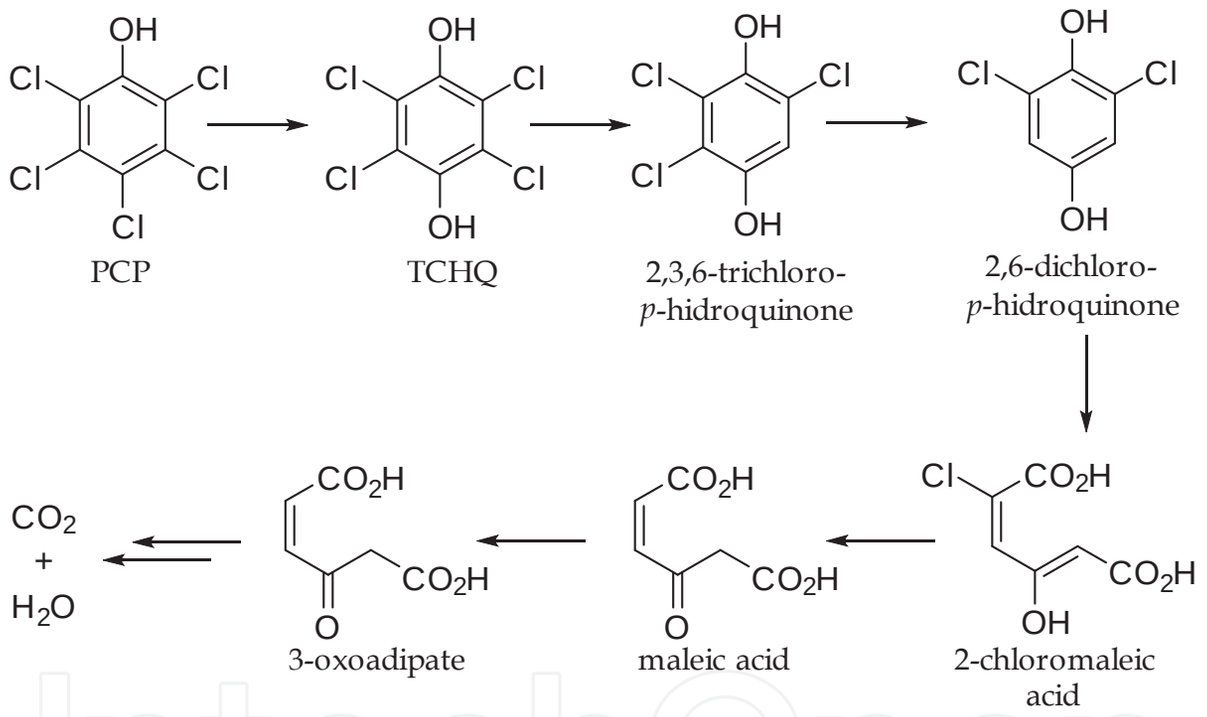

Figure 2. Biodegradation pathway of PCP by the *Sphingobium chlorophenolicum* ATCC 39723 bacteria (adapted from Cai and Xun [86]).

Usually, metabolic transformations in biological systems can be divided into two phases. The reactions of phase I promote changes in xenobiotics such as oxidation, reduction, hydrolysis, and other reactions. After this step, the phase II reactions known as conjugations occurs, in which endogenous groups, which are usually polar and present in abundance *in vivo*, are added to the xenobiotic resulting in more polar products (except in alkylation reactions) and therefore, more easily eliminated compounds. It is noteworthy that conjugated xenobiotics can undergo inverse reactions and regenerate the original compound [87]. Thus, the compound can be degraded (into smaller molecules which can be toxic or not), absorbed, adsorbed, or conjugated during the biodegradation [88].

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There are many reports involving the use of terrestrial fungi in the biodegradation of PCP. Among them, white-rot fungi are highly tolerant to toxic compounds and are widely used in biodegradation techniques [71]. These fungi are effective in the degradation of PCP by having ligninolytic and peroxidase enzymes [89] that act by generating free radicals [90], which can also degrade a variety of recalcitrant pollutants (Figure 3) [91].

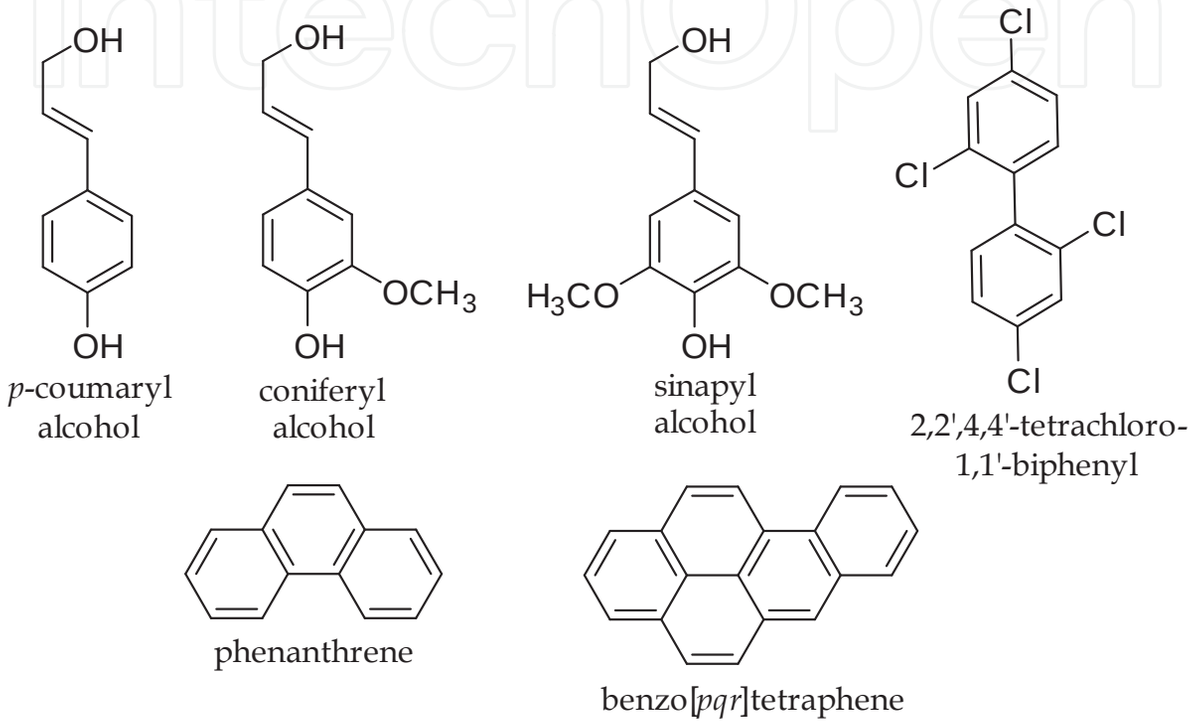

Figure 3. Examples of recalcitrant compounds biodegraded by ligninolytic and peroxidase enzymes: lignin monomers, polycyclic aromatic hydrocarbons (PAHs), and halogenated compounds (Adapted from Pointing [90]).

The ligninolytic extracellular activity of some fungal enzymes is considered a promising method for PCP degradation [92, 94]. The *Phanaerochaete chrysoporium* [95, 96], as well as *Phlebia brevispora* [97], *Phlebia radiata*, *Trametes versicolor* [98], and *Mucor plumbeus* [99] showed great ability to degrade organopollutants (including PCP). Fungal species belonging to the genus *Trichoderma*, such as *T. virgatunil* [100] and *T. harzianum* [74], were efficient in the mineralization of PCP and *Anthracophyllum discolor* mineralized this polutant in reactors containing soil slurry according to Rubilar [101]. Figure 4 shows the PCP biodegradation pathway by *A. discolor*. It is noteworthy that this pathway is different from that by *S. chlorophenolic* ATCC 39723.

The use of filamentous fungi in biodegradation is increasing considerably in recent years, due to the high rates of biodegradation, sortion, and resistance in adverse environmental conditions [102]. According to Sankaran et al. [103], the interest in the use of filamentous fungi in bioremediation is due to high species diversity, high resistence for recalcitrant compounds, and high production of extracelular enzymes.

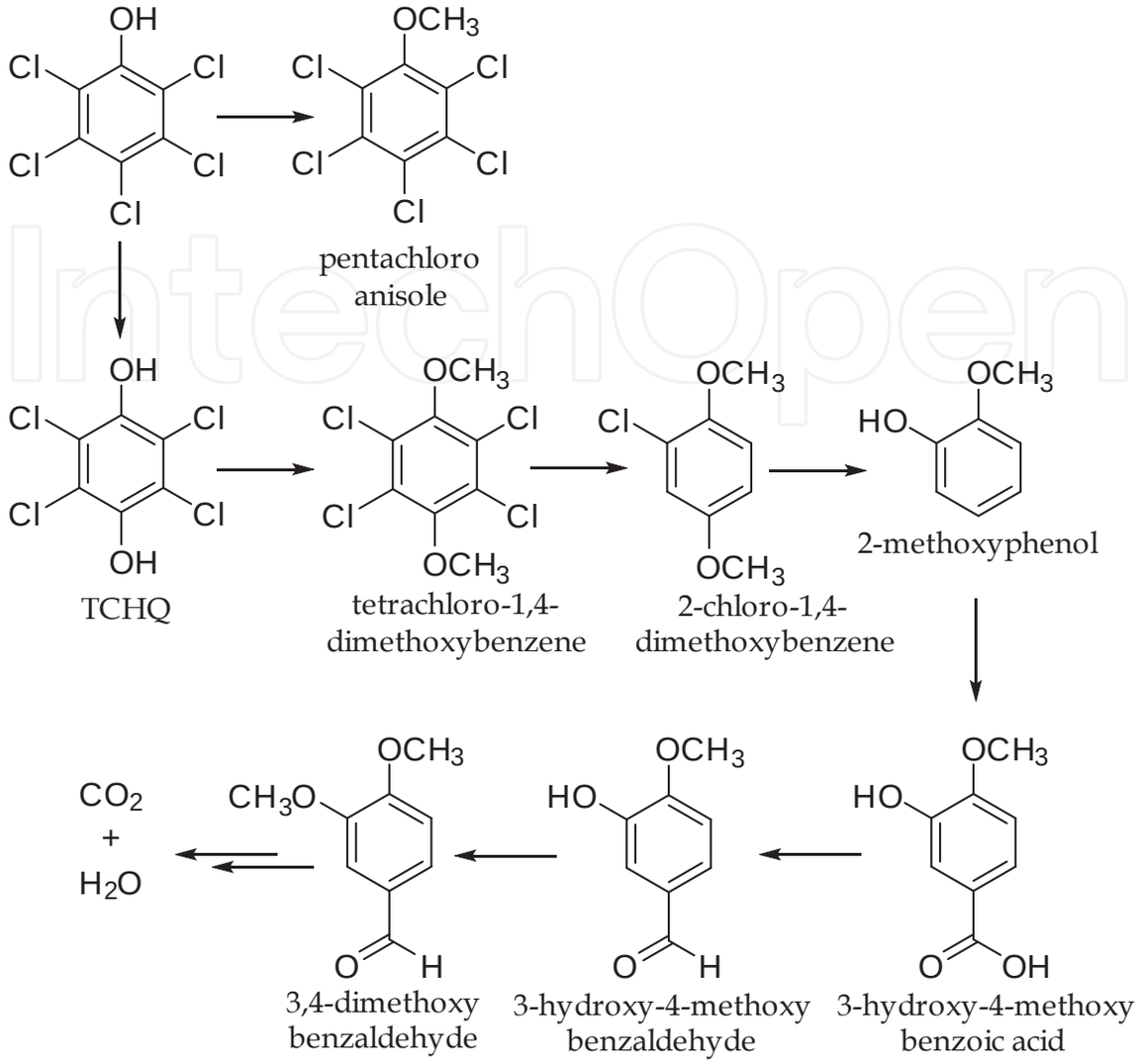

Figure 4. Degradation of PCP by the fungus *Anthracophyllum discolor* (Source: modified from Rubilar et al. [101]).

1.6. Marine fungi

The marine environment covers more than three quarters of the Earth's surface and is a promising source of new enzymes [104]. These enzymes show great potential for use in biocatalytic reactions by possessing unique characteristics related to the marine environment. In recent years, a wide variety of enzymes and microorganisms with specific activities have been isolated from marine environments [105] and have been extensively studied, particularly proteases, carbohydrases, oxidoreductases, peroxidases [106].

The words “marine fungi“ are not derived from a taxonomic class and they are not classified by their physiological characteristics. These fungi considered as an ecological group, and the most suitable definition was proposed by Kohlmeyer and Kohlmeyer [107]: "Mandatory marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat; facultative marine fungi are those from freshwater or terrestrial water environments and are able to grow and even sporulate in the marine environment " [108]. In the marine environment,

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many fungi strains can be found in a wide variety of habitats such as open sea, sediment, mangroves, surface of wood, shells of molluscs, corals, marine vertebrates and invertebrates, on the surface or interior of algae and even in hydrothermal vents. The variety of habitats also influences their metabolic diversity, which contributes to their potential use as source of enzymes and bioactive molecules [109].

Unlike terrestrial fungi, which were initially exploited for drug discovery, marine fungi have attracted the attention of researchers as a source of new natural products and enzymes [110]. Marine fungi are adapted to high salinity and extreme conditions, developing attributes that give them the ability to produce a different enzymatic metabolism from their respective representatives from the terrestrial environment [111]. Researches had been generally focused on biological activities such as antibiotic and by marine fungi [112]. However, recently they have been investigated for dechlorination and detoxification of effluents [113], biodegradation of polycyclic aromatic hydrocarbons [114], lignin [115], pesticides [116, 117], and polyethylene [118], and more recently, studies on biocatalytic reactions for organic synthesis [106].

Filamentous fungi *Aspergillus sydowii*, *Penicillium raistrickii*, *Trichoderma* sp., and *Penicillium miczynskii* isolated from marine environment and cultured in artificial sea water were capable of catalyzing the hydrolysis of benzyl glycidyl ether and allyl glycidyl ether [119, 62]. Bonugli-Santos et al. [120] found interesting results in a study of ligninolytic enzyme production by the marine fungi *Aspergillus sclerotiorum* CBMAI 849, *Cladosporium cladosporioides* CBMAI 857, and *Mucor racemosus* CBMAI 847. The marine fungi *Microsphaeropsis* sp., *Acremonium* sp., and *Westerdykella* sp. promoted the biodegradation of esfenvalerate (pyrethroid pesticide) with formation of several metabolites [121].

The enzymatic reactions catalyzed by marine-derived fungi can be carried out in laboratory using artificial seawater. Studies have shown that marine bacteria and fungi cultured in laboratory have specific requirements of salts, especially the sodium ions, potassium, magnesium, and chloride [122, 119]. According to Rateb and Ebel [123], for biotransformation studies and production of secondary metabolites, marine-derived fungi strains have been isolated mainly from inorganic substrates, plants, marine invertebrates, and vertebrates. In this context, studies on enzyme production by filamentous marine-derived fungi are important for future applications in bioremediation techniques. Thus, this work aimed the exploration of the potential biodegradation of the pesticide PCP by strains of marine-derived fungi isolated from a marine invertebrate, the ascidian *Didemnun ligulum*.

2. Materials and Methods

2.1. Isolation of fungi strains

Marine-derived fungi were isolated from the ascidian *Didemnum ligulum* according to the method described by Kossuga et al. [124]. The ascidian samples were collected in São Sebastião, South Atlantic Ocean, in September 2005 at the northern coast of São Paulo state, Brazil, by Prof. Roberto G.S. Berlinck (IQSC-USP, Brazil). After the isolation and purification of the

strains, the marine-derived fungi were deposited in the microbiology laboratory of the Department of Ecology and Aquatic Microbiology supervised by Mirna H.R. Seleghim (UFSCar, Brazil). They were preserved by two techniques: in distilled water according to Castellani [125] and in inclined tubes containing agar, both stored under refrigeration. The strains were reactivated for the experiments by streaking or aseptic transfer of mycelial discs to solid culture media (3% malt).

In the laboratory, samples collected from the ascidian were subjected to surface sterilization by successive washes with 0.001 g.L^{-1} solution of $HgCl_2$ in 5% ethanol for 1 minute, followed by 3 washes with sterile sea water [126]. Then, portions of about 1 cm^2 were taken from the inside of the ascidian with a sterile scalpel. These fragments were inoculated in Petri dishes containing agar medium with artificial sea water (ASW - Artificial Sea Water) and the broad-spectrum antibiotic rifampicin (0.3%) to inhibit bacterial growth [127]. Plates were incubated for 7 d at 25° C. Eight culture media were prepared (Table 3) in order to expand the possibilities of obtaining different strains that may be associated with the ascidian *D. ligulum*.

Culture media	**Composition**
2% Malt Extract Agar (MA2%)	Malt extract (20 g L^{-1}), agar (15 g L^{-1}) in artificial seawater
3% Malt Extract Agar (MA3%)	Malt extract (30 g L^{-1}), mycological peptone (5 g L^{-1}) and agar (15 g L^{-1}) in artificial seawater
Glucose agar, Peptone, and Yeast extract (GPY)	Glucose (1 g L^{-1}), soy peptone (0.5 g L^{-1}), yeast extract (0.1 g L^{-1}), agar (15 g L^{-1}) in artificial seawater
Potato Carrot Agar (PCA)	Cooked and mashed potatoes (20 g L^{-1}), cooked and mashed carrots (5 g L^{-1}), agar (20 g L^{-1}) in artificial seawater
Corn Meal Agar (CMA)	Maize flour (42 g L^{-1}) stirred in 500 mL of distilled water at 60°C for 12 h, filtered, and then the supernatant was diluted with artificial seawater to 1 L with agar (15 g L^{-1})
Oat Meal Agar (OMA)	Rolled oats (30 g) were boiled in 500 mL of distilled water for 1 h, filtered, and then diluted with artificial seawater to 1 L with agar (20 g L^{-1})
Tubaki Agar (TA)	Glucose (30 g L^{-1}), yeast extract (0.5 g L^{-1}), peptone (1.0 g L^{-1}), dibasic potassium phosphate (1.0 g L^{-1} KH_2PO_4), magnesium sulfate heptahydrate (0, 5 g L^{-1}, $MgSO_4.7H_2O$), 0.01 g of iron(II) sulphate heptahydrate (0.01 g L^{-1} $FeSO_4.7H_2O$), agar (15 g L^{-1}) in artificial seawater
Cellulose Agar (CA)	Cellulose (10 g L^{-1}), yeast extract (1 g L^{-1}), agar (15 g L^{-1}) in artificial seawater

Table 3. Culture media composition for isolation of marine-derived fungi from *Didemnun ligulum* [124].

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2.2. Purification

The Petri dishes with different culture media containing the filamentous fungi strains were examined periodically. The isolated strains were subjected to successive inoculations to obtain pure cultures. Initially, the pure cultures were described by morphological method and coded as DL. The DL code was related to the organism from which the strains were isolated, the ascidian *Didemnum ligulum*, and the abbreviation for the culture medium used in the isolation. Eight different culture media for strain isolation were used; however, fungi growth was not observed in the cellulose agar and Tubaki agar media.

The 15 isolated strains were coded as; DL5A, DL6A, DL11A (oatmeal agar medium), DL2B, DL5B (potato carrot agar medium), DL1F, DL2F, (corn meal agar medium), DL5G, (glucose agar, peptone, and yeast extract culture medium), DL3M2, (2% malt extract agar medium), DL1M3, DL4M3, DL6M3, DL7M3, DL8M3, and DL9M3 (3% malt extract agar medium). The detailed methodology for the isolation and purification were described by Kossuga et al. [124]. The procedures were performed at the Department of Ecology and Evolutionary Biology at UFSCar, São Carlos, Brazil.

2.3. Identification of strains by molecular biology

The 15 fungal strains were characterized and identified by techniques based on the molecular identification of genes rRNA, ITS1 and ITS4. These analyzes were carried out under the supervision of Prof. Dr. Suzan Pantaroto de Vasconcellos at the Federal University of São Paulo (UNIFESP), Campus Diadema.

The isolates were grown on yeast extract sucrose agar (YES) (10 g yeast extract, 75 g sucrose, 10 g agar, and 500 mL distilled water). Then, DNA was extracted with the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The DNA concentration and purity (relative to proteins and salts) were determined by optical density at 260 nm (OD260) and ratios of OD260/280 and OD260/230, respectively. The internal transcribed spacer (ITS) region of rDNA were amplified with primer pairs and ITS1/ITS4 using the protocol described by Gonçalves et al. (2012). The reactions were performed with PCR master mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. After amplification, the fragments were sequenced following the protocol provided with the BigDye reagent kit (Applied Biosystems, Foster City, CA, USA) in an ABI 3130 (Applied Biosystems, Foster City, CA, USA) automatic sequencer. PCR products were sequenced with the same primers used for amplification. Contig assembly and editing were performed with Sequencher DNA sequence assembly software 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Successful assembly of the contigs required a minimum match percentage of 85 and a minimum overlap of 20.

Complete ITS consensus sequences were used to conduct BLAST search analysis for species identification from the NCBI genomic database (http://blast.ncbi.nlm.nih.gov/).

For all regions analyzed by BLAST search, the sequences that were presented with high identity (99%), queries, and E values of $e10^{-5}$ were considered for the final species identification using the sequencing method.

2.4. Growth of fungi strains in solid medium

The strains of marine-derived fungi were cultivated on Petri dishes containing 3% malt solid medium using artificial sea water with the following composition: malt extract (30.0 g L^{-1}), soy peptone (3.0 g L^{-1}), and agar (20.0 g L^{-1}). The pH was adjusted to 8 with KOH solution (0.1 mol L^{-1}), similar to the pH of the marine environment [124]. Artificial seawater composition was: $CaCl_2.2H_2O$ (1.36 g L^{-1}), $MgCl_2.6H_2O$ (9.68 g L^{-1}), KCl (0.61 g L^{-1}), NaCl (30.0 g L^{-1}), Na_2HPO_4 (0.014 mg L^{-1}), Na_2SO_4 (3.47 g L^{-1}), $NaHCO_3$ (0.17 g L^{-1}), KBr (0.10 g L^{-1}), $SrCl_2.6H_2O$ (0.04 g L^{-1}), and H_3BO_3 (0.03 g L^{-1}).

2.5. Selection of fungal strains resistant to PCP in solid culture medium

Fifteen fungal strains were grown in Petri dishes and inoculated in solid medium containing 3% malt extract medium without PCP (control) and with different concentrations of the organochlorine pesticide; 10, 25, 30, 40, and 50 mg L^{-1} per plate (98%, analytical standard commercially obtained from Sigma-Aldrich, Brazil). The experiments were prepared in triplicate. Ethyl acetate was used as solvent to prepare the stock solution of the pesticide in the proportion of 5.0 mg of PCP / 100 μL of ethyl acetate.

The culture media were sterilized in an autoclave at 121 °C for 20 minutes, cooled to about 40-50 °C, and then the pesticide stock solution was added, according to the desired concentration. The mixture was homogenized and then added in Petri dishes. The inoculation of fungi was made by transferring the mycelium of pure cultures precultivated in 3% malt medium after 5 d of growth by a platinum needle insertion point into the plate center. The plates were incubated at 32 °C (B.O.D. 411D, Nova Ética) and the radial growth of the fungus were observed for 21 d. The diameter of the colony formed was measured at 7 d intervals, as performed by Birolli et al. [128]. The strain that showed the highest radial growth was selected for the PCP biodegradation in a liquid medium. The experiments were performed in triplicates.

3. Results and Discussion

The aim of this chapter was the isolation and selection of marine-derived fungi with potential for PCP biodegradation. So the PCP biodegradation details will not be discussed because they already were published. Figure 5 shows the 15 fungi strains isolated from *Didemnum ligulum* cultivated in 3% malt extract medium in absence of pesticide.

The isolated fungi were identified by molecular biology and exhibited a variety of genera and species illustrating the fungi diversity in marine environment (Table 4): *T. harzianum* CBMAI 1677 was deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI - http://webdrm.cpqba.unicamp.br/cbmai/, WDCM 823).

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Strains	Identification	CBMAI deposit
DL1M3	*Stagonosporopsis cucurbitacearum*	
DL4M3	*Penicillium citrinum*	
DL6M3	*Mycosphaerella crystallina*	
DL7M3	*Didymella phacae*	
DL8M3	*Phoma* sp.	
DL9M3	Not identified*	
DL1F	*Pleosporales* sp.	
DL2F	*Cladosporium cladosporioides*	
DL3M2	*Cladosporium cladosporioides*	
DL5B	*Cladosporium cladosporioides*	
DL5G	*Cladosporium cladosporioides*	
DL6A	*Aspergillus versicolor*	
DL5A	*Aspergillus versicolor*	
DL11A	*Fusarium fujikuroi*	
DL2B	*Trichoderma harzianum*	1677

* Not cultured strain

Table 4. The codification and identification of the strains employed in this study.

For the evaluation of the fungi inhibition caused by the presence of the xenobiotic compound, radial growth experiments were performed. The marine-derived fungi were cultivated in various concentrations of PCP (10, 25, 30, 40, and 50 $mg.L^{-1}$ per plate). The inoculation was carried out by a central insertion point using an inoculation needle. After incubation, the colonies' diameters were measured at 7, 14, and 21 d. The results are summarized in Tables 4-6.

All marine-derived fungi showed excellent growth after 7 d of cultivation in solid culture medium (3% mat extract agar) without PCP. The results showed that 3% malt extract medium was suitable for growth of marine-derived fungi as suggested by Kjer et al. [130]. After 21 d of incubation, 60% of the strains have grown throughout the plate surface, reaching 8.0 cm of colony diameter (diameter of the employed Petri dish). The cultivation of the fungus in the absence of PCP was important to assess the development of the pure cultures isolated from the sponge *D. ligulum*.

In the presence of the organochlorine pesticide, the strains coded as DL1M3, DL4M3, DL6M3, DL7M3, DL8M3, and DL9M3 failed to grow in any of the plates containing PCP, showing low

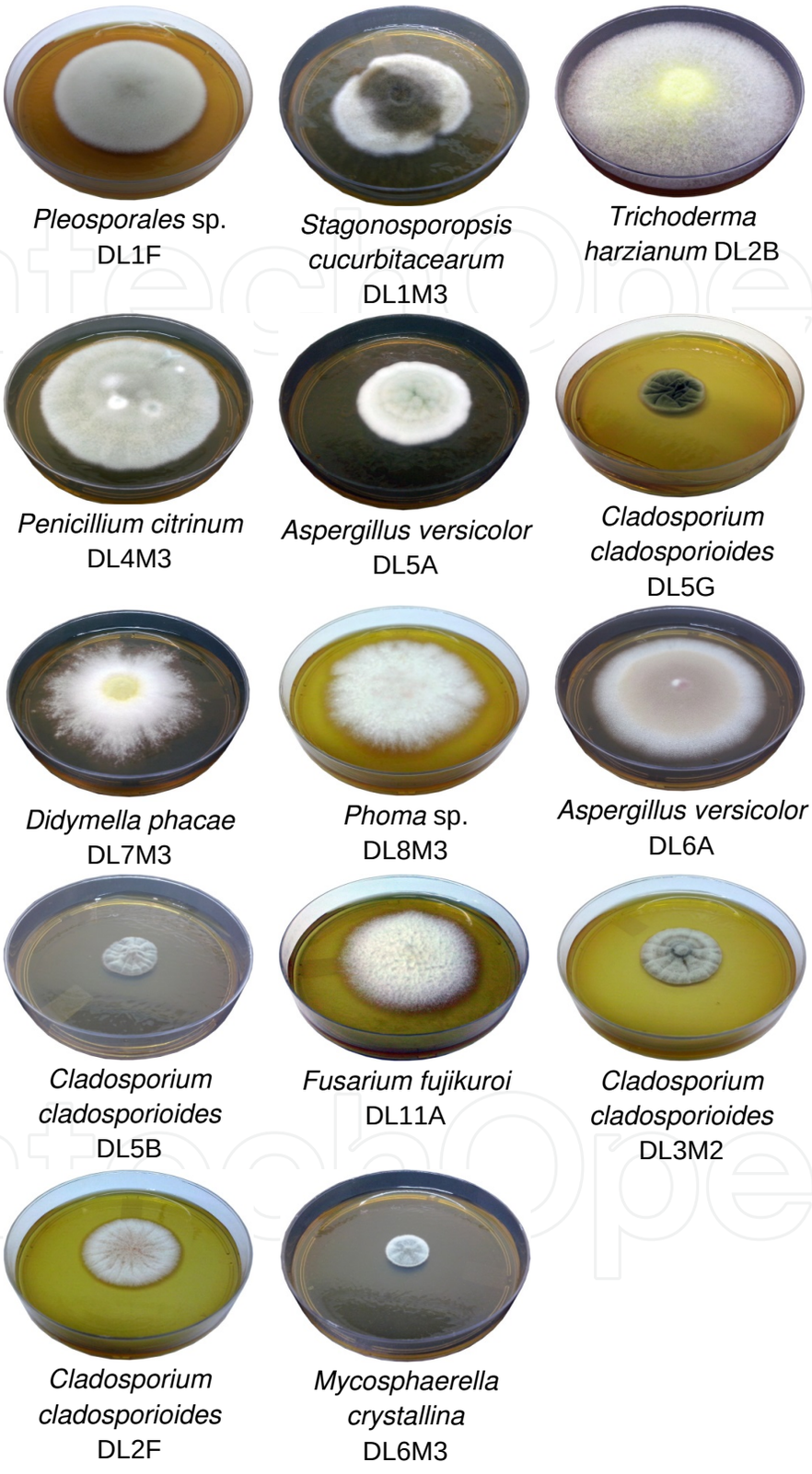

Figure 5. Colonies of marine-derived fungi isolated from the ascidian *Didemnum ligulum* grown in 3% malt extract medium.

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resistance and adaptation to the organochlorine presence, and thus suggested low potential for biodegradation. It is noteworthy that these microorganisms were isolated from environments without PCP contamination; therefore its presence caused growth inhibition because this is a very toxic compound for living organisms and these strains were not adapted to its effects on their metabolism.

Colony diameter (cm)[a]						
Strains	**Petri dishes without PCP**	**Concentration of PCP in Petri dishes with PCP**				
		10 (mg.mL^{-1})	**25 (mg.mL^{-1})**	**30 (mg.mL^{-1})**	**40 (mg.mL^{-1})**	**50 (mg.mL^{-1})**
S. cucurbitacearum DL1M3	4.2	-	-	-	-	-
P. citrinum DL4M3	1.5	-	-	-	-	-
M. crystallina DL6M3	1.9	-	-	-	-	-
D. phacae DL7M3	0.9	-	-	-	-	-
Phoma sp. DL8M3	0.6	-	-	-	-	-
Not identified DL9M3	3.9	-	-	-	-	-
Pleosporales sp. DL1F	1.9	0.2	-	-	-	-
C. cladosporioides DL2F	2.1	0.4	-	-	-	-
C. cladosporioides DL3M2	2.5	0.5	-	-	-	-
C. cladosporioides DL5B	1.6	0.6	-	-	-	-
C. cladosporioides DL5G	1.5	0.9	-	-	-	-
A. versicolor DL6A	5.1	1.8	0.7	-	-	-
A. versicolor DL5A	3.1	1.6	1.0	0.1	-	-
F. fujikuroi DL11A	6.6	2.2	0.8	0.7	-	-
T. harzianum CBMAI 1677	7.8	4.1	3.0	1.8	0.7	0.9

[a]Standard deviation: minimum (0.07 cm) and maximum (0.4 cm).

- not grown.

All experiments in plates were performed in triplicate.

Table 5. Average diameter of fungi colonies isolated from the ascidian *D. ligulum* after 7 d of growth (32° C, 3% malt extract medium) in the presence and absence of PCP.

Colony diameter (cm)[a]						
Strains	**Petri dishes without PCP**	**Concentration of PCP in Petri dishes with PCP**				
		10 (mg.mL^{-1})	**25 (mg.mL^{-1})**	**30 (mg.mL^{-1})**	**40 (mg.mL^{-1})**	**50 (mg.mL^{-1})**
S. cucurbitacearum DL1M3	6.6	-	-	-	-	-
P. citrinum DL4M3	3.6	-	-	-	-	-
M. crystallina DL6M3	4.2	-	-	-	-	-
D. phacae DL7M3	3.1	-	-	-	-	-
Phoma sp. DL8M3	1.5	-	-	-	-	-
Not identified DL9M3	5.4	-	-	-	-	-
Pleosporales sp. DL1F	2.6	0.4	-	-	-	-
C. cladosporioides DL2F	4.3	1.2	-	-	-	-
C. cladosporioides DL3M2	4.3	1.3	-	-	-	-
C. cladosporioides DL5B	3.1	1.5	-	-	-	-
C. cladosporioides DL5G	4.8	3.4	-	-	-	-
A. versicolor DL6A	6.7	3.6	1.7	0.1	-	-
A. versicolor DL5A	4.8	3.0	1.6	0.3	-	-
F. fujikuroi DL11A	8.0	4.9	1.8	1.6	-	-
T. harzianum CBMAI 1677	8.0	6.6	5.9	2.7	1.4	2.1

[a]Standard deviation: minimum (0.0 cm) and maximum (0.2 cm).

- not grown.

All experiments in plates were performed in triplicate.

Table 6. Average diameter of fungi colonies isolated from the ascidian *D. Ligulum* after 14 d of growth (32° C, 3% malt extract medium) in the presence and absence of PCP.

As shown in Tables 5-7, some strains did not grow in the presence of PCP. In addition, the strains capable of growth in the employed conditions showed that the more concentrated the PCP, the less growth presented in the culture medium. These results indicated that PCP causes a toxic effect on these microorganisms. However, the fact that the majority of the strains subjected to this experiment grew, at least, in one of the tested concentrations indicates that

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Strains	Colony diameter (cm)[a]					
	Petri dishes without PCP	Concentration of PCP in Petri dishes with PCP				
		10 ($mg.mL^{-1}$)	25 ($mg.mL^{-1}$)	30 ($mg.mL^{-1}$)	40 ($mg.mL^{-1}$)	50 ($mg.mL^{-1}$)
S. cucurbitacearum DL1M3	8.0	-	-	-	-	-
P. citrinum DL4M3	6.5	-	-	-	-	-
M. crystallina DL6M3	8.0	-	-	-	-	-
D. phacae DL7M3	6.2	-	-	-	-	-
Phoma sp. DL8M3	2.8	-	-	-	-	-
Not identified DL9M3	7.1	-	-	-	-	-
Pleosporales sp. DL1F	6.1	0.9	-	-	-	-
C. cladosporioides DL2F	8.0	3.0	-	-	-	-
C. cladosporioides DL3M2	8.0	3.2	-	-	-	-
C. cladosporioides DL5B	8.0	3.3	-	-	-	-
C. cladosporioides DL5G	8.0	6.3	-	-	-	-
A. versicolor DL6A	8.0	5.4	2.5	0.2	-	-
A. versicolor DL5A	6.6	4.5	3.2	0.4	-	-
F. fujikuroi DL11A	8.0	7.2	3.1	2.8	-	-
T. harzianum CBMAI 1677	8.0	7.6	7.2	3.8	2.2	3.5

[a]Standard deviation: minimum (0.1 cm) and maximum (0.5 cm).

- not grown.

All experiments in plates were performed in triplicate.

Table 7. Average diameter of fungi colonies isolated from the ascidian *D. Ligulum* after 21 d of growth (32 C, 3% malt extract medium) in the presence and absence of PCP.

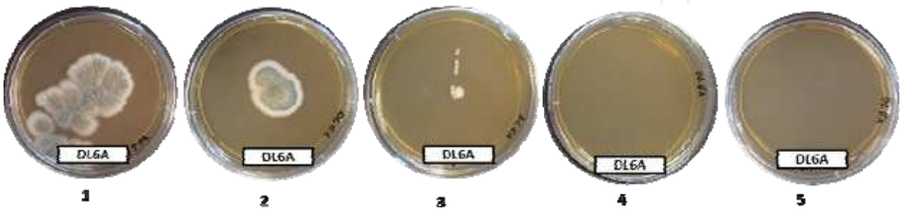
the toxic effect exerted by the compound was not enough to prevent fungal resistance and consequently, biodegradation potential.

According to Bonugli-Santos et al. [129] and Ortega et al. [116], marine-derived microorganisms tend to be resistant when subjected to adverse conditions and can be used in bioremediation techniques because they have enzymes adapted to complex environments such as those with extreme pressure, salinity, and temperature variations. They are able to develop impor-

tant metabolic and physiological activities, for example, degradative potential of organochlorine pesticides.

The best adapted strain to the presence of PCP were by DL6A, DL5A, DL11A, and DL2B strains, which were capable to grow at concentrations above 10 mg L^{-1}. The colony diameter of the strains DL6A and DL5A increased in the concentrations of 10, 20, and 30 mg L^{-1}, but the sizes were inferior in comparison with DL11A and DL2B strains in the same concentrations (Figure 6).

Aspergillus versicolor DL6A



Aspergillus versicolor DL5A

Fusarium fujikuroi DL11A

Trichoderma harzianum DL2B

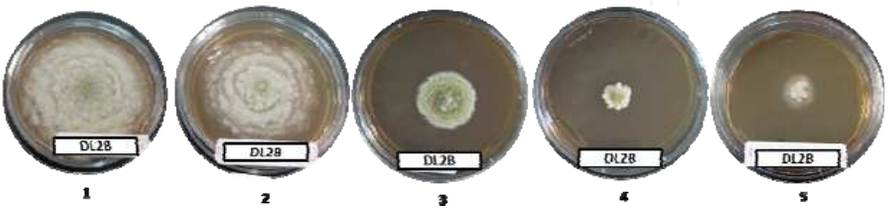


Figure 6. Growth of marine-derived fungi (DL6A, DL5A, DL11A, DL2B) in 3% malt extract agar containing different concentrations of PCP after 21 d at 32° C. The plate numbers of 1, 2, 3, 4, and 5, respectively, correspond to 10, 25, 30, 40, and 50 mg L^{-1} of PCP.

The most part of microorganisms show increasing growth inhibition in increasing xenobiotics concentrations, especially on those with high toxicity. However, the strain DL2B, which showed the best results in the solid media experiment also grew well at the highest pesticide concentration (50 mg L^{-1}). Thus, this fungus showed resistance to toxicity, adaptive capacity, and biodegradation potential for PCP, even at high concentrations. Creswell and Curl [131] achieved similar results assessing the growth of the fungus *Trichoderma harzianum* in the presence of herbicides such as prometryn, norflurazon, and ciazine. In this work, the fungal growth was significantly increased at the highest dose of the herbicide norflurazon. According to Tomasini et al. [132] fungi need a period of adaptation in high toxicity conditions and, if they were resistant, in the final period of cultivation they tend to grow more. If a group of microorganisms can proliferate efficiently in environments with high concentrations of certain pollutants, it is an indication that these microorganisms have a metabolism adapted to the presence of these contaminants [62]. The increased growth in the presence of the xenobiotic can occur because of its use as nutrient, especially carbon source.

Earlier studies have shown that adaptation experiment with fungi in solid culture medium is a simple and important methodology to screen microorganisms for pesticide biodegradation [133]. After the adaptation experiments with the 15 isolated strains, *Trichoderma harzianum* DL2B (CBMAI 1677) was selected for studies of biotransformation and biodegradation of PCP. In a later study, Vacondio et al. [134] observed that after 7 d of incubation with 20 mg L^{-1} of PCP in liquid medium, it was no longer detected in the presence of PCP in the samples, showing the biodegradation of the pesticide by *Trichoderma harzianum* CBMAI 1677. In addition, the metabolites pentachloroanisole (PCA) and 2,3,4,6-tetrachloroanisole (2,3,4,6-TeCA) were identified. *T. harzianum* was also able to biodegrade PCA and 2,3,4,6-TeCA in liquid medium (Figure 7). These results confirmed the efficiency of marine-derived fungi in the biodegradation of persistent compounds and contributed to the improvement of decontamination techniques. Detailed results were published recently in the literature [134].

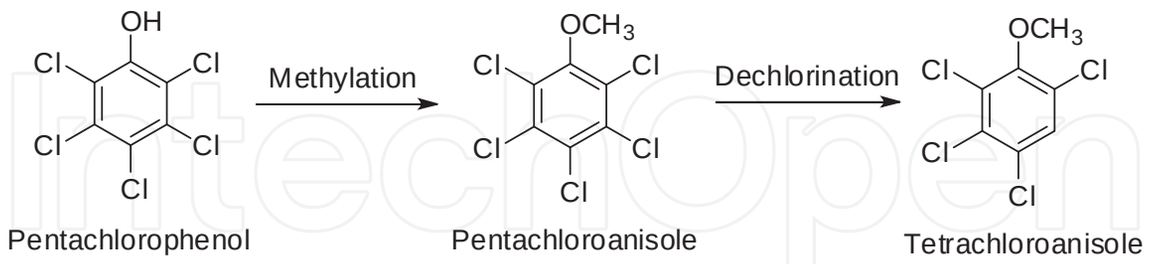


Figure 7. Proposed PCP biodegradation pathway by marine fungus *T. harzianum* DL2B (CBMAI 1677).

4. Conclusions and perspectives

Fifteen marine-derived fungi associated with the ascidian *Didemnun ligulum* were isolated and identified by molecular techniques based on the genes rRNA ITS1 and ITS4. They were tested

for toxicity resistance and biodegradation of PCP, and promising results were obtained. Experiments with these strains using culture medium containing 3% malt extract agar in the presence of PCP enabled the selection of a resistant strain (*Trichoderma harzianum* CBMAI 1677) capable of biodegrading this compound. This fungus grew well in high concentrations of PCP; therefore, showed resistance to its toxicity and potential for the biodegradation of this xenobiotic. This work showed the great potential of microorganisms from marine environment for biotransformation and biodegradation of anthropogenic compounds. The biomethylation and dechlorination of PCP gave the pesticide metabolites PCA and 2, 3, 4, 6-TeCA.

Acknowledgements

BV and WGB thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for their scholarships, respectively. The authors wish to thank Prof. RGS Berlinck (Instituto de Química de São Carlos USP) for providing the marine sponge ascidian *Didemnun ligulum* and MCM Peret for the technical support. ALM Porto and MHR Seleghim are grateful to CNPq and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) for financial support.

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