Potential use of fungal-bacterial co-cultures for the removal of organic pollutants

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Abstract

Fungi and bacteria coexist in a wide variety of natural and artificial environments which can lead to their association and interaction – ranging from antagonism to cooperation – that can affect survival, colonization, spatial distribution and stress resistance of the interacting partners. The use of polymicrobial cultivation approaches has facilitated a more thorough understanding of microbial dynamics in mixed microbial communities, such as those composed of fungi and bacteria, and their influence on ecosystem functions. Mixed (multi-domain) microbial communities exhibit unique associations and interactions that could result in more efficient systems for the degradation and removal of organic pollutants. Several previous studies have reported enhanced biodegradation of certain pollutants when using combined fungal-bacterial treatments compared to pure cultures or communities of either fungi or bacteria (single domain systems). This article reviews: (i) the mechanisms of pollutant degradation that can occur in fungal-bacterial systems (*e.g.,* co-degradation, production of secondary metabolites, enhancement of degradative enzyme production, and transport of bacteria by fungal mycelia); (ii) case studies using fungal-bacterial co-cultures for the removal of various organic pollutants (synthetic dyes, polycyclic aromatic hydrocarbons, pesticides and other trace or volatile organic compounds) in different environmental matrices (*e.g.,* water, gas/vapors, soil); (iii) the key aspects of engineering artificial fungal-bacterial co-cultures, and (iv) the current challenges and future perspectives of using fungal-bacterial co-cultures for environmental remediation.

Keywords: fungal-bacterial interactions; co-cultures; soil remediation; wastewater treatment; waste gas treatment

Introduction

Microorganisms do not live under axenic conditions but rather they are part of complex microbial communities in the environment. The co-habitation of fungi and bacteria is common in soils, foods, animals, and even the human body [1-3]. Fungi and bacteria, along with their associations and interactions, are key players for diverse terrestrial ecosystem processes (*e.g.,* element cycling, geochemical reactions) and services (*e.g.,* plant production) [2, 4].

The co-occurrence of fungi and bacteria in different environments could be the result of an intimate physical association, in which the partners interdependently develop or could be the result of random mixing within the microbial community with no relevance for either partner [5]. Depending on the effect induced by fungal-bacterial interactions (FBIs), fungi and bacteria can develop relationships that can range from synergistic (cooperative) to antagonistic (competitive) [6], resulting in: (i) changes for either partner affecting growth, survival, colonization, spatial distribution, resistance and tolerance to stress and pathogenesis [5], and (ii) alteration of the surrounding environment through different physical, biological and chemical processes (*e.g.,* pH conditions, composition of the substrate) [4, 5, 7]. For example, the development of fungal hyphae can facilitate the growth and dispersal of specialized bacteria, which can affect the bacterial community structure [8]. Besides, the production of fungal exudates (*e.g.,* glycerol [9]) can stimulate bacterial activity, whereas bacteria can aid hyphal growth (*e.g.,* production of metabolites such as auxofuran that can stimulate hyphal growth [10]), detoxify compounds inhibitory to fungi, and protect fungi against parasites [11]. More detailed discussions on the ecology, mechanisms and biochemical changes that occur during FBI in the field of medicine, food, agriculture and environmental engineering can be found in several recent reviews [5, 7, 12, 13] and therefore, these aspects will not be discussed in this review.

Fungi and bacteria are well known for their ability to degrade a wide range of different organic compounds, from simple substrates (*e.g.,* sugars) to complex molecules (*e.g.,* cellulose, lignin), which makes them good candidates for the degradation of organic pollutants including pesticides, hydrocarbons and other xenobiotics [14-17]. In mixed microbial co-cultures, fungi and bacteria can develop synergistic metabolic or co-metabolic activities that can potentially lead to a more complete and faster degradation of organic compounds and increased resistance to changing or fluctuating environmental conditions (Table 1). Mixed microbial cultures composed of multiple species possess a wider range of genes and metabolic capabilities in comparison to pure cultures due to their ability to interact with each other in various ways [18].

Interactions among different microbial species in mixed communities can lead to the development of emergent communal properties such as stability, robustness (ability to survive stress) and division of labor (specialization of microbial partners to perform different tasks within a microbial community) [19-21]. Hence, due to these unique properties, there is great interest in engineering artificial consortia (*i.e.,* collections "of unmodified populations that have been assembled through manual intervention and are not thought to interact in natural habitats" [22]), particularly fungal-bacterial co-cultures, for environmental remediation applications (Table 1). In this review, the term 'co-culture' refers to mixed cultures of fungi and bacteria growing together regardless of their sequence of inoculation (*i.e.,* concurrent or sequential).

The use of fungal-bacterial systems for the degradation and removal of organic pollutants from different environmental matrices (*e.g.,* soil, water, gas/vapors) has shown promising results (*e.g.,* increased removal efficiencies compared to their monocultures), but challenges remain associated to their application. For example, when bioreactors treating wastewater or waste gases are inoculated with pure cultures of fungi and/or bacteria, during long term operation, it is difficult to maintain the purity and stability of the original inoculum. In such cases, the original strain or one of the microbial partners used as inoculum might become dominant for a specific period of time depending on the pollutant load and the composition of the wastewater/waste gas, the presence of competing ions or inhibiting chemicals, the availability of nutrients, and the prevailing process conditions (*e.g.,* pH, temperature and oxygen concentration). In pilot and full-scale systems microbial contamination cannot be avoided and as long as the bioreactor is able to demonstrate stable and high removal efficiencies of the target pollutants, it is somewhat challenging to maintain pure co-cultures in the reactors [23, 24]. Depending on the operating strategy/conditions and feeding regimes, the desired co-culture composition and growth conditions can be maintained using automated process control systems.

This article reviews the potential mechanisms of pollutant degradation that can occur due to the association and interaction between fungi and bacteria, as well as some examples of how fungal-bacterial systems have been used for the treatment of different contaminated environmental matrices. Finally, this review will also discuss the challenges of engineering fungal-bacterial co-cultures for environmental remediation and the scope for future investigation.

Mechanisms of degradation in fungal-bacterial systems

Independently, *i.e.,* as monocultures, fungi and bacteria have shown great potential for the degradation of organic pollutants as these microorganisms can utilize organic pollutants as sources of energy and carbon, or by co-metabolizing them in the presence of other substrates [25]. Bacteria produce many intra- and extracellular oxidoreductive enzymes (*e.g.,* azo-reductases, laccases, dichlorophenolindophenol-reductase) for the degradation of organic pollutants [26]. Due to their ability to utilize different electron acceptors, bacterial degradation of pollutants is possible in both aerobic and anaerobic environments [27]. However, whereas bacteria possess the ability to adapt and grow on multiple organic pollutants, bacterial degradation is often limited by low moisture, low pH, and nutrient-limitations [109] as well as the heterogeneity of the environmental matrix (*e.g.,* in unsaturated porous media the access to pollutants is limited). Many fungi possess extracellular, non-specific, and non-stereoselective enzymes (*e.g.,* lignin peroxidases, laccases, manganese peroxidases) that can degrade different organic pollutants [14, 15]. However, most fungal degradation of organic pollutants occurs via oxidation under aerobic conditions, although fungal degradation under anaerobic conditions has also been reported [28, 29]. Fungi often co-metabolize these organic pollutants, by utilizing other available carbon sources such as plant or bacterial exudates or plant debris [25]. Fungi can typically degrade monoaromatic pollutants, but certain fungal species have been shown to also co-metabolize more complex compounds (*e.g.,* PAHs with up to six benzene rings) [30]. Perhaps from a practical view point, the greater advantage to employ fungi for the degradation of organic pollutants is their ability to adapt and survive in harsh environmental conditions such as low moisture content, low pH, fluctuating pollutant loads and its resilience ability to dry and wet feeding cycles (*e.g.,* in biotrickling filters) (Table 2).

Fungal-bacterial systems rely on different synergistic mechanisms driven by FBIs for the degradation of organic pollutants, including dispersal of degradative bacteria by fungal hyphae, co-degradation of pollutants, and enhancement of degradative enzyme production and secondary metabolites (Fig. 1). While there is evidence that fungi and bacteria in the plant microbiome can be important ‘hidden’ players in the phytoremediation process (use of plant systems for the removal of pollutants), plant-fungi-bacteria interactions to improve plant growth are out of the scope of this review. For further information on this topic see [25, 31, 32].

Dispersal of degradative bacteria by fungal hyphae

Microbial degradation of pollutants in porous media (*e.g.,* soil) is often limited by a lack of contact between microbes and their substrates, and microbial dispersal can aid in overcoming the distance that separates pollutants from microorganisms. Movement of bacteria in porous media is controlled by physical factors such as heterogeneity and pore-space complexity of the media, availability of surfaces for bacterial attachment, water availability (the lack of continuous water paths restricts bacterial motility) and oxygen availability [33, 34]. Unlike bacteria, movement of fungi through porous media is not significantly affected by the heterogeneity and dynamics of the prevailing hydrological conditions. The production of hydrophobins (hydrophobic proteins) [35] allows fungal hyphae to breach the air-liquid interface by reducing the surface tension, thus, allowing fungal growth into unsaturated pores [36]. The presence of filamentous fungi (and fungus-like microorganisms) can enhance bacterial dispersion in both saturated and unsaturated environments by offering surfaces and channels along which bacteria could move [37-39]. Kohlmeier et al. [37] suggested that growing hyphae serve as "highways" through which bacteria can translocate (active movement in the range of centimeters), rather than "subways" in which bacteria just attach to the hyphae and become passively displaced. Translocation or migration of bacteria depends on bacterial motility and fungal hydrophobicity [37, 40], where the presence of liquid films around hydrophilic hyphae allows bacteria to swim or swarm around and along the hyphae [41]. The characteristics of the porous media (*e.g.,* soil type) in which fungi and bacteria are co-cultivated, can also affect the growth and activity of fungi and bacteria and thus the fungal hyphae-mediated migration of bacteria [42]. The pH is another factor that can influence the migration of bacteria along fungal hyphae; some studies have reported that acidic or semi-acidic soil environments (pH 3.8–4.5) tend to restrict bacterial growth as well as the fungal hyphae-mediated migration of bacteria [43, 44].

Research on fungal hyphae-mediated migration of bacterial cells is still in its infancy and is mostly focused on studies simulating soil systems [37, 42, 43, 45-49]. Fungal-mediated migration of bacteria seems to be feasible for 'single-strain migrators' (*i.e.,* bacteria able to migrate using hyphae when grown in pure culture) as well as for 'community-migrators' (*i.e.,* members can only migrate through hyphal growth as part of a bacterial consortium) [43, 48]. Migrating bacteria can 'travel' along different fungal hosts, whereas fungi appear to have a specific 'carrying capacity' limited to a certain bacterial density that they can support [47]. While translocation of bacteria does not seem to negatively affect the prevalence and growth

of fungi, certain fungal strains display negative effects due to the presence of bacteria, including inhibition of growth and reduced competitiveness with other fungi present in the environment [49]. Besides, the active translocation of bacteria along the "fungal highways" can enhance the access to organic pollutants in unsaturated porous media and is therefore an important mechanism in the degradation of organic pollutants by fungal-bacterial co-cultures.

Co-degradation of pollutants

Biodegradation is the process by which complex organic compounds are transformed by microorganisms into simpler molecules/end-products (*e.g.,* water and carbon dioxide). Single species or communities of either fungi or bacteria might not possess the metabolic capabilities necessary to achieve complete degradation of complex pollutants (*e.g.,* high molecular weight [HMW] organics, such as those having >3 ring-PAHs). However, some mixed multi-domain communities have been shown to successfully achieve the complete degradation of such pollutants (Fig. 2). Each microorganism in these mixed microbial cultures might have a specific role in the degradation process, in which intermediates produced by certain microorganisms are utilized by other members. Several studies investigating fungal-bacterial co-cultures concurred that HMW-PAHs (*e.g.,* benzo[*a*]pyrene, pyrene, fluoranthene) degradation is the result of a co-degradation processes between fungi and bacteria, in which fungi initiate the oxidation step that allows bacteria to have access to the resulting less complex and more water soluble compounds [50-53]. PAHs do not seem to be used as sole source of carbon and energy by fungi; however, some fungi can produce extracellular enzymes (*e.g.,* lignin peroxidases, manganese peroxidases, laccases, cytochrome P450 monooxygenase-like enzymes) that oxidize PAHs to more polar metabolites (water-soluble and bioavailable) which can be degraded further by bacteria [16, 50].

Certain organic pollutants (*e.g.,* pharmaceutical compounds, endocrine disrupting chemicals) can be present at very low concentrations (<10 μg L^{-1}) in different environmental compartments (water, soil and sediments). These organic pollutants are usually toxic and strongly resistant to microbial degradation. In such cases these pollutants cannot support microbial growth and their removal is mostly attributed to co-metabolic degradation and physical mechanisms (*e.g.,* sorption in soil) [54-56]. To promote co-metabolism external growth-supporting nutrients (*e.g.,* a readily available carbon source) are required to stimulate microbial growth and enzyme production (see section below). Co-metabolic degradation can

result in the production of intermediate products that can be mineralized by other microbes but can also yield by-products that are toxic to the co-metabolizing microorganism [57, 58].

Enhancement of degradative enzyme production and secondary metabolites

FBIs in co-cultures can result in (i) the increase of enzyme production by either one of the microbial partners, (ii) the release and combination of different enzymes produced by the partners, which can contribute to the complete mineralization of the pollutants, or (iii) the production of secondary metabolites that can aid pollutant degradation.

The production of degradative enzymes (*e.g.,* lignin peroxidases, laccases, manganese peroxidases) can be increased due to synergetic and/or antagonistic interactions in fungal-bacterial co-cultures [59, 60]. Fungal-bacterial co-cultivation can result in the competition for nutrients and space, as well as oxidative stress, which can accelerate a fungal metabolic switch to secondary metabolism [61], resulting in the production of oxidative enzymes. Fungal laccases are oxidative enzymes effective for the degradation of persistent pollutants such as endocrine disruptors [62]. Induction and increase of laccase activity due to interspecies interactions seems to be a widespread phenomenon for various species of white-rot fungi (WRF) [59, 60]. In laboratory experiments, a 2–25 fold increase of laccase activity by *Trametes versicolor* was observed as a response to interactions with other soil microorganisms, including other fungi and bacteria [59]. Furthermore, laccase activity was increased when cultures of *T. versicolor* were grown with *E. coli*, bacteria not commonly found in the same environment [59]. A similar increase of laccase activity was observed in *Pleutorus ostreatus* during interactions with bacteria from activated sludge [60]. Induction of enzyme activity in fungi might be enzyme-specific; *e.g.,* when *T. versicolor* was grown with other soil microorganisms, only laccase activity was affected, but there was no significant change observed in the manganese peroxidase activity [59].

The combination of fungal and bacterial enzymes can result in the synergistic degradation of organic pollutants. Tran et al. [58] suggested the combination of fungal laccases and bacterial oxygenases as a plausible strategy to improve the degradation of emerging trace organic contaminants. For instance, bacterial oxygenases can oxidize aromatic pollutants resulting in smaller phenolic compounds [63]. These phenols can be toxic to bacteria, however, fungal laccases can promote degradation of these phenolic compounds [64].

Microbial secondary metabolites (*e.g.,* pigments, antibiotics, alkaloids and carotenoids) can facilitate external interactions among partners in microbial communities [65]. Secondary

metabolites could result from close interactions of microorganisms, from microbial crosstalk or chemical defense mechanisms that results in the induction of metabolic pathways due to the presence (signal) from another microorganism [61]. Previous studies have suggested that intimate physical interactions of bacteria and fungi are required to promote the production of secondary metabolites [66, 67]. Co-inoculation of *Aspergillus fumigatus* with the bacterium *Streptomyces bullii* resulted in the production of several fungal secondary metabolites (*e.g.,* metabolites belonging to the diketopiperazine alkaloid family) that were undetectable when the fungus was grown by itself [68].

Removal of organic pollutants by fungal-bacterial co-cultures

As described in the previous section, the combination of fungal and bacterial systems could lead to synergistic collaborations, which can facilitate the degradation of organic pollutants. The advantages and disadvantages of using either fungal, bacterial, or fungal-bacterial co-cultures for the degradation of organic pollutants are summarized in Table 2. This section describes defined fungal-bacterial co-cultures that have been used to degrade a broad range of pollutants including synthetic dyes, pesticides, polycyclic aromatic hydrocarbons (PAHs), trace organic contaminants (TrOCs), and volatile organic compounds (VOCs). Table 1 summarizes recent literature reports, wherein different fungal-bacterial co-cultures were used for the removal of organic pollutants from diverse environmental matrices.

Synthetic dyes

Synthetic dyes are largely used in the textile industry, and they are known for their resistance to degradation and persistence in the environment [69]. Treatment of textile wastewater is challenging, besides containing synthetic dyes, due to its fluctuating pH (~5.5–11.8, but most typically alkaline), high chemical oxygen demand (~150–30,000 mg L^{-1}) and suspended solids content (~15–5000 mg L^{-1}), high temperatures (~30–45 ºC) and presence of metals and salts/competing ions [70, 71]. Biological systems are typically efficient at dealing with effluents containing high chemical oxygen demands (COD), but they have some challenges at removing color.

Fungi and bacteria have been used for the treatment of textile dyestuff and effluent. Bacteria possess intra- and extracellular oxidoreductive enzymes (*e.g.,* azo-reductases, laccases, dichlorophenolindophenol-reductase) that can degrade dyes [26]. Some bacterial strains have achieved complete mineralization of anthraquinone textile dyes [71]. However, bacterial degradation of azo dyes (containing ≥ 1 azo group, R1-N=N-R2) is usually not

complete and it leads to the production of toxic aromatic amines or secondary pollutants [72, 73]. Diverse yeast and filamentous fungi have been used for the decolorization of synthetic dyes with the most widely used being *Phanerochaete chrysosporium* and *T. versicolor* [74, 75]. Fungal decolorization occurs via adsorption, enzymatic degradation, or a combination of both [75]. Fungal extracellular enzymes (*e.g.,* lignin peroxidases, laccases, manganese peroxidases) can degrade synthetic dyes (including azo dyes) without producing toxic aromatic amines [76]. However, the use of fungi in continuously operated reactors usually requires long hydraulic retention times for decolorization, and optimal growth generally occurs at low pH (*i.e.,* values <6.0), which might be a disadvantage considering the fact that most of the effluents containing synthetic dyes are alkaline in nature [70, 71, 77] (Table 2).

The use of fungal-bacterial co-cultures has been shown to enhance the decolorization of various synthetic dyes compared to their monocultures [23, 60, 73, 78-83] (Table 1). Most of the published studies used synthetic wastewater containing single dyes [60, 73, 78, 81-83], although the successful decolorization of real textile effluents was also reported [73]. As an example, Lade et al. [73] reported enhanced degradation of Rubine GFL (100 mg L^{-1}) using a co-culture of the fungus *Aspergillus ochraceus* with a *Pseudomonas* sp. and achieved 95% removal compared to pure cultures of *A. ochraceus* (46% removal) and *Pseudomonas* sp. (63%) after 30 h of incubation (at pH 8.5). The use of pure cultures resulted in the incomplete degradation of the azo dye and formation of toxic aromatic amines while complete removal of amines was achieved with the fungal-bacterial co-culture, suggesting the complete mineralization of the dye was due to synergistic reactions of fungal and bacterial oxidoreductases (*e.g.,* laccases, veratryl alcohol oxidase, azo reductases) [73]. Both, mono-cultures and the fungal-bacterial co-culture were able to remove Rubine GFL at a broad range of pH conditions, but maximum removal efficiency for the co-culture was observed in the range of pH 8.0–9.0 and at pH 8.0, respectively, for the single cultures. The removal efficiency decreased at lower (3.0–7.0) and higher (>9.0) pH values for the single and the fungal-bacterial co-cultures, respectively. The same fungal-bacterial co-culture was used to treat a textile effluent, reaching significantly higher reduction of the organic load (COD:96%, BOD: 82%, TOC:48%) and color (98% ADMI -American Dye Manufactures Institute- units removal) compared to the single cultures (Table 1). These results suggest that fungal-bacterial co-cultures could be a good alternative to bioremediate textile effluents. However, it is important to highlight that the experiments performed by Lade et al.[73] were carried out axenically, similarly to most studies investigating the application of fungal-bacterial co-cultures for

removing dyes from wastewater. From a practical perspective, it would be of interest to perform experiments under non-axenic conditions to assess the stability and long-term of the co-culture when in the presence of other microorganisms. Anew, it should be noted that most of the experiments performed on the laboratory scale focus on the decolorization of single dyes, whereas the removal efficiency of effluents containing mixed pollutants (either multiple dyes of other co-pollutants such as metals) is limited.

Pesticides

Pesticides are pollutants mostly comprised of organophosphate, organochlorine and carbamate compounds characterized by their high persistence in the environment and bioaccumulation potential [84]. Bacterial degradation of pesticides is usually only partial; few bacterial species (*e.g., Enterobacter*, *Stenotrophomonas* sp., *Sphingomonas* sp*., Bacillus cereus* and *Aminobacter* sp.) have been used to degrade chlorpyrifos (a widely used organophosphate insecticide) [85-88], as well as 2,6-dichlorobenzamide (BAM), a degradation product of the herbicide dichlobenil (2,6-dichlorobenzonitrile) [89]. Pesticide-degrading fungi have been extensively reported including WRF (*T. versicolor*, *P. chrysosporium*, *Pleutorus ostreatus*), brown-rot (*Gloeophyllum trabeum, Fomitopsis pinicola* and *Daedalea dickinsii*) and other filamentous fungi (*Penicillium citrinum*, *Aspergillus fumigatus*, *Aspergillus terreus* and *Trichoderma harzianum*) [90-96]. While the ability of fungi to degrade pesticides is often being associated with the presence of ligninolytic enzymes [94], fungal degradation of pesticides is often not very efficient and requires long incubation periods and acclimation time.

The use of fungal-bacterial co-cultures for the degradation of organochlorine and organophosphate pesticides has been reported in both, aqueous and porous media [97-103] (Table 1). While pesticide degradation in porous media using fungal-bacterial co-cultures was attributed to the activity of degrading bacteria associated with the fungal mycelium [97, 99, 103], in aqueous systems the pesticide degradation was mostly attributed to the degradative activity of both [98, 100-103]. 1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) has been the most common pesticide used in studies of synergistic fungal-bacterial degradation, reaching degradation removal efficiencies in the range of ~75-86% by co-cultures promoting synergistic fungal-bacterial degradation, co-cultures contained *P. ostreatus – P. aeruginosa* [100], *F. pinicola – B. subtilis* [102], *Pleurotus eryngii – R. picketii* [104], and *G. lingzhi – B. subtilis* [105]. Purnormo et al. [100] used the fungus *P. ostreatus* and the biosurfactant-producing bacterium *P. aeruginosa* for the degradation of DDT (0.25 μM) in an aqueous system. After 7

days of incubation, 19%, 36% and ~ 86% DDT degradation were observed by the fungus, bacteria and the fungal-bacterial co-cultures, respectively. Increased DDT degradation by the fungal-bacterial co-cultures was suggested to be due to the ability of *P. aeruginosa* to produce rhamnolipid biosurfactants, which can increase the solubility of DDT, thus facilitating degradation by the fungus [106]. In another study by Purnomo et al. [104], the WRF *P. eryngii* and the biosurfactant-producing bacterium *R. picketii* were used for DDT degradation. DDT degradation by the co-culture reached 78% after 7 d of incubation, whereas only 43% degradation was observed by the single culture of fungi. The authors observed that the presence of the bacteria stimulated the fungal growth. Analysis of the metabolic products from the degradation of DDT indicated that a pure culture of bacteria produced DDE (1,1-dichloro-2,2-bis(4-chlorophenyl) ethylene), the pure fungal culture produced DDE and DDMU (1-chloro-2,2-bis(4-chlorophenyl) ethylene), while the fungal-bacterial co-culture produced DDE, DDMU and DDD (1,1- dichloro-2,2-bis(4-chlorophenyl) ethane). The authors suggested a synergistic effect that resulted in the "transformation of DDT into DDD via dichlorination, followed by the formation of DDE through dehydrogenation followed by the formation of DDMU via dehydrochlorination" [104].

Trace organic contaminants (TrOCs)

Trace organic contaminants are a challenging group of pollutants due to their diverse chemical composition and their relatively low concentrations found in wastewater and water bodies (few ng L^{-1} to several µg L^{-1}). Common sources of TrOCs include pharmaceutically active compounds, personal care products, and endocrine disruptors. Bacterial systems as monocultures or mixed activated sludge often do not remove TrOCs efficiently from wastewater [107]. Fungi produce non-specific oxidative extracellular enzymes that can degrade various TrOCs, even those that are not efficiently removed by wastewater treatment plants (*e.g.,* pharmaceuticals like carbamazepine and diclofenac) [108, 109]. Some WRF can metabolize TrOCs [110]; for instance, *T. versicolor* is able to utilize diclofenac and benzophenone-3 as substrates for amino acid production [111]. Although fungi can metabolize TrOCs, the low concentration of these pollutants is usually insufficient to maintain fungal growth, thus a secondary source of carbon is required. Furthermore, the transformation of TrOCs by fungi could lead to the production of by-products that can be even more toxic than the parent TrOCs; *e.g.,* degradation of ibuprofen by *T. versicolor* generates 1,2-hydroxy ibuprofen, a metabolite that is more toxic than ibuprofen [112].

Few studies have reported the use of fungal-bacterial co-cultures for the removal of TrOCs [113, 114] (Table 1). Li et al. [113] studied the degradation of sulfamethoxazole (an antimicrobial) using a co-culture of the fungus *Pycnoporus sanguineus* and the bacterium *Alcaligenes faecalis*. After 48 h of incubation, the co-culture degraded 73% of sulfamethoxazole, whereas the fungal and bacterial monocultures degraded 23% and 53%, respectively. The enhanced removal efficiency by the co-culture was attributed to an increase in laccase activity (laccase plays a major role in the degradation of pharmaceuticals) by *P. sanguineus* in the presence of *A. faecalis*. The authors indicated that catabolism of sulfamethoxazole by *A. faecalis* might produce metabolites (not specified by the authors) that induce and enhance fungal laccase production [113]. Nguyen et al. [114] studied the degradation of 30 TrOCs in synthetic wastewater (5 μg L^{-1} of each TrOC) in a membrane bioreactor augmented with the fungus *T. versicolor* and activated sludge. Overall, the fungus-bacteria augmented bioreactor had greater TrOC removal (~80% for almost all TrOC) compared to a conventional membrane bioreactor containing only activated sludge. In that study, the enhanced TrOC removal was attributed to synergistic degradation by the fungus and the bacteria. Furthermore, the fungus-bacteria augmented bioreactor removed fenoprop, clofibric acid, pentachlorophenol, ketoprofen, diclofenac and naproxen, all TrOCs whose bacterial degradation is commonly not very efficient [115, 116]. The authors also showed that there was no increase in toxicity of the synthetic wastewater treated by the fungal-bacterial bioreactor, suggesting that toxic by-products did not accumulate to significant levels during the biodegradation of the TrOC [114].

Polycyclic aromatic hydrocarbons (PAHs)

PAHs are released into the environment mostly due to the incomplete combustion of organic materials, and they are of great concern due to their toxicity, mutagenicity and carcinogenicity [117, 118]. Although the degradation of PAHs by single fungal and bacterial co-cultures has been studied extensively [119, 120], complete mineralization of PAHs is usually limited to low-molecular-weight PAHs (≤ four-benzene ring). Bacteria can utilize PAHs as a sole source of carbon and energy generating oxidized products. Four-benzene-ring PAHs are frequently degraded by bacteria, while just some bacterial strains (single and in consortium) have been able to partially degrade five-benzene-ring PAHs [121]. Unlike bacteria, fungi transform PAHs by co-metabolism using different fungal enzymes (cytochrome P450 monooxygenase enzymes, lignin peroxidases, manganese peroxidases, and laccases)

depending on species and growth conditions [119]. Typically, fungi can degrade four to five-benzene-ring PAHs, although a yeast consortium (*Rhodotorula* sp., *Debaryomyces hansenii*, and *Hanseniaspora valbyensis*) was able to degrade benzo[ghi]perylene (a six-benzene-ring PAH) [30].

Fungal-bacterial co-cultures, mostly relying on commensalism and co-metabolism, have been able to degrade larger than four-benzene-ring PAHs as well as increased the degradation efficiency for low molecular-weight PAHs in porous media and aqueous systems [38, 50-53, 122-124] (Table 1). For instance, fungi can produce extracellular enzymes capable of degrading poorly soluble compounds and convert them into more soluble and less toxic compounds, that can in turn be utilized by them or other community members (including bacteria). Fungal-bacterial co-cultures can cooperatively degrade benzo[*a*]pyrene, (a 5-benzene-ring compound) as a result of sequential breakdown by fungi and bacteria, with the fungi performing an initial oxidation step producing metabolites that are available for bacterial degradation [50, 124] (Fig. 2). Bhattacharya et al. [50] showed the degradation of benzo[*a*]pyrene (10 μg mL^{-1}) using a co-culture of *P. ostreatus* PO-3 and *Pseudomonas aeruginosa*. In that study, after 30 days of incubation, benzo[*a*]pyrene degradation efficiency corresponded to 75% and 65%, for the fungal-bacterial co-culture and the fungal monoculture, respectively. Bhattacharya et al. [50] suggested that increased benzo[*a*]pyrene degradation by the fungal-bacterial co-culture was attributed to the cleavage of benzo[*a*]pyrene by ligninolytic fungal enzymes, which results in the formation of polar and more water-soluble compounds that could be further degraded by bacterial enzymes (*e.g.,* salicylate hydroxylase, 2-carboxybenzaldehyde dehydrogenase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase) [50]. Another study reported the degradation of 2-naphthol (pollutant derived from dye and pharmaceutical production [125]) by a co-culture of the fungus *A. niger* and the bacterium *Bacillus subtilis* [126]. After 10 days of incubation, 71%, 49% and 41% of the 2-naphtol (100 mg L^{-1}) were removed from the treated effluent using the fungal-bacterial co-culture, fungal and bacterial monocultures, respectively. The authors suggested that 2-napthol is first partially oxidized by *A. niger* resulting in the production of metabolites (1,2-naphthalene-diol and 1,2-naphthoquinone) that were then mainly degraded by *B. subtilis* [126] (Fig. 2). Boonchan et al. [124] observed increased degradation of five-benzene-ring PAHs in soil and utilization of benzo[*a*]pyrene as the only source of energy and carbon by a fungal-bacterial co-culture (*Penicillium janthinellum* with a bacterial consortium retrieved from a contaminated site). Moreover, the authors observed the decrease of mutagenicity in extracts

from soil treated with the co-culture compared to soil treated with single species and indigenous microbiota.

Volatile organic compounds (VOCs)

Volatile organic compounds are carbon-containing chemicals emitted as vapors and they are often characterized by their low-molecular-weight, high volatility, and low water solubility [127]. VOCs (*e.g.,* propane, butane, formaldehyde, benzene, toluene, acetone) are among the most common air pollutants in waste streams from chemical, petrochemical, and other industries [128]. Many microbial species have been used for the treatment of VOCs in both, gas and liquid phases. Bacterial biofilters are robust and are commonly used for off-gas treatment; however, their performance usually deteriorates under low moisture, low pH, nutrient-limited conditions and when the inlet waste gas concentration is > 5 g m^3 [129]. Bacteria often exhibit low performance for the degradation of hydrophobic VOCs under these conditions, which is mostly attributed to the low hydrophobicity of the bacterial cell surface, and the uneven distribution of free-water in biofilters, which can decrease the accessibility of gaseous substrates for bacteria [129, 130]. Fungi have been used for the removal of VOCs, including hexane, toluene, alpha-pinene, pentene and styrene in both biofilters and biotrickling filters [131-135]. Fungi seem to have some advantages over bacteria for the treatment of VOCs in biofilters: (i) aerial, fungal mycelia generally provide larger surface area in the gas phase than bacterial biofilms, which could facilitate the uptake of VOCs from the gas phase [136]; (ii) greater fungal cell surface hydrophobicity, which facilitates the adsorption of hydrophobic compounds [130]; (iii) fungi can tolerate hostile/harsh environmental conditions encountered in biofilters, such as low pH and low moisture content of the filter bed [137]; and (iv) the ability of fungi to grow under low moisture content can increase the mass transfer of hydrophobic VOCs from the gas phase to the fungal surface [129]. The use of fungi can improve the removal efficiency of pollutants in biofilters and biotrickling filters by at least 2-fold compared to biofilters inoculated with bacteria [138]. However, their low specific growth rates, long degradation times and operational problems such as filter bed clogging and pressure drop limit the use of fungi for VOC degradation.

The use of fungal-bacterial co-cultures to remove VOCs from waste-streams has shown promise [139-142] (Table 2). Enhanced biodegradation of VOCs by fungal-bacterial co-cultures is attributed to synergistic effects between fungi and bacteria in the consortium. Cheng et al. [139] observed a much faster degradation of chlorobenzene (220 mg L^{-1}) using a mixed

culture of *Trichoderma viride* and *Ralstonia pickettii* (100% removal efficiency within 60 h) compared to the single strains in liquid medium (100% removal efficiency in 96 h and 72 h for fungi and bacteria, respectively). The authors suggest that while *R. pickettii* was able to transform chlorobenzene to 2-chlorophenol, the accumulation of 2-chlorophenol becomes inhibitory to the bacteria. *T. viride,* besides being able to biodegrade chlorobenzene directly, is also able to rapidly utilize 2-chlorophenol, therefore, decreasing its concentration and limiting its toxicity to the bacteria [139] (Fig. 2).

Engineering fungal-bacterial co-cultures

There is a growing interest in engineering artificial microbial consortia to capitalize on the unique communal properties (*e.g.,* stability, robustness and division of labor [19-21]) developed in mixed microbial communities for environmental remediation applications. Increasing the microbial diversity in a system can lead to an increase of functional responses to perturbations disturbances over time, which can result in enhanced degradation of pollutants and more stable system performance, something that is necessary for bioreactor design and operation for remediation purposes [18]. Several recent reviews discuss the factors relevant to engineering (and studying the interactions in) artificial microbial consortia for different biotechnological applications [143-148]. Here, we discuss the factors that affect the successful establishment of artificial fungal-bacterial systems for environmental applications, including selecting appropriate microbial partners, inoculation strategies, nutritional and cultivation conditions to create adequate and stable ecological niches [143] that favor the degradation of organic compounds.

Selection of microbial species

Selection of the interacting microbial species is key when constructing fungal-bacterial co-cultures. The choice of microorganisms determines the overall success of the strategy to remove pollutants from different environmental matrices (air, water, soil and sediments). Certain microbial species will be better suited to survive and/or grow in the presence of specific partners, as well as in the presence of a certain pollutant or mixture of pollutants, depending on the nature and composition of the compounds (hydrophobic, hydrophilic, recalcitrant and xenobiotic). The combination of certain species can envisage the following changes: (i) create synergistic effects, thereby enhancing the degradation capability of the mixed cultures, (ii) lead to antagonistic interactions, which could inhibit the growth of one of the partners, thus being

detrimental to the biodegradation of pollutants, or (iii) trigger a secondary metabolism in one of the partners or in both, thus increasing the production of beneficial enzymes or metabolites (see 'Mechanisms of degradation in fungal-bacterial systems' above).

Selection of a suitable bacterial partner should be based on the ability of bacteria to develop traits that confer to them advantages to colonize on or in association with fungi, such as the ability to use nutrients available in fungal exudates (*e.g.,* carbohydrates, polyols, organic acids), or the ability to tolerate antibacterial metabolites [4, 149]. Most of the work with fungal-bacterial co-cultures for environmental applications has been performed using predefined combinations of pure microbial cultures (Table 1). However, the choice of strains should also consider naturally co-occurring microorganisms isolated from environmentally relevant matrices (*e.g.,* polluted soils or waters)[150]. Acclimation of microorganisms or directed evolution are strategies that can also be used, in which the selected microbial partners are cultured in the presence of different concentrations of the targeted pollutants with the expectation that the microorganisms will eventually display physiological, anatomical or morphological changes to better perform or survive the prevailing environmental conditions or environmental stress [151].

Inoculation

When constructing fungal-bacterial systems, the size of the inoculum as well as the sequence of inoculation could be determining factors influencing the effectiveness (*e.g.,* colonization and growth, degradation of pollutants) and the stability/activity of the final community. Most studies using fungal-bacterial co-cultures for the degradation of pollutants used single strains and constructed an artificial fungal-bacterial consortium by mixing the known strains together, in well-defined proportions. However, most studies do not provide detailed descriptions of the process of consortium development and long-term survival of the dominant species. The most recognized inoculation factors that affect the establishment of mixed microbial communities are the fungal-bacterial ratio and the sequence of inoculation and the acclimation conditions in batch or continuous bioreactors.

Fungal-bacterial ratio - The initial concentration and ratio of microorganisms in the mixed cultures needs to be optimized since the excess of one of the partners can result in an antagonistic relationship inhibiting the establishment of the other partner. Purnomo et al. [100] tested two different fungal-bacterial systems using *P. ostreatus* in combination with either *Pseudomonas aeruginosa* or *Bacillus subtilis* for the degradation of DDT. Different

concentrations of bacteria (1, 3, 5 and 10 mL of bacteria; 1 mL~1.25 $\times 10^9$ cfu mL^{-1}) were added to *P. ostreatus* cultures (no information regarding fungal concentrations was provided). Based on the maximal DDT removal efficiency, optimal inoculum size was 3 mL for *P. aeruginosa* and 5 mL for *B. subtilis*. When adding high concentrations of bacteria (> 5mL inoculum), decreased DDT degradation by the fungal-bacterial co-cultures was observed. The authors suggested that this could be presumably due to increased competition between the bacteria and *P. ostreatus* for nutrients in the supplied growth medium. Moreover, *P. aeruginosa* and *B. subtilis* produce biosurfactants that might solubilize DDT for degradation, but such biosurfactants can exhibit antifungal activities, and hence, high concentrations of bacteria could result in high production of biosurfactants that could potentially inhibit or hinder the activity of *P. ostreatus* [100]. Due to different growth rates, it is possible that over time one partner outcompetes the other(s); besides controlling the fungal-bacterial ratio when inoculating, another strategy to help maintaining the stability of the culture is to intermittently re-inoculate the 'underdog' partner to extend the period of co-cultivation [144].

Sequence of inoculation - The general protocol to develop fungal-bacterial co-cultures consists of growing single strains as cell/spore suspensions (in batch vessels) and then mixing them concurrently. However, the sequence of addition of partners can influence biomass growth, biofilm formation, the composition of extracellular polymeric substances (EPS) and enzymes [152-155]. Válková et al. [152] observed that the order of inoculation affected the formation of biofilms comprised of *Pleurotus ostreatus* and the bacterium *Pseudomonas fluorescence*. Addition of *P. fluorescence* to pre-grown *P. ostreatus* biofilms resulted in biofilms comprised of fungal mycelium and bacterial cells associated with the hyphae or in clusters within the biofilm. In this mixed biofilm, the enzymatic activity of the fungus was not hampered, whereas adding *P. ostreatus* to pre-grown *P. fluorescence* biofilms resulted in reduced fungal biomass growth and lower production of extracellular enzymes [152]. Velmourougane et al. [153] reported similar findings with sequential inoculation of the fungus *Trichoderma viride* and the bacterium *Azotobacter chroococcum*. After 18 days of incubation, maximal biofilm formation was observed when *Azobacter* was added to pre-formed fungal biofilms (48 h-old), which was attributed to greater attachment of bacterial cells to the already established fungal matrix. The addition of bacteria to pre-formed fungal biofilms also resulted in significantly higher content of total carbohydrates, proteins, acetyl- and uronic acid-residue content in the EPS matrix compared to biofilms formed adding *Trichoderma* to pre-formed bacterial biofilms or when inoculated concurrently [153]. The effect of sequence of inoculation

on the establishment and growth of the fungal-bacterial co-cultures is strain-dependent. Triveni et al. [155] studied biofilm formation as the result of the sequential inoculation of *T. viride* with bacterial strains of *Azotobacter chroococcum*, *Bacillus subtilis* and *Pseudomonas fluorescens*. The quality of the biofilms formed was better (in terms of the amount of biomass formed and the population counts of the respective partners) when *Trichoderma* was inoculated into 48 h-old bacterial cultures of *B. subtilis* and *P. fluorescens* compared to the biofilms developed when the bacteria were inoculated to already established *Trichoderma* biofilms. On the contrary, better biofilm development was observed when *A. chroococcum* was added to 48 h-old *Trichoderma* biofilms.

Growth media and culture conditions

One of the main challenges in constructing fungal-bacterial communities is to achieve media compositions and culture conditions (*e.g.,* pH, temperature, oxygen) that can successfully support multiple species. Medium composition and culture conditions are key factors that can influence the growth, stability, physical association, and composition of the fungal-bacterial community.

Medium composition - Medium composition can affect: microbial growth [156], the ability to form biofilms [153], and microbial interactions [157]. Velez et al. [157] studied the effect of diverse nutrient scenarios on the interactions between fungi (*Coprinellus micaceus*, *Cladosporium* sp., and *Aspergillus niger*) and bacteria (*Aeromonas* and *Vibrio* spp.) isolated from liquid samples in an oligotrophic desert oasis. When cultivated under low-nutrient conditions, close physical association among fungi and bacteria occurred, and most interactions were beneficial (enhanced growth) for both, fungi and bacteria [157]. Whereas when grown in rich media (carbohydrate- and peptide-rich) physical associations between fungi and bacteria were seldom observed and antagonistic interactions dominated. Under intermediate level nutrient conditions (media containing carbohydrates and peptides), moderate physical associations were observed, and antagonistic interactions were dominant [157]. While most research on media optimization has been done at the laboratory scale using response surface methodology [158] or other statistical optimization techniques, for field-scale applications, it is not recommended to add pure chemicals as carbon sources (*e.g.,* glucose, ethanol, methanol) for microbial growth. Thus, it is also important to consider the use of less expensive nutritional supplements to stimulate fungal-bacterial communities *in situ; i.e.,* the use of alternative carbon sources such as agro-waste should be considered as co-substrates for the degradation of

pollutants in practice [159].

pH conditions - pH is a well-known parameter that affects microbial growth, it can influence the fungal-bacterial relationship by promoting or inhibiting the growth of one of the partners and it can also have an effect on the fungal hyphae-mediated migration of bacteria [44, 160]. Rousk et al. [160] studied the effect of pH on the competitive interaction between fungi and bacteria in soil. In this study, when co-inoculated and in the presence of suitable substrates, at pH <5.0 bacterial growth rate was slow and fungal growth was promoted; whereas, in higher pH soils (pH 6.5−8.0) low fungal growth rate was observed while bacterial growth increased. When bacterial growth was suppressed, increased fungal growth was observed even in high pH soils, which suggests that bacteria were causing competitive pressure inhibiting fungal growth at high pH [160]. The pH conditions also seem to affect the fungal hyphae-mediated migration of bacteria [44]. Yang et al. [44] observed that low soil pH (3.8-4.2) significantly limited the survival of *Paraburkholderia terrae* as well as its dispersal along the hyphae of a *Lyophyllum* sp. Moreover, the initial pH conditions can influence the direction of bacterial migration along the fungal hyphae; Warmink et al. [43] reported that migration of *P. terrae* along the hyphae of *Lyophyllum* sp. at low soil pH (4.1-4.5) was only observed in the fungal growth direction, as opposed to being bidirectional under more alkaline conditions as reported in other studies [44].

Bioreactor systems

Most studies related to fungal-bacterial co-cultures and their applications in organic pollutant degradation are performed using batch co-culture systems (*e.g.,* using flasks containing liquid media or soil/sand contaminated with the desired pollutant) while fewer studies use column systems, membrane bioreactors, biofilters or biotrickling filters. At the laboratory scale tests, batch systems have provided relevant insights into establishing fungal-bacterial co-cultures and their potential for organic pollutants degradation However, these systems might not be suitable for establishing a stable co-culture in the long term considering that the fairly homogeneous conditions prevailing in batch systems can promote the dominance of a specific member in the co-culture. Thus, for the successful co-cultivation of fungal-bacterial communities, it is necessary to develop novel bioreactor configurations that consider the metabolic diversity present in mixed microbial consortia and the different growth requirements of the interacting partners (*e.g.,* oxygen, temperature, light intensity, pH, oxidation and reduction potential). One strategy to address these challenges is to design

reactors that allow for the establishment of controlled heterogeneity with the spatial niches required for optimal growth and activity of the partners [143]. Creating bioreactor systems that promote the formation of different microenvironments, for example by introducing gas permeable membranes that allow for the establishment of oxygen gradients, can provide the conditions necessary to promote the cultivation of aerobic and anaerobic microorganisms [143]. Another strategy is compartmentalization of reactors using separate modules that provide optimal growth conditions for each interacting partner, the partners can then 'communicate' with each other through controlled fluxes of exchanged medium containing metabolites, enzymes or byproducts of the degradation of complex xenobiotics [161]. Compartmentalized reactors could be used, for example, for the degradation of emerging TrOCs; complex aromatic pollutants could be initially oxidized in a module containing bacteria, while the phenolic compounds produced as a result of this initial degradation could be transferred to a second module containing fungi, which should be able to promote the degradation of phenols via fungal laccases [59].

Challenges and scope for further research

Fungal-bacterial co-cultures offer a broad range of potential applications in environmental remediation technologies. However, there are still challenges when constructing and maintaining fungal-bacterial systems for environmental applications (Fig. 3). ***The maintenance and stability of the fungal-bacterial co-culture*** during long-term operation is one of the main challenges. Selection of random or non-compatible species in a mixed culture can result in unstable populations, dominance of one species, or loss of one or all partners, or reduced performance of the system to remove the pollutants [162]. As mentioned in the section "Engineering fungal-bacterial co-cultures", partners in a mixed culture might have different growth requirements and growth rates, thus optimization of the medium composition and growth conditions might be essential for the survival and growth of both partners. The successful establishment of mixed cultures depends on the correct selection of the microbial species, the optimization of the inoculation process, and maintenance of optimal co-cultivation conditions. Common approaches to ensure balanced growth of the subpopulations include, among others: (i) determination of the optimal fungal-bacterial inoculation ratio, and (ii) the intermittent re-inoculation of the system with the potential "underdog" subpopulation [144]. Furthermore, it is important to *design and test suitable bioreactor configurations* that will favor fungal and bacterial co-habitation, or that can allow for sequential treatment, *e.g.,* a first stage

of fungal growth followed by a second stage with bacterial growth, or vice-versa. Most of the studies reported so far have used homogenous batch laboratory scale systems, thus it is important to consider up-scaling challenges for fungal-bacterial systems into semi-industrial or full-scale systems. There certainly is a need to develop novel systems that could aid in: (i) providing more insights into the fungal-bacterial interactions (*e.g.,* visualization of physical association, microbial migration, metabolic pathways of degradation), (ii) building ecological niches [143] that can favor degradation of pollutants under different conditions appropriate for each interacting partner, and (iii) studying degradation of pollutants under realistically heterogenous conditions to promote the necessary growth conditions required for all of the microbial partners.

Another challenge of using artificial fungal-bacterial mixed cultures arises when ***working under environmentally relevant, non-axenic conditions***. Most of the studies using fungal-bacterial co-cultures in batch or continuous flow bioreactors report experimental data obtained under axenic conditions, which raises concerns regarding the biodegradation performance under more realistic environmental conditions containing indigenous microbes. The few studies performed under non-axenic conditions do not typically monitor the composition of the microbial community during the biodegradation process; thus, it is difficult to understand the stability of fungal-bacterial co-cultures over time. In well-controlled laboratory conditions, axenic conditions can be maintained in continuously operated bioreactors fed with wastewater or waste gas, but only for short periods of time. Badiat-Fabregat et al. [24] studied the effect of the bacterial and fungal communities present in real wastewater containing pharmaceuticals in a non-axenic bioreactor inoculated with *T. versicolor*. The authors showed that indigenous fungi (*e.g., Trichoderma asperellum*) and bacteria can outcompete the inoculated fungus. Furthermore, the composition of the wastewater and the operational conditions seemed to have a stronger influence on the microbial community than the inoculation with *T. versicolor* [163]. Thus, there is a need to explore the effect of non-axenic conditions on artificial fungal-bacterial co-cultures. Some questions that arise include: *Can the established fungal-bacterial co-culture outcompete autochthonous microbes in wastewater or other environmental matrices? Does the presence of invading microbes affect the activity of the fungal-bacterial consortia and therefore the degradation of pollutants?*

The ***co-occurrence of various types of pollutants*** in the environment is common and it is a factor that needs to be considered when exploring the biodegradation potential of fungal-

bacterial systems. Ma et al. [164] observed that fluoranthene degradation by fungal-bacterial co-cultures (*Acremonium* sp. and *Bacillus subtilis)* in porous media is affected by the presence of heavy metals. The fungal-bacterial co-culture was able to remove fluoranthene more efficiently (~65% total removal) compared to the monocultures, but the presence of heavy metals influenced the fluoranthene degradation. The presence of Cu^{2+} inhibited bacterial growth and reduced bacterial translocation by the fungal hyphae, which resulted in a 10% decrease in the fluoranthene removal efficiency. Whereas, the presence of Mn^{2+} increased bacterial growth and bacterial translocation on fungal hyphae, leading to enhanced fluoranthene degradation by the fungal-bacterial co-culture (total removal efficiency ~80%) [164]. Elucidating the mechanisms by which co-existing pollutants affect fungal-bacterial systems could aid in (i) understanding microbial interactions in polluted matrices, and (ii) engineering artificial fungal-bacterial co-cultures (*i.e.,* selecting species tolerant to different pollutants).

Although some studies have reported the use of fungal-bacterial systems for remediation purposes and certain mechanisms of pollutant removal have been identified, there is still ***little understanding of the dynamics and interactions of fungal-bacterial populations***, limiting their use in real-world applications. For example, many aspects of the fungal-mediated translocation of bacteria and its impact in pollutant degradation remain to be explored. *How does the migration of motile bacteria along fungal hyphae affect the assembly, microbial diversity, and stability of multispecies consortia? How do multiple motile bacteria interact and compete on "fungal highways"? What factors affect the 'carrying capacity' of fungi to support bacteria, and is it specific to certain bacterial species?* Moreover, little is known about the pollutant degradation pathways (*e.g.,* intermediate and end products) and the specific metabolites and enzymes used by fungi and bacteria during the degradation process. Furthermore, it is unclear how the composition and structure of the organic pollutants might influence fungal-bacterial interactions. The use of 'next-generation physiology approaches' [165] using a combination of multi-omic techniques (*e.g.,* proteomics, metabolomics, transcriptomics, metagenomics), high-resolution advanced microscopy techniques (*e.g.,* Raman microspectroscopy, cryo-electron microscopy), isotope and substrate analogue probing (*e.g.,* incubation of co-cultures with ^{13}C-glucose or $^{15}NH_4^+$, biorthogonal non-canonical amino acid tagging [BONCAT]) and polymicrobial culturing approaches could provide more thorough understanding of fungal-bacterial dynamics and their spatial relationships, and thus, their influence on pollutant degradation.

Conclusions

Fungal-bacterial biotechnology for environmental remediation has a wide range of potential applications. The use of fungal-bacterial co-cultures for the removal of pollutants in different environmental matrices has shown promising results on the laboratory scale by demonstrating enhanced biodegradation and removal efficiency of pollutants compared to their respective monocultures. However, this is still an emerging subject. Fungal-bacterial co-cultures rely on various mechanisms for the degradation of pollutants including dispersal of degradative bacteria by fungal hyphae, co-degradation of pollutants, and enhancement of degradative enzyme production and secondary metabolites. These mechanisms depend on the microbial species selected and the developing physical associations and interactions among the partners. Further developments towards full scale application of fungal-bacterial bioreactors depend on maintaining a stable fungal-bacterial community, which can successfully establish under non-axenic conditions robust enough to simultaneously treat multiple contaminants.

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Disclosure of interest

The authors report no conflict of interest.

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Figures

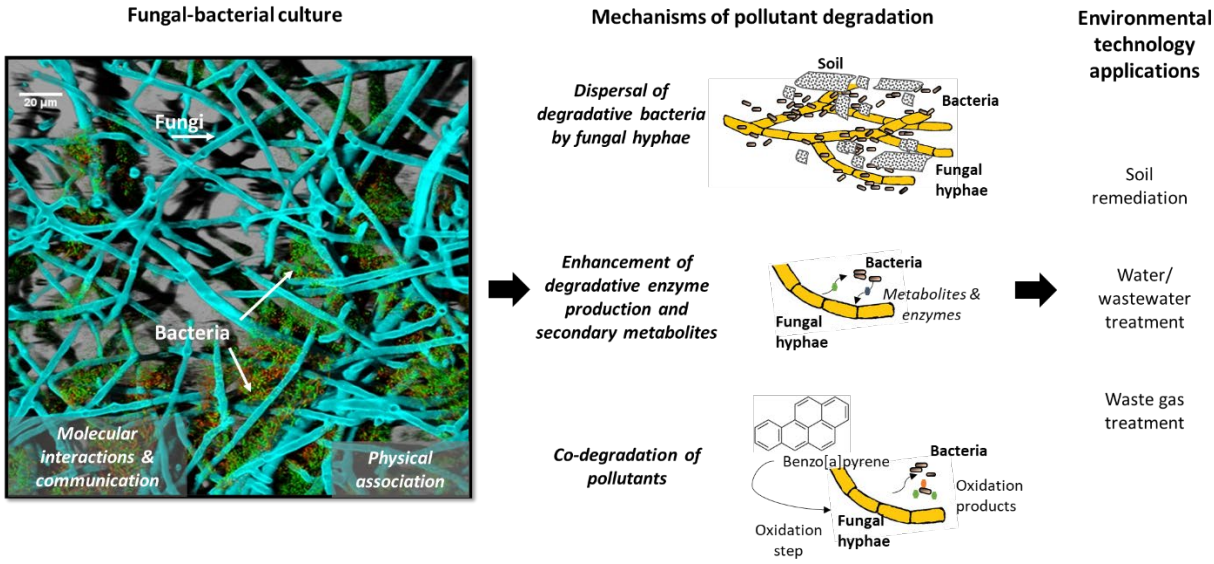

Figure 1. Use of fungal-bacterial co-cultures for environmental remediation. Association of fungi and bacteria can result in interactions that can promote the degradation of organic pollutants in different environmental matrices. Fungal-bacterial interactions result in different pollutant degradation mechanisms including fungi acting as vectors to disperse bacteria in unsaturated porous media, enhancement of degradative enzyme production and secondary metabolite production, as well as co-degradation of pollutants. Fungal-bacterial co-cultures have been successfully used at the laboratory scale to demonstrate soil remediation, water/wastewater and waste gas treatment.

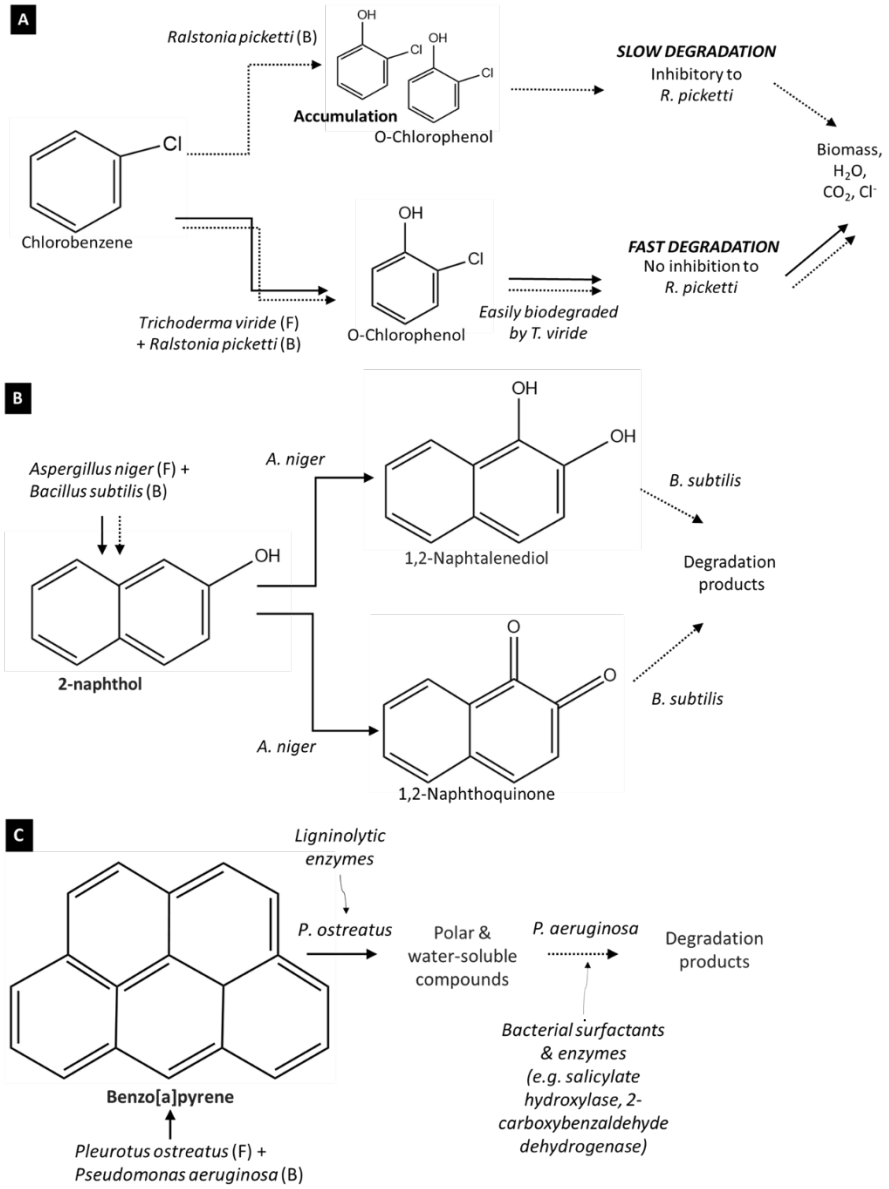

Figure 2. Examples of synergistic degradation of organic pollutants by fungal-bacterial co-cultures. (A) Degradation of chlorobenzene by a co-culture of the fungus *Trichoderma viride* and the bacterium *Ralstonia picketti* [139]. *R. picketti* is able to transform chlorobenzene to 2-chlorophenol, an intermediate that can be inhibitory to the bacteria, resulting in slow degradation of chlorobenzene. Faster degradation of chlorobenzene is observed when using the fungal-bacterial co-culture. *T. viride*, besides being able to degrade chlorobenzene, is also able to rapidly utilize 2-chlorophenol, decreasing its concentration and therefore limiting its toxicity to *R. picketti*. (B) Degradation of 2-napthol was achieved using a co-culture of the fungus *Aspergillus niger* with the bacterium *Bacillus subtilis* [126]. It is suggested that *A. niger* partially degrades 2-naphthol to 1,2-naphthalene-diol and 1,2-naphthoquinone, metabolites that are mostly degraded by *B. subtilis*. (C) Degradation of benzo[*a*]pyrene was achieved using a co-culture of the fungus *Pleurotus ostreatus* with the bacterium *Pseudomonas aeruginosa* [50]. It is suggested that fungal ligninolytic enzymes break down benzo[*a*]pyrene to more polar and water soluble compounds (not identified) available for bacterial degradation.

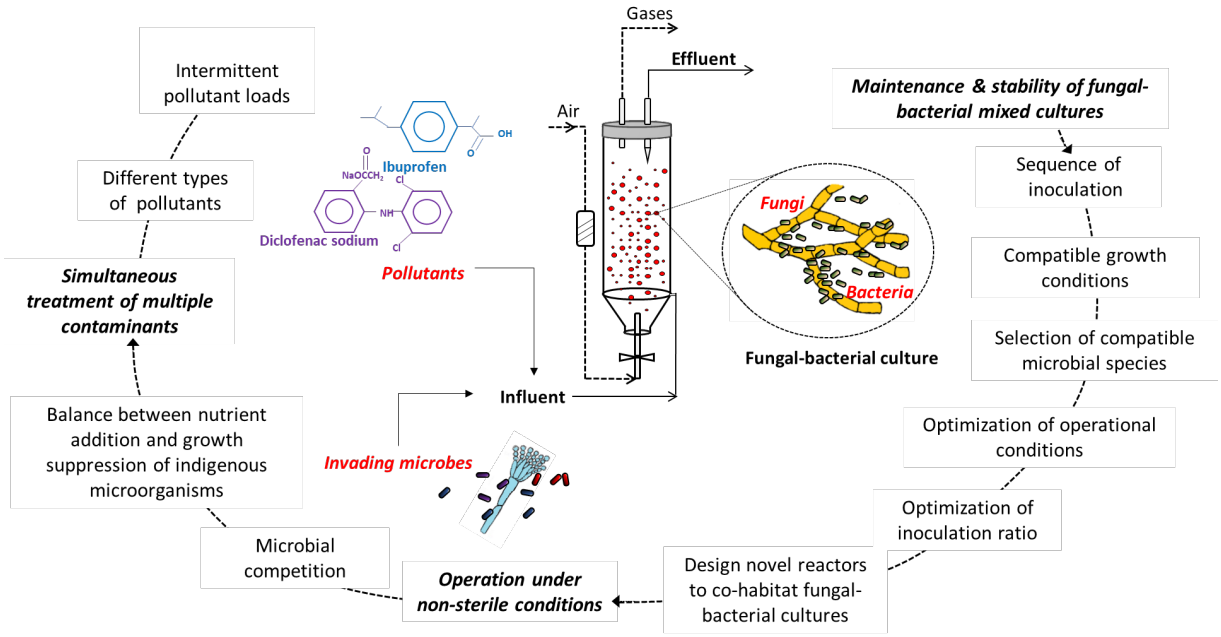

Figure 3. Overview of the challenges in engineering fungal-bacterial co-cultures for the treatment of contaminants in different matrices: maintenance and stability of the co-cultures, operation during non-axenic conditions and simultaneous treatment of various contaminants.

Tables

Table 1. Fungal-bacterial co-cultures for the removal of pollutants in different environmental matrices.

Pollutants (Initial concentration)	Microbial co-culture (Fungus + Bacteria)	Treatment conditions	Pollutant removal efficiency†	References
Dyes				
Azo dyes				
Rubine GLF (100 mg L^{-1})	*Aspergillus ochraceus* (F) + *Pseudomonas* sp. (B)	Matrix: liquid Operation time: 30 h pH: 8.5, T: 37 ºC	95% (F+B), 46% (F), 63% (B)	[73]
Reactive red 120 (50 mg L^{-1})	*Aspergillus niger* (F) + *Bacillus* sp. (B)	Matrix: liquid Operation time: 24 h pH: 7.0, T: 35 ºC, 130 rpm	90% (F+B), 50% (F), 30% (B)	[82]
Reactive Brilliant Red X-3B (235 mg L^{-1})	*Penicillium* sp. (F) + *Exiguobacterium* sp. (B)	Matrix: liquid Operation time: 12 h pH: 6.5, T: 33 ºC	96% (F+B)	[81]
Reactive Brilliant Red X-3B (50 mg L^{-1})	*Penicillium* sp.(F) + *Sphingomonas xenophaga* (B)	Matrix: liquid Operation time: 3 d pH: 3.0, T: 30 ºC	88% (F+B)	[78]
Dark Blue K-R (200 mg L^{-1})	*Penicillium* sp. (F) + *Exiguobacterium* sp. (B)	Matrix: liquid Operation time: 24 h pH: 7.0, T: 30 ºC	85% (F+B), 70% (F), 57%(B)	[83]
Anthraquinone dyes				
Remazol Brilliant Blue R (100 mg L^{-1})	*Pleurotus ostreatus* (F) + activated sludge (B)	Matrix: liquid; solid-bed bioreactor Operation time: 24 h pH: 7.6; RT	95% (F+B), 77% (F)	[60]
Textile effluent				
Textile industry effluent [1]BOD (260 mg L^{-1}) [2]COD (3920 mg L^{-1}) [3]TOC (4175 mg L^{-1}) [4]Color (% ADMI removal)	*Aspergillus ochraceus* (F) + *Pseudomonas* sp. (B)	Matrix: liquid Operation time: 35 h pH: 8.5, T: 37ºC Micro-aerophilic	[1]82% (F+B), 15% (F), 76% (B) [2]96% (F+B), 11% (F), 90% (B) [3]48% (F+B), 5% (F), 37% (B) [4]98% (F+B), 5% (F), 44% (B)	[73]
Textile effluent (NR, ADMI*)	*Aspergillus ochraceus* (F) + *Providencia rettgeri* (B)	Matrix: liquid Operation time: 30 h	92% (F+B)	[80]

		pH: 7.0, T: 30 ºC Micro-aerophilic		
Synthetic effluent (NR, ADMI*)	*Galactomyces geotrichum* (F) + *Brevibacillus laterosporus* (B)	Matrix: liquid Operation time: 48 h pH: 9.5, T: NR	95% (F+B), 89% (F), 75% (B)	[79]
COD (6755 mg L^{-1}) BOD (2005 mg L^{-1}) Color units (1367) TSS (566 mg L^{-1})	*Trametes versicolor* (F) + *Trametes* sp. (F) + *Pleurotus ostreatus* (F) + *Pseudomonas* sp. (B) + *Enterobacter xiangfangensis* (B)+ *Bacillus subtillis* (B)	Matrix: liquid Operation time: 147 h pH: 7.0, T: 20 ºC Aerobic	COD, 75% (F+B) BOD, 95% (F+B) Color, 92% (F+B) TSS, 99% (F+B)	[23]
Pesticides				
2,6-dichlorobenzamide (100 µg kg^{-1})	*Mortierella* sp. (F) + *Aminobacter* sp. (B)	Matrix: sand Operation time: 60 d T: 20 ºC, 5% WHC	50% (F+B), 0% (F), 19% (B)	[99]
Chlorpyrifos – organophosphate pesticide (50 mg L^{-1})	*Trichosporon* sp. (F) + *Serratia* sp. (B)	Matrix: liquid pH: 8.0; T: 30 ºC	100% (F+B) in 18 h 100% (F) in 7 d 100% (B) in 5 d	[103]
DDT (4.43 mg L^{-1})	*Fomitopsis pinicola* (F) + *Bacillus subtilis* (B)	Matrix: liquid Operation time: 7 d pH: NR; T: 30 ºC	86% (F+B), 42% (F) 35–86% (B) depending on inoculum size	[102]
DDT (0.088 mg L-^{1})	[1]*Pleurotus ostreatus* (F) + *Pseudomonas aeruginosa* (B); [2]*Pleurotus ostreatus* (F) + *Bacillus subtilis* (B)	Matrix: liquid Operation time: 7 d pH: NR, T: 25 ºC	[1]86% (F+B), 19% (F), 25% (B) [2]30% (F+B), 19% (F), 20% (B)	[100]
DDT (0.088 mg L-^{1})	*Fomitopsis pinicola* (F) + *Bacillus subtilis* (B)	Matrix: liquid Operation time: 7 d pH: NR, T: 30 ºC	86% (F+B), 42% (F), 86% (B)	[101]
Diuron – phenylurea herbicide (100 µg kg^{-1})	*Mortierella* sp. LEJ702 (F) + *Varovorax* sp. SRS16 (B) + *Arthrobacter globiformis* D47 (B)	Matrix: sand Operation time: 54 d pH: NR, T: 20 ºC	32.2% (F+B), 0.4% LEJ702 (F), 4.4% SRS16 (B), 4.8% D47 (B)	[97]
[1]Aldicarb (10 mg L^{-1}) [2]Atrazome (10 mg L^{-1}) [3]Alachlor (10 mg L^{-1})	*Trametes versicolor* (F) + activated sludge (B)	Matrix: liquid Operation time: 14 d pH: NR, T: 28 ºC, 80 rpm	[1]47% (F+B), 20% (F) [2]98% (F+B), 0% (F) [3]62% (F+B), 0% (F)	[98]
Polycyclic aromatic hydrocarbons (PAHs)				
Naphthalene (6.4 g L^{-1})	*Acremonium* sp. (F) + *Bacillus subtilis* (B)	Matrix: liquid	100% (F+B), 97% (F), 95% (B)	[51]

Fluorene (10 g L^{-1}) Phenanthrene (8.9 g L^{-1}) Anthracene (8.9 g L^{-1}) Fluoranthene (10 g L^{-1})		Operation time: 10 d pH: NR, T: 28 ºC, 80 rpm	89% (F+B), 72% (F), 86% (B) 82% (F+B), 63% (F), 72% (B) 71% (F+B), 55% (F), 57% (B) 60% (F+B), 53% (F), 27% (B)	
Fluoranthene (80 mg kg^{-1})	*Acremonium* sp. (F) + *Bacillus subtilis* (B)	Matrix: sand Operation time: 14 d pH: NR, T: NR	64% (F+B), 58% (F), 30% (B)	[164]
Anthracene (121.4 mg kg^{-1}) Phenanthrene (106.2 mg kg^{-1}) Pyrene (94.8 mg kg^{-1})	*Aspergillus terreus* (F) + *Rhodococcus* sp. (B); *Penicillium* sp. (F) + *Rhodococcus* sp. (B)	Matrix: soil Operation time: 10 w pH: NR, T: NR Dark conditions	100% for all PAH's with both co-cultures	[52]
2-Naphthol (100 mg L^{-1})	*Aspergillus niger* (F) + *Bacillus subtilis* (B)	Matrix: liquid Operation time: 10 d pH: NR, T: NR	71% (F+B), 49% (F), 41% (B)	[126]
Phenanthrene (200 mg kg^{-1})	[1]*Penicillium* sp. (F) + *Pseudomonas cepacia* (B); [2]*Penicillium* sp. (F) + *Ralstonia pickettii* (B); [3]*Penicillium* sp. (F) + *Pseudomonas aeruginosa* (B)	Matrix: soil Operation time: 10 d pH: NR, T: 30 ºC	[1]72% (F+B), 35-50% (F), 20% (B) [2]73% (F+B), 35-50% (F), 20% (B) [3]69% (F+B), 35-50% (F), 20% (B)	[122]
Pyrene (100 mg kg^{-1})	*Fusarium* sp. (F) + *Bacillus* sp. (B) + *Sphingomonas* sp. (B)	Matrix: soil Operation time: 63 d pH: NR, T: 30 ºC, 150 rpm	87% (F+B), 60% (F), 65% (B)	[53]
Benzo[*a*]pyrene (10 µg mL^{-1})	*Pleurotus ostreatus* (F) + *Pseudomonas aeruginosa* (B) *Pleurotus ostreatus* (F) + *Bacillus cereus* (B)	Matrix: liquid Operation time: 30 d pH: NR, T: 30 ºC	75% (F+B), 64% (F) 72% (F+B), 64% (F)	[50]
Benzo[*a*]pyrene (50 mg L^{-1})	*Penicillium* sp. (F) + *Serratia marcescens* (B)	Matrix: liquid Operation time: 20 d pH: NR, T: 25 ºC, 175 rpm	60% (F+B), 45% (F), 50% (B)	[123]
Hydrocarbons				
Oil (21,552 mg kg^{-1} TPH)	*Candida tropicalis* (F) + *Bacillus subtilis* (B) + *Pseudomonas fluorescens* (B) + *Streptococcus faecalis* (B)	Matrix: soil Operation time: 48 d pH: NR, T: 25 ºC	56% (F+B), 37% (F), 40% (B)	[166]
Crude oil (1% v/v)	*Scedosporium boydii* (F) + indigenous bacterial consortium (B)	Matrix: soil	81% (F+B), 25% (F), 61% (B)	[167]

		Operation time: 7 d pH: NR, T: 30 ºC, 150 rpm		
Crude oil (50,000 mg kg^{-1} TPH)	*Acremonium* sp. (F) + *Bacillus subtilis* (B)	Matrix: soil Operation time: 180 d pH: NR, T: NR	74% (F+B), 71% (F), 60% (B)	[168]
Crude oil (31.5 mg kg^{-1} TPH – soil) (21.5 g L^{-1} TPH – soil slurry)	*Geomyces pannorum* (F) + *Geomyces* sp. (F) + *Bacillus cereus* (B), *Bacillus thuringiensis* (B)	Matrix: soil & slurry Operation time: 30d; 50% WHC; RT	79.5% (F+B) in soil 87.7% (F+B) in slurry	[169]
Hexadecane (7652.53 mg L^{-1})	*Aspergillus flavus* (F) + *Bacillus cereus* (B)	Matrix: liquid Operation time: 14 d pH: NR, T: 40 ºC	99.5% (F+B), 52.9% (F), 9.6%(B)	[170]
Trace organic contaminants (TrOCs)				
Fenoprop (5 µg L^{-1}) Clofibric acid (5 µg L^{-1}) Pentachlorophenol (5 µg L^{-1}) Ketoprofen (5 µg L^{-1}) Diclofenac (5 µg L^{-1}) Naproxen (5 µg L^{-1})	*Trametes versicolor* (F)+ activated sludge (B)	Matrix: liquid Operation time: 90 d pH: 4.5; 28 ºC; membrane bioreactor (MBR); aerobic	57% (F+B), 20% abiotic MBR 65% (F+B), 25% abiotic MBR 92% (F+B), 60% abiotic MBR 94% (F+B), 65% abiotic MBR 50% (F+B), 15% abiotic MBR >99% (F+B), 45% abiotic MBR	[114]
Sulfamethoxazole (50 mg L^{-1})	*Pycnoporus sanguineus* (F) + *Alcaligenes faecalis* (B)	Matrix: liquid Operation time: 48 h pH: NR, T: NR	73% (F+B), 23% (F), 53% (B)	[113]
Volatile organic compounds (VOCs)				
Chlorobenzene (220 mg L^{-1})	*Trichoderma viride* (F) + *Rasltonia pickettii* (B)	Matrix: liquid pH: 7.0; T: 30 ºC; 160 rpm	100% (F+B) in 60 h 100% (F) in 96 h 100% (B) in 72 h	[139]
Toluene (271.4–1073.6 mg m^{-3})	Activated sludge – Key species identified *Phialophora* sp. (F) + *Alicyclobacillus* sp. (B)	Matrix: gas; biofilter Operational time: 125 d >98% RH; RT	92% (F+B)	[141]
Toluene (200-300 mg m^{-3} start up, 300-1000 mg m^{-3} steady state)	*Trichoderma viride* (F) + activated sludge (B)	Matrix: gas; biofilter Room temperature, 45% RH	95–100% (F+B), 18 d, start up >90% (F+B), 71 d, steady state	[140]

NR=Not reported; RT=Room temperature; TSS=Total suspended solids; TPH=Total petroleum hydrocarbons; COD=Chemical oxygen demand; BOD=Biological oxygen demand; DDT=1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane; WHC=water holding capacity; RH=Relative humidity. *ADMI=American Dye Manufactures Institute units; † Pollutant removal efficiency is presented for the fungal-bacterial co-culture (F+B), and fungal (F) and bacterial (B) monocultures when experiments were performed under the same conditions for all the treatments.

Table 2. Advantages and disadvantages of using fungal, bacterial, and fungal-bacterial co-cultures for the degradation of organic pollutants.

Microbial culture	Advantages	Disadvantages
Fungi	• Possess extracellular, non-specific, and non-stereoselective enzymes (e.g. lignin peroxidase, laccase, manganese peroxidase) that degrade a broad range of pollutants, generally under aerobic conditions. • Potential of fungal by-product recovery from wastewater treatment [171]. • Can co-metabolize organic pollutants, usually dependent on other available carbon sources (e.g. plant exudates) [25] • Can tolerate hostile growth conditions (e.g., low pH and limited nutrient availability) [137]. • Can grow under low water activity conditions, favored by the production of hydrophobins (hydrophobic proteins existing on the hyphal surface). • High cell surface hydrophobicity, which facilitates the adsorption of hydrophobic compounds to the fungal cells [130]. • Mycelia provide larger surface area for pollutant degradation. • When grown as biofilms or pellets, fungi allow for easy solid-liquid separation (biomass separation). • Capable of degrading monoaromatic pollutants, but certain fungal species are also able to co-metabolize PAHs (4-5 benzene rings). • Can degrade synthetic dyes without the formation of toxic aromatic amines [76].	• Require longer growth incubation periods compared to bacteria, which extends the start-up period of reactors. • Require long hydraulic retention times for degradation of pollutants. • Addition of secondary carbon source might be required for the treatment. • Filamentous growth may cause operational issues in bioreactors (e.g. clogging, high pressure drop, pressure increase in biofilters for gas treatment) [138]. • The degradation of organic pollutants under anaerobic conditions is uncommon [14, 28].
Bacteria	• Production of intra- and extracellular oxidoreductive enzymes (e.g. azo-reductases, laccases, dichlorophenolindophenol-reductases) for the degradation of pollutants [26]. • Degradation of organic pollutants can occur under oxic and anoxic conditions [27]. • Able to use low-molecular-weight PAHs (≤4 benzene rings) as a sole carbon source and energy, generating oxidized products. • Rapid growth and fast degradation rates. • Easy to cultivate.	• Incomplete degradation of dyes resulting in the formation of toxic aromatic amines [72, 73]. • Degradation performance deteriorates under low moisture, low pH, and nutrient-limited conditions [129]. • Degradation range of action can be limited by physical or chemical entrapment in heterogeneous matrices (e.g. soil) [25]. • Low performance for the degradation of hydrophobic VOCs.
Fungi-Bacteria	• Increased resistance to fluctuating environmental conditions [172]. • Short degradation times. • Increased production of enzymes. • Enhanced settleability, resulting in an improved separation of the biomass and effluent [173].	• Successful establishment of fungal-bacterial consortia depends on microbial selection, growth media, culture conditions and sequence of inoculation [152, 153, 157].