Can white-rot fungi be a real wastewater treatment alternative for organic micropollutants removal? A review

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Abstract

Micropollutants are a diverse group of compounds that are detected at trace concentrations and may have a negative effect on the environment and/or human health. Most of them are unregulated contaminants, although they have raised a concern in the scientific and global community and future regulation might be written in the near future. Several approaches have been tested to remove micropollutants from wastewater streams. In this manuscript, a focus is placed in reactor biological treatments that use white-rot fungi. A critical review of white-rot fungal-based technologies for micropollutant removal from wastewater has been conducted, several capabilities and limitations of such approaches have been identified and a range of solutions to overcome most of the limitations have been reviewed and/or proposed. Overall, this review argues that white-rot fungal reactors could be an efficient technology to remove micropollutants from specific wastewater streams.

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1. Overview

Micropollutants can be defined as substances that may be bioaccumulative, persistent and toxic and may have a negative effect on the environment and/or human health, even at trace concentrations. This diverse group contains, but is not restricted to: pharmaceutically active compounds (PhACs), personal care products, endocrine disruptors, pesticides and industrial chemicals. Several authors have referred to them also as emerging contaminants, preferably termed *contaminants of emerging concern* (Sauvé and Desrosiers, 2014). Most of them are unregulated pollutants, although future regulation might be written depending on research (Verlicchi et al., 2010). These contaminants remain biologically active even at concentrations of few ng·L-1, may be accumulated through the food chain and can have negative effects on the environment, fauna and human health. The World Health Organization (2016), for example, raised concern on the development of antibiotic resistance on target bacteria due to exposure to non-lethal concentrations of antibiotics.

The origin of these pollutants is diverse: from industrial waste streams to human-excreted metabolized and non-metabolized medicaments. Typically such compounds enter the environment through municipal or industrial effluent, but they are not completely removed in wastewater treatment plants (WWTPs), which are mainly designed for removing macropollutants such as organic matter, nutrients and suspended solids (Evgenidou et al., 2015; Frédéric and Yves, 2014; Kaiser et al., 2014). In fact, micropollutants have been found in surface water, groundwater, drinking water and sewage (Dai et al., 2015).

Answering to these concerns, the scientific community has devoted extensive research into mechanisms to degrade, transform and /or remove micropollutants from wastewater. Among the possible treatments, white-rot fungi (WRF) are regarded as an effective possibility due to their capacity to transform most of the compounds studied so far thanks to their versatile enzymatic machinery.

This manuscript reviews the bioremediation capabilities of WRF and the success examples of application with different types of micropollutants, primarily focusing on continuous treatments. Some drawbacks of the technology, largely related to the non-sterility of wastewater, are analyzed and solutions discussed.

1. Bioremediation capabilities of white-rot fungi
	1. White-rot fungi and their enzymatic machinery

The term *white-rot fungi* is not a taxonomical grouping but rather a collection of fungal species that are able to degrade lignin (Dashtban et al., 2010). WRF are mainly basidiomycetes and some relevant species include *Pleurotus ostreatus, Phanerochaete chrysosporium, Trametes versicolor, Ganoderma lucidum* and *Irpex lacteus*.

In the environment, WRF efficiently break down lignin to release the more easily metabolized carbohydrates hemicellulose and cellulose –oxidation of lignin yields no net energy gain (Leonowicz et al., 1999). To do so, they rely on a combination of extracellular ligninolytic enzymes, organic acids, mediators and accessory enzymes. A bold feature of this enzymatic machinery is its non-specificity, due to its action via the generation of radicals. This property makes the extracellular white-rot fungal enzymes capable of transforming a wide range of organic molecules, including micropollutants.

White-rot fungi secrete lignin modifying enzymes (LMEs) and other compounds for lignin degradation. LMEs include laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). The main difference between laccases and peroxidases is that the former uses molecular oxygen whilst the others use hydrogen peroxide (H2O2) as electron acceptor.

Enzyme characteristics and their action mechanisms are widely described previously (Camarero et al., 1999; Harvey et al., 1992; Hofrichter, 2002; Jones and Solomon, 2015; Reddy, 1995; Ruiz-Duenas et al., 1999), as well as, their biotechnological applications (Bogan and Lamar, 1996; Rodríguez Couto et al., 2006; Van Driessel and Christov, 2001).

The composition of the growth medium and culture conditions highly condition the production of ligninolytic enzymes (Nerud and Misurcova, 1996). In addition to LMEs, WRF can also produce and secrete redox mediators that act as vehicles for electron transfer and further expand the range of substrate for the ligninolytic enzymes (Cañas and Camarero, 2010; Marco-Urrea et al., 2010b; Morozova et al., 2007; Pointing, 2001). In spite of the extraordinary extracellular enzymatic system of WRF, it is not the only responsible of microcontaminant degradation. Cytochrome P450 constitutes a superfamily of intracellular heme-containing monooxygenases ubiquitous in all biological kingdoms. In fungi, they play a role in housekeeping biochemical reactions, detoxification of xenobiotics and adaptation to hostile ecological niches (Durairaj et al., 2016). The involvement of cytochrome P450 in degradation of several micropollutants has been largely described: trinitrotoluene (Spiker et al., 1992), polycyclic aromatic hydrocarbons (Yadav and Reddy, 1993), the dye malachite green (Cha et al., 2001), the organochlorine compounds polychlorinated dibenzodioxins and dichloro-diphenyl-trichloroethane (Kamei and Kondo, 2005; Xiao et al., 2011), carbamazepine and clofibric acid (Marco-Urrea et al., 2009), ketoprofen (Marco-Urrea et al., 2010b), the UV filter 4-methylbenzylidene camphor (4-MBC) (Badia-Fabregat et al., 2012) and several agrochemicals (Mir-Tutusaus et al., 2014).

Fungal cytochrome P450, shares some similarities with its mammalian and human counterparts (Stojan et al., 2014). These similarities include the capacity of forming glucuronides and conjugates in general (Bezalel et al., 1996). In humans, conjugation increases water solubility of xenobiotics so they can be excreted via urine (Dalgaard and Larsen, 1999; Lynn et al., 1978). WRF, however, have been consistently reported to reverse such modifications and deconjugate human conjugates (Badia-Fabregat et al., 2015a; Mir-Tutusaus et al., 2017).

* 1. Advantages and disadvantages of WRF systems vs. bacterial treatment

The fungal enzymatic systems are an important capability that supports WRF’s suitability for bioremediation of micropollutants from wastewater, but it is not the only one –and they come with some disadvantages too.

Micropollutants are typically found in wastewater streams at trace concentrations. This fact poses a difficulty for bacterial degradation as bacteria typically use the contaminants as growth substrates. If the pollutant is present at a low concentration, the bacterial species that is supposedly able to degrade it will not be able to colonize the matrix (Harms et al., 2011). Degradation of organic pollutants in white-rot fungi, on the other hand, is part of the secondary metabolism. In other words, fungi need a carbon source other than the contaminant to grow, meaning that WRF transform micropollutants co-metabolically (Wen et al., 2011). This does not mean that WRF cannot metabolize the micropollutant: *T. versicolor* could metabolize, mineralize and integrate some micropollutants such as diclofenac and benzophenone-3 into the fungus’ amino acids (Badia-Fabregat et al., 2014; Marco-Urrea et al., 2010b, 2010c). However, the concentration of micropollutants is insufficient to maintain fungal growth and a secondary carbon source is therefore needed. On one hand, this feature enables WRF to attack the micropollutants present in the wastewater even at low concentrations. On the other hand, the need for an additional carbon source constitutes a drawback over bacterial treatment.

Municipal and municipal-like wastewater commonly contains a mixture of a wide range of trace organic pollutants: from caffeine and insect repellents such as N,N-diethyl-meta-toluamide (DEET) to sunscreens, preservatives, antibiotics, hormones and other pharmaceutically active compounds (Wang et al., 2014; Yang et al., 2017). It is noteworthy that although they are found at trace concentrations, they retain high biological activities. Bacteria are usually less versatile when treating combinations of pollutants: a specific bacterial species can be a good degrader of a single or a small subset of similar micropollutants and this constitutes an advantage when treating a waste stream contaminated with a single micropollutant. But bacteria in general have difficulties when removing mixtures of contaminants. Conventional activated sludge, for instance, does no degrade most of pharmaceuticals and personal care products in municipal wastewater (Verlicchi et al., 2015, 2012). Recently an interesting review has been published about the organic micropollutants removal in conventional biological wastewater treatment where the requirement of hybrid treatment is pointed out, including the use of WRF (Grandclément et al., 2017). Authors suggest the need of studying the influence of the operational conditions, which is one of the objectives of this review. White-rot fungi’s non-specific enzymatic machinery, on the other hand, is especially well suited for coping with this scenario, as their ability to degrade mixtures of several contaminants has been widely demonstrated (Mir-Tutusaus et al., 2014; Shreve et al., 2016; Valentín et al., 2007). However, the pH of municipal and municipal-like wastewater is commonly around 7, while an effective fungal treatment usually requires pH 4.5. This drawback could be easily solved at expense of increasing the process cost.

In regards to the interaction between fungal and bacterial species, studies about the evolution of the microbial communities are scarce. However it has been found that bacteria and fungi can show a positive synergistic effect. This was hypothesized between fungal and bacterial enzymes that led to an increase removal percentage of several pollutants in non-sterile wastewater treatment in contrast to sterile treatment (Gros et al., 2014). This is regarded as a key aspect that requires further research in the future.

Additionally, although fungal systems have been regarded as a cost-effective solution for micropollutant removal, it is important to note that the application cost strongly depends on several factors: cost for inoculum and biomass production, requirement for operating conditions adjustment (e.g., pH adjustment), need for adding unit processes and hydraulic retention time among others.

Finally, some waste effluents cannot be treated with fungi: WRF systems are not good candidates for anoxic groundwater bioremediation, for example, where oxygen is scarce. WRF indeed need aerobic conditions for survival and activity whilst some bacterial species thrive in such environments and some can effectively degrade pollutants –e.g., dichloromethane fermentation in anaerobic conditions (Trueba-Santiso et al., 2017). However, aerobic waste effluents with concentrated pollutants pose a problem for conventional wastewater treatment processes: pulp and paper bleach industry effluent contains chlorinated and phenolic compounds; olive oil mill effluent is acidic and contains toxic phenols; textile and dyestuff industry effluents contain structurally distinct dyes; pharmaceutical industry effluent might contain residues of the active compound produced (Harms et al., 2011). White-rot fungal processes, on the other hand, have been reported to survive these conditions and degrade the pollutants in such waste streams (Nogueira et al., 2015; Ntougias et al., 2015; Van Driessel and Christov, 2001; Zhuo et al., 2011). Therefore, white-rot fungal based treatments can be regarded as a good option for on-site treatment of these wastewaters.

1. White-rot fungi and continuous wastewater treatment for micropollutants removal

In this section several continuous fungal operations treating a variety of micropollutants, summarized in Tables 1-3, are reviewed. Special interest is invested in works carried out using whole-cell cultures in non-sterile conditions because they portrait a more realistic picture of the technology.

* 1. Pharmaceutically active compounds

Pharmaceutically active compounds, or PhACs, are molecules that enter the environment and remain active, either as unmetabolized parent compounds or as pharmaceutically active metabolites (also referred as transformation products, or TPs). Drugs are administered to humans or animals and reach the environment via excretory systems in an unmodified, partially metabolized or completely metabolized state (Ebele et al., 2017). These molecules can promote drug tolerance or resistance to the original target organisms (e.g. antibiotic resistance in bacteria, or analgesic tolerance in humans) and unwanted effects in non-target organisms (e.g. alteration of sex ratio and decreased fertility) (Annamalai and Namasivayam, 2015; Jorgensen and Halling-Sorensen, 2000) even at a very low concentration. The intended biological activity allowed scientists to categorize several compounds into families: analgesics and anti-inflammatories, antibiotics, psychiatric drugs, beta-blockers or lipid regulators, among many others. In this section continuous PhAC removal is reviewed (and summarized in Table 1), opening with sterile and defined matrices and moving on to non-sterile and complex matrices such as wastewater.

Although several fungal species have been found to have PhAC degradation capabilities and showed promising results (Castellet-Rovira et al., 2018), continuous bioreactor treatments focused mainly on *Trametes versicolor* and *Phanerochaete chrysosporium*. *P. chrysosporium* was investigated in several operation modes and reactor configurations for the continuous removal of analgesics and anti-inflammatories diclofenac (DCF), ibuprofen (IBU) and naproxen (NPX), and psychiatric drugs carbamazepine (CBZ) and diazepam in sterile defined media. Nearly complete removal of DCF, IBU and NPX was achieved when biomass was auto-immobilized in the form of pellets and stirred tanks were used with a hydraulic retention time (HRT) of 1 d (Rodarte-morales et al., 2012; Rodarte-Morales et al., 2011). The fungus was not able to remove diazepam and an unstable CBZ removal of 0-63% was achieved when spiking at 0.5 mg·L-1. Similar results were achieved when operating a fixed bed reactor, even in a 100-day long operation: complete removal of analgesics and anti-inflammatories and limited and unstable removal of diazepam (0-30%) and CBZ (0-40%) (Rodarte-Morales et al., 2012). These series of studies exemplified a general trend in fungal PhAC degradation: analgesics and anti-inflammatories are usually well removed whilst the psychiatric drugs family is more recalcitrant. The possibility of CBZ and the sulfonamide antibiotics sulfamethazine (SMT), sulfathiazole (STZ) and sulfapyridine (SPY) removal by *T. versicolor* pellets was investigated in a sterile fluidized bed bioreactor treating defined media. Jelic et al. (2012) and Rodríguez-rodríguez et al. (2012) obtained a 54% removal of CBZ when spiking with 200 µg·L-1 and >94% removal of the sulfonamides spiked at 5 mg·L-1.

Some studies used non-sterile defined media, sometimes referred as non-sterile synthetic wastewater, as an approach to real application. Nguyen et al. (2013) and Yang et al. (2013a) used this approach to study the behavior of a membrane bioreactor (MBR) inoculated with *T. versicolor* lumps with an HRT of 2 d. Again, analgesics and anti-inflammatories were highly removed (salicylic acid, ketoprofen, ibuprofen, naproxen), with the exception of diclofenac, with an unstable removal of 0-60%. CBZ and the antibiotic metronidazole were poorly removed at 21 and 38% removal, respectively. Psychiatric drugs amitriptyline and primidone were also well removed. Long-term operations of 165 d and 160 d were achieved by Li et al. (2016, 2015b) using immobilized *P. chrysosporium* in a countercurrent seepage bioreactor and a rotating suspension cartridge reactor treating naproxen and carbamazepine spiked non-sterile defined media. The operations removed up to 70-90% of carbamazepine, value not achieved in any other study reviewed. A similar non-sterile media was compared with the use of non-sterile spiked municipal wastewater in a plate bioreactor described in Zhang and Geißen (2012). Immobilized *P. chrysosporium* removed in that operation an 80 and 60% of CBZ in the defined media and wastewater, respectively.

In order to shed light on the effect of sterility, Gros et al. (2014) operated the same 10 L fluidized bed reactor with sterile and non-sterile hospital wastewater (two wastewaters were collected on different days) inoculated with *T. versicolor*. The X-ray contrast agent iopromide and the antibiotic ofloxacin were removed up to 87 and 98.5%, respectively, in the sterile reactor and 65.4 and 99%, respectively, in the non-sterile reactor. Further approaching real-life application, several studies were carried out using real wastewater. Badia-Fabregat et al. (2015b) operated a fluidized bed reactor with *T. versicolor* pellets treating non-spiked, non-sterile veterinary hospital wastewater. Some compounds in the analgesics and anti-inflammatory family were well removed, but some exhibited an increase in their concentration (ketoprofen, piroxicam, diclofenac, indomethacine). An impressive 83% removal was obtained for diazepam and complete removal of ranitidine, clopidrogel and the antibiotic ciprofloxacin were achieved, but other pharmaceuticals were poorly removed. In a hospital wastewater spiked with ketoprofen and ibuprofen, 80 and 100% removal values were achieved using a similar fungal system (Mir-Tutusaus et al., 2016). Comparing both studies, it is interesting to note that ketoprofen was well removed in the spiked matrix, but its concentration rose when the matrix was not spiked. This was related to conjugation/deconjugation processes, which are briefly discussed in sections 2.1 and 5. A similar non-spiked study used non-sterile hospital wastewater pretreated with a coagulation-flocculation treatment to feed a similar fluidized bed bioreactor inoculated with *T. versicolor* pellets (Mir-Tutusaus et al., 2017). The reactor was operated for 56 d and nearly complete removal was achieved for the analgesics and anti-inflammatories family with the exception of ketoprofen, whose concentration rose, which was in accordance to Badia-Fabregat et al. (2015b). Around 60% of antibiotics were removed and psychiatric drugs were well removed overall.

* 1. Endocrine disruptors

Previous studies have confirmed significant removal of various trace organic contaminants by white-rot fungal cultures under sterile batch test conditions. However, little is known about endocrine disruptor compounds’ removal in fungal reactors operating in continuous mode; such studies are summarized in Table 2. *Trametes versicolor* was the most investigated white-rot fungus for the removal of these contaminants. Among the various types of pollutants, endocrine disruptors are receiving increasing attention as they are widespread and can pose serious risks to the environment and public health, even at low concentrations (Auriol et al., 2006). Indeed, these chemicals interfere with the hormone systems and produce adverse developmental, reproductive, neurological, and immunological effects in mammals. These compounds can be found in many products including plastic bottles, metal food cans, detergents, flame retardants, food, toys, cosmetics, and pesticides (Yang et al., 2017).

Estrogen compounds including natural ones, estrone (E1), 17β-estradiol (E2), estriol (E3), and synthetic 17α-ethinylestradiol (EE2) are commonly detected in sewage effluents and considered to be significant contributors to the estrogenic activity of wastewaters due to their high endocrine disruptor activity even at extremely low concentrations (Cabana et al., 2007; Shreve et al., 2016). Removal of these compounds in continuous mode using white-rot-fungi has been reported by some authors. Blánquez and Guieysse (2008) explored the potential of the white-rot fungus *Trametes versicolor* to biodegrade E2 and EE2 in a fluidized bed bioreactor operated during 26 days at a hydraulic retention time of 120 h. The results showed that E2 and EE2 were completely removed at volumetric removal rates of 0.16 and 0.09 mg l−1 h−1, respectively, when fed at 18.8 and 7.3 mg l−1, respectively. Shreve et al. (2016) explored the potential of the same fungus *T. versicolor* using the strain NRRL 66313 to continuously remove E1, E2 and EE2 from a mixture of nine trace organic contaminants with 350 µg·L-1 concentration each and during 8 days. The results showed that *T. versicolor* was able to decrease the estrogenic activity of the mixture and especially of the target contaminants (more than 71%) with the following trend E2 > E1 > EE2. Nguyen et al. (2013) studied the continuous removal of 30 trace organic contaminants, E1, E2 EE2, E3 and 17-b-estradiol-17-acetate among them, in a fungus-augmented bioreactor. The reactor contained the white-rot fungus *T. versicolor* and activated sludge and was operated for 110 d. It was fed continuously with synthetic wastewater spiked with the selected contaminants each with a concentration of approximately 5 µg·L-1. Data from this study highlighted the high removal of these compounds (> 90%) by the fungus-augmented bioreactor. The degradation of the same endocrine disrupting compounds, except 17-b-estradiol-17-acetate, was also recently investigated by Křesinová et al. (2017) using *Pleurotus ostreatus* HK 35. The strain was first, tested in a laboratory-scale continuous-flow reactor and then in a pilot bioreactor under non-sterile conditions. Results revealed that the EDC degradation in the trickle-bed bioreactor containing the mixed culture of the fungus and wastewater-autochthonous bacteria was very efficient in both cases. In the same work, the authors investigated also the bioreactor inoculated with the same strain as a tertiary treatment step to remove EDC, including E1 and EE2, from effluent of secondary treatment. Results also showed the potential of *P. ostreatus* HK 35 to remove these compounds and that 100 and 71% of E1 and EE2 were removed, respectively, within 24 hours.

Phenolic compounds, mainly bisphenol A (2,2-bis (4-hydroxyphenol) propane), nonylphenol (4-nonylphenol), and triclosan (5-chloro-2(2,4-dichloro-phenoxy)phenol) are xenobiotic compounds frequently detected in receiving waters downstream of areas of intense urbanization (Boyd et al., 2003; Kolpin et al., 2002). These chemicals are classified as endocrine disruptors since they can mimic or interfere with the hormonal system of different organisms (Cabana et al., 2009; Naylor, 1995). Although they have many orders of magnitude lower estrogenic activity, their elevated concentrations in wastewater drew attention to these EDC. Bisphenol A is used as raw material for the production of polycarbonates and epoxy resins; nonylphenol mainly originates from the degradation of nonylphenol polyethoxylates, a widely used industrial surfactant and triclosan is widely used in soaps, mouthwashes, toothpastes and other products in household personal care and hospital applications. The application of white-rot fungi in continuous mode for the treatment of these phenolic compounds has been scarcely described. Continuous removal of Bisphenol A was studied by Yang et al. (2013) in a membrane bioreactor (MBR) inoculated with *T. versicolor* and operated in non-sterile conditions for three months. Results showed that the performance of the fungal MBR was dependent on trace organic contaminants loading. Indeed, 80 to 90% were removed at an HRT of two days and bisphenol A loading of 475 mg·L-1d-1. Continuous removal of Bisphenol A was also reported in other studies and reached 75% in a fungus-augmented bioreactor operated during 110 d (Nguyen et al., 2013) and 61.9 % in the conditions of the study described previously by Shreve et al. (2016).

Regarding the antibacterial agent triclosan, it has been reported to be well removed (>95%) in continuous mode using *T. versicolor* at an initial concentration of 5 µg·L-1 in synthetic medium (Nguyen et al., 2013). However, low (34%) or no removal was observed using the strains *T. versicolor* NRRL 66313 and *P. ostreatus* HK 35 at initial concentrations of 25 ng·L-1 and 350 µg·L-1 respectively, in an effluent from secondary treatment (Kresinová et al., 2017; Shreve et al., 2016). Nguyen et al. (2013) also reported the removal of benzophenone, octocrylene and oxybenzone (three UV filters) with values of 68, 90 and 96%, respectively. However, Shreve et al. (2016) observed no removal of oxybenzone.

* 1. Pesticides

Few studies have investigated pesticide removal in continuous mode using different white-rot fungi and conditions, and they are summarized in Table 3. The potential of the white-rot fungus *Bjerkandera adusta* for the degradation of the insecticide hexachlorocyclohexane (HCH) in a spiked soil in a slurry system was investigated by Quintero et al. (2007). Bioremediation studies in the reactor were performed for 30 d and the operational conditions tested were the solid load (10% and 30%) and concentration of the pollutants in the soil (25 and 100 mg·kg-1). The results showed that higher degradation percentages were obtained for a solid concentration of 10% and a concentration for each isomer of 25 mg·kg-1 and were of 94.5%, 94.5%, 78.5% and 66.1%, for α-, γ-, δ- and β-HCH isomers, respectively.

The performance of a continuous packed bed bioreactor degrading the organophosphorus insecticide chlorpyrifos by the fungus *Aspergillus* sp. was studied at varying insecticide loading rates by Yadav et al. (2015). *Aspergillus* sp. is not a white-rot fungus but it was found to be quite efficient in the biodegradation of chlorpyrifos and its removal efficiency varied from 68 to 89% with the flow rate ranging from 10 to 40 mL·h-1 and the HRT from 24 to 100 h. Results also showed that the continuous packed bed bioreactor was able to regains its performance quickly after the perturbation in the flow rate. The potential of the same fungus *Aspergillus niger* to degrade continuously an herbicide, atrazine, in wastewater was evaluated by Marinho et al. (2017).

*T. versicolor* showed potential in the biodegradation of clofibric acid in a fluidized bed bioreactor. The study operated for 24 d a continuous reactor with an HRT of 4 days and achieved a 80% removal (Cruz-Morató et al., 2013b). Interestingly, the identification of transformation products and a toxicity assessment showed that the treated effluent was more toxic than the initial feed, probably due to the presence of hydroxyl-clofibric acid. Continuous removal of six pesticides, namely, atrazine, propoxur, fenoprop, ametryn, clofibric acid and pentachlorophenol, was investigated by Nguyen et al. (2013) with the same fungus in a MBR treating synthetic medium. The main results showed that fungus-augmented reactor achieved good removal of fenoprop (57%), clofibric acid (65%) and pentachlorophenol (92%) compared to conventional MBR. Toxicity assays were not performed in this case. The effect of a continuous dosing of a mediator (1-hydroxy benzotriazole, HBT) to the fungus-augmented MBR was also investigated during the last 30 days of operation. The results showed no significant difference in removal of atrazine and ametryn by the MBR, even after doubling the mediator dose to 10 µM. Shreve et al. (2016) observed no removal of atrazine and N,N-diethyl-3-methylbenzamide (DEET) within a mixture of nine contaminants spiked on sterile WWTP effluent.

* 1. Industrial chemicals

Continuous treatment of industrial chemicals has been also examined only by few researchers and the studies are summarized in Table 3. Palli et al. (2016) investigated the biodegradation of 2-naphthalensulfonic acid polymers (NSAP) in a wastewater in a continuous packed bed bioreactor working for three months under non-sterile conditions. The bioreactors were inoculated by *B. adusta* and *P. ostreatus* immobilized on straw. The results showed that the fungus *B. adusta* exhibited a limited enzymatic activity and was not able to remove the tested contaminant. However, the reactor inoculated with *P. ostreatus* showed a stable laccase activity during the whole experiment and noticeable NSAP biodegradation was achieved after two weeks of work and remained until the end of the experiment (30 to 60 %). In another study, high removal (> 95%) of two industrial chemicals, namely 4-tert-Butylphenol and 4-tert-Octylphenol, among thirty contaminants, was observed in an augmented fungal MBR (Nguyen et al., 2013).

1. Limitations of fungal based systems and how to overcome them

Despite all the potentialities of WRF, and the high amount of interesting studies about fungi being used for micropollutant removal, fungal systems for wastewater treatment are not being commonly applied at industrial scale. In this section, we review the main drawbacks of the technology and how can they be overcome.

* 1. Need for nutrient addition

As discussed in section 2.2, although organic micropollutants contain carbon, some WRF need an additional assimilable carbon source for growth and survival. Wastewater usually contains organic carbon and nitrogen (Verlicchi et al., 2010), both needed for microbial growth, and bacteria are perfectly capable of assimilating both. In the case of WRF, most experiments used glucose-based or malt extract-based spiked media (a.k.a. synthetic wastewater) and few studies can be found using real wastewater. The need for nutrient addition in real wastewater treatments by WRF was identified only after using real wastewater. Cruz-Morató et al. (2013) and Badia-Fabregat et al. (2015a) highlighted the need of glucose and ammonium tartrate addition for maintaining pelleted *T. versicolor* biological activity and enzymatic production in a fluidized bed bioreactor treating wastewater. Zhang and Geißen (2012) also found that glucose and ammonium tartrate addition were required for carbamazepine removal in a plate bioreactor inoculated with polyether foam-immobilized *P. chrysosporium* treating WWTP effluent. Other studies using fluidized bed bioreactors and treating flocculated hospital wastewater obtained similar results when adding ammonium chloride instead of ammonium tartrate (Mir-Tutusaus et al., 2017, 2016). In the reviewed literature, common nutrient addition rates ranged between 343 – 1453 mg·g dry cell weight (DCW)-1·d-1 of glucose and 0.77 - 1.98 mg·g DCW-1·d-1 of ammonium tartrate. However, some WRF were able to assimilate organic components (measured as chemical oxygen demand, COD) from wastewater: Palli et al. (2017) operated a fluidized bed reactor with *Pleurotus ostreatus* and observed significant growth of the fungus and reduction in the COD concentration. In those cases where a fungal species able to assimilate wastewater COD is used, there is no need for nutrient addition. This in turn could reduce bacterial growth, but overgrown fungal biomass should then be purged regularly. Nevertheless, it can be fairly accepted that nutrient addition can be needed to operate a white-rot fungal reactor for the treatment of wastewater. This poses a problem to full scale application, as the cost of glucose and nitrogen addition would be high, especially taking into account the large volumes of wastewater treated in WWTPs, and potentially increase the COD and nitrogen load.

This limitation could be partially overcome (i) by optimizing the nutrient addition, because when nutrients are added at consumption rate lower quantities are needed and nutrients’ concentration in the effluent remains very low, therefore not increasing COD or nitrogen load. This in turn prevents overgrowth of fungal biomass; (ii) by replacing the glucose and ammonium tartrate/chloride by cheaper products; or (iii) by reimagining the use of the technology: white-rot fungal systems could be viable, even taking into account the costs of nutrient addition, when smaller volumes of micropollutant-contaminated wastewater are treated. This is the case, for example, of hospital wastewater, veterinary hospital wastewater and several industrial wastewaters (Verlicchi et al., 2010). A fourth answer to this limitation is (iv) the immobilization of fungal biomass onto lignocellulosic materials. These substrates act also as carbon and nitrogen sources for WRF, thus avoiding the need of nutrient addition (Ehlers and Rose, 2005; Lu et al., 2009; Torán et al., 2017). It is worth noting that the use of a lignocellulosic material may lead to the release of recalcitrant compounds, e.g. tannins or phenolic compounds (Ramos et al., 2013). However, an advantage is that lignocellulosic materials are very abundant and are usually byproducts of other industries, reducing their price (Dashtban et al., 2010; Leonowicz et al., 1999).

* 1. Immobilization of fungal biomass

Fungal dispersed mycelium usually causes bioreactor operation difficulties such as growth on the reactor walls and agitators, foaming and increased need of mixing and oxygen supply. The immobilization of fungal biomass overcomes most of these difficulties –or reduces them.

The immobilization can be accomplished by the growth of the fungus in form of pellets (auto-immobilization). Fungal pellets are spherical aggregates of interweaved hyphae with a size usually in the range of several hundred micrometers to several millimeters (Espinosa-Ortiz et al., 2015). This immobilization is usually accomplished by growing the fungus in Erlenmeyers with liquid media under shaking conditions. Quite a few studies have dealt with the pelletization of different fungal species, the optimal pellet diameter and the study of mass and oxygen transfer into the pelleted biomass (Borràs et al., 2008; Casas López et al., 2005; Feng et al., 2004; Leštan and Lamar, 1999; Sharma and Padwal-Desai, 1985; Sitanggang et al., 2010; Wittier et al., 1986). Some studies have reported successful pellet production in a fluidized bed reactor, even in a pilot-scale bioreactor, thus enabling the upscaling of the technology (Borràs et al., 2008; Mir-Tutusaus et al., 2017). Additionally, Espinosa-Ortiz et al. (2015) reviewed several fungal pelleted reactor configurations with the perspective of treating wastewater.

The immobilization can also be carried out by growing the fungus onto a carrier. Some studies have done so using inert carriers such as polyurethane foam cubes (Li et al., 2016; Yadav et al., 2015). Gao et al. (2008) listed amongst the advantages of immobilizing *P. chrysosporium* in polyurethane foam the improved survival and increased enzymatic activity of the fungus in non-sterile cultures. But taking into account WRF’s ability of degrading lignin, cellulose and hemicellulose, several other authors have looked into the immobilization onto non-inert carriers such as wood chips, serving both as support and carbon source (Li et al., 2015; Pedroza-Rodríguez and Rodríguez-Vázquez, 2013; Rodarte-Morales et al., 2012; Sirtori et al., 2009). Interestingly, when Ehlers and Rose (2005) immobilized several WRF in pine chips, fungi were shown to penetrate the wood, possibly using the cellulose and hemicelluloses as carbon source. In this case, WRF not only benefited from the immobilization, but bacteria were not able to use the carbon source, hence avoiding substrate competition. Recent studies have also reported improved micropollutant degradation and fungal survival with *T. versicolor* immobilized in wood chips, even when treating real wastewater (Torán et al., 2017).

In general, immobilization and auto-immobilization leaded to more robust operations in non-sterile conditions (Hai et al., 2013; Leidig et al., 1999; Nilsson et al., 2006; Tang et al., 2011). Experiments with immobilized biomass tend to use fixed-bed column reactors (their low shear stress helps the adhesion of the biomass on the support) rather than the stirred-tank or fluidized bed reactors usually used with pelleted biomass.

* 1. Competition with autochthonous microorganisms

The decline in micropollutant removal observed in several studies has been largely attributed to bacterial contamination and it has been identified as the main bottleneck of the technology (Espinosa-Ortiz et al., 2015; Gao et al., 2008; Hai et al., 2013, 2008; Libra et al., 2003). Indeed, bacteria has been shown to exert competitive pressure for the substrate, thus leading to the loss of fungal biomass, and to destabilize fungal enzymes (Hai et al., 2008; Libra et al., 2003). For that reason, researchers have since proposed a wide range of alternatives for dealing with this limitation.

* + 1. Favoring fungal growth

Favoring fungal growth usually involves supplying the conditions that distinctively favor WRF over bacteria. These strategies include operation at optimal fungal pH, immobilization of fungal biomass, periodical biomass renewal and optimizing the carbon-to-nitrogen ratio (C/N ratio) of the nutrients supplied.

* **Operation at optimal fungal pH**. Most white-rot fungi’s optimal pH is acidic; not surprisingly, lignin modifying enzymes’ optimal pH is also acidic (Pazarlioglu et al., 2005). Although a specific bacterial species might find it difficult to grow at acidic pH, bacteria is a diverse domain and acidic pH does not suppress bacterial growth. However, pH too acidic ceased enzyme production of *T. versicolor* and led to the loss of pelleted morphology in a fluidized-bed reactor (Borràs et al., 2008; Libra et al., 2003). Therefore, acidic pH does not *distinctively* favor fungi over bacteria, but it does improve the viability and activity of WRF.
* **Partial biomass renovation**. When growth-limiting culture conditions are implemented the biomass concentration is nearly constant because the low nutrient addition is used mainly for biomass maintenance. In this case the biomass retained in the bioreactor ages over time. In this scenario, partial biomass renovation was developed as a strategy for stabilizing the age of fungal biomass in a sterile treatment, thus extending the operational time (Blánquez et al., 2006). They purged 1/3 of the fungal biomass in the reactor and added the same amount of fresh biomass every week, obtaining a solids/cells retention time of 21 d. The work concluded that partial biomass renovation helped in maintaining a biomass age distribution constant as well as their activity. So a pseudo steady state was obtained for the fungus in the bioreactor. The strategy was continued in several sterile operations (Blánquez and Guieysse, 2008) and in a non-sterile treatment of wastewater by Badia-Fabregat et al. (2015b) in an attempt to improve the enzymatic production and integrity of pellets. It was also successfully applied in a non-sterile operation of wastewater pretreated with a coagulation-flocculation process, allowing for a 56-day treatment (Mir-Tutusaus et al., 2017). In summary, the substitution of old biomass by fresh one allowed for a more stable fungal population in the reactors, in turn maintaining enzymatic activity for a longer period of time and favoring white-rot fungal colonization.
* **Carbon-to-nitrogen ratio**. In systems where nutrient addition is needed –i.e. where biomass is not immobilized in lignocellulosic substrates and/or the fungus is not able to assimilate nutrients present in the wastewater–, the ratio between carbon and nitrogen may play a role in favoring fungal over bacterial populations. On the one hand, high C/N ratios mimic ligninolytic conditions (wood has a high C/N ratio), increasing white-rot fungal production of lignin modifying enzymes (Eggert et al., 1996); and not contrarily, limiting conditions of carbon or nitrogen have also been reported to enhance LME production (Viswanath et al., 2014). On the other hand, lower carbon-to-nitrogen ratios favor fungal growth over bacterial growth (Demoling et al., 2007; Rousk and Bååth, 2007). It is important to notice that lower ratios do not favor white-rot fungi exclusively, but rather the growth of fungal species in general. Therefore, a compromise must be found between favoring fungal growth over bacteria and favoring LME production. However, one should take into account that LME production has rarely been linked to an increase of micropollutant removal. For example, in a recent publication treating flocculated wastewater in a fluidized bed bioreactor, a rather low C/N ratio of 7.5 was chosen in terms of PhAC degradation and biomass integrity (Mir-Tutusaus et al., in press).
* **Immobilization.** In addition to the advantages of immobilization discussed in section 4.2, auto-immobilization of WRF in the form of pellets allows a high concentration of fungus inside the reactor, thus hindering bacterial colonization. If the immobilization is carried out on lignocellulosic carriers the fungal concentration tends to be lower, but most bacterial species find it difficult to grow on lignocellulosic substrates.
	+ 1. Washing out bacteria

Another strategy for overcoming the competition with native microorganisms is by means of decoupling the hydraulic retention time (HRT) and solids retention time (SRT), sometimes also referred as cellular retention time (CRT). The purpose of these strategies is to keep the fungal biomass in the reactor while washing out the bacteria and other microorganisms, therefore increasing the retention time of WRF while keeping an HRT able to wash out the other microorganisms.

In order to achieve this decoupling, some authors auto-immobilized the WRF, typically in the form of pellets while others immobilized the fungi on inert carriers or lignocellulosic substrates, as discussed in section 4.2. A third option for decoupling HRT and fungal retention time is by the use of membrane technology. Membranes are widely used and can be found at industrial scale in several WWTPs (Joss et al., 2006; Rubirola et al., 2014). They allow for higher SRT and have been successfully applied with fungal biomass for the removal of organic micropollutants at laboratory scale (Hai et al., 2009; Nguyen et al., 2013; Yang et al., 2013).Van Leeuwen et al. (2003) described a technology using 100 µm microscreens that allowed for production of the fungus Rhizopus (not a white-rot fungus) under non-aseptic conditions thanks to the manipulation of HRT and SRT.

These four approaches allow for the retention of fungal biomass inside the reactor, therefore permitting the decrease of the HRT without affecting the SRT. A lower HRT leads to the washout of non-attached microorganisms, and bacterial concentration has been linked with the loss of degradation capacity, enzymatic production and viability of WRF (Blánquez et al., 2008; Hai et al., 2013, 2009; Mir-Tutusaus et al., 2016). Therefore, lower HRT favor white-rot fungal viability by washing out non-attached microorganisms. However, it is noteworthy that bacteria can attach to virtually everything, including pellets, immobilized fungal biomass, inert carriers and reactor surface (Fletcher, 1994). While fungal survival might be improved, lower HRTs often meant lower degradation of several contaminants by WRF: for example, Blánquez et al. (2007) reported reduced decolorization of a textile dye when lower HRTs were applied, similarly to Asses et al. (2009). Moreover, washing out of bacteria comes inevitably with the washout of extracellular enzymes and mediators produced by the fungus (Badia-Fabregat et al., 2017; Nguyen et al., 2013). However, as reviewed in sections 2 and 3, not only extracellular enzymes play a role in microcontaminant degradation. In fact, several authors reported concentration of LMEs not being crucial to maintain good removal percentages (Anastasi et al., 2010; Blánquez et al., 2004; Yang et al., 2013). In spite of that, maintaining a sufficient concentration of LMEs in the reactor is desirable for compounds whose biotransformation is LME-dependent.

In summary, both HRT and SRT must be optimized in order to achieve a compromise between bacteria-and-enzyme washout, micropollutant removal and fungal survival.

* + 1. Suppressing bacteria

Another strategy for assisting fungi in the competition with autochthonous microorganisms is the direct suppression of bacteria. This could obviously be achieved by sterilization, but it is not feasible in the wastewater treatment industry. Regardless, two approaches have been studied in order to reduce the bacterial count.

Sankaran et al. (2008) suggested the use of ozone (O3) as a selective disinfectant in order to decrease bacterial contamination in a non-sterile continuous fungal cultivation on corn-processing wastewater. The aim of the work was the production of fungal biomass, rather than COD or micropollutant removal; that is why the researchers used very high dosages of ozone (57 mg·L-1), while ozone doses in full scale WWTPs range between 5 and 15 mg·L-1 (Ternes et al., 2003; Verlicchi et al., 2010). Ozonation behaves similarly to acidic pH in the sense that it favors most fungal species over bacteria. In fact, Sankaran et al. (2008) inoculated the reactor with *R. oligosporus* but the fungal population was replaced by a wastewater-native fungus. Cheng et al. (2013) used ozone as a bactericide in a white-rot fungal dye-decolorization continuous operation, thus maintaining the bacterial concentration at around 105 CFU·mL-1. The study reported a 99.4% inhibition of contaminating bacteria and the involvement of ozone in the degradation of the Acid Blue 45 dye. Indeed, ozone has been reported to improve biodegradability of refractory organic matter and to degrade several micropollutants (Contreras et al., 2003; Fujioka et al., 2014; Gomes et al., 2017; Kusvuran and Yildirim, 2013; Ternes et al., 2003; Yang et al., 2016). Therefore, care must be taken when using ozonation as a disinfectant in assigning removal efficiency to the WRF and to the ozonation itself.

The addition of pretreatments can potentially reduce the inlet concentration of bacteria. A recent study successfully extended the operation of a *T. versicolor* fluidized bed reactor treating hospital wastewater from 10 to 28 days (Mir-Tutusaus et al., 2016). Specifically, a coagulation-flocculation pretreatment reduced the bacterial count of the influent wastewater from 107-108 to 103-105 CFU·mL-1, allowing for a longer-term operation. Coagulation and flocculation processes have been largely applied in WWTPs and are regarded as cost-effective (Liu et al., 2012; López-Maldonado et al., 2014). Therefore, the addition of this and other pretreatments might be a noteworthy strategy that enables WRF to operate with urban-like wastewaters.

* + 1. A final note on non-sterility

Some studies in non-sterile conditions have been reviewed in this section. However, two groups can be distinguished: studies operating in non-sterile conditions with defined medium or tap water and studies using wastewater. The studies using defined medium or tap water usually rely on contamination by air-borne microorganisms and microorganisms present in non-sterile surfaces. Such contamination could be regarded as mild and operations tend to be longer. The other group, using wastewater, deals with the contamination due to growth of native wastewater microorganisms. Bacterial count in those cases tends to be very high, the contamination could be regarded as heavy and the reactor operations tend to be shorter. The latter studies should be encouraged, because in addition to be a more reliable representation of real conditions, consortia formed in those operations could play a role in degradation of micropollutants and fungal metabolism intermediate products.

* 1. Fungal treatments require high HRTs

As discussed in section 4.3.2, low hydraulic retention times produced lower degradation for some micropollutants and higher loss of extracellular enzymes. Fungal treatments usually require a HRT of around 1-3 days for the removal of microcontaminants (Blánquez et al., 2008, 2007, Hai et al., 2009, 2008). In fact, micropollutant removal is usually improved by increasing HRT (when toxic compounds are not accumulated). Generally, WRF require higher HRTs to remove micropollutants than bacteria to remove organic matter. This adds a difficulty on combining a fungal treatment step on a conventional WWTP, reinforcing the idea of using white-rot fungal operations as on-site treatments in specific contaminated streams (enumerated in section 2.2 and 4.1). In those streams, the fungal process would be a treatment to decrease micropollutant concentration prior to discharge to the WWTP. If a fungal treatment were to be included in a conventional WWTP, some options could be considered: first, the increase of SRT or fungal concentration in the reactor could be optimized in order to allow higher removal efficiencies, thus enabling the coupling; second, low hydraulic retention times, between 6 to 12 h, are enough to remove several families of compounds such as analgesics, anti-inflammatories (Marco-Urrea et al., 2010a, 2009) and endocrine disruptors (Kresinová et al., 2017; Shreve et al., 2016), although enzyme washout should be taken into consideration. Therefore, wastewaters containing mainly these families of pollutants could be treated with fungal systems at low HRTs.

1. Conclusions and future outlook

The fungal treatment of effluents containing organic micro-pollutants is a feasible alternative. However, the best strategy will depend on the wastewater to be treated, the final use of the treated wastewater and consequently the cost of the treatment.

In order to advance the technology towards industrial scale, sterility must be discarded. Wastewater sterilization is not feasible from an economic and environmental point of view, so fungal research in applied science should focus on non-sterile conditions. The difficulty of non-sterile fungal operations has been discussed, and it greatly shifts the focus on the research field: from establishing WRF’s biodegradation capabilities to guaranteeing the fungus’ survival and activity during the fungal operation. The biomass in the reactors are usually retained or immobilized. Therefore the biomass concentration in a continuous treatment, three different operation mode can be distinguished: (a) growth conditions due to high concentration of nutrients (either present in the wastewater or artificially supplied), where periodic purge is required to maintain the biomass level and good performance of the reactor; (b) growth limiting conditions with low nutrient supply, where biomass level remains constant but periodic partial biomass renovation is required to maintain the distribution of biomass age in the reactor and consequently maintaining the degradation capacity; and finally (c) biomass pre-grown on a ligninolytic material with no other nutrient addition, where the biomass concentration is lower than in the previous operation modes.

Besides favoring fungal survival over other microorganisms, some studies have focused in the microbiological community evolution during fungal treatments. Such studies should be encouraged, as bacterial and fungal interspecies interactions and its consequences in micropollutant removal are not fully understood.

Similarly, the journey towards full scale operation requires the use of real, non-spiked matrices. This should be no surprise, as the complexity of a real matrix –microbial diversity, chemical composition, trace contaminants, etc. – is impossible to replicate in a defined medium. In addition, the bacterial contamination problems when using real wastewater will be more difficult to deal with, but they will be more similar to a real operation. Lastly, because real matrices are a source of variability, successful fungal operations using real wastewater greatly increase the systems’ robustness.

The use of non-spiked real matrices, however, poses a big pressure on analytical techniques. Not only are micropollutants found at a very low concentration, but they are also commonly found in the form of glucuronides and other conjugated forms. Conjugated microcontaminants are not usually detected by the current analytical techniques, thus undervaluing the concentration of the pollutant studied. This in turn underestimates the removal capacity of WRF, as they have been consistently described to deconjugate such compounds. Therefore, an effort should be made to analyze all compounds in any form.

Reported experiences in pilot plant are still too scarce and consequently, the results obtained in bench-scale reactors need to be validated at pilot plants before a full-scale application can be considered.

Finally, the drawbacks of fungal wastewater treatment for the removal of recalcitrant organic micropollutants can be technologically overcome and the strategy will be established depending on the effluent quality required. WRF can be an alternative for the removal of organic micropollutants from real wastewater but further studies are necessary at pilot plant to full adapt the process to the real application.

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Table 1. Removal efficiencies of fungal systems for pharmaceutically active compounds.



Table 2. Removal efficiencies of fungal systems for endocrine disruptors.

Table 3. Removal efficiencies of fungal systems for pesticides and industrial chemicals.

