Journal of Water Process Engineering 55 (2023) 104105

Contents lists available at ScienceDirect

Journal of Water Process Engineering

journal homepage: www.elsevier.com/locate/jwpe

Effect of dissolved oxygen on the degradation activity and consumption capacity of white-rot fungi

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ARTICLE INFO

Keywords:
Pesticide
Flame retardant
Oxygen limitation
Chemical oxygen demand removal
White rot fungi
Bioremediation

ABSTRACT

In recent decades, bioremediation using white rot fungi (WRF) has become an attractive alternative for the removal of xenobiotics from water. However, WRF are aerobic microorganisms whose degradative capacity may be reduced when operating in oxygen-restricted environments. This work determines the limiting dissolved oxygen level of *Trametes versicolor* in terms of degradation of two target micropollutants: bentazon and tributyl phosphate. When the dissolved oxygen concentration was set below 15 % saturation (1.3 mg $O_2 \cdot L^{-1}$), the results revealed a considerable decrease in degradation capacity and laccase synthesis. Hence, 15 % dissolved oxygen was established as a reference value of aerobic conditions to assess dissolved oxygen profiles in both a rotating drum bioreactor (RDB) and a fixed-bed bioreactor (FBB). Restrictive oxygen conditions were achieved after an operating period of 24 h in the RDB and an empty bed contact time of 44 min in the FBB. In addition, growth kinetics on *Q. ilex* wood and organic matter removal (in terms of COD) were studied, obtaining 0.059 mg ergosterol·g wood $DW^{-1} \cdot day^{-1}$ and 16.23 mg $O_2 \cdot L^{-1} \cdot h^{-1}$, respectively. Therefore, *T. versicolor* has demonstrated a remarkable ability to assimilate complex carbon sources and a high micropollutant degradation activity, especially when operating in non-limiting oxygen regimes.

1. Introduction

Micropollutants in the aquatic environment have become a worldwide issue of rising environmental concern. These compounds constitute a diverse and expanding group of anthropogenic and natural pollutants that include pharmaceuticals, personal care products, steroid hormones, industrial chemicals, pesticides and flame retardants, among others [1]. Even though these xenobiotics are generally detected at low concentrations, many of them raise significant toxicological concerns in both wildlife and human beings [2,3]. Therefore, remediation techniques must be developed and applied to remove these toxic compounds from the environment.

The most established micropollutant treatments are physical-chemical, as they are considered relatively fast, simple and effective methods against micropollutants. However, these techniques have important limitations, such as the formation of residues in sorption, fouling in membrane technology, and high operational costs and the generation of transformation products in advanced oxidation processes [4]. In contrast, bioremediation is considered a sustainable and environmentally friendly technology that, despite possible technical difficulties related to biomass maintenance, presents increasing evidence of its potential application in micropollutant abatement. Particularly, white rot fungi (WRF) are basidiomycetes known for their ability to degrade a wide range of micropollutants through their powerful enzyme system, which is composed of extracellular enzymes, such as laccase, and the intracellular enzyme system known as cytochrome P450 [5]. Among WRF, *T. versicolor* is considered one of the best candidates because of its excellent degradation efficiency for many xenobiotics [6].

Like other WRF, *T. versicolor* is an aerobic fungus whose growth and metabolic activity have traditionally been considered to be affected by the dissolved oxygen (DO) concentration in the medium [7,8]. However, some investigations have shown that this fungus can operate under anaerobic conditions, e.g. in the conversion of xylose to ethanol [9], or even degrade recalcitrant compounds such as industrial dyes [10]. Even though *T. versicolor* seems to degrade micropollutants to some extent under oxygen restricted conditions, this fungus may be oxygen-limited and hence its degradative activity may be reduced. In fact, a common practice in the field of fungal bioremediation is to overlook the DO level as long as the fungus exhibits some degradation activity. In any case, no study has quantitatively determined the lower level at which a WRF (e.

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https://doi.org/10.1016/j.jwpe.2023.104105
Received 7 May 2023; Received in revised form 20 July 2023; Accepted 26 July 2023
Available online 2 August 2023

g., *T. versicolor*) is oxygen-limited for pollutant degradation. DO content is an essential parameter and must be properly monitored, otherwise oxygen may be under-supplied, reducing treatment performance, or over-supplied, increasing operating costs.

Another key parameter in wastewater treatment is chemical oxygen demand (COD). WRF generally degrade recalcitrant micropollutants via co-metabolism, thus requiring an easily assimilated carbon supply for maintenance. In addition, micropollutant concentrations are too low to support metabolic requirements [11]. Nevertheless, some WRF, such as *Pleurotus ostreatus* and *Phanerochaete chrysosporium*, have been reported to successfully assimilate intrinsic COD from high organic load wastewaters without the addition of supplementary nutrients [12,13]. In contrast, *T. versicolor* has shown a doubtful ability to reduce COD [14,15], although there is still scarce evidence and many information gaps to investigate.

Therefore, the aim of this study was to determine the DO concentration at which *T. versicolor* was oxygen-limited in terms of pollutant degradation, where bentazon and tributyl phosphate (TBP) were used as target compounds. Two different types of chemicals, a pesticide (bentazon) and a flame retardant compound (TBP), were chosen to cross-sectionally study the effect of DO on the degradation of micropollutants. Bentazon was chosen as the target pesticide because it has recently been detected in surface waters of the Ebro River Delta, is rapidly degraded by *T. versicolor* and has a low sorption propensity [16–18]. Besides, bentazon has been associated with potential health problems and is considered of particular concern according to the Directive 2008/105/EC on Environmental Quality Standards of the EC [19]. Meanwhile, TBP has been shown to pose toxicological risks in several organisms [20,21]. The limiting DO level was used as a reference to assess whether limiting conditions were reached in two well-established fungal bioreactors, an FBB and an RDB. Thus, this study allows to determine the aeration requirements in common fungal bioreactors, a key information gap to date. In addition, this study analyses *T. versicolor* kinetic growth and capacity to remove organic matter (in terms of COD) under sterile conditions.

2. Materials and methods

2.1. Agricultural wastewaters

Agricultural wastewater (AW) was collected from an agricultural drainage channel in the Llobregat River Basin in Gavà in December 2020 and stored in the fridge at 4 °C until use. Table 1 summarizes the AW physicochemical parameters.

2.2. Chemical and materials

Bentazon, TBP and sodium acetate anhydrous were purchased from Sigma Aldrich (Barcelona, Spain). High-performance liquid chromatography (HPLC) grade acetonitrile, methanol and formic acid were supplied by Merck (Darmstadt, Germany). D(+)-Glucose was supplied by Acros Organics (New Jersey, USA). Ammonium tartrate was acquired from Scharlau (Barcelona, Spain). All chemicals were of high purity grade.

Table 1
Physicochemical characterization of the agricultural wastewater. Values are means ± standard deviation for triplicate samples.

Parameter	Agricultural wastewater (Llobregat River Basin, 2020)
pH	8.06 ± 0.02
Conductivity ($mS \cdot cm^{-1}$)	3.17 ± 0.01
Colour at 650 nm	0.10 ± 0.011
Chloride ($mg\ Cl \cdot L^{-1}$)	653.7 ± 6.1
Sulphate ($mg\ S \cdot L^{-1}$)	86.3 ± 1.6
Nitrite ($mg\ N \cdot L^{-1}$)	0.4 ± 0.1
Nitrate ($mg\ N \cdot L^{-1}$)	0
Ammonia ($mg\ N \cdot L^{-1}$)	0
TSS ($mg \cdot L^{-1}$)	16 ± 2
COD ($mg\ O_2 \cdot L^{-1}$)	44 ± 4
TOC ($mg \cdot L^{-1}$)	16 ± 1
Heterotrophic plate count ($CFU \cdot mL^{-1}$)	$3.50 \cdot 10^4 \pm 1.50 \cdot 10^4$

2.3. Fungal strain and culture conditions

T. versicolor ATCC 42530 was purchased from the American Type Culture Collection. *T. versicolor* was maintained by subculturing on agar plates of malt extract (2 % w/v) every 30 days at 25 °C. *T. versicolor* mycelial suspension and pellets were prepared as previously described elsewhere [22]. *T. versicolor* immobilized on *Q. ilex* wood chips was prepared as described by Beltrán-Flores et al. [23].

2.4. Bioreactor set-ups and procedures

2.4.1. Stirred tank bioreactor: biodegradation

Maintenance medium used in the bioreactor was prepared (per litre) with 3 g glucose, 21 mg ammonium tartrate, 10 mL micronutrients and 100 mL macronutrients, as described in previous works [24]. The medium was sterilized at 120 °C for 30 min. Glucose and ammonium tartrate were supplied at the consumption rate, approximately 1 $g \cdot d^{-1} \cdot L^{-1}$ and 7 $mg \cdot d^{-1} \cdot L^{-1}$, respectively. The glucose and ammonium tartrate stock solutions were 0.3 $g \cdot mL^{-1}$ and 3.2 $mg \cdot mL^{-1}$, respectively.

Experiments were performed in a stirred tank bioreactor Applikon model ez-Control (Applikon Biotechnology, Netherland) of 2 L maximum useful volume, with 1.5 L operating volume. The pH was set to 4.5 and controlled using 1 M HCl and NaOH. The temperature was fixed at 25 °C. DO levels were set at 5, 10, 15 and 30 %, depending on the experiment, so that the aeration rate was automatically adjusted to maintain these percentages, keeping a constant agitation of 200 rpm. Experiments were conducted at different DO levels in order to evaluate the effect of oxygen on the fungus degradation ability. The DO set-point was fixed between 5 and 30 % saturation. This operational range was established based on the fact that most wastewater treatment plants (WWTP) work above 25 % saturation, which is considered as non-oxygen limited region [25]. Hence, a saturation of 30 % DO was initially selected to work under non-oxygen limiting conditions, at which *T. versicolor* was supposed to maintain its intrinsic degradation capacity intact, while lower DO levels of 15 %, 10 % and 5 % were chosen to assess the effect of oxygen limitation on degradation activity.

A total of 80 $g\ L^{-1}$ wet weight of pellets (equivalent to 3.5 $g\ DW \cdot L^{-1}$) were inoculated to the reactor. In each cycle, a pulse of 10 ppm bentazon or 5 ppm TBP from their respective stock solutions (10 $mg \cdot mL^{-1}$ in methanol) was injected into the bioreactor every 24 or 48 h, respectively. A total of 3 cycles were performed for each DO level, which means a total injection of 30 ppm bentazon and 15 ppm TBP. Separate experiments were conducted for each DO level, i.e., with fresh medium and biomass. Samples of 2 mL withdrawn from the bioreactor were centrifuged at 15.000*g* during 1 min to separate the supernatant from the pellets.

2.4.2. Stirred tank bioreactor: OUR estimation

The reactor was set up as described in Section 2.4.1. In this case, 1 L of maintenance medium (without micropollutants) was saturated above 50 % DO, when aeration was stopped. The specific oxygen uptake rate (sOUR) measures the rate at which oxygen is consumed by a microorganism/consortium and is defined as the mass of oxygen consumed per unit of fungal biomass and time ($g\ O_2 \cdot g\ biomass DW^{-1} \cdot day^{-1}$). The sOUR was obtained by dividing the value of the slope of the linear regression of the DO concentrations over time by the biomass amount. The operating conditions were the same as in Section 2.4.1.

2.4.3. Stirred tank bioreactor: organic matter removal

Regarding the study of the organic matter removal, the consumption rate of glucose was compared with that of another less assimilable source of COD, sodium acetate. For this purpose, 1 L of maintenance medium was treated but replacing glucose with an equivalent amount of sodium acetate in terms of COD (2 g $O_2 \cdot L^{-1}$). Thus, a pulse of acetate was added in the medium from a stock solution of 0.2 g·mL^{-1}. Once the acetate was almost consumed, a new acetate pulse was injected, obtaining two complete cycles. In addition, a control experiment with glucose was conducted. In these cases, the DO level was maintained at 30 % and the medium was not spiked with any micropollutant. Samples of 2 mL withdrawn from the bioreactor were centrifuged at 15.000*g* for 1 min to separate the supernatant from the pellets. The resulting supernatant was used to measure glucose or acetate. The operating conditions were the same as in Section 2.4.1.

2.4.4. Fixed-bed bioreactor

The FBR consisted of a cylindrical methacrylate tube (4.4 cm diameter and 62 cm height) assembled with 250 g DW of inoculated wood (Fig. 1). DO measurements were performed by inserting the oxygen probe through a series of holes, plugged with septums, which were made in the column. Thus, the DO was measured at 0, 7 (P-1), 19 (P-2), 34 (P-3) and 49 (P-4) cm height (bed volume = 0.74 L, useful volume = 0.45 L) using an OXROB10 robust oxygen probe coupled to a FireSting-PRO (4 channels) fiber-optical multi-analyte meter (Pyroscience, Germany). The reactor was fed with an upward stream of AW saturated at 100 % DO at flow rates of 12, 23 and 40 mL·min^{-1}, equivalent to empty bed contact times (EBCT) of 59, 31 and 18 min, respectively. The pH of the AW was previously adjusted to 4.5. In this case, the sOUR was calculated for each reactor section according to Eqs. (1) and (2).

$$\text{sOUR} = \frac{[DO_E - DO_S] \cdot Q}{M_{biomass} \cdot 1000} \quad (1)$$

$$M_{biomass} = \frac{X_{ergo}}{R_{E-B}} \cdot M_{wood} \quad (2)$$

where DO_E and DO_S are the DO concentrations at the inlet and outlet of each section (mg $O_2 \cdot L^{-1}$), respectively, Q the flow rate (L day^{-1}), $M_{biomass}$ the fungal biomass of each section (g DW), X_{ergo} the ergosterol content in the wood (mg ergosterol·g wood DW^{-1}), $R_{E\text{-}B}$ is the correlation between ergosterol mass and fungal biomass of *T. versicolor* and corresponds to 6.61 (mg ergosterol·g biomass DW^{-1}) [26], and M_{wood} the wood mass in each section (g).

2.4.5. Rotating drum bioreactor

The RDB was constructed with a methacrylate tube (length: 45.0 cm

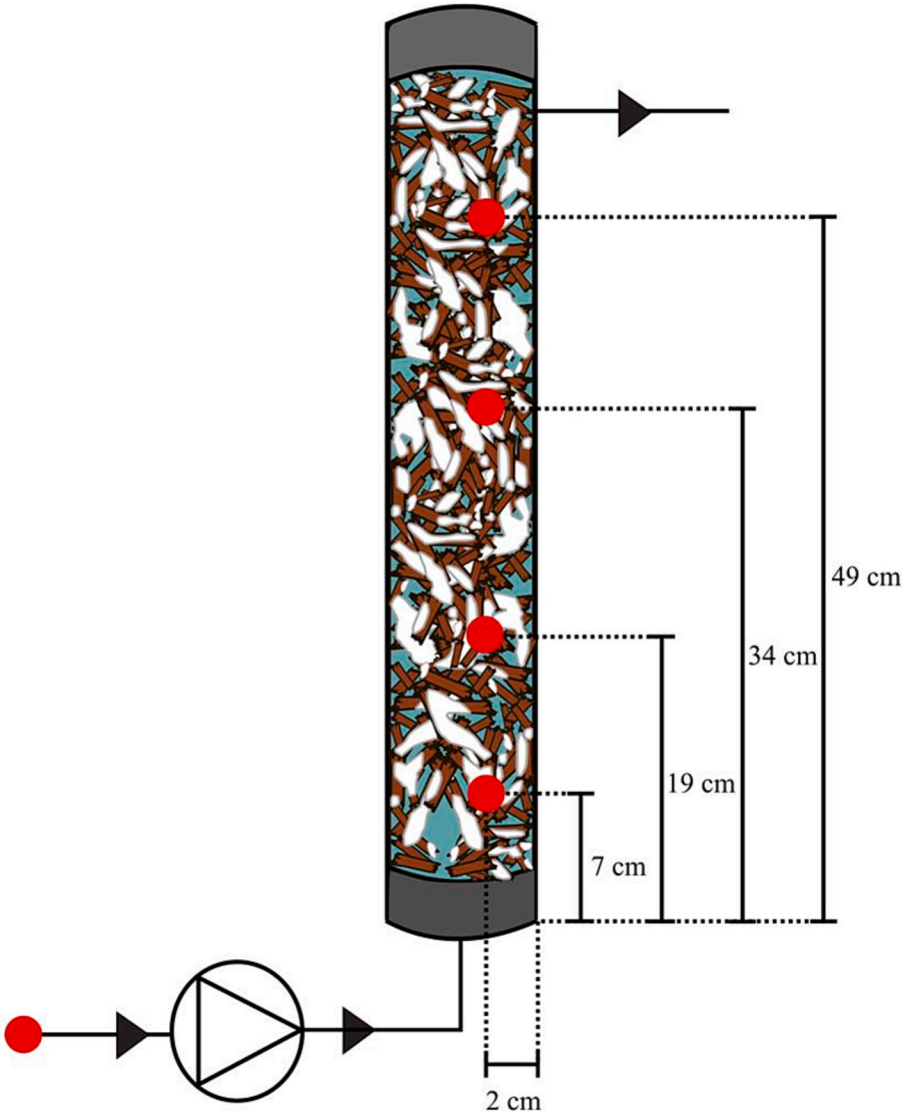

Fig. 1. Representation of the FBB and DO measurement points.

× radius: 4.2 cm) supported on a polyvinylchloride gutter (length: 51.0 cm × radius: 7.0 cm). The wastewater was contained within the channel while the colonized wood chips were placed inside the tube. The tube was provided with multiple 8 mm diameter holes to ensure adequate contact between the biomass and the wastewater and it was covered with a plastic mesh to prevent biomass loss. Approximately 30 % of the biomass was submerged in the liquid phase, while the rest was in direct contact with air. The inner tube was connected to an electric motor (Worm Gear Motor, model: 4632-370, 12 V) that rotates to alternate the submerged biomass fraction. The working volume was approximately 2.3 L. No additional substrate or nutrient was added to the reactor.

DO concentrations were analysed in a RDB that had been previously used for AW treatment [27,28]. The feed tank was stirred during the entire operation, from which water (100 % DO) was pumped to one side of the reactor (HRT = 5 days). The water left the reactor from the other side by overflow. An external recirculation loop (4.7 $L{\cdot}day^{-1}$) was required for pH adjustment, which was performed in a recirculation tank (≈0.4 L). The pH was automatically controlled at 4.5 by adding either 1 M HCl or NaOH. A total of 545 g DW of inoculated wood was introduced into the inner tube, which was rotated one and a half turns every 24 h. Aeration was initially provided through a diffuser located in the reactor until reaching oxygen saturation. Afterwards, aeration was turned off, and the dynamic profile of DO uptake was monitored until equilibrium, which was reached after 30 h of operation. The oxygen level was measured at 3 different points of the reactor: inlet, intermediate, and outlet; and at two depths: 4 and 6 cm (reactor dimensions are length: 51 cm × radius 7 cm), as shown in Fig. 2.

2.5. Pesticide analysis in liquid phase

Residual concentration of bentazon and TBP were analysed by LC, with a limit of detection of 0.5 ppm for both compounds. Liquid samples were initially filtered through Millipore Millex-GV PVDF filters (0.22 μm). In the case of bentazon, the analysis was conducted by a Dionex Ultimate 3000 HPLC system with a UV detector operating at 254 nm. The separation was performed in a C18 reversed-phase column (Phenomenex®, Kinetex® EVO C18 100, 5 μm, 4.6 mm 150 mm) with a mobile phase of 0.01 % formic acid solution (v/v) (A) and acetonitrile (B) at 30 °C. The organic gradient used in chromatographic separation was: 35 % B from 0 min to 5 min, linear increase to 45 % B from 5 min to 15 min, restoration of initial conditions in 1 min and maintenance of initial conditions for 2 min more (total runtime: 18 min) [16]. The flowrate was 0.9 $mL{\cdot}min^{-1}$, and the sample injection volume was 40 μL.

TBP chromatography study was adapted from a method previously reported in the literature [29]. This analysis was performed using a Prominence UFLC that was controlled by an LCMSsolution Chromatography Data System software, consisting of a SIL/20A autosampler and LC-20AD solvent-delivery system and equipped with a LCMS-2010A detector, all of them from Shimadzu (Japan). Chromatographic separation was achieved by an Acclaim Organic Acid (OA) column from Dionex, OA 5 μm, 120 Å, 4.0 × 150 mm (Thermo Scientific, Waltham, USA). The column temperature was 30 °C, injection volume was 2 μL and the mobile phase consisted of 75 % methanol and 25 % ultrapure water (v/v), which was pumped isocratically at 0.25 $mL{\cdot}min^{-1}$ (total runtime: 14 min). The mass spectrometer was operated in the selected-ion monitoring (SIM) positive mode electrospray ionization (ESI+) and was set up to monitor the ions m/z 267.10 Da. Data in the tuning file were selected for parameters which contains the lens voltage values, Interface, Q-array and others. High purity nitrogen was used as nebulizing gas at 1.50 L/min and drying gas pressure was zero. The detection voltage was set at 1.5 kV. The other operation parameters for MS were optimized as follows: capillary voltage: 4.50 kV; heat block temperature: 200 °C; CDL temperature: 250 °C.

2.6. Acetate measurement

The samples were filtered prior measurement with 0.22 μm pore-size Millipore Millex-GV PVDF filters and, when required, diluted with ultrapure water to have concentration between the calibration ranges. Acetate was measured by HPLC (Ultimate 3000, Dionex Integrion HPLC, USA) calibrated to measure volatile fatty acids (VFA) in the range of 20 to 1000 $mg{\cdot}L^{-1}$ (acetate, propionate, formate, isobutyrate, valeric, butyrate and isovaleric). The separation was performed in an ICSep ICE-CPREGEL 87H3 column (height: 150 mm, diameter: 7.8 mm, Transgenomic, Omaha, NE, USA) at 30 °C working with a sulphuric acid (6 mM H_2SO_4) as mobile phase pumped at a constant flow rate of 0.5 $mL{\cdot}min^{-1}$. Total run time was 25 min. The HPLC was equipped with a refractive index detector (Waters 2410) with an IR of 1024 and an autosampler (Ultimate 3000 Autosampler). Under these conditions, the retention time was approximately 18.31 min.

2.7. Other analyses

Laccase activity was determined through the absorbance changes induced by the oxidation of 2,6-dymetoxyphenol (DMP) and expressed in activity units per litre ($UA{\cdot}L^{-1}$), as described elsewhere [30]. Ergosterol content was used as an indicator to quantify fungal biomass and was analysed by homogenizing and extracting inoculated wood in triplicate based on a modified method previously described [31]. Briefly, 0.5 g of each homogenized sample was transferred to a test tube to be extracted with 1 mL cyclohexane and 3 mL KOH-methanol solution (10 %, w/v) by 15 min sonication followed by heating at 70 °C for 90 min. After adding 1 mL of distilled water and 2 mL of cyclohexane, the tube was vortexed for 30 s and then centrifuged at 3.500 rpm for 5 min. The upper organic phase was recovered while the aqueous phase was washed twice with 2 mL cyclohexane. The organic phases were pooled and evaporated to dryness with nitrogen. The residue was reconstituted in 1 mL methanol at 40 °C for 15 min, vortexed for 30 s, transferred to Eppendorf vials and centrifuged at 6000 rpm for 3 min. The resulting solution was transferred to 2 mL amber vial for quantification by HPLC (Ultimate 3000, Dionex, USA) equipped with a UV detector at 282 nm, using a C18 reverse phase column (Phenomenex®, Kinetex® EVO C18

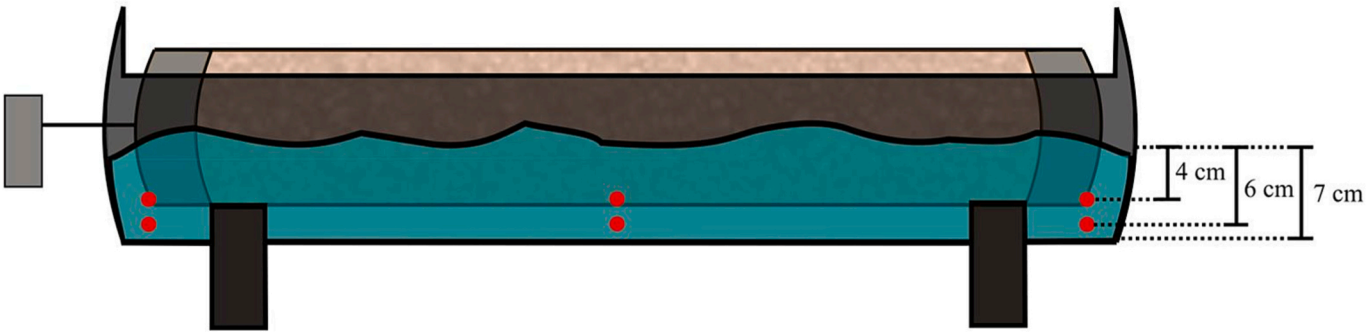

Fig. 2. Representation of the RDB and DO measurement points.

100 Å, 4.6 mm × 150 mm, 5 µm). Acetonitrile was isocratically supplied at 1 mL·min^{-1} as eluent, obtaining a retention time of approximately 7.6 min. The injection volume was 40 µL and the oven temperature was 35 °C.

Conductivity was determined using a CRISON MicroCM 2100 conductometer. An UNICAM 8625 UV/VIS was used to detect colour as absorbance at a wavelength of 650 nm. The commercial kits LCK114 or LCK314m and LCH303 were used to quantify COD and ammonia concentrations respectively (Hach Lange, Germany). Total suspended solids (TSSs) and volatile suspended solids (VSSs) were determined using standard procedures 2540 D and 2540 E respectively, and HPC was calculated according to standard method 9215 [32]. Chloride, nitrite and sulphate concentrations were quantified by ion chromatography using a Dionex ICS-2000. Glucose concentration was measured with a YSI model 2700 biochemical analyser (Yellow Spring Instruments & Co., USA) after filtering the sample through a 0.22 µm Millipore Millex-GV PVDF filter.

3. Results and discussion

3.1. Stirred tank bioreactor

3.1.1. Effect of dissolved oxygen on the biodegradation of two model micropollutants

The relative remaining concentrations of the pollutants are shown in Fig. 3. *T. versicolor* was only able to completely eliminate both micropollutants in the first cycle studied when working at 30 % DO. This result indicates that the maximum performance of the fungus was accomplished at the highest DO level. However, the degradation efficiencies obtained when working at 30 % and 15 % DO were clearly better than those achieved under more restricted oxygen conditions in all cycles. In this regard, Leisola (1983) and Li (2016) demonstrated that the presence of oxygen was a critical factor in the removal of lignin and carbamazepine respectively by the WRF *P. chrysosporium* [33,34]. Nevertheless, *T. versicolor* was able to partially degrade both compounds, bentazon and TBP, even at a very low DO level of 5 %, which is in line with other research, in which the fungus remained metabolically active in anaerobic environments [10], e.g., being able to bioconvert xylose to ethanol without oxygen [9].

This finding was further analysed by studying the biodegradation kinetic constants of bentazon and TBP, as shown in Table 2. The equations used for the determination of the kinetic constants are shown in the Supplementary Material. According to these results, the fungus was found to be in an oxygen-limiting region when the DO was decreased below 15 %, i.e., 10 % and 5 %. In this region, the degradation capacity was dramatically reduced by up to 84 %. In contrast, it cannot be clearly concluded whether the fungus is oxygen-limited when the DO was restricted to 15 % since the biodegradation rate was only moderately reduced for bentazon and remained high for TBP. At 30 % DO, *T. versicolor* was even able to completely remove bentazon at 10 ppm after 6 h and TBP at 5 ppm after 32 h. Note that the degradation profiles changed slightly between cycles, which was mainly attributed to the accumulation of metabolites and extracellular enzymes. This variation could also be associated with changes in the morphology of the fungal pellets, although the stirring speed was relatively low (200 rpm) and apparently no morphological alteration was observed on a macroscopic scale.

The results of the degradation kinetics of bentazon are in line with those reported previously [16], in which bentazon was eliminated in less than 3 days. Regarding TBP, no data have been found on its degradation by fungi, but some bacteria such as *Sphingomonas* sp. have been able to degrade TBP with similar kinetics [35].

Furthermore, laccase activity was analysed as an indicator of fungal activity. This extracellular enzyme was only monitored for TBP, since a recent study has shown that, although laccase is slightly involved in bentazon degradation, the main mechanism of degradation is via cytochrome P450 [16]. Fig. 4 shows the evolution of laccase activity for each DO value. Enzyme activity reached maximum values at 30 % DO, while it was substantially limited as DO decreased, especially below 15 %. These results show that the enzymatic activity of *T. versicolor*, and thus its degradation potential, is inhibited when working near anoxic conditions. In this regard, Pinheiro et al. (2020) evaluated laccase production by *T. versicolor* in three different reactors, detecting higher activity values when aeration was incorporated into the system [36]. Therefore, laccase production was found to be correlated to DO concentration during the process, which is in good agreement with the results of the present study. Additionally, a higher DO concentration may not only lead to an increase in laccase production by *T. versicolor*, but could also enhance TBP degradation efficiency, as oxygen is an essential electron acceptor in certain laccase-mediated oxidation reactions [37].

Bettin et al. (2020) also reported maximum laccase activity working at 30 % DO for another WRF, *Pleurotus sajor-caju* PS-2001 [38]. When the DO concentration was higher than 30 % DO, i.e., more favourable for the fungus, instead of promoting laccase production, it stimulated

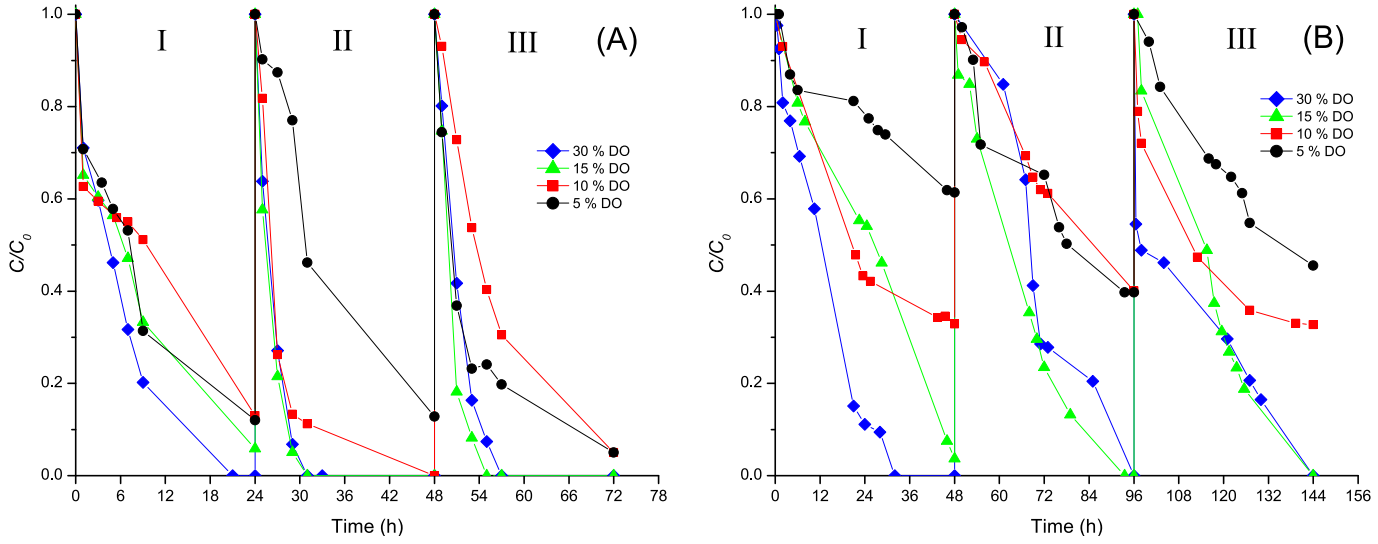

Fig. 3. (A) Bentazon concentration profiles for three batches of 24 h and (B) TBP concentration profiles for three batches of 48 h at different DO levels. Initial concentrations are 10 ppm bentazon and 5 ppm TBP.

Table 2
First-order kinetic constants of bentazon and TBP biodegradation at four different DO levels (30, 15, 10 and 5 %) and three cycles. Average kinetic constants and degradation decline were calculated from the regression of the point cloud of all cycles. R^2 is the correlation coefficient of the first-order kinetics.

	k (h^{-1})							
	Bentazon				TBP			
DO (%)	30	15	10	5	30	15	10	5
Cycle								
1	0.287	0.119	0.086	0.095	0.125	0.099	0.026	0.010
2	0.397	0.355	0.296	0.086	0.077	0.106	0.019	0.020
3	0.348	0.397	0.126	0.144	0.082	0.051	0.027	0.017
Average (point cloud)	0.335 ± 0.055 (R^2 = 0.914)	0.288 ± 0.150 (R^2 = 0.789)	0.164 ± 0.112 (R^2 = 0.767)	0.109 ± 0.031 (R^2 = 0.798)	0.096 ± 0.026 (R^2 = 0.753)	0.096 ± 0.030 (R^2 = 0.847)	0.025 ± 0.004 (R^2 = 0.898)	0.015 ± 0.005 (R^2 = 0.790)
Degradation decline (%)	0	14.0	51.04	67.5	0	0	74.0	84.4

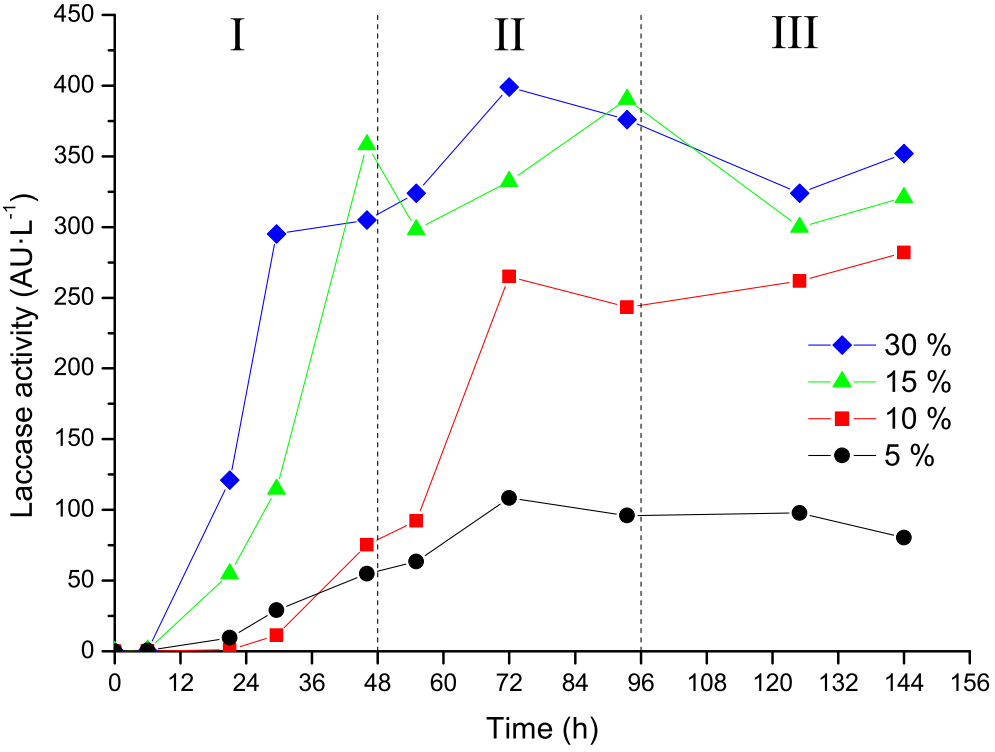

Fig. 4. Time course profile of laccase activity for TBP degradation at different dissolved oxygen concentrations.

biomass growth. In the present study, biomass growth was limited by the use of a maintenance medium (1 g·L^{-1} of glucose) instead of a growth medium (8 g ·L^{-1} of glucose), remaining in all cases below 4.5 g DW·L^{-1} of fungal biomass at the end of the experiments (initial biomass concentration was 3.50 ± 0.40 g DW·L^{-1}).

Therefore, the DO level was found to be a critical variable to control and, according to these results, at least 15 % DO is desirable to remain the maximum degradative potential of *T. versicolor*. Aeration represents the largest contribution to the costs of most WWTPs cost [39], thus reducing the oxygen content from 30 to 25 % (typically used in WWTP) to 15 % can result in large energy savings. This is the first study that quantitatively determine the DO level at which *T. versicolor*, as an example of WRF, is oxygen-limited in a bioremediation process.

3.1.2. Specific oxygen uptake ratio (OUR) estimation

A fundamental parameter for the design of any aerobic bioprocess is the sOUR. The sOUR can be estimated from the oxygen uptake of the fungus in a specific period. This study requires working under non-oxygen limiting conditions. In this case, the DO level was set at approximately 52 %. From the slope of its linear regression, the oxygen consumption was found to be 3.00 % min^{-1} approximately (Fig. S.1 of the Supplementary materials). Considering that the oxygen saturation (100 % DO) is 8.30 mg O_2·L^{-1} (at 25 °C) and that the inoculated biomass was 3.50 g DW·L^{-1} (at 25 °C), a sOUR of 0.10 g O_2·g biomass DW^{-1}·day^{-1} was obtained. This consumption was predictably lower than that (1.39–2.05 g O_2·g^{-1}·day^{-1}) obtained by Thiruchelvam and Ramsay (2007) since in the latter case a growth medium was used instead of a maintenance medium [40].

3.1.3. Organic matter removal

WRF degrade micropollutants mainly by co-metabolism, as these fungi require a more abundant carbon source for their maintenance [41]. Carbon sources that have been traditionally used in experimental studies as substrate for WRF include glucose, which is an easily assimilable nutrient [42], straw and wood, which are natural substrates for these fungi [23]. These substrates are generally required because the intrinsic organic matter in the water usually consists of complex compounds that are difficult to be assimilated by WRF. In fact, COD reduction from wastewater by WRF is inconsistent and scarcely reported [43,44]. In this section, the ability of *T. versicolor* to reduce COD under sterile and non-oxygen-limited conditions was evaluated. For this purpose, acetate (sodium acetate) was added to the medium as a less

assimilable carbon source for *T. versicolor*, which is commonly used in synthetic waters to simulate COD [45]. In this case, there are no pesticides nor any other organic compound in the medium and DO was set at 30 % to operate in the non-oxygen limiting region, preventing these factors from influencing acetate consumption.

Fig. 5 shows the results of two degradation experiments using either acetate and glucose (control) as COD sources. As expected, *T. versicolor* was able to completely consume glucose in a short period, obtaining a decreasing trend that was adjusted to a straight line ($R \approx 1.00$) whose slope was 83.28 mg $O_2 \cdot L^{-1} \cdot h^{-1}$. For 3.5 g pellets DW·$L^{-1}$, the glucose consumption rate was 15.86 mg $O_2 \cdot L^{-1} \cdot$g pellets $DW^{-1} \cdot h^{-1}$. Interestingly, the fungus was also able to consume an equivalent amount of acetate in terms of COD, although over a considerably longer period (approximately 130 versus 26 h). Once consumed, a second acetate pulse was added to verify whether the fungus was able to maintain its metabolic activity over time in an additional cycle. In the second pulse, *T. versicolor* was also able to almost completely degrade acetate for the same period of time (130 h). The decreasing acetate trends of both cycles were fitted to straight lines, giving a slope of 16.23 mg $O_2 \cdot L^{-1} \cdot h^{-1}$ ($R = 0.96$) for the first pulse, and 15.91 mg $O_2 \cdot L^{-1} \cdot h^{-1}$ ($R = 0{,}90$) for the second pulse. For 3.5 g pellets DW·L^{-1}, the acetate consumption rates were 3.09 and 3.03 mg $O_2 \cdot L^{-1} \cdot$g pellets $DW^{-1} \cdot h^{-1}$ for the first and second cycle, respectively. Therefore, the consumption rate of acetate was approximately 5 times lower than that of glucose, which was attributed to an energetically less efficient assimilation of the former compound by this fungus [46,47]. Although acetate was found to be a carbon source not as readily biodegradable as glucose by *T. versicolor*, the fungus was able to consume acetate for 264 h (11 days).

In addition, soluble COD analyses were performed at the beginning and end of each cycle to ascertain whether the acetate was completely mineralized or whether some transformation products (TPs) were formed. In the first cycle, soluble COD was reduced from approximately 2000 to 892 mg $O_2 \cdot L^{-1}$. This result indicates that although *T. versicolor* was able to completely remove acetate, some TPs that contribute to COD were probably generated. Anyway, *T. versicolor* was found to be able to reduce COD by 55 % at the end of the first cycle. After spiking the medium with approximately 2 g·L^{-1} of acetate at the beginning of the second cycle, the soluble COD increased to 3072 mg $O_2 \cdot L^{-1}$, which progressively decreased throughout this last cycle to 1480 mg $O_2 \cdot L^{-1}$ (52 % removal). These results contrast with other studies that have reported the inability of *T. versicolor* to reduce COD. In this regard, Badia-Fabregat et al. (2017) studied several fungal treatments under non-sterile conditions in continuous operational mode with veterinary hospital wastewater and reverse osmosis concentrate with COD concentrations of 245–264 mg $O_2 \cdot L^{-1}$ and 65 mg $O_2 \cdot L^{-1}$ respectively [15]. In both cases, significant increases in COD concentration were reported, which were attributed to the possible production of metabolites. Anastasi et al. (2012) examined the treatment of textile wastewater by different fungal strains and concluded that fungal treatment was more effective in decolourisation than in COD reduction [48]. In particular, *T. versicolor* was the worst performing strain in terms of COD reduction, considerably raising the initial level. Hu et al. (2021) also reported a considerable increase in COD concentration during the treatment of agricultural water by *T. versicolor* in a fluidized bed reactor [49]. In this case, glucose was used as substrate, thus the COD increase was attributed to the addition of antifoam (Tween 80), which could not be consumed by *T. versicolor*. By comparing the results of the reported studies with those obtained in the present work, it can be deduced that although the fungus is capable of consuming acetate, hence reducing COD, the efficiency of the treatment can depend on other factors, such as the characteristics of the organic content, the matrix, the operating conditions and the reactor type.

3.2. DO study in a fixed-bed bioreactor

The wood was inoculated with *T. versicolor* in a box under sterile conditions, reaching 0.395 ± 0.034 mg·g DW^{-1} of ergosterol content after 50 days of culture (Fig. S2 of the Supplementary materials). Fig. 6 shows the DO evolution over time in the FBB working in continuous mode for different flow rates. The results showed that the DO consumption rate of the system varied over time until reaching the equilibrium (maximum). This transition period was attributed to the interaction between water and residual air during the column filling stage. This evolution was hardly observed when working at a flow rate of 40 mL·min^{-1} (Fig. 6 (C)) as the column was rapidly filled up.

Fig. 7 shows two graphs that allow a better interpretation of the

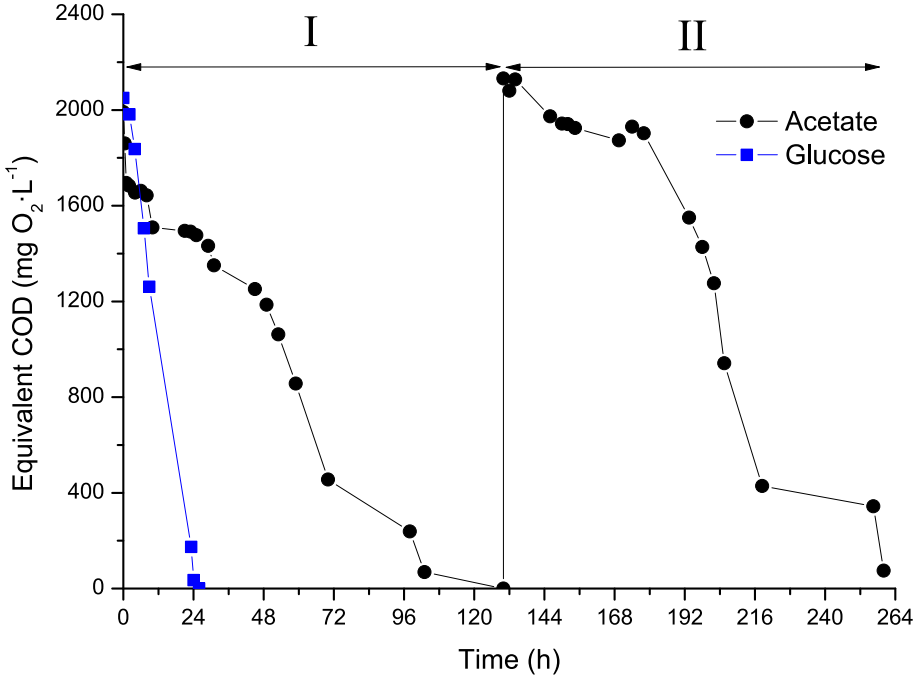

Fig. 5. Acetate (black circle) and glucose (blue circle) consumption profiles in terms of equivalent COD level over time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

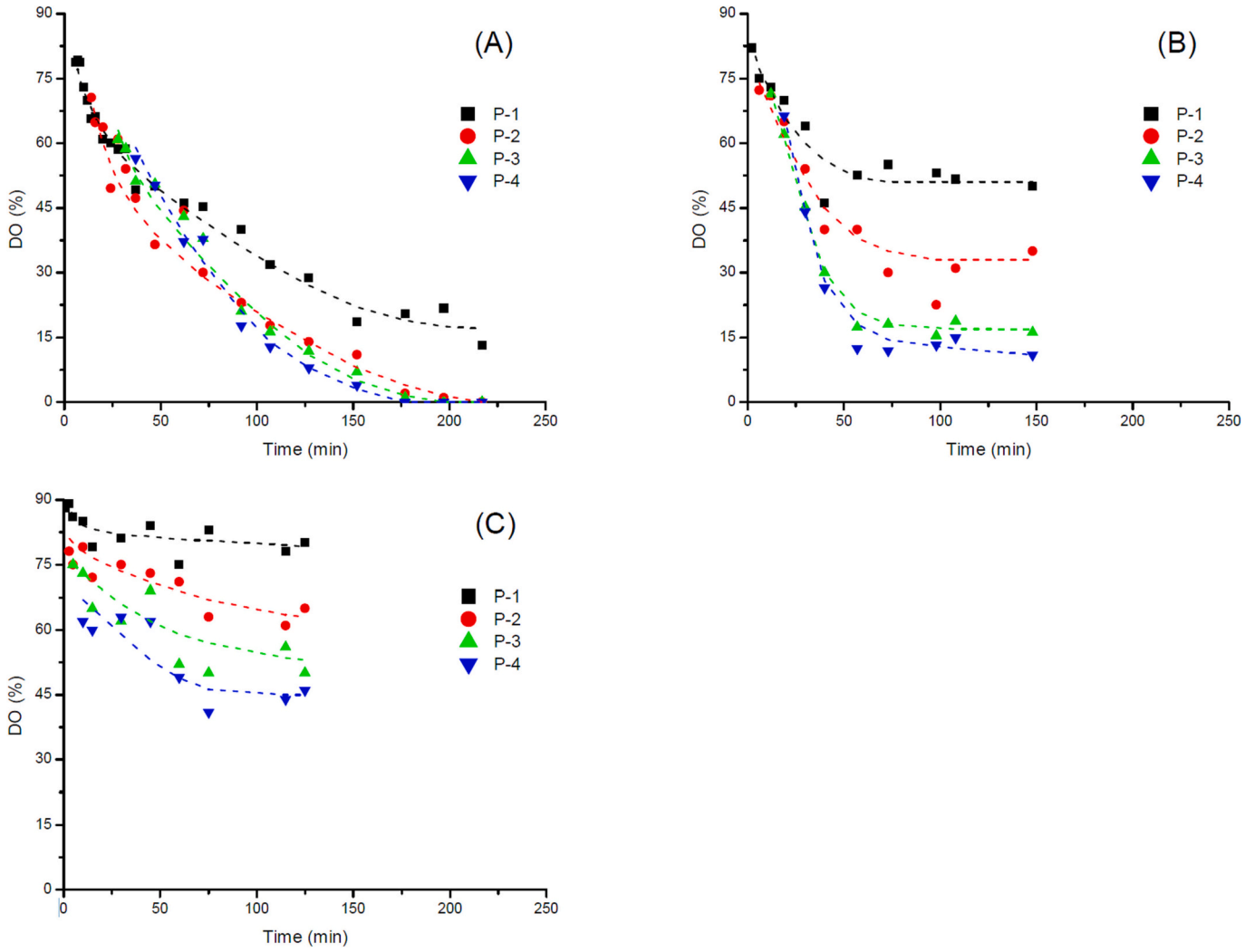

Fig. 6. Dissolved oxygen depletion profiles in the FBB at flow rates of 12 (a), 23 (b) and 40 (c) mL·min^{-1}. The reactor measurement points were located at 7 (P-1), 19 (P-2), 34 (P-3) and 49 (P-4) cm heights.

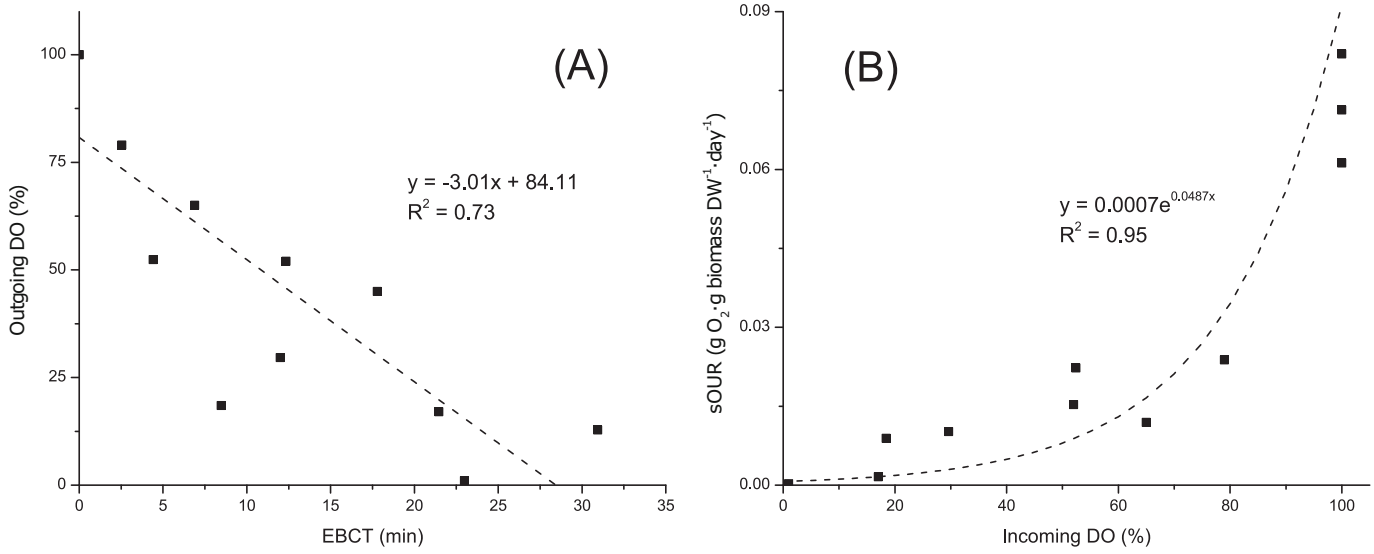

Fig. 7. Correlations between outgoing dissolved oxygen and EBCT (a), and between sOUR and incoming dissolved oxygen. Dashed lines are linear (a) and exponential (b) regressions.

results in the steady-state. In this case, the steady-state results in the FBB have been unified to form point cloud plots that allow a better correlation between variables. Fig. 7 (A) shows that there is a strong correlation ($R^2 = 0.73$) between the EBCT and the DO level in the reactor. The higher the EBCT, the longer period the water remained inside the reactor and thus, the more DO was assimilated by *T. versicolor*. This correlation indicates that an FBB with the characteristics described in the present study operated under limiting DO concentrations (i.e., less than 15 % DO, Section 3.1.1) from 43 min of EBCT onwards. These results show that, even if the influent is fed at 100 % saturation, an FBB may require internal aeration in order to guarantee aerobic conditions for the fungus.

The sOUR can be calculated from DO results, the level of ergosterol in the wood and the amount of wood in each reactor section [Eqs. (1) and (2)]. DO consumption was basically attributed to the activity of *T. versicolor* since the presence of other microorganisms was considered negligible. Different sOURs were obtained for each flow rate and reactor section studied. As shown in Fig. 7 (B), an exponential correlation ($R^2 = 0.95$) was found between the DO concentration and the sOURs of the system. Thus, the existence of different sOURs was mainly attributed to the DO level measured in each reactor section. These results contrast with those obtained in the stirred-tank bioreactor (see Section 3.1.2). In the latter case, sOUR was considered constant in the non-oxygen limiting region. However, it should be noted that in the stirred-tank bioreactor there was a much better oxygen transfer than in the FBB. In the FBB, low DO liquid interfaces, or even dead zones were probably created around the fungus with diffusional and oxygen transfer limitations that, consequently, restricted oxygen availability to the fungus [50]. Therefore, delocalized oxygen supply along the column and/or working in more turbulent conditions should be considered to favour a better transfer and availability of oxygen to the fungus.

3.3. DO study in a rotating drum bioreactor

The RDB was inoculated with wood colonized by *T. versicolor*, which was previously cultured for 24 days (0.24 mg ergosterol·g wood DW^{-1}). The RDB is a reactor with a rotating mechanism that maintains aerobic conditions using two strategies: alternation of the submerged biomass and aeration of the liquid medium by agitation.

Consequently, high speeds and long periods of rotation promote the aeration and thus the DO saturation in the system. In this regard, a constant and relatively high rotational speed (12 rpm) maintained high DO saturation (8.0–6.5 mg $O_2 \cdot L^{-1}$) in a rotating biological contactor inoculated with *T. versicolor* immobilized on polypropylene discs [51]. In contrast, continuous rotation (6 rpm) has also been reported to produce fungus detachment from wood [52], which is probably attributed to the generation of shear forces that damage the mycelial structure or/and to a better performance of the fungus in this substrate when working under static conditions. Regarding the latter, another recent study that worked with discontinuous rotation showed better biomass maintenance when the rotation frequency was changed from 1.5 turns every 4 h to every 24 h [28]. Although operating under more static conditions could favour biomass immobilization on wood and thus increased treatment yield, the lack of agitation may cause the submerged biomass to be oxygen limited. In this section, DO was measured at different points of the reactor (at the inlet, middle and outlet) to evaluate whether the fungus was under limiting conditions in the RDB (Fig. 8).

DO depletion profiles were fitted to first-order kinetics (Table 3). The equations used for the determination of the kinetic constants are shown in the Supplementary Material. The oxygen limiting conditions (15 %) were reached at all studied points of the RDB before 30 h of operation. According to the kinetics, oxygen limiting conditions were reached for the first time in the RDB (intermediate point, 6 cm depth) after approximately 9.7 h; and at all studied points after 30 h. Furthermore, rotation performed after 24 h (1.5 turns) did not result in an appreciable increase in DO concentrations. These results indicate that if the RDB is operated under the same conditions for a period longer than 9.7 h (conservative scenario) without additional aeration, the submerged fungal fraction can be oxygen limited. However, fungal biomass has proven to remain active and immobilized on wood in the RDB for a long period of operation [28]. These results indicate that the alternation of the submerged fraction may mitigate the effects of anoxic conditions and contribute to maintaining the metabolic activity of the fungus.

Although DO concentrations were considerably reduced after 30 h in the channel, it remained around 82 % in the recirculation tank. Other reported studies have also periodically monitored the DO concentration in the recirculation tank of the RDB and it remained above 30 % throughout the treatment [23,52]. This higher DO level in the recirculation tank compared to the channel may be due to aeration during liquid dripping in the recirculation tank, a larger liquid-air contact surface and stirring of the recirculation tank. Accordingly, measurements were also conducted in the feed tank, obtaining an average value of 34.5 ± 4.1 % DO, but it is feasible to assume that the actual DO concentration at which the effluent entered the reactor was significantly increased by dripping.

DO concentrations varied as a function of depth and axial location, as demonstrated by fitting the data to a first-order kinetics of oxygen depletion (Table 3). The consumption curves were approximately fitted to first-order kinetics, from which the kinetic constants were calculated. DO decrease was faster at 6 cm than at 4 cm depth, probably due to worse oxygen diffusion, and at the middle point than at the extremes of the RDB, which was attributed to a lower air-water contact surface.

These results indicate that the submerged biomass operates under oxygen limiting conditions from the first 30 h onwards (in some points even earlier), and therefore, the incorporation of an additional aeration system in the RDB would be highly recommended to enhance the treatment performance. This improvement should be explored in future

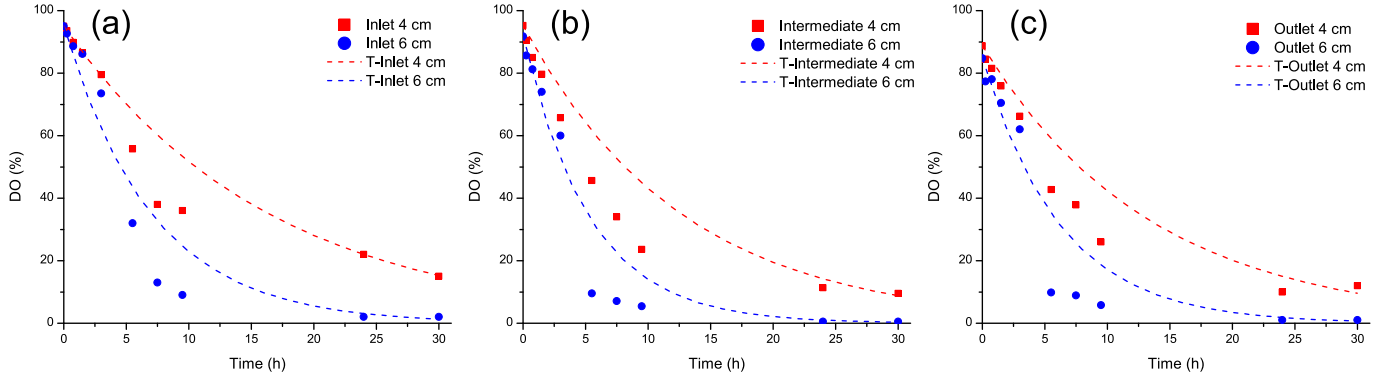

Fig. 8. Dissolved oxygen depletion profiles measured at two depths (4 and 6 cm) at the inlet (A), intermediate zone (B) and outlet (C) of the RDB. Dashed lines are the curves described by the fitted first-order kinetics (T: theoretical).

Table 3
Kinetics of dissolved oxygen consumption in the RDB.

	Inlet		Intermediate		Outlet	
Depth (cm)	4	6	4	6	4	6
Kinetic rate (h^{-1})	$6.09{\cdot}10^{-2}$	$1.42{\cdot}10^{-1}$	$7.93{\cdot}10^{-2}$	$1.88{\cdot}10^{-1}$	$7.41{\cdot}10^{-2}$	$1.60{\cdot}10^{-1}$
R^2	0.93	0.91	0.93	0.91	0.92	0.88

work on the RDB, along with the evaluation of operational costs, prior to its eventual implementation at full-scale.

According to the results obtained in the stirred tank bioreactor, at least 15 % DO is required to avoid limiting the degradative activity of *T. versicolor*. Therefore, the results obtained in the FBB and RDB highlight the need for aeration in bioreactors using *T. versicolor*. In fact, this aeration is hardly reducible or adjustable, since oxygen consumption is relatively fast and the limiting oxygen region was reached in only an EBCT of 43 min in the FBB and 9.7 h in the RDB.

4. Conclusions

T. versicolor was able to completely remove bentazon at 10 ppm after 6 h and TBP at 5 ppm after 32 h under non-limiting oxygen conditions. The limiting dissolved oxygen concentration was found to be 15 % of saturation (1.3 mg $O_2{\cdot}L^{-1}$). Based on this result, two well-studied reactors in fungal bioremediation, the fixed-bed and the rotating drum bioreactors, were found to be oxygen limited for an EBCT longer than 43 min and an operational period longer than 9.7 h, respectively. In case these limits are exceeded, aeration should be added to ensure full aerobic conditions. Moreover, *T. versicolor* was able to consume acetate as a poorly assimilable carbon source, without requiring the addition of any supplementary nutrients. This result indicates that *T. versicolor* is not only able to remove micropollutants co-metabolically but also to consume other organic matter as substrate, thus decreasing the COD of the broth. Therefore, our findings suggest that bioremediation using *T. versicolor* is a promising strategy to degrade different micropollutants and reduce COD when working in the non-limiting oxygen conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Project PID2019-103989RB-100 financed by MCIN/AEI/10.13039/501100011033. Eduardo Beltrán-Flores acknowledges support from a MINECO predoctoral research grant (BES-2017-080500).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2023.104105.

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