Research Paper

Advanced bio-oxidation of fungal mixed cultures immobilized on rotating biological contactors for the removal of pharmaceutical micropollutants in a real hospital wastewater

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ABSTRACT

Hospital wastewater represents an important source of pharmaceutical active compounds (PhACs) as contaminants of emerging concern for urban wastewater treatment plants. This work evaluates a fungal biological treatment of a hospital effluent before discharging in the municipal sewer system. This treatment was performed in rotating biological contactors (RBCs) covered with wooden planks in order to promote the attachment of the fungal biomass. These bioreactors, initially inoculated with *Trametes versicolor* as white rot fungi, have created biofilms of a diversified population of fungal (wood-decaying fungi belonging to *Basidiomycota* and *Ascomycetes*) and bacterial (*Beta-proteobacteria*, *Firmicutes*, and *Acidobacteria*) microorganisms. The mixed fungal/bacterial community achieved a stable performance in terms of carbon, nitrogen, and phosphorous reductions for 75 days of continuous operation. Moreover, a remarkable removal of pharmaceutical micropollutants was accomplished especially for antibiotics (98.4 ± 0.7, 83 ± 8% and 76 ± 10 for azithromycin, metronidazole and sulfamethoxazole, respectively). Previous studies have proven a high efficiency of fungi for the removal of microcontaminants as a result of advanced bio-oxidation processes mediated by oxidizing hydroxyl radicals. This study evidences the development of a stable fungal-bacterial mixed culture over wooden-modified RBCs for in-situ removal of pharmaceutical compounds of hospital wastewater under non-sterile conditions and non-strict temperature control, avoiding periodical fungal inoculation due to destabilization and displacement of fungal cultures by indigenous wastewater bacteria.

1. Introduction

Nowadays, hospital wastewaters (HWWs) represent an essential input of residual pharmaceutical active compounds (PhACs) into the environment (Serna-Galvis et al., 2019). HWW's composition is usually similar to urban wastewaters (UWWs) and they are generally discharged into the municipal sewer system without any specific pre-treatment (Oliveira et al., 2018; Verlicchi et al., 2015). However, HWWs contain a concentration of PhACs between 3 and 150 higher than domestic urban ones, and composition may change significantly depending on the therapeutic and clinic specialities of the hospital (Chonova et al., 2016). Antibiotics, analgesics, anti-inflammatories, and iodinated contrast media are the most frequent therapeutic groups detected in these effluents (Daouk et al., 2016; Oliveira et al., 2018). Unfortunately, conventional wastewater treatment plants (WWTPs) have not been designed for the effective removal of pharmaceutical compounds, and

the pharmaceutical micropollutants can reach to the aquatic ecosystems (Tondera et al., 2018).

Several works have studied separated and dedicated treatments to decrease the pharmaceuticals' loading of hospital wastewaters. Advanced oxidation processes, such as ozonation (Souza et al., 2018), Fenton (Cruz del Álamo et al., 2020a; Munoz et al., 2017) or UV treatments (Graumans et al., 2021), have been highly effective removing PhACs from HWWs. However, they usually have remarkable operation costs due to the addition of oxidant reagents (ozone, hydrogen peroxide) or the energy required. Biological treatments are low-cost technologies in comparison to advanced oxidation processes, but the removal of some pharmaceutical micropollutants is not still completely satisfactory. The performance of conventional activated sludge (CAS) has shown a remarkable dependence on the pharmaceutical compounds' properties and the operating conditions (Couto et al., 2019). Thus, although some pharmaceutical compounds (ibuprofen, paracetamol or caffeine)

showed a high removal efficiency during the CAS treatment (Verlicchi et al., 2013), other compounds such as carbamazepine (Martín et al., 2012), macrolide antibiotics (Deblonde and Cossu-Leguille, 2011), or diclofenac (Thiebault et al., 2017) exhibited a low and variable removal. As it has been mentioned, these recalcitrant compounds belong to the most frequent therapeutic groups detected in hospital effluents. Membrane bioreactors (MBRs) have shown promising results in the removal of pharmaceuticals (especially antibiotics) working with a hydraulic retention time (HRT) of 31 h and a total membrane area of 1600 m^2, although some compounds (carbamazepine, diclofenac) still evidence a very refractory behaviour (Beier et al., 2011). Moreover, their application at the industrial scale is still conditioned by membrane fouling and the associated operational costs. Alternatively, fungal bioreactors using white rot fungi (WRF) such as *Trametes versicolor* have shown promising results for the removal of a wide range of biorecalcitrant pollutants, including PhACs (Mir-Tutusaus et al., 2018a). Despite the good performance of WRF for the removal of PhACs, several limitations have been identified for wastewater treatment (Mir-Tutusaus et al., 2018a). The previous works usually propose reactors under strict control of the temperature and high hydraulic retention times between 3 and 7 days, where the initial composition of the hospital wastewater was modified by the addition of carbon and nitrogen biodegradable sources and the biomass was periodically replaced by fresh one to ensure the maintenance of the fungal communities and their predominance in the reactor (Jaén-Gil et al., 2021, 2019; Mir-Tutusaus et al., 2018a; Tormo-Budowski et al., 2021).

Different operational strategies have been proposed to increase the fungal activity and stability inside the reactor under non-sterile conditions, which are the major drawbacks for the implementation of fungal biological treatments (Mir-Tutusaus et al., 2018b, 2016). One of them is the immobilization of fungal biomass, which provides a more robust operation in a continuous mode, in order to favor the wash-out of the suspended bacteria, reducing the competition inside the bioreactor (Mir-Tutusaus et al., 2018a). In this sense, several authors have immobilized WRF onto wood structures as support and carbon source due to the natural ability of WRF to degrade lignocellulosic substrates (Atilano-Camino et al., 2020; Torán et al., 2017). Likewise, recent studies have demonstrated the degradation of different pollutants (herbicides and PhACs) using *Trametes versicolor* immobilized over wooden substrates (Beltrán-Flores et al., 2020; Tormo-Budowski et al., 2021). Nevertheless, the occurrence of bacteria has been confirmed in natural decaying wood regardless of the use of wood as a carbon source (Kielak et al., 2016). Consequently, the interactions between fungi and bacteria may be important inside the biological reactor and should be carefully established.

Recently, the efficiency of *Trametes versicolor* immobilized on polypropylene discs in rotating biological contactors (RBCs) was demonstrated for the treatment of UWWs and the removal of PhACs (Cruz del Álamo et al., 2020a). In that work, the addition of advanced bio-oxidation promoters, gallic acid as a lignin-derived mediator and metal complexes with redox activity, enhanced the performance of the fungal biological treatment for the removal of PhACs. Gallic acid used as promoter was completely degraded during the treatment with no signs of toxicity of this compound or oxidized by-products.

The aim of this work has been the evaluation of the fungal biomass immobilized on wooden-modified RBCs for the continuous treatment of a real HWW effluent using bio-oxidation promoters with non-control of temperature and any external air supply. In this case, polypropylene RBCs' discs were covered by superficial pine wood planks as support for the immobilization of *Trametes versicolor* to prevent destabilization of the fungal biomass under non-sterile conditions. This study evaluates the performance of the modified RBCs for the removal of 23 PhACs of different therapeutic families along macroscopic parameters related to C, N and P removals and the evolution of fungal and bacterial communities in the biofilm during 75 days of operation. The reproducibility and robustness of the fungal treatment was assessed by the operation of two identical and independent RBC units.

2. Materials and methods

2.1. Hospital wastewater

Wastewater samples were taken from a hospital located in the south of Madrid (Spain). The hospital facilities generate three streams from different therapeutic units. Thus, three automatic water sampler devices were used to collect 330 mL of wastewater (110 mL at each point) every 10 min through 7 days. All the collected samples from the different streams were mixed up altogether in an Intermediate Bulk Container (IBC) of 1 m^3. The sampling process was carried out twice, in January 2019 and April 2019.

2.2. Cultivation of Trametes versicolor

The strain *Trametes versicolor* (CECT 20817) was collected from the "*Colección Española de Cultivos Tipo (CECT)*" and maintained by subculturing as described elsewhere (Vasiliadou et al., 2016). The fungal mycelial suspensions used as inoculum for the bioreactors were prepared, as described by Cruz Cruz del Álamo et al., 2018.

2.3. Chemical reagents

Malt extract, saccharide, ammonium tartrate, sodium phosphate dibasic anhydrous, sodium phosphate monobasic monohydrate, for incubation and growth; and Fe^{3+}-oxalate hexahydrated, Mn^{2+}-nitrate tetrahydrated, and gallic acid as advanced bio-oxidation promoters were purchased from Sigma-Aldrich (Spain). LC-MS quality methanol and the reagents used to adjust the pH were also obtained from Sigma-Aldrich.

2.4. Rotating biological contactors (RBC) modified by attaching wooden sheets

Two identical RBC units (*R1* and *R2*) were simultaneously operated independently in parallel in order to assess the reproducibility and robustness of biological treatment of hospital wastewater. The bioreactor, based on the authors' bench-scale RBC unit of 10 L of working volume and total surface area of 0.71 m^2 distributed in 5 propylene discs, is described by Cruz del Álamo et al., 2020a. In this work, the propylene discs were covered by wooden pine sheets of the same diameter and 0.5 cm thickness Additionally, an impeller joined to the mechanical discs' shaft was set up to improve the homogenization of the vessel's content. Fig. 1 shows different schematic views of the modified RBC units.

Initially, the twin RBC units were started-up for the biofilm generation and acclimation in the wooden surface (experimental procedure is included in the supplementary material). After the start-up, the HWW was treated under continuous operation for 75 days in the twins wooden-modified RBC units. The discs' immersion was set to 40% using a working volume of 10 L and a constant rotational speed of 12 rpm. Gallic acid (85 mg/L) as a quinone-like mediator and metallic species in the form of Fe^{3+}-oxalate hexahydrated (145 mg/L) and Mn^{2+}-nitrate tetrahydrate (20 mg/L) were continuously added to the inlet as promoters of the advanced bio-oxidation process according to the values established in previous works (Cruz del Álamo et al., 2018; Vasiliadou et al., 2019). The inlet stream's pH was adjusted to 4.5, a common practice in fungal bioreactors in order to inhibit the bacteria cellular transport, promoting the fungal activity in the bioreactor (Mir-Tutusaus et al., 2018a). The HRT was fixed at 1 day using the minimum value studied in the literature for continuous reactors based on WRF (Cruz del Álamo et al., 2020a; Mir-Tutusaus et al., 2018a).
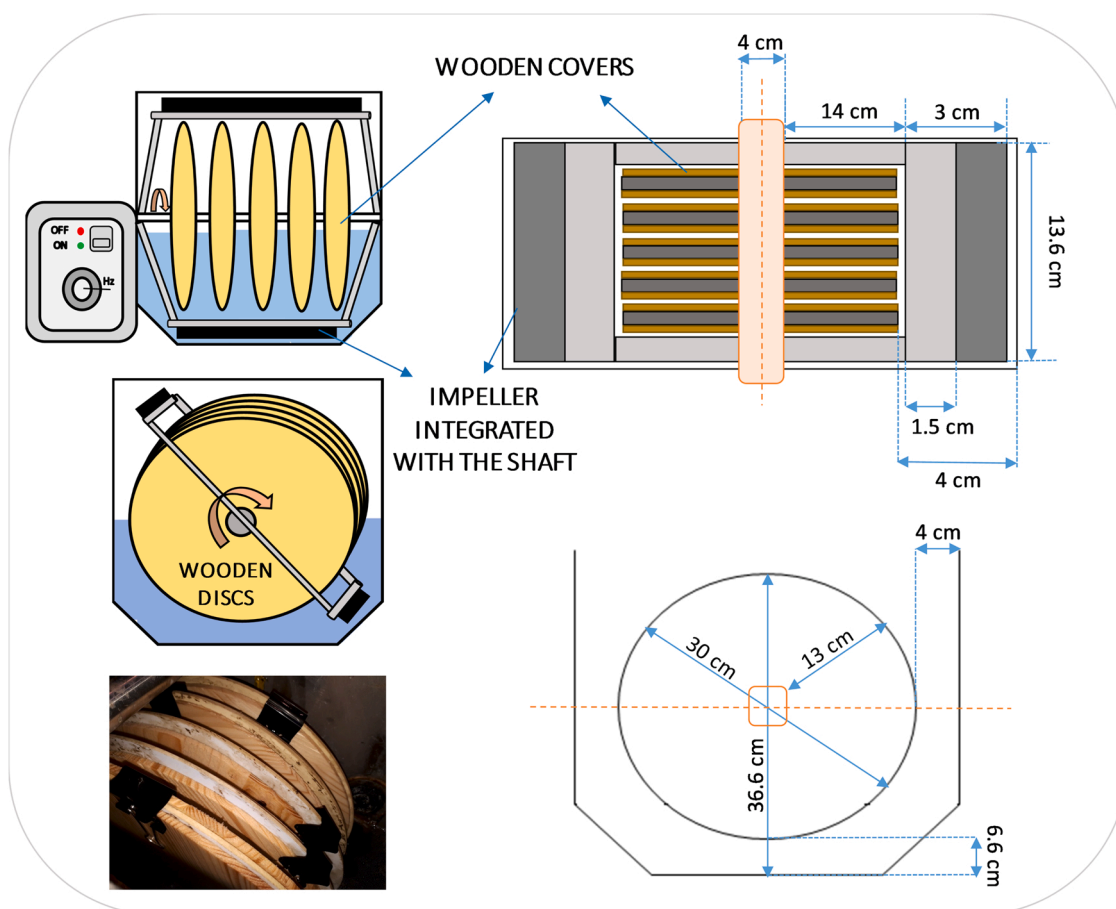
Fig. 1. Wooden-modified RBC units.

2.5. Characterization techniques

Samples of the inlet and outlet streams were monitored daily to follow the performance of the wooden-modified RBC units. Total organic carbon (TOC) was estimated using a combustion/non-dispersive infrared gas analyzer model TOC-V from Shimadzu. Chemical oxygen demand (COD), nitrogen concentration as ammonium (N-NH_4^+), total Kjeldahl nitrogen (TKN), and P concentration as phosphate (P-PO_4^{-3}) were determined following the standardized APHA methods (APHA, 2005). Dissolved oxygen (DO) and pH were monitored with a CellOx 325 and a Sentix 81 probes from WTW-Xylem. The quinone-like mediator of the advanced bio-oxidation process (gallic acid) was measured by High Performance Liquid Chromatography (HPLC) in a Varian Prostar equipped with a Phenomenex C18 column (3×150 mm) and a UV-Vis detector at 254 nm. A mixture of methanol (49.5%), ultrapure water (49.5%), and glacial acetic acid (1%) at a pH of 2–2.5 was used as a mobile phase at 0.15 mL/min. The Fe and Mn contents of the treated effluent were determined by ICP-AES analysis collected in a Varian Vista AX Pro-720ES spectrometer.

2.6. Pharmaceutical micropollutants analysis

The concentration of pharmaceutical micropollutants in the inlet and outlet streams of the reactors was carried out after solid-phase extraction (SPE) by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-ESI-MS/MS) using a vortex electrospray ionization interface (Bruker UHPLC/MSMS EVOQ™ QUBE). Twenty-three PhACs of twelve different therapeutic families were analysed according to the method described elsewhere (Cruz del Álamo et al., 2020b; details are included in the Supplementary material). The detection and

quantification limits (LOD and LOQ, respectively) of the PhACs are shown also in Supplementary material (Table S1). All the samples were analysed by triplicate.

2.7. Assessment of fungal/bacterial communities developed on RBC units

2.7.1. Metagenomics of biomass

Assays of quantitative polymerase chain reactions (qPCR) for samples taken from the biofilm of RBCs were performed for quantification of *Trametes versicolor*, total fungi and total bacteria. Samples were analyzed by duplicate. Total DNA was extracted using the Microbial DNA isolation kit (Soil DNA Isolation Kit, CANVAX, Córdoba, Spain). The DNA yield range was between 103.57 and 675.57 ng/µL. Different primers were defined to detect *Trametes versicolor* (ITS1 and ITS2; Badia-Fabregat et al., 2017), total fungi (FR1 and FF390; Chemidlin Prévost-Bouré et al., 2011) and total bacteria (16S V3–V4; Klindworth et al., 2013). Further details of the sequences of the specific primers are included in Table S2.

Typically, 10 µL were used for the qPCR assays, which include 4 µL of the diluted sample (1/400), 1 µL of Primer Mix (5 µM of each primer) and 5 µL *Power* SYBR® Green PCR Master Mix (Thermo Fisher Scientific, CN 4367659). The latter includes AmpliTaq Gold® DNA Polymerase, dNTPs and the rest of reagents needed to perform the PCR. qPCR assays were performed in a CFX384 Real-Time System C1000 Thermal Cycler (Bio-Rad), in hard-Shell® 384–Well PCR Plates White Well Clear shell (Bio-Rad CN HSP-3805), using the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min. A melting curve from 60 °C to 95 °C (0.5 °C/s) was added at the end of the program to verify the specificity of the PCR. The qPCR products were purified with the Real clean spin kit (Real, Ref: RBMCS02) following the

manufacturer's instructions. Individual efficiency of each primer pair was determined, and it is shown in Table S3. DNA samples were sequenced on an Illumina MiSeq Instrument under a 2×300 protocol. Sequencing runs were performed using the following specific sequencing primers: **16SV3-V4-CS1:** CCTACGGGNGGCWGCAG; **16SV3-V4-CS2:** GACTACHVGGGTATCTAATCC; **CS1-ITS4:** TCCTCC GCTTATTGATATGC; **ITS86F-CS2:** GTGAATCATCGAATCTTTGAA.

The V3-V4 region of the 16S rRNA gene was amplified as described previously (Caporaso et al., 2011), including extensionśtails (CS1, CS2) in PCR products (35–301 bp). It allowed sample barcoding and the addition of specific Illumina sequences in a second low-cycle number PCR. However, a different primer pair (ITS86F/ITS4) was used to amplify the ITS2 region, as it has shown an efficient amplification of that region of a broad range of fungal taxa in environmental soil samples (Op De Beeck et al., 2014). After sequencing, quality analyses were performed over reads using the FastQC software. The primers sequences were trimmed from both sides of two reads: 3′ and 5′ ends of forward and reverse reads, respectively, using the Cutadapt software, and joined into one single sequence using PANDAseq Assembler. It allows correcting sequencing errors in the overlap region and discarding those pairs that do not align between them or that have a low quality (less than 10% of the joined reads). The trimmed and assembled reads were the input for the core analysis. The GreenGenes and UNITE databases were used for mapping 16S and ITS2 sequencing reads, respectively, through the Qiime2 software.

2.7.2. Activity tests of fungal/bacterial biomass

Activity tests were performed with the biofilm developed into the wooden discs after 75 days of operation in order to assess contribution of fungal or bacterial communities developed in the biofilm of the RBCs on the performance of the continuous treatment of HWW under non-sterile conditions. The activity tests were carried out according to the protocol previously described by Cruz del Álamo et al., 2020a. In these bioassays, 200 mg of dry-based VSS/L of biomass collected from the biofilm was inoculated in 100 mL of a synthetic wastewater containing a mixture of micro- and macro-nutrients. Additionally, Gram- and Gram+ bacteric ides (ampicillin, 4 mg/L, and tetracycline, 128 mg/L) or fungicide (nystatin, 200 mg/L) were added and maintained for 24 h under continuous stirring at 25 ºC to inhibit the bacterial or fungal activity, respectively. Finally, 250 mg/L of TOC as sodium acetate was added to the medium to start the bioassays and monitored the TOC consumption during 4 days of incubation at 25 ºC (further details of experimental procedure is included in the Supplementary Material).

3. Results and discussion

3.1. General performance of wooden-modified RBCs

Prior to the assessment of the removal of pharmaceutical micro-pollutants of HWW by the wooden-modified RBCs, the performance of the system was analyzed in terms of the elimination of the organic matter, nitrogen, and phosphorous nutrients. Most of the works found in the literature have used operational strategies that involve the addition of carbon and nitrogen sources to control the C/N ratio (Mir-Tutusaus et al., 2018a). This practice makes difficult to establish the efficiency of the fungal treatment in terms of these parameters as some fungal processes maintained or even increased the chemical oxygen demand (COD) after the treatment (Badia-Fabregat et al., 2015).

Fig. 2 shows the concentration of TOC, $N-NH_4^+$, $P-PO_4^{3-}$ and TKN of the inlet and outlet streams in *R1* and *R2* wooden-modified RBCs for 75
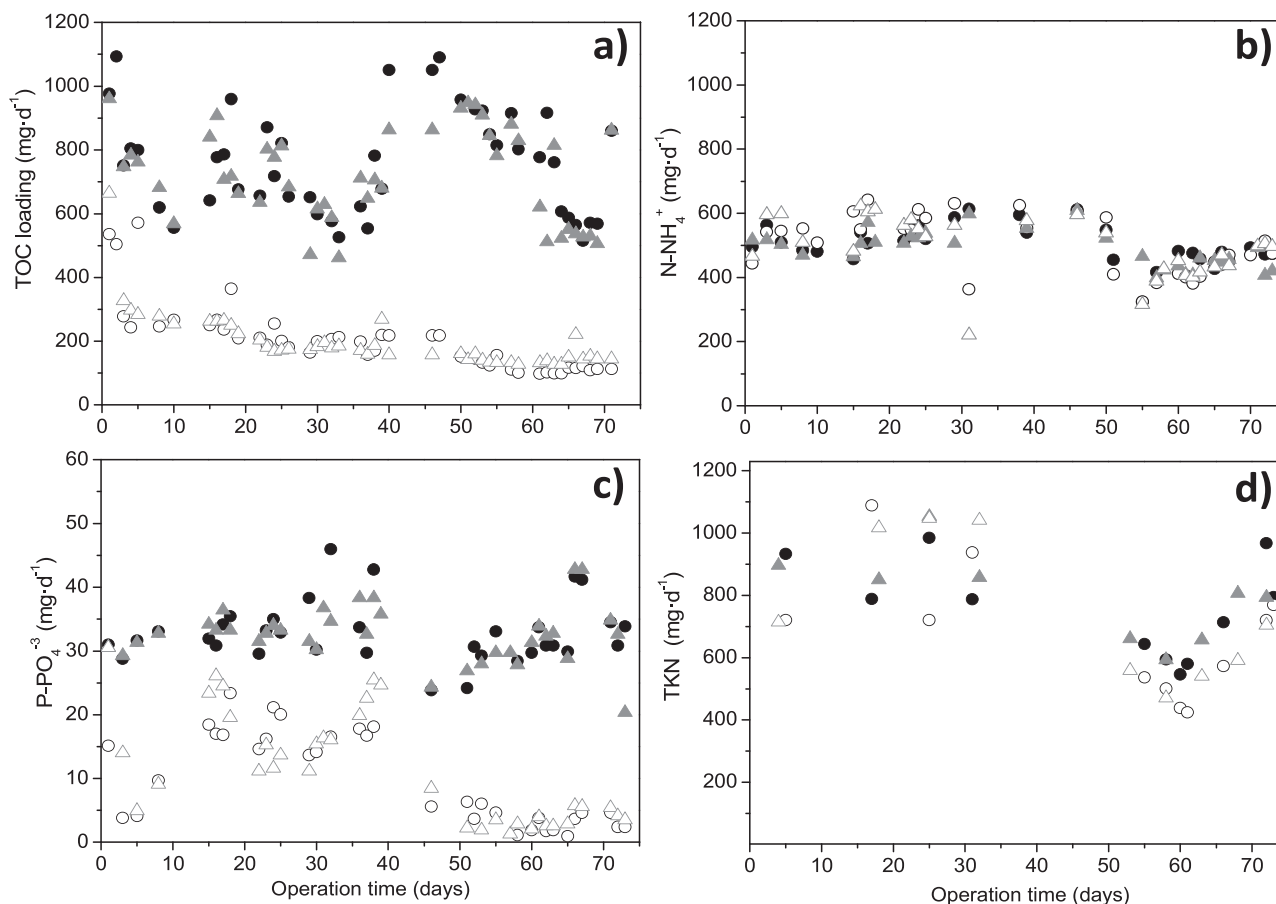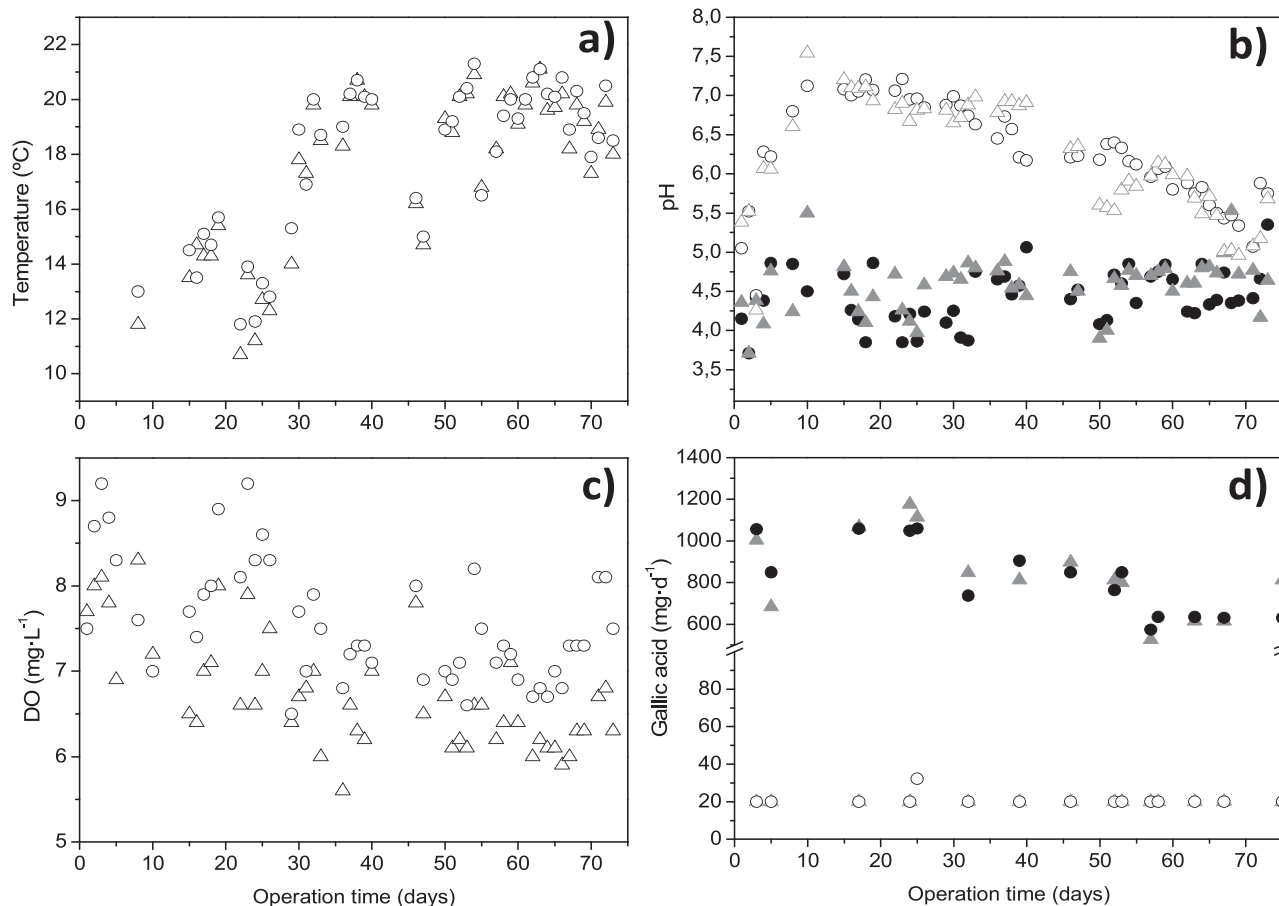

Fig. 2. Removal of (a) TOC, (b) $N-NH_4^+$, (c) $P-PO_4^{3-}$ and (d) TKN obtained in the wooden-modified RBCs: *R1* (●,○) and *R2*(▲,△). Full symbols indicate data of the inlet stream and empty symbols of the outlet stream in both reactors.

days of continuous operation. Average concentration and removal percentage of these parameters are also summarized in Table S4. The results of both RBC units were quite similar. Average TOC removals of $78 \pm 4\%$ and $82 \pm 6\%$ were obtained in *R1* and *R2* reactors, respectively. Total COD concentrations of 68 ± 41 and 88 ± 56 mg L^{-1} in the outlet stream were also obtained, which are below the discharge limit (125 mgL^{-1}) established for this parameter by current European legislation (Council Directive 91/271/EEC of 21 May 1991). Phosphorous as P-PO$_4^{3-}$ was also significantly removed ($73 \pm 25\%$ and $75 \pm 22\%$), reaching concentrations of 0.8 ± 0.8 and 0.9 ± 0.7 mg L^{-1} in the effluents of *R1* and *R2* units, respectively. In contrast, the reduction N-NH$_4^+$ was not statistically significant ($10 \pm 10\%$ in both units) and the TKN showed reductions of $26 \pm 15\%$ and $15 \pm 15\%$ in *R1* and *R2* units, with TOC/TKN consumption ratios between 3 and 5.4 (see Table S4). These results can be attributed to physiological aspects of wood decay microorganisms such as white rot fungi, which are adapted to use wood as substrate with C/N ratio up to 1250/1 (Watkinson et al., 2006). These microorganisms can develop nitrogen-conserving strategies based on selective depletion of cells for nitrogen recycling and re-assimilation from old to new mycelium (Paustian and Schnürer, 1987). Some studies have demonstrated that nitrogen is not completely assimilated under nitrogen limitation conditions due to these recycling strategies (Watkinson et al., 2006). It has been also reported that these wood decay microorganisms are able to uptake nitrogen from different sources (Marzluf, 1996), but the presence of ammonia as a readily available nitrogen source represses enzymes involved in the use of other nitrogen sources, such as those responsible for the transport of urea or glutamate and histidine usage (Lee et al., 2006). All these factors seem to be responsible of the limited nitrogen removal of the RBC units. Likewise, these results are in agreement with our previous work performed for the removal of pharmaceutical micropollutants in urban wastewaters using *Trametes versicolor* (Cruz del Álamo et al., 2020a).

Likewise, it must be pointed out that the bioreactors were operated under non-controlled temperature for 75 days. During this period, the temperature increased from ca. 11–14 °C to 18–22 °C during the days 20 and 30 of operation due to seasonal fluctuations of the region (Fig. 3a). The temperature is an important factor accelerating biochemical reactions (Atilano-Camino et al., 2020). Previous works reported in literature always control the temperature between ca. 25–30 °C to maintain the activity of the fungal systems (Jaén-Gil et al., 2019; Mir-Tutusaus et al., 2021). The increase of temperature seems to improve the performance of RBC units promoting higher elimination of TOC (from $67.3 \pm 7\%$ to $77.7 \pm 7\%$; Fig. 2a) and phosphate (from $53.1 \pm 16\%$ to $77 \pm 19\%$; Fig. 2c) after day 35 of operation. This increase in the activity due to biomass growth and consequently oxygen uptake rate is denoted by a reduction of the dissolved oxygen (from ca. 8–6.5 mg L^{-1}; Fig. 3c). The pH of the effluent also varied from ca. 7–7.5 for a first initial period of operation (15–40 days) to decrease until values of 5.5–5 (Fig. 3b). The high pH of the effluent could be responsible of the ammonium released from fungal endogenous metabolism as the ammonium concentration increased in the outlet stream of RBC units as compared to the inlet concentrations (Fig. 2b).

Finally, the mediators of the advanced bio-oxidation process were also monitored. The quinone-like compound as gallic acid (ca. 850 mgd^{-1}) was reduced to ca. 20 mgd^{-1} (Fig. 3d), which corresponds to a concentration of gallic acid lower than 2 mgL^{-1} and a consumption of $98 \pm 2\%$. Likewise, the iron oxalate and manganese nitrate loadings (1.45 gd^{-1} and 0.20 gd^{-1}, respectively) corresponding to iron and

Fig. 3. Evolution of (a) temperature, (b) pH, (c) DO and (d) Gallic acid in the wooden-modified RBCs: *R1* (•,○) and *R2*(▲,△). Full symbols represent data of the inlet stream and empty symbols represent of outlet stream in both reactors.

manganese concentrations of 16.7 mgL^{-1} and 5.5 mg L^{-1}, respectively, evidenced different results. The iron concentration in the effluent decreased to less than 1 mg L^{-1}, whereas the manganese concentration hardly varied between the inlet and outlet streams. Interestingly, the iron content of the biofilms developed in the RBC units was of ca. 12 mg $_{Fe}$/g biomass. White rot fungi and other wood-decaying fungi are able to retain heavy metals by different mechanisms (Tyler, 1982; Bayramoğlu et al., 2003). The precipitation of phosphorous as iron-hydroxy-phosphate, can be also responsible of the iron removal. The addition of Fe^{3+} after aerobic–anoxic activated sludge process has been proposed in literature as method for phosphorus removal from domestic wastewater (Fulazzaky et al., 2014), using a Fe/P ratio and a pH range similar than that achieved in this work. Nevertheless, it should be mentioned that the iron and manganese concentrations in the influent (ca. 17 and 5.5 mg L^{-1}, respectively) are below their toxic limits in fungi (Baldrian, 2003). Thus, the potential negative effects of both metals are discarded.

3.2. Pharmaceuticals removal efficiency

Up to 19 out of the 23 pharmaceutical micropollutants studied in this work were detected in the hospital wastewater: 4 antibiotics (amoxicillin, AMX; azithromycin, AZM; metronidazole, MDZ; and sulfamethoxazole, SMX), 3 psychiatric drugs (carbamazepine, CPZ; sulpiride, SPD; and caffeine, CFN), 2 β-blockers (atenolol, ATN; and metoprolol, MTP), 2 nonsteroidal anti-inflammatory drugs (diclofenac, DCF; and ibuprofen, IBP), 1 analgesic (4-Acetamidoantipyrine, 4-AAA), 1 cytotoxic (cyclophosphamide, CPD), 1 contrast agent (iohexol, IHX), 1 lipid regulator (gemfibrozil, GFZ), 1 chemical diuretic (hydrochlorothiazide, HCT), 1 steroid hormone (progesterone, PGT), 1 H$_2$ histamine receptor antagonist (ranitidine, RNT) and 1 endocrine disruptor (bisphenol A, BPA). The major contribution to the total concentration of micropollutants in the RBC units (16,320 and 16,511 µg L^{-1} for *R1* and *R2*, respectively) corresponds to cyclophosphamide (10,539 and 10,582 µg L^{-1}) and atenolol (5038 and 5193 µg L^{-1}). In less concentration were detected azithromycin (405 and 339 µg L^{-1}), sulpiride (338 and 397 µg L^{-1}) and iohexol (159 and 121 µg L^{-1}). The rest of the pharmaceutical micropollutants appeared in much lower concentrations from 55 to 0.030 µgL^{-1}.

Fig. 4 shows the removal, including the maximum and minimum values, of each pharmaceutical compound during the 75 days of continuous operation in both RBC units (*R1* and *R2*). These results correspond to the average removal considering seven samples collected at different operation times during the steady-state period (days 58, 61,
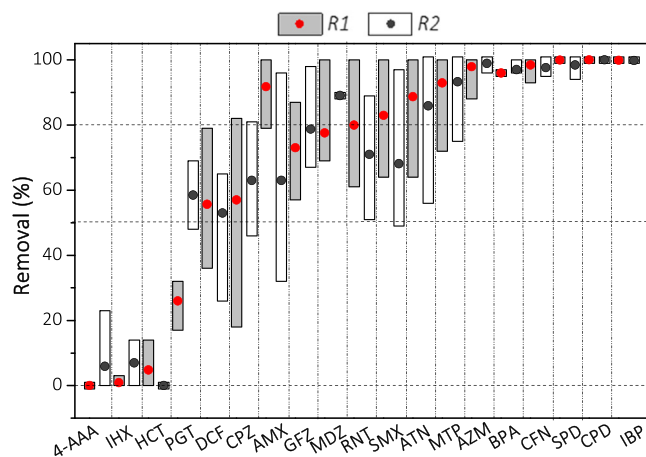
63, 66, 68, 72 and 74) of both RBCs units. The average concentration and standard deviation of inlet and outlet streams are summarized in Table S6. In general, both RBC units showed similar removal of PhACs. Only the efficiency of amoxicillin and progesterone were statistically different (92% and 26% in *R1* and 63% and 58% in *R2*, respectively). Removals higher than 80% were observed for 8 out of the 19 micropollutants and only three of them evidenced values below 40%. The most abundant pharmaceutical compounds detected in the hospital wastewater (cyclophosphamide, atenolol, azithromycin and sulpiride) were removed in a high extension (>99.9%, from 85% to 87%, >97.8% and >98.5%, respectively). On the other hand, 4-acetamidoantipyrine, iohexol and hydrochlorothiazide were the most refractory compounds. Low removal of 4-acetamidoantipyrine and hydrochlorothiazide has been also reported in the treatment of urban wastewaters for non-modified RBCs using *Trametes Versicolor* (Cruz del Álamo et al., 2020a). This behaviour is attributed to their hydrophilic nature (log Kow −0.13 and −0.07, respectively) which make them highly refractory to the biological treatment. In the case of the iohexol, the presence of strong electron acceptor groups of amide and iodine hinders their biological oxidation (Cruz del Álamo et al., 2020a; Haiß and Kümmerer, 2006).

The results of the PhACs′ removal in the wooden-modified RBCs were similar or even better than those reported in the literature for the treatment of a real hospital wastewater in fluidized bioreactors under non-sterile conditions using *Trametes versicolor* (Mir-Tutusaus et al., 2017). The anticancer drug, cyclophosphamide, was highly removed in contrast to the previous works (Ferrando-Climent et al., 2015). Higher removals have been also achieved for β-blocker (atenolol), antibiotics (azithromycin, sulfamethoxazole, and metronidazole), H$_2$ histamine receptor antagonist (ranitidine), and psychiatric drug (carbamazepine). Similar removal degrees were observed for others such as a chemical diuretic (hydrochlorothiazide) and an anti-inflammatory drug (ibuprofen) (Mir-Tutusaus et al., 2017). It must be also noted that the fluidized bioreactors used in that works were operated with a higher HRT (3 days), addition of biodegradable substrates to achieve an optimal C:N ratio for fungi (7.5) and refreshment of 1/3 of the fungal culture every 7 days. Therefore, the immobilization of the fungi in wooden-modified RBCs seems to improve the PhACs removal degrees of hospital wastewaters without external additions of biodegradable substrates and absence of aeration at a lower HRT (1 day).

3.3. Characterization of microbial communities

Fig. 5 displays the amount of copies of specific white-rot fungi (*Trametes versicolor*) as well as total fungal and bacterial communities determined by qPCR assays. These samples were taken of the biofilm generated during the operation of the RBCs after: i) the acclimation period of the fungal reactors to the hospital wastewater and ii) 21, 46 and 75 days of the continuous treatment of the HWW. Interestingly, the biofilm after the acclimation period (0 days) is majorly colonized by other fungi, different to the initial inoculated *T. versicolor*, as well as bacterial communities. These microorganisms are associated to common wood-inhabiting microorganisms (Clausen, 1996), which can appear due to the wood planks that cover the RBCs providing an ideal lignocellulosic substrate for the growth of these decomposer microorganisms. Initially, wood-decay fungi, typical decomposer of lignocellulosic substrate, would have colonized the wooden-RBCs due to their highly competitive behaviour (Boddy, 2000). Additionally, the indigenous bacteria of non-sterilized hospital wastewater would have also developed specific communities that coexist under the conditions of the HWW treatment and the competitive interactions between fungal and bacterial species may have a strong and deterministic impact on the community composition during the RBCs operation (Kielak et al., 2016). Thus, the wood-decay fungi and bacteria displaced *Trametes versicolor* during the acclimation period, becoming predominant communities in the biofilm during the continuous treatment of the HWW in both RBC units. Note
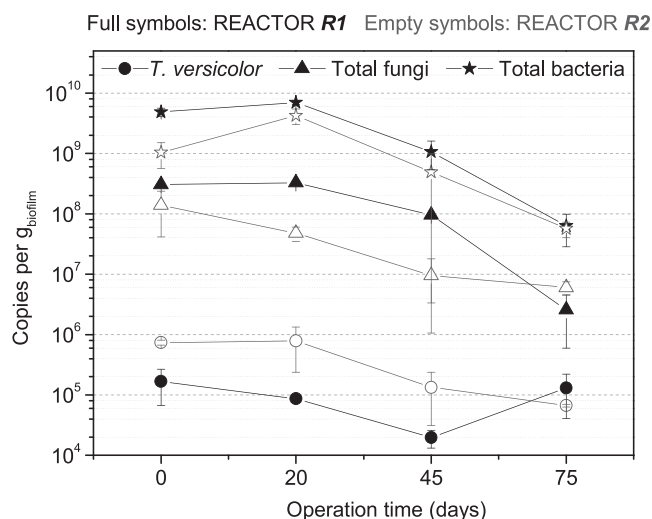

Fig. 4. Removal of PhACs by the wooden-modified *R1* and *R2* RBC units. Average values in dots and range (minimum and maximum values during operation) in bars.

Fig. 5. Total bacteria, fungi and specific *Trametes versicolor* white-rot fungi quantified by qPCR analyses for samples of biofilm of RBCs during the treatment of the hospital wastewater.

that the amount of copies quantified for *Trametes versicolor* and total fungal and bacterial communities are slightly different in the RBC units after the acclimation period, but they followed analogous trends, reaching similar distribution of communities after 75 days of operation. The decrease of total fungal and bacterial communities from 20 to 75 days is probably due to the lower availability of $P-PO_4^{3-}$ and $N-NH_4^+$ nutrients, as the $P-PO_4^{3-}$ was almost completely disappeared (Fig. 2b) and the initial $N-NH_4^+$ release ratios decreased (Fig. 2c). Another important factor is the decrease of pH in the outlet effluents of RBCs (Fig. 3b), which can reduce the development of bacterial communities.

3.3.1. Evaluation of bacterial communities

Fig. 6a shows a general distribution of the taxonomic affiliations of the bacterial communities (by phylum) identified in the RBC units during the continuous treatment of HWW. Fig. 6b and c summarizes the most abundant bacterial communities in terms of phylum/class/order at the initial and final periods of the treatment of HWW. After the acclimation period (0 days), *Proteobacteria* was the dominant phylum in *R1* and *R2* bioreactors (68.1 ± 14.8%) with a remarkable presence of *Sphingomonadales* belonging to *Alpha-proteobacteria* class (50.5 ± 14.5%). *Alpha-proteobacteria (Sphingomonadales* or *Rhodospirillales)* are related to lignin degradation in wood decay (Bugg et al., 2011; Kielak et al., 2016). *Actinobacteria* (14.5 ± 12.1%) and *Bacteroidetes* (13.6 ± 2.1%) were also identified in both RBC units with a significant relative abundance. All these bacterial communities are typical wood-inhabiting microorganisms. In contrast, after 75 days, *Sphingomonadales* decreased up to (6.5 ± 0.7%), and other classes such as *Beta-proteobacteria* (*Burkholderiales*, 14.3 ± 0.8%), *Firmicutes* (*Clostridiales*, 20.2 ± 0.4%), *Acidobacteria* (6.3 ± 2.3%) and *Actinobacteria* (4.6 ± 1.0%) prevailed in both *R1* and *R2* bioreactors. *Beta-proteobacteria* (*Burkholderiales*) and *Acidobacteria (Rizhobiales)* are associated with fungi in the removal of toxic wood compounds (Kielak et al., 2016; Vasiliadou et al., 2019). In the case of *Firmicutes* such as *Clostridiales*, they use to produce the cellular lysis of fungi and are usually considered a predator of fungal communities (Vasiliadou et al., 2019). According to previous work published by Mir-Tutusaus et al. (2017), a similar phylum distribution (*Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes* and *Proteobacteria*) were reported for the continuous fungal treatment of non-sterile real hospital wastewater pretreated by coagulation-flocculation in absence of any wooden support for fungi. These results reveal that the bacterial distribution observed in this work seems to be also a consequence of the HWW wastewater nature.

3.3.2. Evaluation of fungal communities

The fungal taxonomic affiliations identified in the biofilm developed at 0, 21, 46 and 75 days of operation of RBCs in terms of the phylum/class and the phylum/gender are shown in Fig. 7a and b, respectively. It must be noted that there was a significant proportion of unclassified reads (relative frequency of 19.8 ± 3.3% (t = 0) and 34 ± 2.8% (t = 75 days) in reactor *R1* and 61.5 ± 2.1% (t = 0) and 28 ± 1.4% (t = 75 days) in reactor *R2*. The Illumina-based DNA metabarcoding does not allow the simultaneous identification of fungal taxa in mixed samples. Likewise, the incomplete state of reference sequences existing in database for many fungal taxa (UNITE in this case) hinders the ITS classification (Heeger et al., 2019). In general, the identified fungal community is characteristic of wood-inhabiting fungi (Yuan et al., 2017). It was observed a remarkable presence of *Ascomycetes* (mainly *Sordariomycetes* class, Fig. 7a; genders *Coniochaeta, Fusarium, Exophiala and Apiotrichum*, Fig. 7b) and *Basydiomycetes* in less extension (mainly *Tremelomycetes* class, Fig. 7a; gender *Rhodotorula*, Fig. 7b). *Coniochaeta* is a well-known wood-degrading soft rot fungi capable of the biomineralization of wood's main structural components (cellulose, lignin, and hemicellulose), especially in wet environments (Shary et al., 2007). During the treatment of the HWW in both RBC units, *Coniochaeta, Exophiala* and Apiotrichum, as *Ascomycetes,* and *Trametes versicolor* as *Basydiomycota,* were the most relevant fungi identified in the developed biofilm of RBCs. Although *Trametes versicolor* is significantly displaced by *Ascomycetes*, the latter are also able to degrade biorecalcitrant compounds (Leonhardt et al., 2018; Ravindran et al., 2012). The ability to drive quinone redox-cycles for the production of hydroxyl radicals that can attack biorecalcitrant organic pollutants has been reported not only for *Trametes versicolor*, but also for other *Basidiomycota* or *Ascomycota* fungi (Krueger et al., 2016). In this sense, the addition of the advanced bio-oxidation promoters (gallic acid and iron and manganese cations) seems to also enhance the activity of the wood-decaying fungi found in the RBCs, promoting the removal of PhACs even in conditions of non-predominance of *Trametes versicolor*.

3.3.3. Activity of fungal/bacterial communities in the performance of RBCs

Fig. 8 depicts the consumption of sodium acetate (in terms of TOC) as readily biodegradable substrate over inoculums of biomass collected from the RBC after 75 days of operation in presence of bactericides (fungal activity) or fungicides (bacterial activity).

As it is shown in Fig. 8, the inoculums from *R1* and *R2* bioreactors achieved in presence of the fungicide a TOC removal of 14 ± 4 and 24 ± 7%, respectively. However, the activity of fungi in bioassays performed in presence of bactericides was much higher obtaining TOC removals of 70 ± 5 and 90 ± 2%, respectively. These results reveal a higher activity of fungi in both inoculums regardless the development of bacterial communities during the treatment of the non-sterile hospital wastewater. These results are in agreement with the results obtained in a previous work where the prevalence of the fungal activity was also demonstrated during the treatment of different urban wastewaters (Cruz del Álamo et al., 2020a).

4. Conclusions

The immobilization of fungal biomass over rotating biological contactors modified with wooden plank convers has showed a stable performance for the treatment of a real hospital wastewater. Removals higher than 80% were observed for 8 out of the 19 pharmaceutical micropollutants and only three of them evidenced values below 40%. Additionally, reductions higher than 78% and 73% were accomplished for TOC and $P-PO_4^{3-}$, respectively. A limited removal of the nitrogen sources is attributed to nitrogen-conserving strategies of the microorganisms attached to the RBCs. The qPCR and metagenomics analyses of the biofilm formed during the treatment of the HWW evidenced the development of a fungal/bacterial mixed culture. The initial inoculated *Trametes versicolor* as white-rot fungi moves to fungal communities
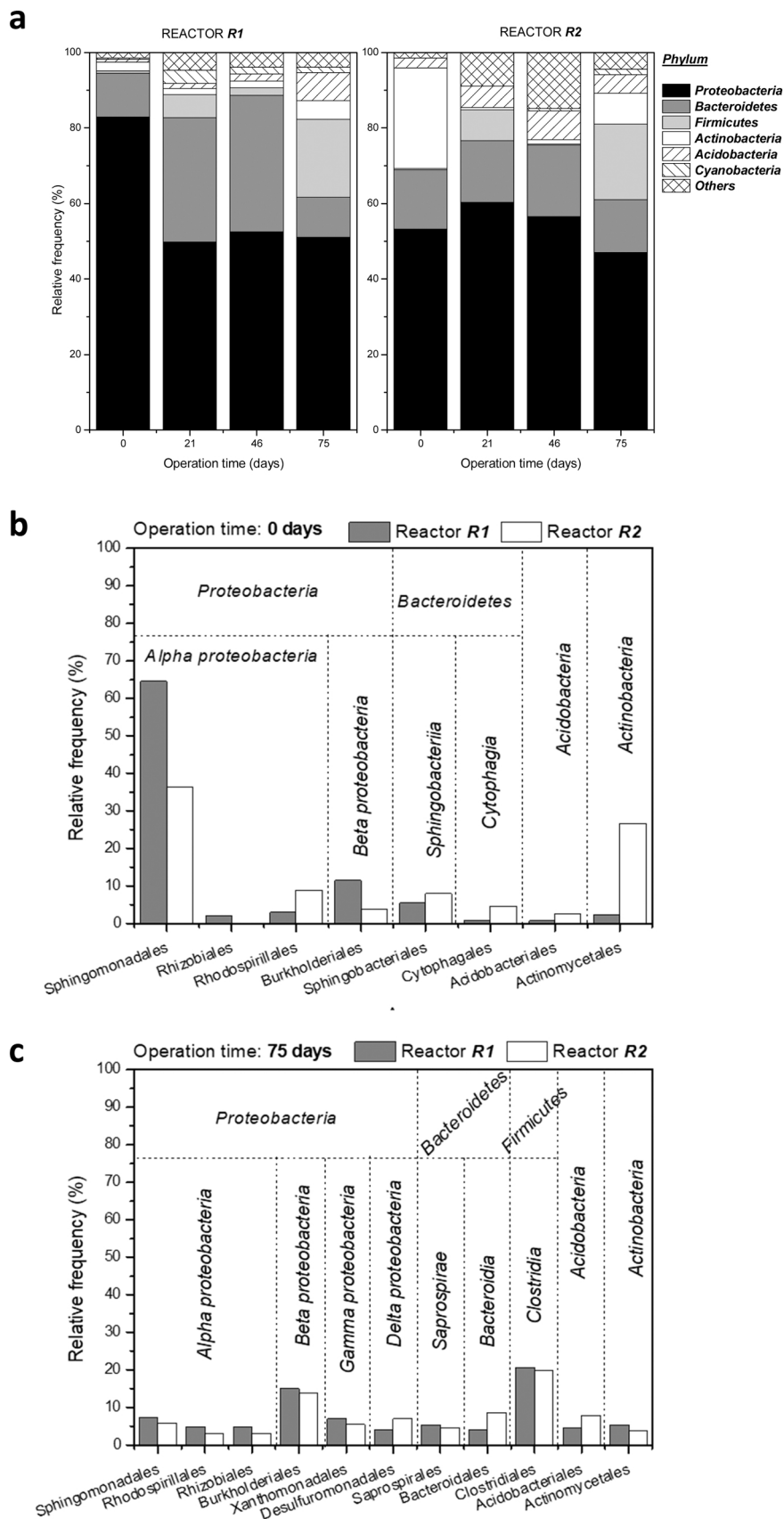
Fig. 6. Taxa at the bacterial phyla distribution at different operation times of the RBCs (a); Taxa at the bacterial order distribution after acclimation (b) and 75 days of continuous treatment of HWW (c).
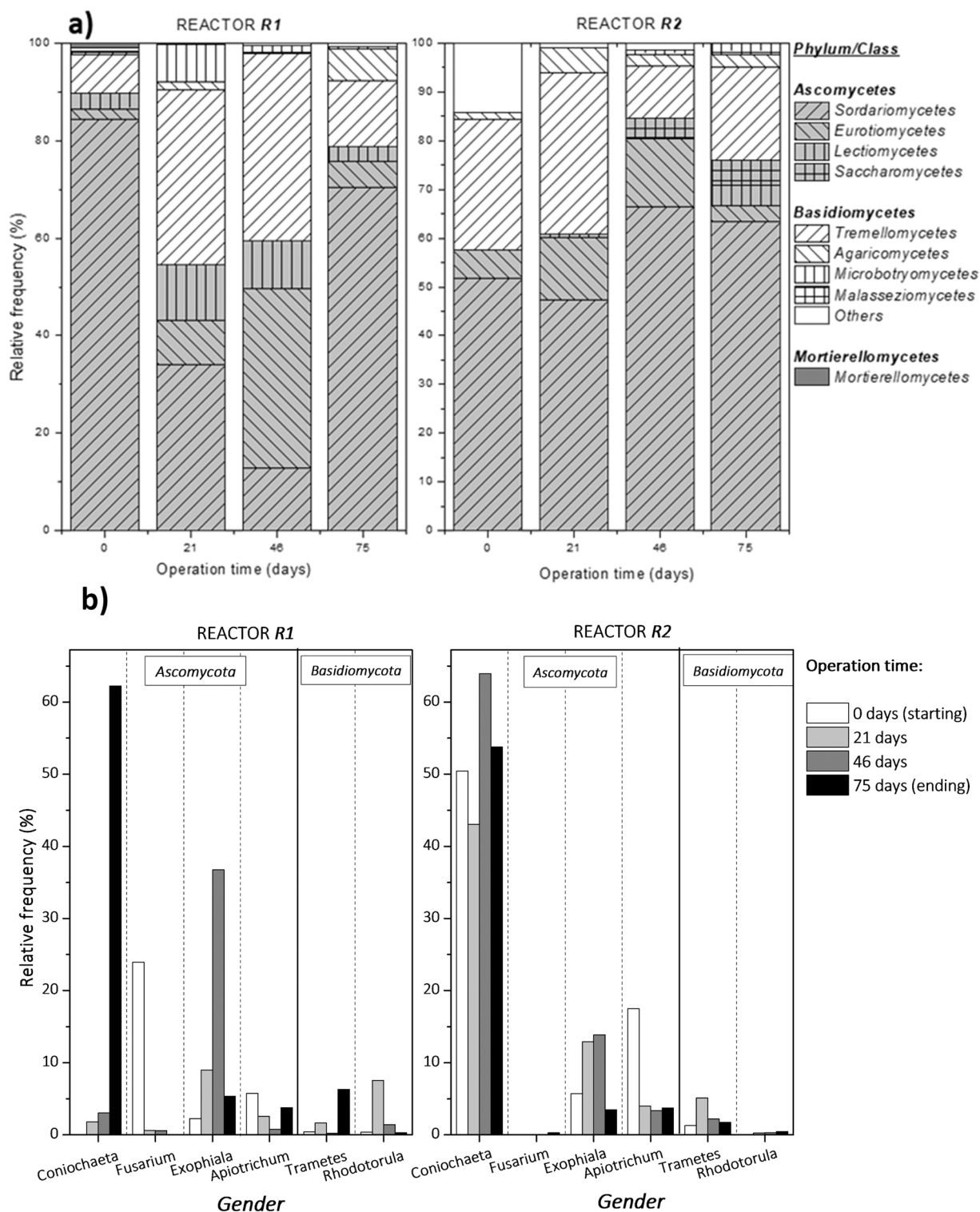
Fig. 7. Fungal communities at different operation times of the RBCs a) taxa at the phylum/class level and b) phylum/gender level.

typically found of the decaying of natural wood. Likewise, the non-sterile conditions of the hospital wastewater promoted the cohabitation of fungal and bacterial communities which are characteristic of the indigenous microorganisms of hospital wastewaters. *Coniochaeta, Exophiala* and Apiotrichum as *Ascomycetes* and *Trametes versicolor* as *Basydiomycota* were the most relevant fungi identified in the biofilm of RBCs during the continuous treatment of the HWW. It was observed a higher contribution of fungal communities to the overall activity of the mixed culture. The ability of fungi to drive quinone redox cycles associated

with the production of hydroxyl radicals enhances the degradation of complex organic molecules as PhACs from the HWW. The steady-state performance of two independent RBC units operating with a HRT of 1-day for 75 days evidenced the potential of this biological technology as robust *on-site* pre-treatment for hospital wastewaters, overcoming typical drawbacks of fungal biological treatments such as the partial biomass renovation or the addition of supplementary biodegradable carbon and nitrogen sources to promote the fungi predominance, and the external air supply or control of temperature.
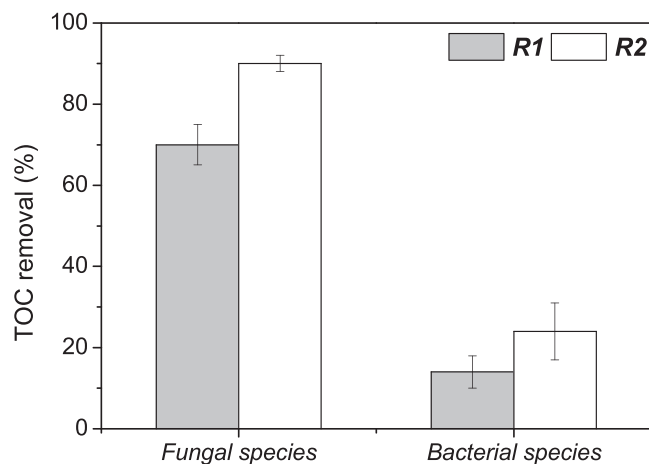
Fig. 8. Fungal and bacterial activity of biofilm inoculums of wooden-RBCs after treatment of HWW for 75 days of continuous operation. Activity test conditions: Inoculum: 200 $mg_{SSV-dry}L^{-1}$; Sodium acetate: 250 $mg_{TOC}L^{-1}$; 4 days of incubations; pH 7; bactericides: 4 mg L^{-1} of ampicillin and 128 mg/L tetracycline (fungal activity test); fungicide: 200 mg L^{-1} nystatin; (bacterial activity test).

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.

CRediT authorship contribution statement

Ana Cruz del Álamo: Investigation, Writing – original draft, Formal analysis, Data curation, Writing – review & editing. **María Isabel Pariente**: Formal analysis, Data curation, Supervision, Validation, Writing – review & editing. **Raúl Molina**: Conceptualization, Writing – original draft, Supervision, Validation, Writing – review & editing. **Fernando Martínez**: Conceptualization, Supervision, Validation, Writing – review & editing, Funding acquisition.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.128002.

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