Biodegradation Potential and Diversity of Diclofenac-degrading Microbiota in an Immobilized Cell Biofilter

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Abstract: Despite that diclofenac has been embodied to the European watch list of priority substances of concern, studies on diclofenac biodegradation are limited and the diversity of diclofenac-degrading microbiota remains unknown. In this work, an immobilized cell biofilter was constructed and operated to evaluate its effectiveness to depure high strength diclofenac wastewater and to identify the diclofenac-degrading community accommodated in activated sludge by employing high-throughput sequencing techniques. After a two-month adaptation period, biofilter removal efficiencies reached values as high as 97.63 ± 0.62%, whereas utilization of diclofenac in the immobilized cell biofilter led to a drastic pH decrease. Based on Illumina sequencing, the major bacterial taxa identified in the immobilized cell biofilter were members of the species *Granulicella pectinivorans* and *Rhodanobacter terrae*, followed by members of the species *Castellaniella denitrificans*, *Parvibaculum lavamentivorans*, *Bordetella petrii*, *Bryocella elongata* and *Rhodopseudomonas palustris*. The ability of such taxa to utilize a wide range of carbon sources and to effectively adapt under acidic conditions seemed to be the main parameters, which favored their prevalence in the immobilized cell biofilter. In addition, *Wickerhamiella* was the predominant fungal taxon in the immobilized cell biofilter, which appears to be actively involved in diclofenac degradation in activated sludge systems.

Keywords: priority substances; pH acidification; wastewater treatment; *Granulicella*; *Rhodanobacter*; *Wickerhamiella*

1. Introduction

The detection of non-steroidal anti-inflammatory drugs (NSAIDs) in aquatic environments is a result of various sources of pollution, exhibiting a negative impact on health and the environment. In particular, pharmaceuticals are disposed of households, veterinary clinics, hospitals and pharmaceutical manufacturing facilities and enter wastewater treatment plants (WWTPs) for processing prior to their final environmental discharge in natural water resources. However, WWTPs are designed mainly to remove conventional pollutants contained in municipal and industrial wastewater and fail to effectively remove NSAIDs [1,2], a fact that has led to the detection of numerous pharmaceuticals in the effluents of WWTPs as well as in surface water and groundwater systems [3,4].

Diclofenac [2-(2,6-dichloranilino) phenylacetic acid] is one of the best-selling NSAIDs worldwide [5]. It is a common environmental contaminant regularly detected in drinking water, river

freshwater and groundwater [6–10]. This along with its documented effects on endocrine systems of humans and on animal reproduction has led the European Commission to include diclofenac in the first watch list of priority substances of concern [11,12]. Toxic effects of diclofenac on bacteria, algae, microcrustaceans and fish have been also reported at concentrations similar to those measured in aquatic habitats [13]. The chronic use of this emerging contaminant can further intensify the negative consequences to ecosystems [14]. Diclofenac toxicity is not limited to aquatic organisms, but also to wildlife via the consumption of contaminated freshwater, like in the case of the oriental white-backed vultures, which suffered a 95% depletion in their population, due to the widespread presence of this pollutant to the surface water [15].

Diclofenac, as other pharmaceuticals, is not effectively removed by conventional WWTPs, a consequence of its large quantities consumed and its low biodegradation potential [4,16,17]. Diclofenac concentration usually ranges between 0.01 and 510 μg/L in the influent of municipal WWTPs [18]. However, experiments with 30 to 50 mg/L have been performed to simulate diclofenac removal from hospital wastewater [19,20]. Lonappan et al. [17] reported that diclofenac removal efficiencies ranged from 30% to 70% in activated sludge systems. In a study performed in 15 Portuguese WWTPs, diclofenac removal efficiencies did not exceed 46% [21]. Similarly, Behera et al. [22] reported diclofenac average removal efficiency of 68% in six WWTPs. Even in the use of non-conventional wastewater treatment configurations, like membrane bioreactors (MBR), residual diclofenac concentrations greater than 50% have been determined [23]. Gurung et al. [24] also reported diclofenac removal efficiency of 38% in an MBR system operating under low temperatures in the Nordic region.

Despite the importance of degrading NSAIDs, scientific findings on diclofenac biodegradation are limited. Such reports include the isolation of a *Brevibacterium* sp. (strain D4) from activated sludge, which was proved partially capable of utilizing diclofenac (by 35%) as the sole carbon source [25]. A *Labrys portucalensis* strain F11 isolated from polluted sediment was capable of completely degrading diclofenac co-metabolically in the presence of acetate [26]. Moreover, the biodegradation potential of diclofenac was investigated through the cultivation of the white-rot fungus *Phanerochaete chrysosporium* in batch bioreactors, reporting complete removal of this pharmaceutical within a day [27].

In this study, an immobilized cell biofilter was installed and operated for an extended time period under diclofenac selection pressure to favor the enrichment of the diclofenac-degrading microbiota that are present in the activated sludge. This set up allowed us to investigate the effectiveness of this biofilter for the depuration of high strength diclofenac wastewater and to identify the diclofenac-degrading microbial community accommodated in the activated sludge through the implementation of high-throughput sequencing techniques.

2. Materials and Methods

2.1. Diclofenac-Based Wastewater Composition

The diclofenac-based wastewater consisted of 400 mg/L diclofenac sodium salt (J&K Scientific, Beijing, China, 97% purity), containing 4 mM NH_4Cl, 0.2 mM $NH_4Fe(SO_4)_2.6H_2O$, 0.5 mM Na_2HPO_4, 5 mM KCl, 2 mM $MgSO_4·7H_2O$, 2 mM $CaCl_2·2H_2O$, 3 μM $NiCl_2·6H_2O$, 1 μM Na_2SeO_3, 3 μM $CoCl_2·6H_2O$, 3 μM $NaMoO_4·2H_2O$, 2 μM $ZnSO_4·7H_2O$ and 1 μM H_3BO_4 [28]. No organic solvent, i.e., methanol, was used, so the pH of the wastewater was initially increased to 8 and then readjusted to 7 to increase the solubility of the pharmaceutical added.

2.2. Bioreactor Configuration

An immobilized cell biofilter of 0.5 L working volume was setup, consisting of a cylindrical bioreactor and a 0.12 L-glass tube for aeration (Figure 1). The main bioreactor was filled with Siran beads, forming a 0.15 L Siran-based column in order to immobilize microbial biomass. At the bottom of the glass tube, an appropriate air pump was connected, providing adequate oxygen to microbial cells by recirculating the liquid medium from the main bioreactor to the air diffuser-containing tank

through the operation of a 33 L/h peristaltic pump. The diclofenac-based wastewater was introduced to the biofilter by a liquid pump, so as a hydraulic retention time (HRT) of 3.5 days was achieved. During start-up, the immobilized cell biofilter was inoculated with activated sludge from a local full-scale WWTP.
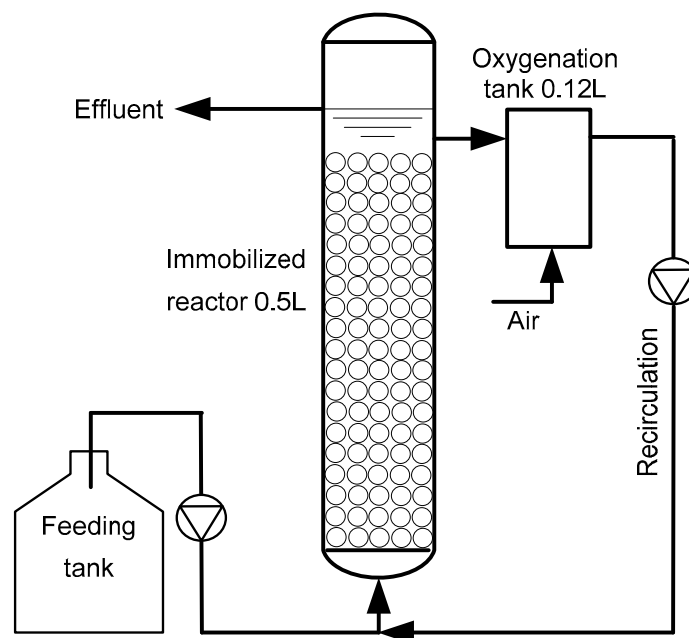
Figure 1. Schematic illustration of the immobilized cell biofilter treating diclofenac-based wastewater.

2.3. Physicochemical Analyses

Dissolved oxygen (DO) was recorded by using a WTW Oxi 320 m, whereas pH and electrical conductivity (EC) were measured by using the Metrohm 632 and Crison CM35 probe apparatus, respectively. Chemical oxygen demand (COD) and biochemical oxygen demand (BOD5) were determined according to the "Standard Methods for the Examination of Water and Wastewater" [29]. Physicochemical parameters were determined on regular basis, e.g., three times per week for pH, EC and COD. Total kjeldahl nitrogen (TKN) and ammonium nitrogen (NH_4^+-N) measurements were performed through the implementation of the Kjeldahl and the ammonium distillation protocol, respectively. To measure NO_3^--N concentration, nitrates were reduced to nitrites through filtration in a Cd-copperized column and subsequently nitrite nitrogen (NO_2^--N) was determined spectrophotometrically at 543 nm as previously described by Clesceri et al. [29]. Determination of orthophosphates as PO_4^{3-}-P was performed through the reduction of the molybdophosphoric acid, deriving from the reaction of orthophosphates with ammonium molybdate under acidic conditions, by stannous chloride solution.

2.4. HPLC Determination of Diclofenac

Prior to the analysis, samples from the influent and the effluent of the biofilter system obtained every 5 to 6 days were dissolved in methanol at concentrations of 1:100 and 1:5 v/v, respectively and passed through 0.45 μm membrane filters. A Shimadzu SCL-10AVP HPLC system (Kyoto, Japan), consisting of a Discovery HS C18 column (5 μm, 250 × 4.6 mm) (Supelco, Bellefonte, PA, USA), a LC-10 ADVP pump, a DGU-14A degassing system, a CTO-10ASVP column oven and a SPD-M10 AVP photodiode array detector was used for the chromatographic determination of diclofenac. The mobile phase consisted of a mixture of acetonitrile and 0.2% formic acid in water at mixing ratio of 60:40 v/v. The flow rate was 0.8 mL/min, the injection volume was 20 μL and the calibration standards or samples were analyzed by applying an isocratic elution program at 80 bar and 30 °C for a period of 20 min.

Photodiode array detection of diclofenac was performed at 200 nm and the limit of detection was 250 µg/L.

2.5. Amplicon Sequencing Analysis of the Diclofenac-degranding Microbiota in the Immobilized Cell Biofilter

Samples for amplicon sequencing analysis were obtained under steady state conditions. Genomic DNA was extracted in triplicates from the Siran beads containing immobilized biomass through the use of "Wizard Genomic DNA Purification Kit" (Promega, Madison, WI, USA), based on the instructions recommended by the manufacturer. In detail, the immobilized on Siran glass beads biomass were initially frozen with liquid nitrogen and pestled to dust. DNA extraction for bacterial amplicon sequencing was performed following the "Wizard Genomic DNA Purification Kit" protocol for bacteria, which includes additional steps for DNA extraction from Gram-positive bacteria, where 60 µL of 10 mg/mL lysozyme and 60 µL of 10 mg/mL lysostaphin were added to achieve cell lysis per each sample preparation. The "Wizard Genomic DNA Purification Kit" protocol for DNA extraction from yeasts, which included a lyticase treatment step by adding 7.5 µL of 75 U/µL lyticase per sample preparation, was employed. Amplification of the V1–V3 fragment of the 16S rRNA gene was performed by using the primer set 27F (5′-AGR GTT TGA TCM TGG CTC AG-3′) and 519R (5′-GTN TTA CNG CGG CKG CTG-3′), whereas amplification of the fungal ITS2 region was performed by primers ITS3F (5′-GCA TCG ATG AAG AAC GCA GC-3′) and ITS4R (5′-TCC TCC GCT TAT TGA TAT GC-3′). Bacterial amplification was carried out at 'Mr DNA' (USA) by employing Qiagen HotStarTaq Plus Master Mix Kit (Qiagen, New York, NY, USA) through a thermocycle program consisted of 3 min at 94 °C for DNA denaturation, accompanied by 28 cycles of 30 s at 94 °C, 40 s primer annealing at 53 °C and 1 min DNA extension at 72 °C, and a final 5-min elongation procedure at 72 °C. Fungal amplicons were obtained by a denaturation step at 95 °C for 2 min, 35 cycles of 30 s denaturation at 95 °C, 30 sec annealing at 55 °C and DNA extension at 72 °C for 1 min, followed by a 10 min final extension step at 72 °C. Purification of the amplified PCR products was performed by Ampure beads (USA). Illumina sequencing was carried out in a MiSeq apparatus and sequencing data was processed through demultiplexing and trimming of the amplicons [30]. Further processing excluded N(s)-containing, abnormal length size (<200 bp) or low-quality score reads ($Q < 30$) [31]. Using USEARCH v.11, the -fastq_filter was employed to improve the quality of the assembled sequences and the -fastx_uniques option was used to select the unique read sequences and their abundances [32]. Clustering of the selected read sequences into operational taxonomic units (OTUs) (number of read sequences ≥ 3) was performed through the use of the -cluster_otus command. Chimeric sequences were discarded by using the -unoise3 option of USEARCH v.11 [33]. Taxonomy was assigned to RDP (Ribosomal Database Project) via SPINGO (Species-level IdentificatioN of metaGenOmic amplicons) algorithm [34]. The 16S rRNA gene copies of the genomes of the bacterial taxa detected in the present study were estimated by using rrnDB version 5.5 [35]. AmpliTAXO within AmpliSAS was employed for clustering fungal reads [36]. The microbial community established in the immobilized cell bioreactor under steady state conditions was evaluated through three independent samplings. A total of 137,244 non-chimeric bacterial amplicons (three replicates of 45,764, 48,249 and 43,231 each) and a total of 184,850 non-chimeric fungal amplicons (three replicates of 62,247, 65,934 and 56,669 each) were sequenced and further deposited into the NCBI database under the BioProject number PRJNA530067.

2.6. Statistical Analysis

Relationships among variables were identified through calculation of Pearson's correlation coefficients using Past v.3.25 [37], whereas the Student's t-test was implemented to assess significant differences between influent and effluent physicochemical traits.

3. Results and Discussion

3.1. Operating Behavior and Effectiveness of the Immobilized Cell Biofilter to Degrade Diclofenac-Based Wastewater

The bioreactor system was fed with synthetic wastewater containing 400 mg/L of diclofenac in order to reveal the specialized members of the activated sludge, which were capable of breaking down this priority pollutant. Statistically significant differences were found between influent and effluent diclofenac concentrations ($p < 0.01$, in Student's t-test) throughout biofilter operation. A further decrease in effluent diclofenac concentration was observed from days 0–75 to 76–141 ($p < 0.01$, in Student's t-test), i.e., from 57.61 ± 6.21 to 7.87 ± 1.84 mg/L diclofenac. During start-up, an adaptation period of over 2 months was needed in order the microbial biomass to be effectively adapted to the new feeding conditions. Thereafter, diclofenac removal efficiency was highly improved, reaching up to $97.63 \pm 0.62\%$ (Figure 2), indicating the existence of an effective diclofenac-degrading population in the activated sludge.
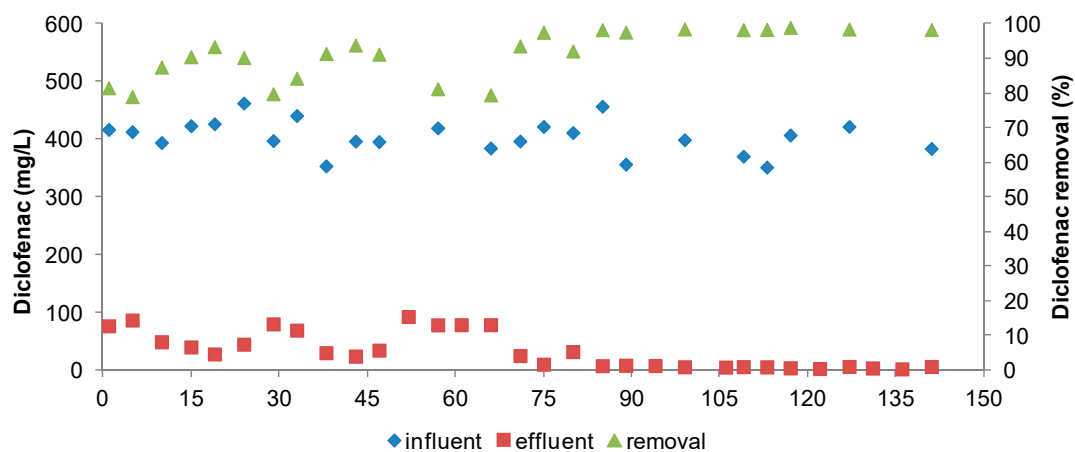
Figure 2. Diclofenac removal pattern in the immobilized cell biofilter treating diclofenac-based wastewater.

Langenhoff et al. [19] used batch reactors containing biomass adapted to high diclofenac concentrations to treat wastewater containing 50 mg/L diclofenac and reported removal efficiencies of 65–70% in a 20 day-treatment period, which are much lower than that achieved in the immobilized cell biofilter operated at HRT of 3.5 days. In contrast to batch reactors, the continuous flow mode of operation employed in our study appears to restrict the accumulation of possible toxic metabolites, permitting the progressive development of an acclimatized biomass capable of effectively degrading high diclofenac concentrations at a shorter HRT. In addition, the immobilization of biomass also resulted in elevated biosystem efficiency, due to the fact that the sludge retention time (SRT) was continuously increasing since no biomass was wasted out. According to the literature, SRT values higher than 20 days are needed in order to reach removal efficiencies higher than 50–60% [10].

Regarding COD removal patterns, the immobilized cell biofilter was adapted in shorter period, resulting in a COD decrease from to 581 ± 6 mg/L in the influent to 125 ± 5 mg/L in the effluent ($p < 0.01$, in Student's t-test), whereas the respective effluent sCOD value was equal to 80.0 ± 4.6 mg/L (Figure 3). This COD decline corresponded to COD removal efficiency of 78.5 ± 0.9 and $86.2 \pm 0.8\%$ in the case of total and soluble COD, respectively (Figure 3). The significant correlation between diclofenac concentration decrease and COD removal (correlation coefficient r = 0.551, $p < 0.01$) points out that both diclofenac and COD removal profiles followed the same trend under steady state conditions.
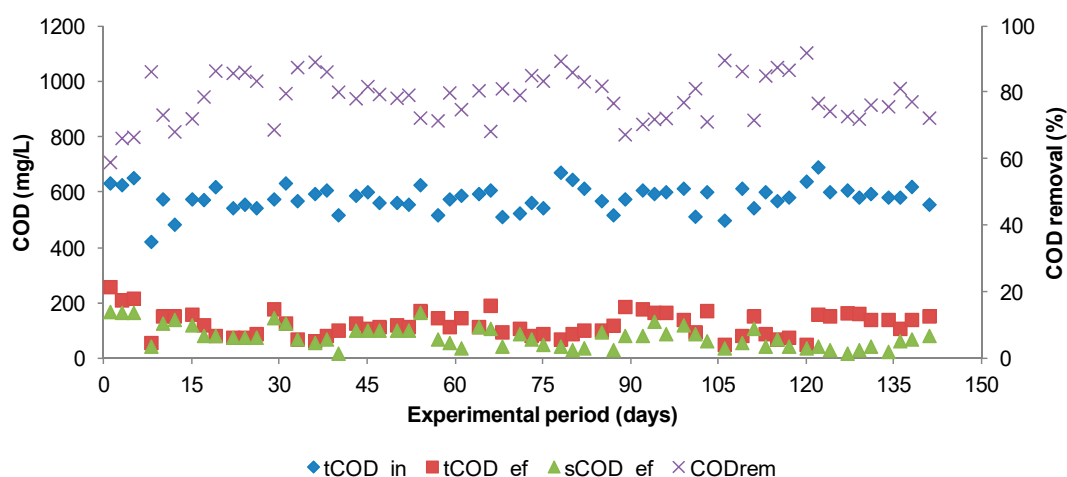
Figure 3. Chemical oxygen demand (COD) removal pattern in the immobilized cell biofilter treating diclofenac-based wastewater.

The pH in the immobilized cell biofilter was dropped drastically to acidic values ($p < 0.01$, in Student t-test) when diclofenac served as the sole carbon and energy source, resulting in a pH decrease from 7.16 ± 0.02 in the influent to 5.67 ± 0.04 in the effluent (Figure 4). Moreover, effluent pH significantly decreased from 5.82 ± 0.06 to 5.51 ± 0.05 from days 0–75 to 76–141 ($p < 0.01$, in Student's t-test). Diclofenac removal highly correlated with ΔpH (pHin-pHef) (r = 0.503, $p < 0.01$), indicating the influence of diclofenac biodegradation on pH drop.
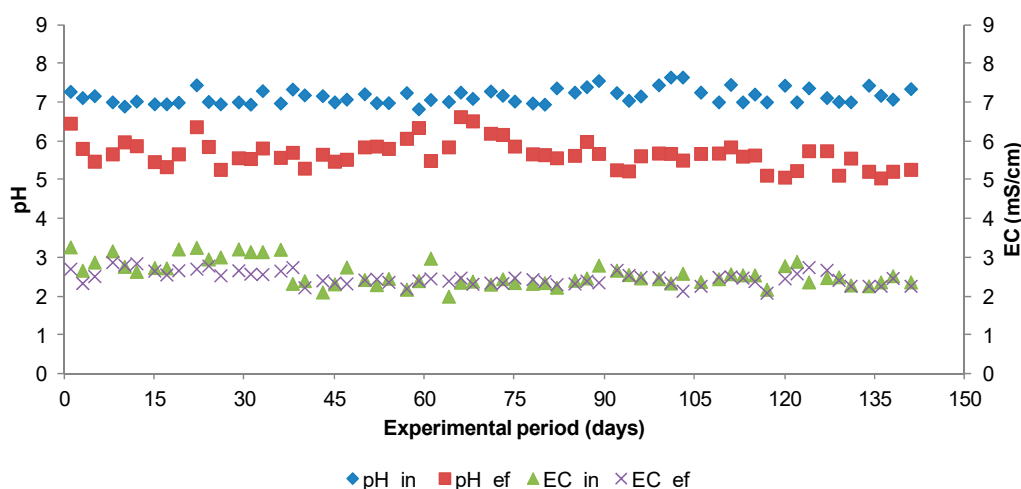
Figure 4. pH and electrical conductivity (EC) patterns in the immobilized cell biofilter treating diclofenac-based wastewater.

A similar observation was also addressed by Trapido et al. [38], who reported that diclofenac degradation during photolysis led to pH acidification, independent of the initial pH value of the diclofenac-containing wastewater. Furthermore, the electrical conductivity (EC) in the influent and the effluent of the biosystem were estimated to be 2.59 ± 0.04 and 2.47 ± 0.02 mS/cm, respectively (Figure 4), due to the fact that the growth medium was supplemented with trace elements. The marginal differences ($p < 0.05$, in Student's t-test), which were observed between influent and effluent EC values, could be attributed to the pooled values determined.

Based on the determination of nitrogenous compounds, it appears that the immobilized biomass could not oxidize ammonium to nitrate. A drastic pH drop to acidic values (below 6) has been reported

to inhibit nitrifying activity [39]. Besides, differences in metabolic capabilities of specific microbial communities, due to the selection pressure applied by the high diclofenac concentration, cannot be excluded. However, organic nitrogen appeared to be mineralized as proven by the determined TKN and NH_4^+-N values in the influent and the effluent of the biosystem (Table 1), since statistically significant differences were identified between influent and effluent TKN ($p < 0.01$, in the Student's t-test).

Table 1. Determination of other physicochemical characteristics in the influent and the effluent of the immobilized cell biofilter treating diclofenac-based wastewater.

Other Physicochemical Parameters	Mean ± SE (n = 6)
NH_4^+-Nin (mg/L)	44.80 ± 6.62
NH_4^+-Nef (mg/L)	21.00 ± 6.93
TKNin (mg/L)	61.60 ± 2.42
TKNef (mg/L)	21.56 ± 3.77
PO_4^{3-}-Pin (mg/L)	13.17 ± 1.36
PO_4^{3-}-Pef (mg/L)	7.07 ± 1.75
NO_3^--Nef (mg/L)	1.81 ± 0.85
NO_2^--Nef (mg/L)	0.21 ±0.12
BODef (mg/L)	8.00 ± 1.22
DO (mg/L)	4.32 ± 0.22

This provides evidence for almost complete mineralization of diclofenac, since the N content of this NSAID was ammonified and its COD was greatly reduced. Even though adequate aeration was provided (4.32 ± 0.22 mg/L) since Langenhoff et al. [19] showed that diclofenac degradation was sufficient only under aerobic rather than anoxic conditions, low levels of nitrate and nitrite nitrogen (1.81 ± 0.85 and 0.21 ± 0.12 mg/L, respectively) were detected as the result of the restricted nitrification process. Indeed, the N content of diclofenac-based wastewater used was mainly assimilated to the microbial cells rather than nitrified (Table 1). Moreover, sufficient phosphorus concentration was provided in the immobilized cells, determining residual phosphorus concentration in the effluent of 7.07 ± 1.75 mg/L (Table 1). As the result of phosphorus assimilation in the biomass, statistically significant differences were found between the influent and effluent PO_4^{3-}-P concentration ($p < 0.05$, in Student's t-test). In addition, the BOD in the effluent of the immobilized cell bioreactor was as low as 8 mg/L (Table 1).

3.2. Bacterial Community Structure in the Immobilized Cell Biofilter Fed with Diclofenac-Based Wastewater

Illumina data revealed that *Acidobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Betaproteobacteria* were the predominant higher bacterial taxa (95.23 ± 1.24% of bacterial reads in total), showing almost equal abundances, i.e., 26.49 ± 0.45%, 25.39 ± 0.40%, 22.10 ± 1.29% and 21.25 ± 1.19% of the total reads, respectively. *Actinobacteria* also accounted for the 3.05 ± 0.45% of the total reads. In addition, amplicon sequencing analysis at genus level showed that the microbial community in the immobilized cell bioreactor fed with diclofenac as the sole carbon and energy source was dominated by bacteria belonging to the genera *Granulicella* and *Rhodanobacter*, which accounted for 20.75 ± 0.56% and 20.56 ± 1.59% of the total reads, respectively (Figure 5a). In particular, the predominant taxa were associated with the species *Granulicella pectinivorans* and *Rhodanobacter terrae* (Figure 5b). Taken into consideration the 16S rRNA gene copies of the genomes of *Granulicella* and *Rhodanobacter*, the *G. pectinivorans* population was double that of *R. terrae* (Figure 5). Little is known for *Granulicella* spp., although, as all members of the family *Acidobacteriaceae*, they are slow growers that are favored by acidic conditions [40] and are capable of utilizing a wide range of carbon sources, even if they are originated from oligotrophic habitants [41]. *Rhodanobacter* is a common habitant of activated sludge systems [42] and has been reported to be an effective degrader of recalcitrant organic compounds, including volatile organics (VOCs) [43] and other NSAIDs like ibuprofen [44]. Moreover,

Rhodanobacter is an acid-tolerant denitrifier, which proliferates under acidic conditions [45]. The drop in the effluent pH can be attributed to the predominance of *Acidobacteria* and *Rhodanobacter* in the adapted to high diclofenac concentration biomass, which are common inhabitants of acidic environments, due to their ability to degrade recalcitrant compounds [46], as well as to the fact that bacteria can adjust environmental pH to their optimum growth [47]. Indeed, the ability of these taxa to utilize a wide range of carbon sources, including recalcitrant organic compounds, and to be effectively adapted under acidic conditions explain their prevalence in the immobilized cell biofilter.
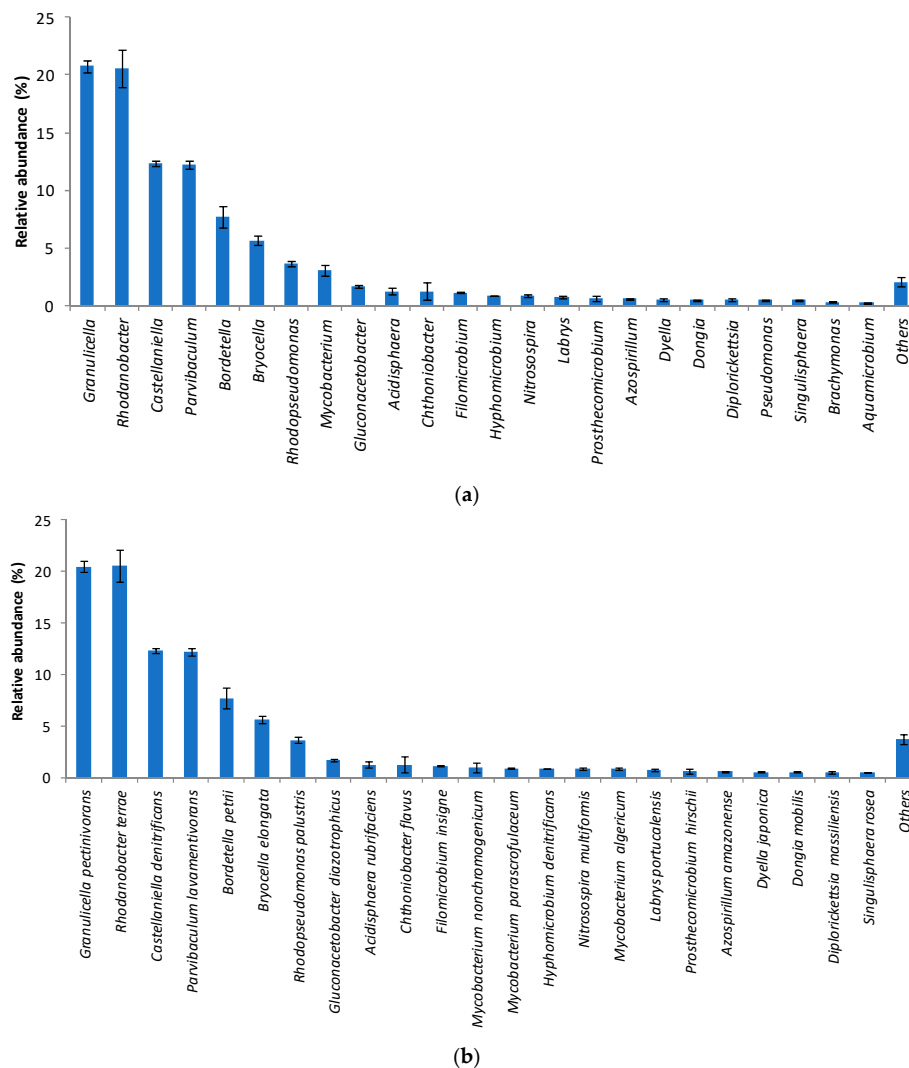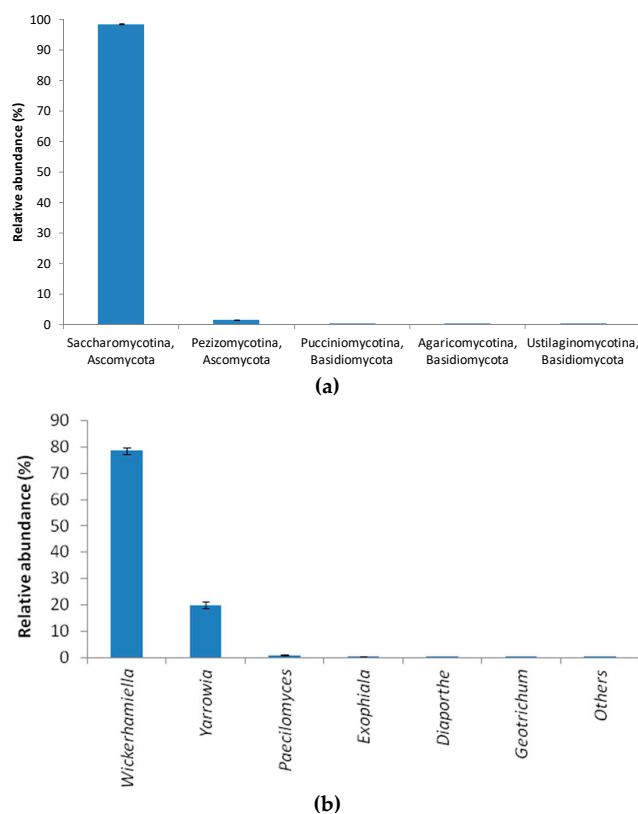

Figure 5. Relative abundances (% of total bacterial reads) at (**a**) genus and (**b**) species level of the major bacterial taxa in the immobilized cell biofilter treating diclofenac-based wastewater. Taxa with relative abundances below 0.25% are not reported. Only 0.25% of the sequences were not assigned to a taxonomically-described species. Genomes' 16S RNA gene copies: *Granulicella*, 1; *Rhodanobacter*, 2; *Castellaniella*, 2; *Parvibaculum lavamentivorans*, 1; *Bordetella petrii*, 3; *Bryocella*; not reported; *Rhodopseudomonas palustris*, 2; *Gluconacetobacter diazotrophicus*, 4; *Acidisphaera*, 1; *Chthoniobacter flavus*, 1; *Filomicrobium*, 1; *Mycobacterium*, 1; *Hyphomicrobium denitrificans*, 1; *Nitrosospira multiformis*, 1; *Labrys*, not reported; *Prosthecomicrobium*, 1; *Azospirillum*, 9; *Dyella japonica*, 2; *Dongia*, not reported; *Diplorickettsia*, not reported; *Singulisphaera*, 8.

Beyond the two most dominant bacterial taxa mentioned above, members of the genera *Castellaniella*, *Parvibaculum*, *Bordetella* and *Bryocella*, showing relative abundance (as a % of total bacterial reads) of 12.32 ± 0.20%, 12.19 ± 0.36%, 7.71 ± 0.97% and 5.67 ± 0.38%, respectively, were detected in

the bacterial community established in the immobilized cell biofilter (Figure 5a). Representatives of *Rhodopseudomonas*, *Mycobacterium*, *Gluconacetobacter*, *Acidisphaera*, *Chthoniobacter* and *Filomicrobium* also corresponded to 12.01 ± 1.25% of the total bacterial reads (Figure 5a). To the best of our knowledge, the endophyte *Microbacterium* sp. MG7 [48], a few soil bacterial strains belonging to *Raoultella-Klebsiella* group [49,50] and *Brevibacterium* sp. D4 [25] isolated from activated sludge are the only bacteria that have been found to be capable of degrading diclofenac. In addition, *Labrys portucalensis*, which was detected in the present study (Figure 5b), has been reported to be capable of completely degrading diclofenac co-metabolically in the presence of acetate [26]. However, no study regarding the diversity of the diclofenac-degrading microbiota, which utilize diclofenac as the sole carbon and energy source, has been performed until now. Moreover, the *Alcaligenaceae* representatives identified in the immobilized biomass, like *Castellaniella denitrificans* and *Bordetella petrii*, which covered 20.03 ± 1.04% of the total reads (Figure 5b), are considered as effective degraders of various recalcitrant compounds [51,52].

Apart from various white-rot fungi like *Trametes versicolor*, which are considered as effective degraders of endocrine disrupting substances [53,54] and a few other allochnonous fungi [55], no information on fungal microbiota from activated sludge that are capable of degrading diclofenac exists. At phylum/subphylum level, the fungal diversity in the immobilized cell biofilter fed with diclofenac as the sole carbon and energy source consisted almost exclusively of members of Saccharomycotina (Ascomycota), representing 98.54 ± 0.08% of the total fungal reads, followed by members of Pezizomycotina, and Ascomycota (1.45 ± 0.07%) (Figure 6a). Thus, Ascomycota accounted for 99.99% of the fungal total relative abundance, whereas the remaining 0.01% consisted of member of the phylum Basidiomycota (subdivisions Pucciniomycotina, Agaricomycotina and Ustilaginomycotina). At the genus level, *Wickerhamiella* was found to be the predominant fungal taxon in the immobilized cell biofilter with relative abundance of 78.56 ± 1.35%, followed by *Yarrowia* (19.96 ± 1.28%), *Paecilomyces* (0.94 ± 0.09%) and *Exophiala* (0.40 ± 0.05%) spp. (Figure 6b).

Figure 6. Relative abundances (% of total fungal reads) at (**a**) phylum/subphylum and (**b**) genus level of the major fungal taxa in the immobilized cell biofilter treating diclofenac-based wastewater.

Wickerhamiella that was proliferated in the immobilized cell biofilter, where diclofenac served as the sole carbon source, was recently found as the dominant fungal taxon in 18 full-scale municipal WWTPs [56]. Moreover, *Yarrowia* has been recently reported to possess the capability of partially degrading diclofenac [55], while this is the first report on the potential involvement of *Paecilomyces* and *Exophiala* spp. on diclofenac degradation.

It is concluded that regarding the efficiency of the immobilized cell biofilter to degrade diclofenac as the sole carbon and energy source, an adaptation period of over two months under prolonged HRT (3.5 days) was required to achieve removal efficiencies as high as 97.63 ± 0.62%. Afterwards, an effective diclofenac-degrading population was enriched from the activated sludge that was used as the inoculum, where both diclofenac and COD removal patterns followed the same trend under steady state conditions. Utilization of diclofenac as the sole carbon and energy source in the immobilized cell biofilter also led to a drastic pH decrease. Evidence is also provided for almost complete mineralization of diclofenac, due to the ammonification of N content of this pollutant and the high COD removal efficiency achieved. Based on amplicon sequencing, the major bacterial taxa identified in the immobilized cell biofilter fed with diclofenac as the sole carbon and energy source were members of the genera *Granulicella* and *Rhodanobacter*. The ability of members of these genera to utilize a wide range of carbon sources and to be effectively adapted under acidic conditions were the main factors favoring their prevalence in the immobilized cell biofilter. *Wickerhamiella* was found to be the predominant fungal taxon in the immobilized cell biofilter, followed by *Yarrowia*, *Paecilomyces* and *Exophiala*. *Wickerhamiella* is a common fungal taxon in WWTPs, which was also favored in the immobilized cell biofilter when diclofenac served as the sole carbon source.

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Conflicts of Interest: The authors declare no conflicts of interest.

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