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Thermophilic compost bacteria as a promising approach for removal of diclofenac and related pharmaceuticals from wastewater

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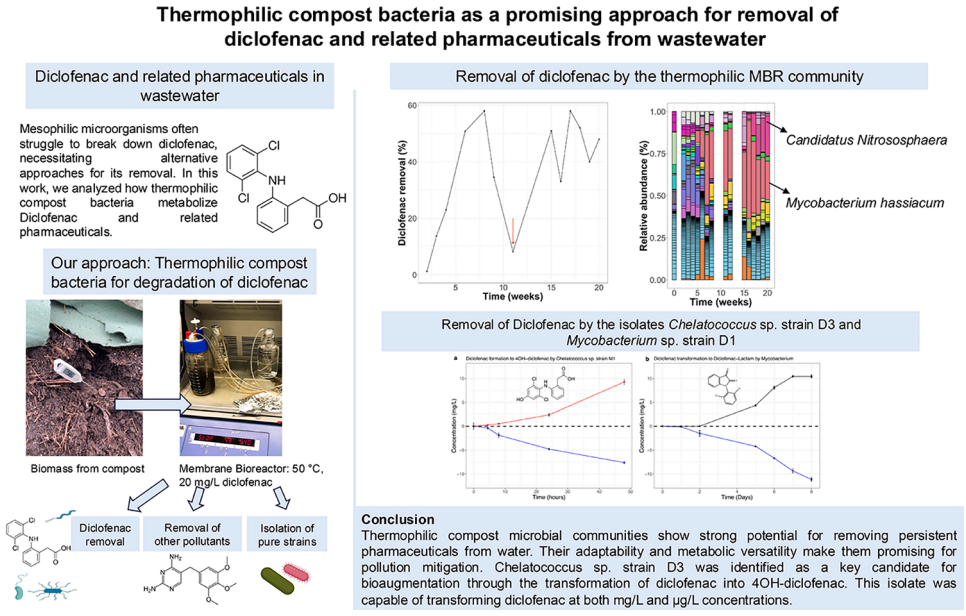
HIGHLIGHTS

- Novel Approach: Application of thermophilic compost-derived microbial consortia in a membrane bioreactor (MBR) to target diclofenac degradation.
- Performance Results: Achieved up to 60 % removal of 2mg/L diclofenac in membrane bioreactor over a 20-week adaptation period.
- Versatility: Adapted microbial community was also able to degrade further pharmaceuticals — sulfamethoxazole, paracetamol, and ciprofloxacin.
- Key Isolate: Chelatococcus sp. strain D3 was isolated and characterized; This strain is capable of transforming diclofenac into 4OH-diclofenac, which is the bottleneck reaction in removal of diclofenac. Moreover, Chelatococcus sp. strain D3 showed versatility on removing target compounds in real wastewater and other similar micropollutants. Finally, Chelatococcus sp. strain D3 was able to remove diclofenac in environmental relevant concentration in the µg/L range.
- Implication: Presents an innovative, sustainable bioremediation strategy for pharmaceuticals resistant to degradation by mesophilic bacteria.

GRAPHICAL ABSTRACT



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ABSTRACT

Diclofenac, a widely used pharmaceutical, poses a significant environmental problem due to its persistence in aquatic systems and resistance to conventional degradation processes. Mesophilic microorganisms, commonly

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employed in wastewater treatment, often struggle to break down diclofenac, necessitating alternative approaches for its removal. In this study, we investigated thermophilic compost microorganisms and their ability to degrade diclofenac. Compost communities were cultivated for 20 weeks at 50 °C in a membrane bioreactor, with a continuous supply of 2 mg/L diclofenac as the sole carbon source. After two weeks, the microbial community steadily enhanced its ability to remove diclofenac, achieving removal rates up to 60%. The consortium demonstrated flexibility in the degradation of further pollutants, namely sulfamethoxazole, paracetamol, and ciprofloxacin, with changes in their community structure depending on the substrates. In addition, thermophilic isolates *Chelatococcus* sp. strain D3 and *Mycobacterium* sp. strain D1 were characterized and demonstrated variation in the first reaction of transforming diclofenac, which is the crucial step in mineralization of this pollutant, resulting in either 4-hydroxy-diclofenac or diclofenac-lactam, respectively. Furthermore, *Chelatococcus* sp. strain D3 demonstrated the capability to catalyze the biotransformation of diclofenac into 4-hydroxydiclofenac in treated wastewater. Notably, this transformation was effectively carried out even at moderate temperature (25 °C and 37 °C), and *Chelatococcus* sp. strain D3 was additionally able to remove diclofenac under environmental relevant concentration in μg/L range. These results show that the use of thermophilic consortia can be applied for efficient bioremediation in wastewater treatment plants, specifically for compounds that mesophilic organisms degrade poorly.

1. Introduction

Water resources are in critical need of protection due to increasing contamination, overuse, and climate change, which threaten the availability and quality of this essential resource for ecosystems and human survival (Geissen et al., 2015; Taylor et al., 2013). One prominent environmental concern is the widespread use of diclofenac, a non-steroidal anti-inflammatory drug (NSAID), which poses risks due to its persistence in the environment and potential toxicity (Sathishkumar et al., 2020; van den Brandhof and Montforts, 2010; Zhang et al., 2008). Diclofenac is recognized as a priority substance both in the EU and Switzerland. In Europe it was included in the Watch List of the Water Framework Directive, with proposed environmental quality standards in the low ng/L range (Leverett et al., 2021). In Switzerland, diclofenac is explicitly regulated under the *Ordinanza sulla protezione delle acque* and is used as a lead indicator for wastewater treatment performance *(OPAc, RS 814.201)* (Swiss Federal Council, 2020). Recent monitoring data confirm that diclofenac frequently exceeds national thresholds in surface waters (Aqua and Gas, 2024), highlighting its environmental relevance. In fact, traditional wastewater treatment processes are often inadequate in completely removing diclofenac, leading to its accumulation in aquatic environments (Vieno and Sillanpää, 2014). Diclofenac concentrations found in wastewater treatment effluencts across all over the world are variable. However, in the upper, most concerning, limit, diclofenac reaches a concentration ranging μg/L in municipal wastewater, and much higher concentration in hospital and industrial wastewater (Demaria et al., 2025; Gómez et al., 2006; Kay et al., 2017).

This persistence of diclofenac is primarily due to the chemical's stability and resistance to biodegradation, allowing it to bypass conventional treatment barriers and enter natural ecosystems. Once in the environment, diclofenac can accumulate and adversely affect aquatic life (Peltzer et al., 2019; van den Brandhof and Montforts, 2010), leading to ecological imbalances and potential human health risks through bioaccumulation in the food chain (Acuña et al., 2015).

Biodegradation by microbial communities, along with activated carbon adsorption (Bhadra et al., 2016), ozonation (Aguinaco et al., 2012), and advanced oxidation processes (AOPs) (Vogna et al., 2004), is a widely used method for removing diclofenac from wastewater in treatment plants. However, while mesophilic bacteria have been reported to degrade diclofenac under certain conditions, their effectiveness is highly debated and often not consistently reproducible (Moreira et al., 2018). Diclofenac and their metabolites can still be detected in effluents from various WWTPs, thus the degradation rates in situ are generally low (Stülten et al., 2008; Zhang et al., 2008). This suggests that relying solely on mesophilic bacterial activity is inadequate for the effective biological removal of diclofenac from wastewater. The transformation of diclofenac to 4-hydroxy-diclofenac is recognized as the crucial step for biodegradation of diclofenac (Bouju et al., 2016a), and various studies showed that microbial cytochrome P450 monooxygenases can catalyze this transformation (Prior et al., 2010; Xu et al., 2015). While diclofenac has a strong recalcitrant character, 4-hydroxy-diclofenac was shown to be easier degradable in various environmental compounds, indicating that 4-hydroxy-diclofenac appears to be the starting point for the efficient breakdown of diclofenac (Bouju et al., 2016a). Besides 4-hydroxy-diclofenac, also diclofenac-lactam (1-(2,6 dichlorophenyl)-2-indoline) was identified as a main transformation product for diclofenac in wastewater (Gómez-Canela et al., 2021). Diclofenac-lactam is frequently detected as a major metabolite in wastewater, suggesting that it may be more resistant to biodegradation and, under certain conditions, result in a dead-end metabolite (Jewell et al., 2016). Nevertheless, further transformation of diclofenac-lactam by activated sludge communities has been observed, with its degradation hypothesized to proceed via the intermediate TP293b, followed by sequential oxidation, decarboxylation, and reductive dichlorination (Jewell et al., 2016). Furthermore, diclofenac-lactam was the main product of degradation when diclofenac was treated with UV/Se NPs/H2O2 nanoparticles (Ameri et al., 2020), as well as in water samples in which diclofenac was treated via acidic extraction (Reddersen and Heberer, 2003). While metabolic pathways for further degradation of 4-hydroxy-diclofenac were discussed (Jewell et al., 2016), the further conversion of diclofenac-lactam remains unknown.

New biological systems need to be investigated to efficiently remove diclofenac from the environment. Therefore, the specific aim of this study is to evaluate the ability of thermophilic compost bacteria to degrade diclofenac and identify major transformation products. By investigating these microbial systems, we seek to provide proof of concept for their potential integration in wastewater treatment processes, ultimately contributing to more effective strategies for mitigating pharmaceutical pollution and safeguarding aquatic ecosystems and human health. We hypothesize that thermophilic compost bacteria could remove diclofenac due to several compelling reasons: (i) Thermophiles are known for their ability to degrade recalcitrant substrates, since their enzyme machineries are extremely robust and could be more effective in breaking down complex pharmaceutical compounds (Peeples, 2014; Suleiman et al., 2020; Zhu et al., 2020). (ii) Compost, which naturally hosts thermophilic bacteria, consists of chemical structures comparable to diclofenac, such as aromatics (Wu et al., 2013) and lignins (Yao et al., 2023), suggesting these bacteria may already possess the enzymatic pathways needed to degrade diclofenac. (iii) Moderate heat could enhance the bioavailability of diclofenac, making it more accessible for microbial degradation (Reid et al., 2000). Thermophilic microorganisms have already been reported for the use in bioremediation and pollutant removal (Nzila, 2018; Peeples, 2014; Qiu et al., 2022; Zhang et al., 2022). We investigated the potential of a compost-derived microbial community for the removal of diclofenac in a membrane bioreactor (MBR). We further evaluated the metabolic

flexibility of this community, once adapted to diclofenac, by testing its ability to degrade other recalcitrant pollutants such as sulfamethoxazole and trimethoprim. Finally, the performance of an isolated MBR-derived strain was assessed for diclofenac degradation under environmentally relevant concentrations in the μg/L range.

2. Materials and methods

2.1. MBR performance and batch cultures

A laboratory-scale membrane bioreactor (MBR) was established with a total volume of 1 L and filled with 500 mL of medium. Brunner mineral medium (BMM) was used, consisting of the following components for 1 L: Na_2HPO_4 2.44 g, KH_2PO_4 1.52 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4$ x 7 H_2O 0.2 g, $CaCl_2$ x 2 H_2O 0.05 g, trace element solution SL-4 10 mL and vitamins solution Schlegel 2.5 mL. In addition, 2 mg/L sodium diclofenac was added as sole carbon source to the medium. After 15 weeks of operation, 0.05 g/L ammonium-acetate was added. Continuous aeration was provided using compressed air at a pressure of 0.5 bar and an oxygen concentration of 20 %. To ensure homogenization, a 3-cm stirrer operated at 400 rpm was employed. The MBRs featured a steel membrane holder equipped with two ultrafiltration membranes, each with a pore size of 0.08 μm, covering a total membrane area of 30 cm^2. The flow rate was maintained at 10 mL/h (hydraulic retention time (HRT) 50 h), allowing the MBRs to operate continuously for a period of 20 weeks at a temperature of 50 °C.

The MBR was inoculated with a 60 °C compost sample taken from a composting site in Switzerland, by adding 10 g compost to the reactor. The MBR was sampled weekly by taking 2 mL culture: the supernatant was used for HPLC analysis, the pellet was used for DNA and for subsequent sequencing the V4 region of the 16S rRNA gene.

Batch cultures were performed using MBR consortia of week 20 for inoculation (1 % v/v) in 20 mL Erlenmeyer flasks, containing of Brunner medium as described above. As carbon source, 20 mg/L sodium diclofenac, ciprofloxacin, sulfamethoxazole, paracetamol, ibuprofen, and trimethoprim were used, respectively. Cultures were incubated for 6–20 days and sampled on day 6 for further microbial community analysis.

Isolates were gained by serial dilution of the MBR consortium, followed by plating on agar plates consisting of BMM medium (mentioned above), containing 20 mg/L diclofenac, 5 mL/L glycerol and 50 mg/L acetate as carbon sources. Plates were incubated at 50 °C for three days, and grown colonies were screened in Erlenmeyer flasks containing 5 mL medium supplemented with 2 mg/L diclofenac at 50 °C, 160 rpm. Two strains, which showed the most efficient removal capacity, were further analyzed.

One isolate, *Chelatococcus* sp. strain D3, was further evaluated in the previously described BMM medium (mentioned above), which was supplemented with 20 mg/L, 750 μg/L and 2 μg/L diclofenac, respectively, and 50 mg/L acetate. The experiments with 20 mg/L were conducted at three different temperatures (25 °C, 37 °C, and 50 °C), and additional tests were performed by introducing 20 mg/L each of sulfamethoxazole and trimethoprim as substrates. Further, diclofenac removal efficiency of the isolate *Chelatococcus* sp. strain D3 was tested in real treated wastewater taken from our in-house wastewater treatment plants (FHNW). The wastewater parameters are shown in Table 1. The batch cultures were set up adding 20 mg/L of diclofenac and OD 0.1 of biomass. Samples were taken at the time of inoculation and after 48 h and measured through HPLC. When *Chelatococcus* sp. strain D3 was inoculated with 750 μg/L and 2 μg/L, respectively, acetate was added in a concentration of 50 mg/L and incubation took place at 50 °C for 48 h.

2.2. DNA extraction, sequencing, and data analysis

DNA extraction was performed using the ZymoBIOMICS DNA Miniprep Kit (ZymoResearch), following the manufacturer's protocol. The V4 region of the 16S rRNA gene was then amplified, and a DNA library was constructed using the Quick-16S™ Plus NGS Library Prep Kit (V4) from ZymoResearch. A 4 pM DNA library, spiked with 25% PhiX, was sequenced in-house using the Illumina MiSeq platform, adhering to the manufacturer's guidelines. Sequencing data were processed based on primer sequences, quality, error rates, and chimeras using the `dada2` R package (Callahan et al., 2016). The resulting sequence table was aligned with the SILVA ribosomal RNA database (Quast et al., 2012), version 138 (non-redundant dataset 99). A phyloseq object was created using the `phyloseq` R package (McMurdie and Holmes, 2013), incorporating the amplicon sequence variant (ASV) table, taxonomy table, and sample data. Prediction of metagenome functions was performed by PICRUSt2 (Douglas et al., 2020). Statistical analysis of microbial strain distribution was conducted by DESeq2 (Love et al., 2014). The phyloseq object, metadata, and detailed R analysis code are available on GitHub (https://github.com/Marcel29071989), and the raw sequencing data can be accessed at NCBI SRA under accession number SUB15191621.

Table 1
Characteristics of the treated wastewater used for inoculation with *Chelatococcus* sp. strain D3.

Parameter	Value
pH	7.637
Conductivity [μS/cm]	1027
Total P [mg/L]	8.16
Nitrate [mg/L]	0.016
Ammonium [mg/L]	0.034
Turbidity [NTU]	−0.01
COD [mg/L]	15
Total N [mg/L]	54.5
Total organic Carbon [mg/L] TOC	7.9
Inorganic Carbon Kohlenstoff [mg/L] IC	17.2
Total carbon [mg/L] TC	25.1

2.3. HPLC analysis

Diclofenac and its metabolites were separated on a ZORBAX RR StableBond C18 column using high-performance liquid chromatography (HPLC) (Agilent Technologies) by applying a flow rate of 0.7 mL/min with water and methanol as mobile phase. Diclofenac was detected using UV/VIS DAD detector. The mobile phase ratio started at 80:20 VV of, respectively, 0.1 % formic acid in Millipore water (A) and methanol (B). The B gradient was programmed to transition from 20 % to 95 % over a span of 15 min, enabling the detection diclofenac at 13.8 min at a detection wavelength of 230 nm. A standard curve was generated for diclofenac (0.1 mg/L–100 mg/L).

2.4. Mass spectrometry

Samples were thawed at room temperature, mixed and centrifuged for 5 min at 21,300 *g* (5425R, Eppendorf). For contaminant precipitation, 300 mL culture sample were mixed with 300 mL methanol and centrifuged for 5 min at 21,300 *g*.

LC-MS/MS parameters: possible metabolites were identified from scans in the *m/z* range of 100–500 and compared with published degradation products. For quantification an LC-MS/MS method was set up using pure substances as reference. The chromatographic separation was done on an InfinityLab Poroshell 120 EC—C18 column (3.0 × 100 mm, 2.7 mm) connected to an Agilent 1100 HPLC system. The column was equilibrated in solvent A (5 % methanol, 95 % H_2O with 0.1 % formic acid) at a flow-rate of 0.5 mL/min. 2 mL of sample were injected and analytes were eluted with a linear gradient from 0 % to 100 % solvent B (95 % (v/v) methanol, 5 % H_2O with 0.2 % (v/v) formic acid) over 15 min. Analysis was performed with an Agilent 6470 QQQ MS equipped with an AJS-ESI ion source in positive or negative ion mode with a capillary voltage of 3500 V (Table 2).

For quantification, standard curves of blank medium spiked with

Table 2
Parameters of the MS method for detection of diclofenac and metabolites.

	m/z Precursor ion	*m/z* Quantifier (collision energy/eV)	*m/z* Qualifier (collision energy/eV)	Ion mode
Diclofenac	296	214.1 (39)	250 (13)	positive
4′-hydoxydiclofenac	310	266.2 (12)	230.3 (8)	negative
1-(2,6-Dichlorophenyl)-2-indolinone	278	214 (33)	208 (29)	positive
2-hydroxyphenylacetic acid	151	107.1 (12)	106.4 (28)	negative
2-aminophenylacetic acid	152	134.4 (5)	77.1 (46)	positive

analyte concentrations between 2 and 2000 ng/mL were measured. Sample preparation procedures were the same as for the culture samples described above. Analyte contents in culture samples were determined using the obtained standard curves.

For relative quantification of diclofenac in the experiment where *Chelatococcus* sp. strain D3 was exposed to 2 µg/L diclofenac, an LC-MS/MS method was developed using a pure reference standard. Chromatographic separation was achieved on a Poroshell 120 EC—C8 column (2.1 × 50 mm, 2.7 µm particle size) with an Agilent 1260 Infinity II HPLC system. The mobile phase consisted of solvent A (5 mM ammonium acetate in water) and solvent B (methanol), delivered at a flow rate of 0.7 mL/min. The HPLC was coupled to an Agilent 6495D Triple Quadrupole mass spectrometer equipped with an AJS-ESI ion source operating in negative ion mode.

3. Results

3.1. Diclofenac degradation by MBR community and possible microbial key players

Diclofenac (2 mg/L) removal was observed after three weeks of cultivation in the MBR, with 13 % of the diclofenac being removed (Fig. 1a). From this point until Week 8, the removal efficiency increased steadily, reaching 58 %. However, by Week 11, the removal capacity dropped to 9 %. Starting at this time, ammonium acetate was added as a co-substrate alongside diclofenac. This addition improved the removal efficiency, which ranged between 33 % and 58 % from Week 15 to Week 20. No metabolites were detected in the effluent by mass spectrometry, except for small concentration of 4-hydroxy-diclofenac (>21 µg/L). This suggests that diclofenac was largely transformed, potentially through mineralization, though the formation of low-abundance or transient intermediate cannot be fully excluded. For each time point of diclofenac measurement, microbial community analysis was performed. The initial compost sample taken at 60 °C was primarily dominated by *Sphaerobacter thermophilus* (30.4 %), followed by unidentified members of *Microtrichales* (13.5 %) and *Gemmatimonadota* (11.4 %), along with numerous unknown genera (Fig. 1B). After six weeks of incubation, a stable microbial community formed within the MBR, predominantly consisting of an unidentified genus from the *Alcaligenaceae* family, *Mycobacterium hassiacum*, and *Brevibacillus*. Subsequently, the relative abundance of *Mycobacterium hassiacum* increased significantly, reaching

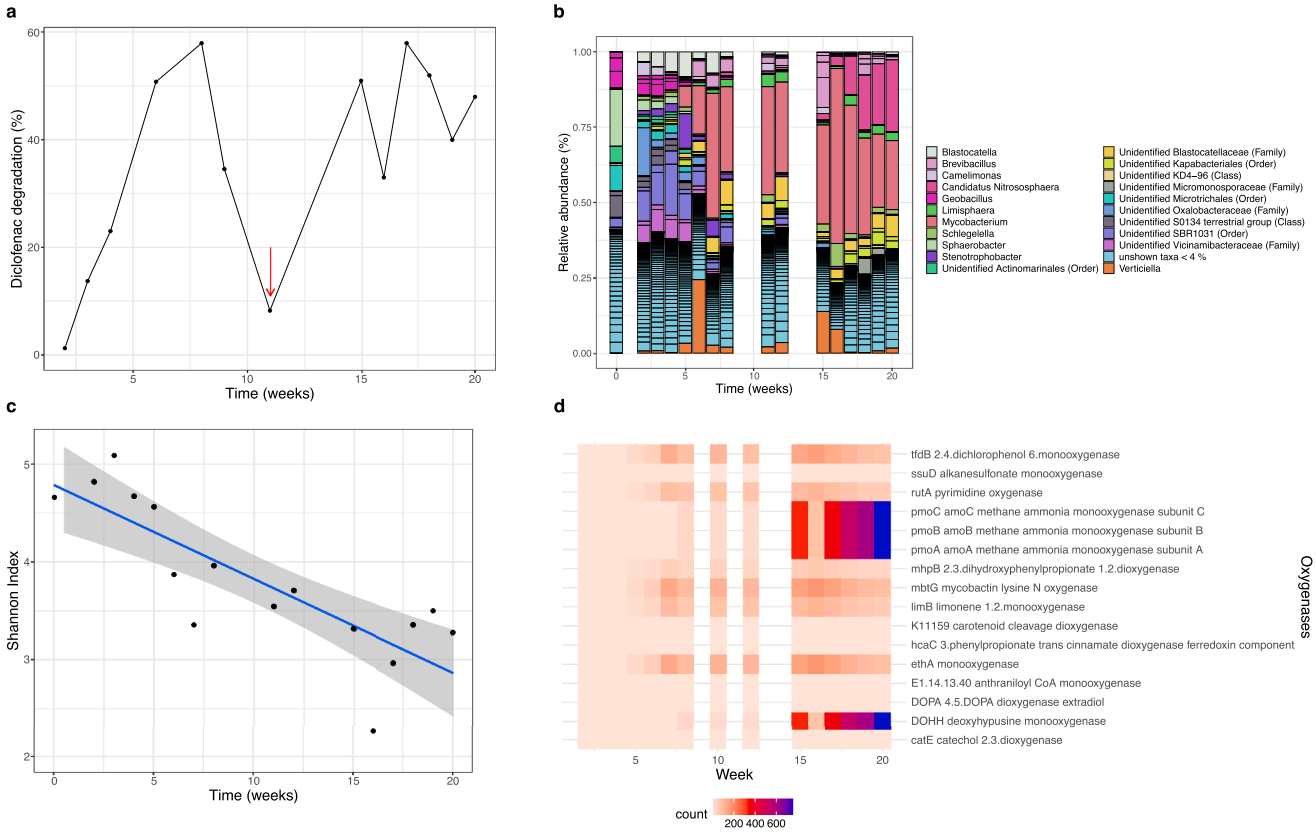

Fig. 1. Performance and microbial community analysis of the diclofenac-degrading MBR. (a) Degradation (in %) of diclofenac within the MBR over time. Diclofenac concentration in the influent of the MBR was constantly 2 mg/L (Hydraulic retention time 50 h). The red arrow indicates the time point of addition of 50 mg/L ammonium-acetate within the influent. **(b)** Microbial community composition (relative abundance in %) within the MBR over time. Only ASVs with relative abundance > 4 % are identified for better visualization. **(c)** Shannon diversity index of the microbial community within the MBR. **(d)** Relative abundance of genomic oxygenases of the microbial community within the MBR. Oxygenases were initially selected based on their abundance (threshold 0.03 %) plotted based on the increase in count over time.

64 % by Week 16. Following the addition of ammonium acetate in Week 11, the ammonium oxidizer *Candidatus Nitrosophaera* also showed a marked increase, reaching 30 % by Week 20. When the MBR reached its maximum degradation capacity in Week 17, the microbial community was composed of *Mycobacterium hassiacum* (42 %), *Candidatus Nitrosophaera* (13 %), an unidentified member of *Blastocatellaceae* (3.5 %) and *Limisphaera* (3.2 %).

The diversity of the incubated microbial community within the MBR decreased over time, with a Shannon Diversity Index of 4.7 in the original compost sample, to a Shannon Diversity Index of 3.3 after week 20 of cultivation, indicating the microbial community became increasingly specialized on the compound diclofenac and its metabolites (Fig. 1c).

Using PICRUSt2, the metabolic pathways of the microbial communities were analyzed, with a special focus on monooxygenases, that are known to play the crucial role to transform diclofenac to 4-hydroxydiclofenac. The most relative abundant oxygenases found in the community were analyzed regarding their initial value at week 0 (Fig. 1d). Notably, the relative abundance of a methane ammonia monooxygenases increased during the 20-week incubation, as well as a DOHH deoxhypusine monooxygenase.

3.2. Degradation of recalcitrant pharmaceuticals by the MBR consortium in batch cultures

As a next step, we tested if the adapted microbial community of the MBR is also able to degrade recalcitrant pharmaceuticals in batch cultures, each incubated with a concentration of 20 mg/L (Fig. 2a). The HPLC results revealed that the communities were able to successfully remove diclofenac (74 % in 20 days), ciprofloxacin (45 % in 20 days), paracetamol (100 % in 7 days), and sulfamethoxazole (33 % in 20 days). However, no removal was detected for ibuprofen and trimethoprim. Further, it was analyzed how the initial microbial community changed during cultivation in relation to the pollutant present in the batch culture (Fig. 2b). Significant changes in the microbial community structures were observed after 10 days of incubation, with the extent of these alterations varying according to the specific pharmaceutical present (Fig. 2b). This shift is further highlighted in the NMDS analysis (Fig. 2c). The taxa *Mycobacterium*, unidentified *Blastocatellaceae*, and *Limisphaera* were among the most abundant in all cultures. Their relative abundances were influenced by the pharmaceutical present, with fluctuations observed depending on the compound.

To analyze in detail which low-abundance microorganisms were significantly enriched in response to different substrates, we performed a DESeq2 analysis based on the relative abundance of taxa across treatments, using diclofenac as the reference condition (Fig. 2d). This approach also allowed us to detect trends in low-abundance taxa. In treatments containing ciprofloxacin, an unidentified candidate from the KD4–96 class and an unidentified member of the *Blastocatellaceae* family were significantly more abundant. In contrast, diclofenac treatments showed significantly higher abundance of the genera *Chelatococcus, Geobacillus, Actinomadura*, and *Microbispora*. When comparing microbial communities in paracetamol treatments to those in diclofenac treatments, *Chelatococcus* remained significantly more abundant, along with *Tepidamorphus, Chelativorans*, and *Bdellovibrio*. The genus *Tepidamorphus* was also significantly more enriched in treatments with sulfamethoxazole compared to those with diclofenac.

These findings suggest that the microbial community adapted in the diclofenac MBR is capable of degrading a wide range of recalcitrant pharmaceuticals commonly found in wastewater. Due to the diverse

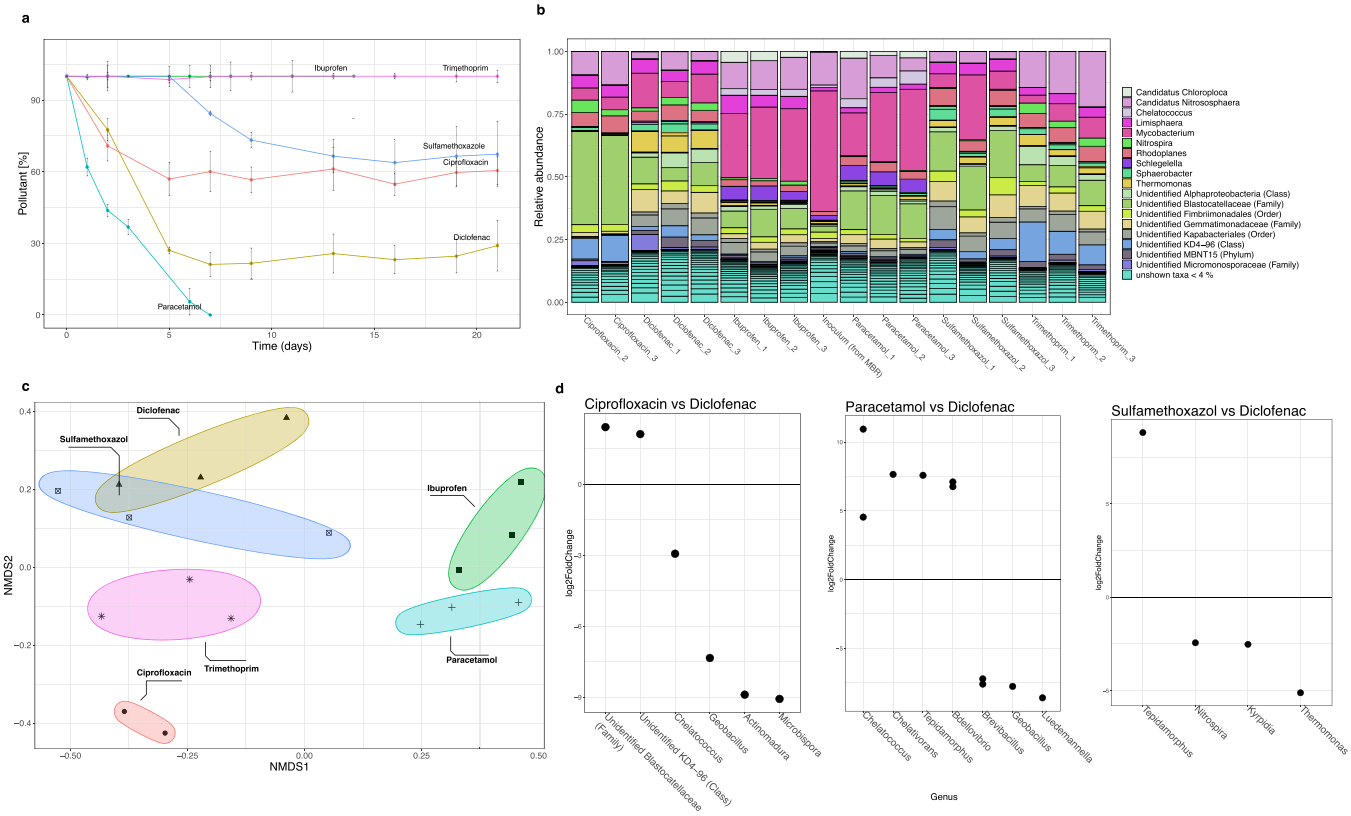

Fig. 2. Performance and analysis of the MBR community within batch cultures facing various pharmaceuticals (a) Concentration (in %) of Ciprofloxacin, Diclofenac, Ibuprofen, Paracetamol, Sulfamethoxazole and Trimethoprim within batch cultures inoculated with the adapted MBR community of week 20. Initial concentration of the pharmaceuticals was 5 mg/L, respectively **(b)** Microbial community composition (relative abundance in %) within the batch cultures on day 10 of cultivation. Just ASV > 0.04 are identified for better visualization. **(c)** NMDS analysis of the batch cultures. Stress is 0.11. **(d)** DESeq2 analysis of significant changes in the abundance of specific taxa between the batch cultures with different compounds. Error bars show ±SE (n = 3).

microbial pool within the community, it demonstrates notable functional flexibility, enabling efficient responses to a variety of pharmaceutical contaminants.

3.3. Isolates from the MBR capable of removing diclofenac

Thermophilic isolates were obtained by plating on diclofenac-containing agar plates through serial dilution of the thermophilic MBR inoculum, and grown colonies were screened for diclofenac removal at 50 °C. Two pure isolates showed the most potential removal activity: *Chelatococcus* sp. strain D3 (100 % identity to *Chelatococcus composti)* and *Mycobacterium* sp. strain *D1 (*100 % identity to *Mycobacterium hassiacum)*. Interestingly, both strains transformed diclofenac into different metabolites. *Chelatococcus* sp. strain D3 transformed 33 % of 20 mg/L diclofenac into equimolar concentrations of 4-hydroxy-diclofenac within 48 h (ammonium acetate present as co-substrate) (Fig. 3a). No growth of the cultures was monitored. *Mycobacterium hassiacum* transformed 100 % of 10 mg/L diclofenac into diclofenac-lactam within 8 days, while growth of the culture was observed under the tested conditions (glycerol as co-substrate) (Fig. 3b). Both strains stopped the metabolization of diclofenac after the mentioned transformation under the tested conditions.

Due to its interesting formation of 4-hydroxy-diclofenac, the isolate *Chelatococcus* sp. strain D3 was further investigate on its performance at different temperatures at 25 °C and 37 °C (and 50 °C for comparison) (Fig. 4a). At all temperatures, diclofenac was transformed to 4-hydroxy-diclofenac with slightly different efficiencies (20 %, 25 %, and 30 % at 25 °C, 37 °C, and 50 °C, respectively, within three days). Furthermore, *Chelatococcus* sp. strain D3 demonstrated the ability to remove diclofenac from real wastewater spiked with 20 mg/L of the compound (Fig. 4b). Impressively, it also degraded the antibiotics trimethoprim and sulfamethoxazole at an elevated temperature of 50 °C (Fig. 4c). Moreover, this isolate was able to remove 55 % of 750 μg/L diclofenac and 23 % of 2 μg/L diclofenac within 48 h, demonstrating its ability to maintain removal efficiency at environmentally relevant lower concentrations (Fig. 4d). These results highlight *Chelatococcus* sp. strain D3 as a highly promising candidate for the removal of diverse pharmaceutical contaminants from wastewater.

4. Discussion

The rising consumption of diclofenac and the low removal efficiency in wastewater treatment plants have led to the presence of this emerging contaminant in various environmental compartments, particularly in water. Diclofenac remains alarmingly detectable in the effluents of numerous wastewater treatment plants, defying attempts to remove it effectively (Sathishkumar et al., 2020; Vieno and Sillanpää, 2014). Furthermore, this persistent contaminant has also been found in lakes and rivers, highlighting its widespread and concerning presence in our water sources (Pheko Amos Sibeko Devrani Naicker and Madikizela, 2019). This persistence is primarily due to the chemical's stability and resistance to biodegradation, allowing it to bypass conventional treatment barriers and enter natural ecosystems. Given the persistent presence of diclofenac in wastewater effluents and aquatic environments, it is crucial to explore innovative biological solutions for its degradation. Our planet hosts a diverse array of microorganisms with complex metabolic pathways that could provide effective strategies for addressing this environmental challenge (Blaser et al., 2016). The study revealed that thermophilic microbial communities from compost can remove diclofenac, which shows new potential for facing the challenge of the tackling of this persistent compound. We added 2 mg/L diclofenac in the MBR to have a stronger selection pressure on the compost microbial community, ensuring that only microorganisms capable of utilizing or degrading diclofenac could proliferate, thereby allowing us to enrich and identify a robust community with the desired biodegradation capability. Furthermore, we showed that *Chelatococcus* sp. strain D3 removed diclofenac under environmental relevant concentrations (750 μg/L and 2 μg/L). These results confirm that the isolate *Chelatococcus* sp. strain D3 retains its degradation activity even at low, real-case scenario concentrations, thereby supporting the potential of this process for actual wastewater treatment applications. Moreover, the community established after 20 weeks within the MBR was also capable of removing the antibiotics sulfamethoxazole and ciprofloxacin, as well as the pain killer paracetamol, which are all present and persistent in wastewater (Githinji et al., 2011; Thiebault, 2020; Wu et al., 2012). This broad substrate range underscores the potential for using these microbial consortia in systems that treat complex pharmaceutical mixtures often found in wastewater effluents. The observation of community structure shifts based on the available pharmaceutical substrate suggests a dynamic response to environmental pressures (Suleiman et al., 2024,

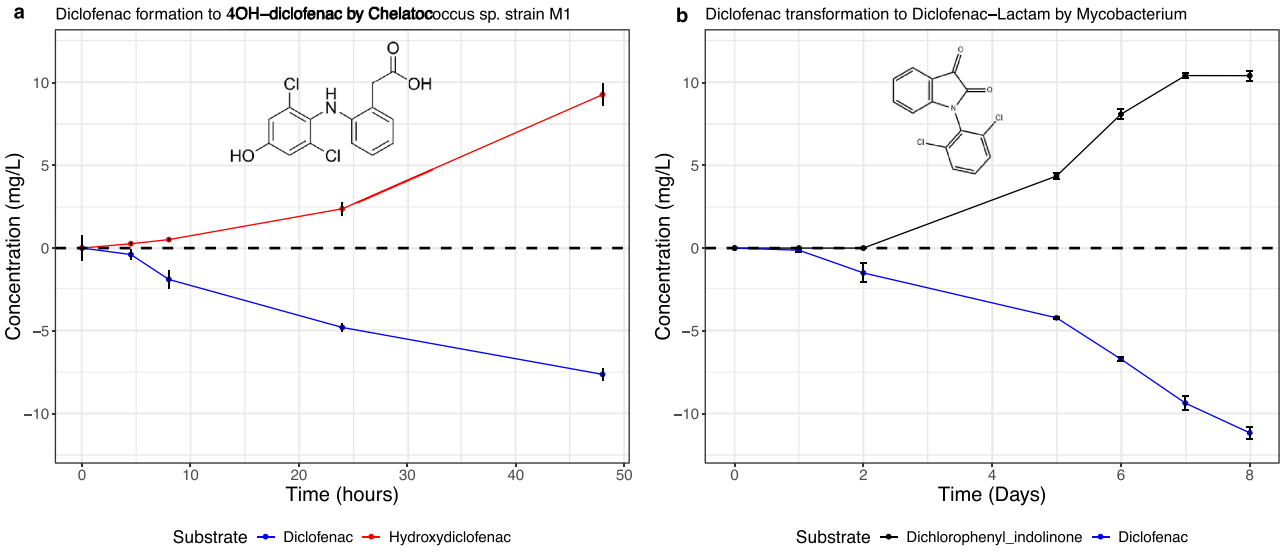

Fig. 3. First reactions of transformation of diclofenac by 2 thermophilic compost bacterial strains at 50°C. (a) Formation of 4-hydroxy-diclofenac by *Chelatococcus* sp. strain D3 within 48 hours **(b)** Formation of diclofenac-lactam by *Mycobacterium* sp. strain D1 after 8 days. Error bars show ±SE *(n = 3)*.

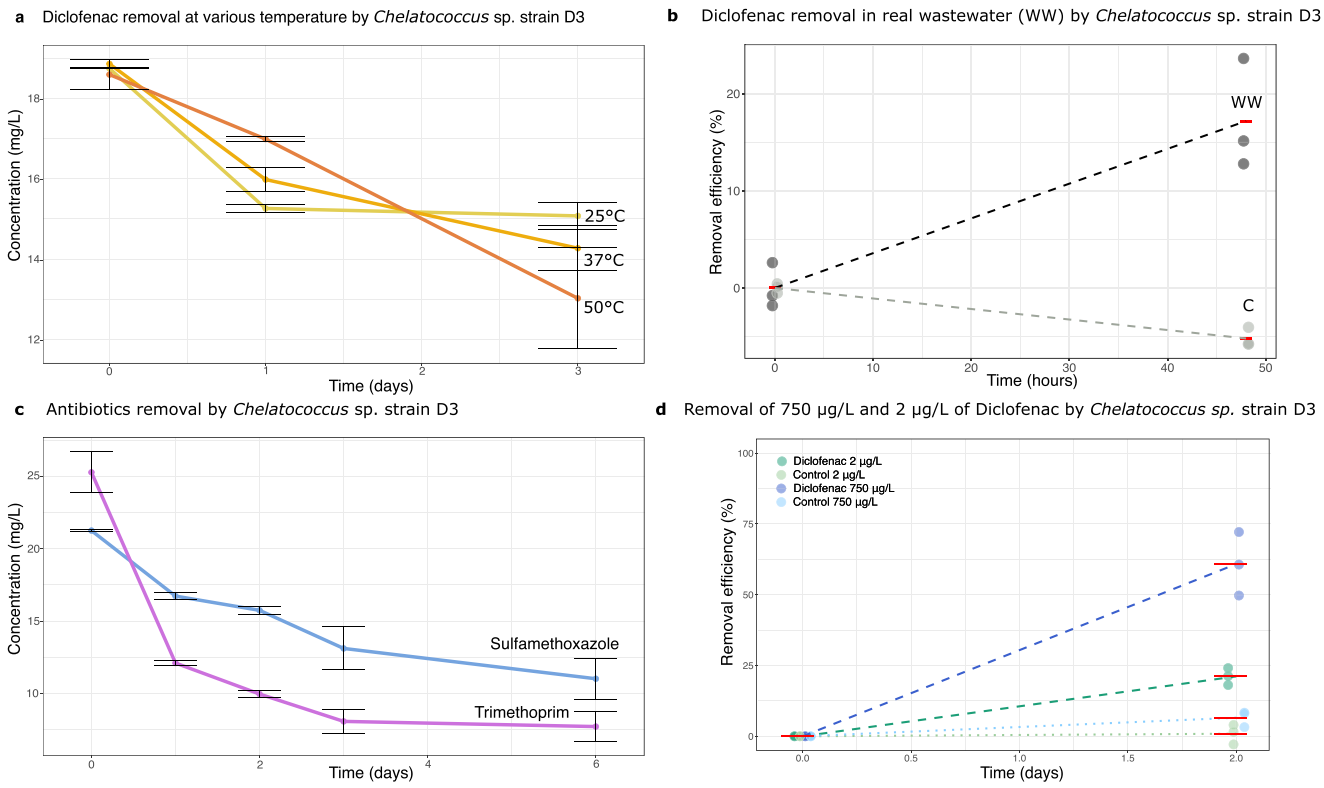

Fig. 4. Characteristics of the degradation of pharmaceuticals by *Chelatococcus* sp. strain D3. (a) Diclofenac removal efficiency by *Chelatococcus* sp. strain D3 cultivated at different temperatures (25°C, 37°C, 50°C). Error bars show ±SE *(n = 3)*. **(b)** Removal efficiency in % of diclofenac by *Chelatococcus* sp. strain D3 in real wastewater (WW) spiked-in with 20 mg/L of diclofenac at 50°C. Abiotic controls represent batch cultures without addition of *Chelatococcus* sp. strain D3. Error bars show ±SE *(n = 3)*. **(c)** Removal efficiency of the antibiotics sulfamethoxazole and trimethoprim by *Chelatococcus* sp. strain D3 at 50°C. Error bars show ±SE *(n = 3)*. **(d)** Removal efficiency of 750 µg/L (dark blue) and 2 µg/L (dark green) diclofenac from water by *Chelatococcus* sp. strain D3. Abiotic controls without the addition of *Chelatococcus* sp. strain D3 with 750 µg/L (light blue), 2 µg/L (light green). The red line represents the average of the triplicates (*n*=3).

2022), further supporting the adaptability and resilience of these microbial communities in real-world wastewater treatment conditions.

Addition of ammonium acetate as a co-substrate significantly improved the removal efficiency in later stages of the MBR operations. Therefore, it is very likely that diclofenac is broken down through co-metabolism (Dalton et al., 1982), which is an often-observed phenomena for degradation of complex pharmaceuticals (Dhamale et al., 2022; Gauthier et al., 2010). It is likely that the first weeks in the MBR operated without acetate, as natural substances from the compost could have served as co-substrates.

The addition of acetate to wastewater treatment plants is a common approach to stimulate microbial activity, as it provides an easily degradable carbon source that enhances the biodegradation of organic pollutants (Zhang et al., 2020). In addition to serving as a carbon source, acetate oxidation generates reduced equivalents (NADH or NADPH) through entering the tricarboxylic acid (TCA) cycle (Yi et al., 2022). These reducing equivalents are crucial for enzymatic reactions mediated by monooxygenases and dioxygenases, including those potentially involved in diclofenac degradation. Notably, the initial step in the diclofenac degradation pathway typically involves a monooxygenase (often a cytochrome P450) that hydroxylates the compound (Bouju et al., 2016b). Moreover, the ammonium released from ammonium acetate stimulates the growth of nitrifiers such as *Candidatus Nitrososphaera*, which catalyze the oxidation of ammonium to nitrate. The activity of these nitrifiers is particularly beneficial in wastewater treatment processes, as municipal effluents typically contain high concentrations of ammonium (Liu et al., 2023). However, it remains to be investigated whether *Candidatus Nitrososphaera* also contribute to the degradation of diclofenac or its transformation products. It is also plausible that the addition of ammonium acetate contributed to a restructuring of the microbial community. At the same time, our results indicate that diclofenac removal efficiency improved progressively during this period. While it is challenging to disentangle the relative contributions of co-substrate-driven shifts versus specific adaptation to diclofenac, the data suggest that both mechanisms may have played a role. Thus, acetate likely facilitated conditions that supported community changes, while the long-term trend points to an enhanced capacity for diclofenac degradation from week 12.

Future research should focus on scaling up these findings, optimizing operational conditions, and further investigating the enzyme systems involved in diclofenac degradation to maximize efficiency. Given the widespread occurrence of diclofenac in the environment, the development of robust and efficient treatment systems for its removal is essential to safeguard water quality and aquatic ecosystems.

A major limitation of this thermophilic wastewater treatment technology is the energy required to heat the system to 50 °C. However, processes with such moderate temperatures can be successfully carried out by utilizing district heating (Schmidt et al., 2017). Furthermore, the isolate *Chelatococcus* sp. D3 was also able to degrade diclofenac at 25 °C and 37 °C, making it a promising candidate for wastewater treatment. Similarly, *Mycobacterium hassiacum* has been described in the literature to grow under mesophilic conditions, such as 35–40 °C [30]. This suggests that it may be possible to operate or adapt the compost community within the MBR to slightly lower temperatures, potentially reducing energy costs and improving environmental sustainability. On the other hand, the higher temperature might be necessary to enhance the bioavailability of diclofenac. The isolation of *Mycobacterium* sp. D1 and *Chelatococcus* sp. D3 is particularly interesting, as they represent both a highly abundant and a low-abundance member of the microbial community. *Mycobacterium* was found to be highly abundant, constituting

more than 40 % of the community, whereas *Chelatococcus* accounted for less than 4 %. However, the importance of *Chelatococcus* becomes evident in the DESeq2 analysis of batch cultures grown with different substrates. This analysis revealed that *Chelatococcus* was significantly more abundant in cultures exposed to diclofenac (and paracetamol), suggesting a specific role in the degradation of these pharmaceuticals despite its overall lower abundance in the microbial community. Moreover, *Chelatococcus* was tested for the degradation of two antibiotics, sulfamethoxazole and trimethoprim. The degradation occurred in a fast fashion within 6 days, demonstrating its potential on bioremediation of further pharmaceuticals. This finding demonstrates that low-abundant bacteria can play a crucial role for the function of ecosystems (Han and Vaishnava, 2023). Even though they make up a small fraction of the microbial population, their specialized metabolic capabilities can significantly influence the overall biodegradation process in complex microbial ecosystems. Further, the finding that both strains produced different metabolites from diclofenac highlights the diversity of metabolic reactions involved in its transformation. While the complete pathway for 4-hydroxy-diclofenac removal has been described in the literature (Bouju et al., 2016a), the metabolism via diclofenac-lactam remains only partially understood. In our experiments, *Mycobacterium* generated diclofenac-lactam as dead-end metabolite. However, since diclofenac-lactam is not present in the MBR effluent of this study, it suggests that further transformation occurs beyond this activation step by using the full potential of a whole microbial community within the MBR. However, diclofenac-lactam has been consistently detected in wastewater treatment plant (WWTP) effluents, emphasizing the need to investigate its role in the transformation and removal processes of diclofenac (Jewell et al., 2016; Wojcieszyńska et al., 2023). This finding raises concerns about the efficiency of wastewater treatment processes in fully breaking down diclofenac and its transformation products. Further research is needed to clarify whether diclofenac-lactam represents a dead-end metabolite or if additional degradation steps exist. Future studies will integrate the detection of diclofenac's principal metabolites with total organic carbon (TOC) measurements, enabling assessment of whether the compound undergoes complete or partial degradation.

5. Conclusion

In conclusion, this study highlights the potential of thermophilic compost-derived microbial communities to remove recalcitrant pharmaceuticals from wastewater treatment systems. The adaptability and metabolic versatility of these communities present a promising avenue for developing more effective strategies to mitigate pharmaceutical pollution in aquatic environments. Thermophilic membrane bioreactors (MBRs) may offer significant advantages for the advanced polishing of pharmaceutical-laden wastewaters, particularly those originating from industrial production and hospital effluents. Notably, *Chelatococcus sp.* strain D3 appears to be a particularly promising candidate for bioaugmentation in WWTPs, as it catalyzes the critical transformation of diclofenac to 4-hydroxy-diclofenac. This strain was also active at lower temperatures and demonstrated additional degradative capacity toward other recalcitrant pollutants, underscoring its potential role in enhancing the efficiency and robustness of biological wastewater treatment processes.

Code availability

R Studio code for the analysis and plotting of figures for the manuscript and supplementary information is available at https://github.com/Marcel29071989

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Data availability

All datasets and metadata are available on the GitHub repository Marcel 29,071,989 (https://github.com/Marcel29071989/), and the raw sequencing data can be found on NCBI SRA archive under ID SUB15191621.

CRediT authorship contribution statement

Francesca Demaria: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Ramona Blattner:** Methodology, Investigation. **Chasper Puorger:** Methodology, Investigation. **Boris Kolvenbach:** Investigation. **Mariana S Cretoiu:** Validation, Software, Data curation. **Timm Hettich:** Methodology, Investigation, Formal analysis, Data curation. **Philippe Corvini:** Writing – review & editing, Supervision. **Georg Lipps:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Marcel Suleiman:** Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Philippe Corvini reports financial support was provided by Horizon Europe. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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