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Ph.D. thesis

Identification of microorganisms capable of biological degradation of pharmaceutical residues (diclofenac, ibuprofen, and carbamazepine) as micropollutants using "omics" methods

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Tartalom

1. Introduction and aims

The harmful substances released into the environment from anthropogenic activities have detrimental effects on both human health and aquatic ecosystems. Pollutants detected in increasing concentrations in freshwater ecosystems are among the most significant and pressing environmental issues facing humanity. Among the emerging organic pollutants present in the environment are pharmaceutically active compounds (PhACs). Since the turn of the millennium, there has been growing attention on the occurrence of pharmaceutical residues in aquatic environments, their environmental impact, and their potential toxicity. Pharmaceutical residues primarily originate from municipal wastewater treatment plants, hospitals, and industrial manufacturing processes. Current wastewater treatment methods are insufficient and inadequate for the removal of these compounds. There is insufficient information regarding the potential long-term environmental and public health effects of pharmaceutical active ingredients. Therefore, it is essential to understand the microbiology, biotransformation, and biodegradation of pharmaceutical residues in the environment. Additionally, it is important to determine their final environmental fate, perform risk assessments, and develop sustainable innovative methods for faster removal. Accordingly, my objectives are:

- To selectively enrich and isolate autochthonous bacterial strains potentially capable of degrading diclofenac, ibuprofen, and carbamazepine from a bacterial biofilm that has been widely studied before.
- To further test the biodegradation of pharmaceutical active ingredients by the bacterial strains.
- To create a bacterial consortium from the most effective pharmaceutical-degrading bacterial strains, which will be tested in both natural water samples and wastewater effluents.

2. Materials and methods

In accordance with my objectives, I carried out the experiments. Previously, I selectively enriched potential pharmaceutical-degrading microorganisms from a bacterial biofilm that had been thoroughly identified. The enrichment, isolation, and species-level identification of microorganisms involved in the degradation of diclofenac, ibuprofen, and carbamazepine were performed, along with the creation of a bacterial strain collection capable of potentially degrading the three examined pharmaceutical compounds. A bacterial consortium was formed from the identified isolates, which was tested in both natural water samples and wastewater effluents. To achieve the above objectives, I applied culture-based microbiological methods as well as cultivation-independent molecular biological techniques, and determined the biodegradation rate of the pharmaceutical active ingredients using advanced analytical methods.

2.1 Biofilm sample and sampling conditions

The bacterial biofilm serving as the basis for the studies is characterized by high phylogenetic and functional diversity and has previously been a source for isolating bacteria capable of biologically degrading simple (BTEX) and polycyclic aromatic hydrocarbons (naphthalene) (Benedek et al., 2016; 2018). It was hypothesized that the biofilm, with its high phylogenetic and functional diversity, could contain bacteria capable of degrading similar aromatic-ring compounds, such as diclofenac, ibuprofen, and carbamazepine.

The biofilm samples were collected in December 2019 from a Pump & Treat system treating petroleum-contaminated groundwater in Hungary's central region, specifically from the BUT-1 well, into 50 mL sterilized tubes. After sampling, the biofilm samples were stored on ice, and upon arrival at the laboratory, they were processed.

2.2 Selective enrichment of degrading bacteria in the presence of pharmaceutical active ingredients

For the selective enrichment of pharmaceutical-degrading bacteria, I used a mineral nutrient medium supplemented with vitamins (100 mL), which contained diclofenac (DCF), ibuprofen

(IBU), or carbamazepine (CBZ) (100 mg/L) as the sole carbon and energy sources. I inoculated the three parallel enrichment cultures with 1 mL of biofilm suspension. The selective enrichment lasted for three months (145 rpm, 25°C), and at the end of each month, 10 mL of the enriched culture was transferred to fresh enrichment medium (90 mL). Additionally, at the end of each month, I prepared dilution series from the enrichment cultures and plated 100 µL from each dilution onto R2A agar plates (protease peptone 0.5 g; casein amino acids 0.5 g; yeast extract 0.5 g; dextrose 0.5 g; soluble starch 0.5 g; dipotassium phosphate 0.3 g; magnesium sulfate $7H_2O$ 0.05 g; sodium pyruvate 0.3 g; agar 15 g; pH 7) for bacterial isolation. After inoculation, the Petri dishes were incubated at 28°C for 48 hours. The colonies with different morphologies were purified using the streak plate technique. For long-term preservation, the purified strains were stored at -80°C in glycerol-R2A solution (30% v/v).

2.3 Identification of isolated bacterial strains based on 16S rRNA

The genomic DNA of the isolates was first extracted using the DNeasy® UltraClean® Microbial DNA Isolation Kit (Qiagen, Germany). In the next step, I amplified the bacterial species-specific 16S rRNA genes by PCR. The PCR amplicons were purified using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). The nucleotide sequences of the obtained 16S rRNA genes were determined by Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA). The sequences were analyzed using the ABI 3130 Genetic Analyzer (Life Technologies, USA). The resulting sequence electropherograms were evaluated using MEGA 11 software, followed by BLAST homology searches in the GenBank database. Based on approximately 1500 base pairs of 16S rRNA sequences read during sequencing, prokaryotic microbes can be identified, even down to the species level.

2.4 HPLC measurements to determine the degradation rate

At concentrations of diclofenac, ibuprofen, and carbamazepine (1.5 mg/L), the mineral media (50 mL) were inoculated with 50 µL bacterial suspensions at an optical density OD600=1. Cometabolic degradation experiments were also conducted, which included not only the

pharmaceutical compounds but also more easily metabolizable carbon and energy sources, such as yeast extract (0.05 g/L or 0.3 g/L), glucose (0.5 g/L or 3 g/L), sodium acetate (0.8 g/L), or sodium citrate (0.8 g/L). The degradation experiments were performed in triplicates, along with abiotic control samples. The test solution was filtered through a 0.45 μm cellulose acetate filter (Whatman) and transferred to HPLC vials. The analysis was performed directly using a Chromaster Hitachi HPLC system with an Ascentis C18 column (150 x 0.46 mm), using 0.02 M KH2PO4-acetonitrile as the mobile phase for isocratic elution (0:50%, v/v; 0.9 mL/min). A diode array detector was used to detect the compounds, with diclofenac detected at 202 nm, ibuprofen at 196 nm, and carbamazepine at 214 nm.

2.5 Formation of the bacterial consortium and testing it in different media

Based on the results of the degradation experiments, bacterial strains and conditions were selected where the consortium showed the highest efficiency in degrading the target pharmaceutical compounds during laboratory tests. To ensure the success of the bacterial consortium, several co-cultivation tests were performed, including cross-streaking, agar diffusion hole tests, and determining the ideal temperature, medium, pH, and salt concentrations. These conditions were then established for the consortium during further degradation experiments.

Degradation experiments were carried out in three different media: Bushnell-Haas mineral medium, natural water samples (Rákos-patak), and a wastewater effluent. The media were supplemented with diclofenac, ibuprofen, and carbamazepine (1.5-1.5 mg/L) in a 1:1:1 ratio. In several setups, the initial test solution was supplemented with yeast extract (0.3 g/L), glucose (3.0 g/L), and, in some cases, sodium acetate (1.0 g/L). The test solutions were inoculated with 100 μL bacterial suspensions at OD600 = 1, which contained the examined bacterial strains in a 1:1 ratio. The experiments were performed in triplicates, with abiotic controls also set up, which contained all the above-mentioned compounds except for the bacterial suspension.

Samples were taken weekly from the solutions during the 4-week incubation period. The natural water sample was taken from the Rákos-patak, Gödöllő, Alsó-park section, on March 16, 2022, while the wastewater effluent sample was collected from the Gödöllő Wastewater Treatment Plant's outlet, with sampling taking place on August 15, 2022. The general physicochemical parameters of the wastewater effluent were determined by Wessling Hungary Kft. using standardized methods.

High-performance liquid chromatography (HPLC) was used to track the concentration changes of the pharmaceutical active ingredients in the samples. The concentrations of the three pharmaceutical compounds were monitored using an HPLC system.

2.6 Testing a potential new bacterial species

During the enrichment process, I was able to culture a bacterial species previously unknown to science. The necessary investigations for species description included the following: bacterial strain isolation and characterization; 16S rRNA gene sequencing, phylogenetic analysis; phylogenetic tree reconstruction; pangenomic analysis; morphological, physiological, and biochemical tests (Barrow & Feltham, 2003); chemotaxonomic analyses (Schumann, 2011); whole genome sequencing; and testing the strain's pharmaceutical compound degradation capacity. These procedures were all carried out in accordance with established methods found in the literature (Cui et al., 2024).

2.7 Determination of the metabolite formed during the biotransformation of diclofenac *Stenotrophomonas humi* DIC_5 and toxicity study

One of the bacterial consortium members, the *Stenotrophomonas humi* DIC_5 strain, was subjected to a more detailed degradation experiment in which I was interested in the generated metabolite and its toxic effects. The sampling process lasted for two weeks, during which two samples were taken daily at 8-hour intervals. Each sample contained 2 mL of liquid, which was extracted from the strain suspension using a medical syringe and filtered through a nylon syringe filter (0.22 μm, AlWSCI Corporation, China). To identify and clarify the structure of the diclofenac metabolites, I used HPLC-Q-TOF-MS, and for further confirmation, I applied UHPLC-TQ-MS. High-resolution analyses were performed using an Agilent 1100 HPLC

system (USA), equipped with a binary solvent delivery system and an automatic sampler. Separation was carried out on a Phenomenex Kinetec 2.6 µm, C18, 150 x 4.6 mm chromatographic column. The injection volume was 20 µL. The eluent consisted of 0.1% HCOOH, 5 mM ammonium formate in water (A) and MeOH (B), with a total run time of 18 minutes at a flow rate of 0.6 mL/min.

To determine the cytotoxicity of the diclofenac biotransformation end-products, I used the ISO 11348-2 (2007) standard for acute *Allivibrio fischeri* bioluminescence measurement. Toxicity was measured by inhibiting the luminescence of the cells after a 30-minute exposure. Special attention was given to controlling the conditions of the controls. The toxicity of pure diclofenac was also determined. A 20 mg/mL diclofenac stock solution was prepared in dimethyl sulfoxide (DMSO, CAS 67-68-5, purity ≥99.99%, Fisher Scientific, USA), and then it was diluted from 1.00E+02 mg/L to 6.25E+00 mg/L in a 2 m/m% NaCl solution containing 1 v/v% DMSO. The biotransformation-derived samples and the inhibition caused by diclofenac were expressed as the percentage change relative to the initial concentration, which resulted in a 50% decrease in luminescence intensity compared to the control sample.

For the zebrafish maintenance and egg collection, I followed the methods described by Csenki et al. (2019). Microinjection was also carried out based on their scientific paper. The injection volumes were as follows: for 75 µm droplet diameter, 0.22 nL; for 100 µm, 0.52 nL; for 150 µm, 1.77 nL; and for 200 µm, 4.17 nL, for the abiotic control, biotic control, and DIC_5 samples. Mortality of the embryos was determined 120 hours post-fertilization (hpf), considering the coagulation of the eggs, absence of somite formation, and the state of heart function. Afterward, the embryos were transferred into Petri dishes containing a 5% methylcellulose solution. Sublethal symptoms were documented in digital photographs of the larvae, from the lateral view, under a 30x magnification stereomicroscope (Leica M205 FA, Leica DFC 7000T camera, and Leica Application Suite X software, Leica Microsystems GmbH, Germany).

3. Results

3.1 Polutation dynamics changes in the bacteriaal community during selective enrichment of biofilm

The bacterial community of the starting biofilm was predominantly dominated by the class *Betaproteobacteria*, followed by *Alphaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, and *Flavobacteriia*. At the genus level, *Thauera*, *Bdellovibrio*, *Acidovorax*, *Azoarcus*, and *Zoogloa* genera were the most represented.

In the diclofenac (DIC) enrichments, the classes *Alphaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* were dominant. While the *Gammaproteobacteria* were more abundant in the DIC enrichments, the *Betaproteobacteria* was the most abundant class in the ibuprofen (IBU) and carbamazepine (CBZ) enrichments. Additionally, the *Actinobacteria* class only became a significant member of the community in the IBU and CBZ enrichments, with a relative abundance of 4–14%.

At the genus level, the bacterial community in the DIC enrichments was predominantly dominated by the genus *Pseudomonas*, followed by *Methyloversatilis*, *Azospirillum*, *Ferrovibrio*, *Rhodanobacter*, and *Flavobacterium.* In contrast, the genus *Methyloversatilis* was the dominant genus in the IBU and CBZ enrichments, while *Pseudomonas* ranked second and third in terms of relative abundance in these two enrichments. Also, in the IBU and CBZ enrichments, representatives of the *Actinobacteria* class, such as *Nocardioides*, *Rhodococcus*, and *Pseudonocardia*, became key members of the community. Notably, in the last two months of the CBZ enrichment, the genus *Azospirillum* reached a significant abundance of nearly 3.4–4%.

Bacterial strains were identified that showed at least a 0.5% increase in relative abundance and at least a 100-fold increase compared to the initial values measured in the starting biofilm community in at least one of the enrichment cultures. In the DIC enrichments, by the third month, *Ferrovibrio*, *Hydrocarboniphaga*, *Zavarzinia*, and *Sphingopyxis* showed the largest relative abundance changes, with increases of 1005-, 957-, 811-, and 628-fold, respectively. In the IBU enrichments, the largest relative abundance increases were observed for *Starkeya* (1022-fold), *Methylibium* (314-fold), and a previously uncultivated, metagenomically

identified bacterium, SCN 62-11 (350-fold). Interestingly, the *Pimelobacter* genus had a relative abundance of only 0.18% in the IBU enrichments, but it showed a 1000-fold increase by the end of the enrichment. In the CBZ enrichments, the most notable increases in relative abundance were observed for *Pseudonocardia*, *Sphingopyxis*, and *Rhodococcus* genera, with increases of 505-, 435-, and 220-fold by the end of the third month.
Surprisingly, the most dominant genera, such as *Pseudomonas*, *Methyloversatilis*, and *Azospirillum*, only showed 11- and 60-fold increases in their relative abundance by the end of the enrichment. mutattak a százalékos relatív abundanciában.

3.2 Bacterial diversity of enriched cultures based ont he culture dependent approach

A total of 31 bacterial isolates were successfully cultured, of which 10 isolates came from the DIC, 9 from the IBU, and 12 from the CBZ enrichments. At the class level, the isolates were grouped as follows: *Gammaproteobacteria* (14 isolates), *Alphaproteobacteria* (7), *Betaproteobacteria* (7), *Actinobacteria* (4), and *Bacillus* (2). The majority of the isolates (13 isolates) belonged to the *Pseudomonas* genus (DIC_4, IBU_5, CBZ_4). Following *Pseudomonas*, *Rhizobium* (DIC_3, IBU_2), *Methyloversatilis* (1 isolate from each enrichment), *Nocardioides* (CBZ_2), *Variovorax* (CBZ_2), and *Bacillus* (IBU_1, CBZ_1) genera were represented. Furthermore, one isolate each was successfully cultured from the Stenotrophomonas (DIC enrichment), Ancylobacter (IBU), Brevundimonas (CBZ), and Rhodococcus (CBZ) genera. The majority of the isolates were Gram-negative bacteria (26 isolates), while 5 were Gram-positive.

The bacterial isolations resulted in the identification of a bacterial species, which was obtained from the CBZ enrichment: this belongs to the Nocardioides genus. The isolate's 16S rRNA gene of *Nocardioides* sp. CBZ_1 showed less than 98% sequence similarity to the closest already cultured relative's 16S rRNA gene. The CBZ_1T isolate was recently described by the authors as a new lineage within the *Nocardioides* genus, named *Nocardioides carbamazepini* (Benedek et al., 2022).

3.3 Screening of isolates capable of biodegrading active pharmaceutical ingridients by HPLC measurment

The HPLC measurements confirmed the following results regarding the pharmaceutical compound degradation capacity of *S. humi* DIC_5 and *R. daejeonense* IBU_18 isolates. After four weeks of incubation, the DIC_5 strain demonstrated near-complete elimination of diclofenac (DCF) (91 ± 0.034% concentration decrease) in the presence of glucose (3 g/l); no higher biodegradation rates were achieved under other experimental conditions. Similarly, after four weeks of incubation, the IBU_18 isolate showed the highest elimination rate of 90.7 ± 0.098% for ibuprofen (IBU) in the presence of yeast extract (0.3 g/l). In carbamazepine (CBZ) degradation, the CBZ_1 isolate exhibited a degradation of over 20% in the presence of yeast extract (0.3 g/l), while the CBZ_3 isolate, with the addition of yeast extract, achieved a degradation efficiency of 22.9 ± 0.042%. The other potential pharmaceutical compound-degrading bacterial strains in the 31-strain collection achieved only 10% degradation efficiency.

The optimal growth conditions for the selected pharmaceutical compound-degrading isolates (DIC_5, IBU_18, CBZ_1, and CBZ_3) were also determined. For subsequent cultivation of the strains, neutral or slightly alkaline R2A medium can be used. The optimal temperature for cultivating the selected strains was 27 °C. During the agar diffusion co-cultivation experiment, no significant inhibition zone was observed for any of the strains. No inhibitory effect was observed among the tested isolates in the cross-streaking method. Based on these results, it can be concluded that a successful bacterial consortium can be formed from the following four bacterial strains:

Stenotrophomonas humi DIC_5
Rhizobium daejeonense IBU_18
Nocardioides carbamazepini CBZ_1
Brevundimonas bullata CBZ_3

3.4 Testing results of the drug degradation ability of the established bacterial consortium in differrent aqueous media

The first test solution was a mineral medium (BHB) enriched with minerals, which did not contain any microorganisms other than those in the consortium. After one week of incubation, the consortium degraded ibuprofen by 93%, while diclofenac was degraded by 39%. The degradation rate increased each week, and by the end of the fourth week, the bacteria in the consortium had degraded 72.6% of diclofenac, 100% of ibuprofen, and 30.6% of carbamazepine. Ibuprofen was completely degraded by the end of the third week, while a significant decrease in carbamazepine concentration was observed between the third and fourth weeks. No significant decrease in the concentration of pharmaceutical compounds was observed in the abiotic controls.

The second test solution was a sample taken from a natural water source (Rákos-patak), which was not filtered before use. When analyzing the degradation of individual pharmaceutical compounds, by the end of the fourth week, the diclofenac concentration decreased by 51.1% in the presence of glucose/yeast extract, while a mixture of glucose/yeast extract/ammonium nitrate achieved nearly 86% degradation. For ibuprofen, in the glucose/yeast extract solution, a similar 50.9% concentration decrease was observed as for diclofenac. However, when the solution was supplemented with ammonium nitrate, ibuprofen was completely eliminated from the test medium within two weeks. The molecular structure of carbamazepine exhibited significant resistance to biodegradation, which was evident during the experiments. As a result, the degradation of carbamazepine remained low across different conditions. Regardless of the test solution type, the consortium achieved 25% degradation results in natural water samples with an initial concentration of 1.5 mg/l. When applying different settings, in natural water samples, the established consortium effectively degraded both diclofenac and ibuprofen in the presence of the autochthonous bacterial community, while the concentration decrease of carbamazepine was slightly detected.

In the final case, a wastewater effluent provided the base for the test solution, which was also not filtered, and the autochthonous bacterial community was present in the experiment. In the samples containing yeast extract and glucose, the degradation of ibuprofen was the most

significant, but diclofenac degradation was also observed in the presence of the consortium. By the end of the four-week incubation period, the consortium introduced into the wastewater effluent achieved 76.29% ibuprofen degradation. In the same sample, the initial intense decrease in diclofenac concentration was followed by stagnation in the second half of the study, resulting in a final degradation of 38.24% by the end of the fourth week. This phenomenon was likely due to other carbon sources present in the wastewater, which were more easily accessible and could interfere with diclofenac degradation. In the wastewater effluent samples containing yeast extract, glucose, and ammonium nitrate, both ibuprofen and diclofenac concentration decreases were observed in the presence of the consortium. In this setting, ibuprofen showed a 79.33% decrease by the end of the study, while diclofenac achieved a 30.85% degradation in the same environment. In summary, the consortium was most effective in degrading diclofenac and ibuprofen in the wastewater effluent, particularly in the setting with yeast extract, glucose, and ammonium nitrate, where nearly 80% ibuprofen and 30% diclofenac degradation were observed. However, no significant concentration change was detected for the third pharmaceutical compound, carbamazepine, in any of the settings.

3.5 Description of a new bacterial species, *Nocardioides carbamazepine* CBZ_1^{T}

During the research period, a species description of the CBZ_1 isolate, which was isolated from carbamazepine-enriched cultures, was completed in accordance with the literature requirements for species descriptions. Based on comprehensive phylogenetic, phenotypic, and biochemical analyses, the new species candidate belongs to the *Nocardioides* genus and has been named *Nocardioides carbamazepine*. Using bioinformatics methods, we identified genes that are exclusively found in the new *Nocardioides* species, distinguishing it from other *Nocardioides* species. The CBZ_1 isolate exhibits good ibuprofen-degrading abilities. More detailed information regarding the species description can be found in the publication by Benedek et al. (2022).

3.6 Biotransformation metabolite of Stenotrophomonas humi DIC_5 isolate, a member of the bacterial consortium and ecotoxicity studies

Based on the high-resolution HPLC results, it can be concluded that in the presence of glucose, the *S. humi* DIC_5 isolate reduced the initial concentration of diclofenac by 75% within 6 days. Among the generated intermediates, nitro-diclofenac was identified. The concentration of nitro-diclofenac increased following the decrease in diclofenac concentration, showing a significant concentration rise starting from day 6. In contrast, the diclofenac concentration did not decrease in the abiotic sample. Biomass studies confirmed that diclofenac did not bind to the cell surface, so the concentration decrease was indeed due to biological transformation and degradation.

Eco-toxicological tests revealed that the by-products of diclofenac biodegradation were not more toxic to the test organisms than diclofenac itself, based on the *A. fisheri* and zebrafish embryo-based assays. According to the *A. fisheri* ecotoxicity test, the toxicity was similar in both abiotic and bacterial inoculated samples, with no significant difference in toxicity levels.

Zebrafish embryo-based toxicity studies also showed no significant difference in toxicity between the abiotic and *S. humi* DIC-5 inoculated samples. Consequently, it can be stated that no more toxic degradation products were formed during the biodegradation process compared to the starting material. Phenotypic examination of the embryos injected with abiotic samples revealed the following changes compared to the control: body shortening, mild pericardial edema, yolk absorption disorders, and closed swim bladders.

4. Conclusons, proposals

The aim of the present study was to identify, isolate, and investigate the biodegradation potential of bacteria capable of degrading the active pharmaceutical ingredients (APIs) diclofenac, ibuprofen, and carbamazepine from the starting biofilm. The initial biofilm had already served as the basis for the enrichment of various bacterial communities (Benedek et al., 2016; 2018; 2020; 2021).

From the enriched cultures supplemented with the three pharmaceutical substances, a bacterial strain collection of 31 members was created (10 DIC, 9 IBU, and 12 CBZ), where the individual strains may potentially be capable of degrading diclofenac, ibuprofen, or carbamazepine. The bacterial strain collection includes bacterial taxa that showed high relative abundance or an increase in their relative abundance during the enrichment process. These strains were successfully cultured. During the enrichment period, the dominance of two genera, *Pseudomonas* and *Methyloversatilis*, was observed; however, the increase in their relative abundance during this period was negligible.

In the diclofenac enrichments, the following genera were identified, which either showed high relative abundance or a notable increase in their relative abundance: *Azospirillum*, *Rhodanobacter* (Navrozidou et al., 2019), *Ferrovibrio, Hydrocarboniphaga, Zavarzinia, Sphingopyxis* (Zhou et al., 2013), and *Prosthecobacter*. In the ibuprofen-enriched cultures, the following genera were present: *Starkeya* (Bessa et al., 2017), and *Rhodococcus*. In the carbamazepine enrichments, the genera showing the greatest increase in relative abundance were *Pseudonocardia*, *Sphingopyxis, Rhodococcus*, and *Achromobacter* (Liang et al., 2021).

I also determined the biodegradation capacity of the 31 bacterial isolates from the collection, capable of degrading diclofenac, ibuprofen, and carbamazepine. After mapping the degradation capabilities, I created a bacterial consortium that can effectively degrade all three pharmaceutical substances. The measurements were conducted using high-performance liquid chromatography (HPLC) analytical methods. The results showed that only a few isolates were capable of degrading the investigated pharmaceutical substances. According to the experiments, the *Stenotrophomonas humi* DIC_5 isolate was able to biologically

transform diclofenac in the presence of glucose (3.0 g/l) during a two-week incubation period. The highest ibuprofen degradation was produced by the *Rhodococcus daejeonense* IBU_18 isolate, using a medium supplemented with yeast extract. The most intense carbamazepine degradation was observed for the *Brevundimonas bullata* CBZ_3 and *Nocardioides carbamazepini* CBZ_1 isolates, in the presence of glucose (3.0 g/l) and yeast extract (1.0 g/l). Literature has also reported that carbon sources can provide energy to support cell growth, thus facilitating the degradation of microcontaminants (Assiaoui et al., 2017).

Based on the results from experiments conducted in different media, the following degradation efficiencies were observed: In BHB mineral medium, with the presence of glucose and yeast extract, 72% diclofenac, 100% ibuprofen, and 30% carbamazepine biodegradation were observed. In a sample of the Rákos- patak water, with the addition of glucose, yeast extract, and sodium acetate, 86% diclofenac, 100% ibuprofen, and 25% carbamazepine degradation were detected. In the sewage effluent, supplemented with the same nutrients, 30% diclofenac and 79% ibuprofen biodegradation were observed, but no significant carbamazepine elimination occurred, likely due to other carbon sources in the sewage, which provided more accessible carbon and energy, thus interfering with the degradation of the pharmaceutical substances.

The CBZ_1 strain isolated from the medium supplemented with carbamazepine as the sole carbon and energy source provided an opportunity for the description of a new bacterial species. Based on phenotypic, chemotaxonomic, phylogenetic, and genomic analyses, the isolate represents a new species belonging to the *Nocardioides* genus. Consequently, the new species was named *Nocardioides carbamazepini*.

The *Stenotrophomonas humi* DIC_5 bacterial strain was able to transform diclofenac at an initial concentration of 1.5 mg/L. Literature also supports that members of the *Stenotrophomonas* genus play an important role in the elimination of pollutants such as polycyclic aromatic hydrocarbons (Ryan et al., 2009), and play a significant role in the nitrogen and sulfur cycles, often forming close relationships with plants, helping them grow and providing protection against pathogens (Heylen et al., 2007). However, this is the first known evidence of diclofenac biotransformation by *Stenotrophomonas humi*. During the

process, a transformation product, nitro-diclofenac, was identified, which was confirmed by mass spectrometry methods. A research group found that ammonium nitrate present in sewage is related to microbial nitrification (Praskova et al., 2011). According to the investigations, the decrease in diclofenac concentration occurred simultaneously with the increase in nitro-diclofenac concentration starting from day 7. Ecotoxicological tests with A. fischeri and zebrafish embryos showed that diclofenac and nitro-diclofenac were not toxic to the test organisms at the applied concentrations.

5. New scientific results

1. Creation of Enrichment Conditions for Biodegradation: I successfully developed enrichment conditions that were suitable for enriching, identifying, and in many cases isolating bacteria capable of biologically degrading diclofenac, ibuprofen, and carbamazepine from the medium I used. My findings were published in the *Environmental Science and Pollution Research* journal (Pápai et al., 2023).

2. Identification of potential drug-degrading isolates and formation of a bacterial consortium: potential drug-degrading isolates were identified, and a bacterial consortium was formed. In the presence of co-metabolic substrates, the developed bacterial consortium effectively degraded both diclofenac and ibuprofen in natural water samples and wastewater effluents.

3. Discovery of a novel *Nocardioides* species: I successfully isolated a previously unknown bacterial species belonging to the *Nocardioides* genus. Based on international guidelines for species description, it was determined that the bacterium, capable of degrading ibuprofen, isolated from carbamazepine-modified selective enrichment cultures, represents a new species within the *Nocardioides* genus, which has been named *N. carbamazepini*. The result was published in the *Systematic and Applied Microbiology* journal (Benedek et al., 2022).

4. Extensive study of the diclofenac-degrading capability of *Stenotrophomonas humi* DIC_5: The diclofenac-degrading ability of the *Stenotrophomonas humi* DIC_5 strain was extensively investigated using modern, high-end analytical methods. One of the intermediate products of the biodegradation process, nitro-diclofenac, was identified. Through the use of two ecotoxicological methods, it was determined that the metabolic products generated during degradation were not more toxic than the original compound. The scientific result was published in the *Applied Microbiology and Biotechnology* journal (Pápai et al., 2024).

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6. Publications

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Pápai, M., Táncsics, A., Hussein, D., Maróti, G., Kriszt, B., Menashe, O., Benedek, T. Screening for diclofenac, ibuprofen and carbamazepine degrading bacteria selectively enriched and isolated from subsurface biofilm- 6th Central European Forum for Microbiology, 2021. 10. 13-15. Abstracts p. 103.

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