PhD Theses

INTENSIFICATION OF PHARMACEUTICAL RESIDUE REMOVAL IN WASTEWATER TREATMENT BY COMETABOLISM AND HIGH-ENERGY IONIZING RADIATION

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I. Introduction

The technological steps of wastewater treatment are currently classified into three generally widespread stages. **Stage 1** is **mechanical treatment**, during which mainly **coarse, undissolved pollutants** are removed. **Stage 2** is **biological treatment**, during which the remaining **suspended organic matter and dissolved and colloidal organic pollutants** are broken down and **biologically transform**ed. The two basic forms of this are **suspended (activated sludge)** and **fixed-bed ("biofilm")** systems. **Stage 3** is a set of **physico-chemical and biological post-treatment technologies** (Tchobanoglous et al. 2014). **Organic micropollutants** (antibiotics, plant protection products etc.) cannot, or can only partially be removed from wastewater with conventional wastewater treatment processes. **Stage 4** aims to reduce the spread of these pollutants.

The rapidly developing high-efficiency oxidation processes (Advanced Oxidation Process, AOP) are promising methods that destroy organic molecules with the help of free radicals and strong oxidants produced through various in situ radical generation processes, thereby promoting their degradation. Ozone treatment is currently the most common. AOP methods also include methods using high-energy ionizing radiation (Co-60 gamma or accelerated electrons) (Wang et al. 2022).

During the **metabolic biological degradation process**, organic molecules serve as a **growth substrate for cell growth and reproduction of microorganisms**, and on the other hand, they **induce the metabolic processes of enzymes**. A significant part of micropollutants, such as **pharmaceutical residues cannot function as the only source of carbon or energy (growth substrate)** (Tran et al. 2013). The substrate-binding site of enzymes is often "loose". This means that it does not chemically bind the substrate tightly, thus, compounds whose chemical structure is similar to that of the substrate have the opportunity to bind as alternative substrates on the enzyme. The phenomenon of **cometabolism** is based on this. Cometabolism is a random process where the enzyme actually makes a mistake and misses its natural substrate. **The cell cannot utilize the inadequate substrate (cosubstrate), but the enzyme performs a chemical reaction on it.** However, for this process, the presence of some growth substrate is essential, which is the natural substrate of the enzyme that commits the error. Thus, cometabolism is a biotransformation process where the transformed material is neither suitable for energy production nor for building the cell structure (Hult and Berglund 2007).

The significance of **heterotrophic cometabolism** by **denitrifying bacteria** has not yet been explored in detail. The functional group of denitrifying bacteria includes **methylotrophic bacteria**, whose **methane monooxygenase (MMO) enzyme** has been proven to be important in cometabolism in other areas (Márialigeti 2013). **Their importance in wastewater treatment has not yet been explored. Furthermore, the effectiveness and prevalence of cometabolism in wastewater treatment (the group of pharmaceuticals for which it works) is not yet clear.**

In the case of the less researched denitrification cometabolism, the presence of an adequate amount of **readily biodegradable organic substrate (rbCOD)**, which is not available in a significant proportion of wastewater treatment plants, is essential (Tardy et al. 2012). During the **thickening and dewatering steps** that are part of the sludge treatment, several types of **sidestreams (supernatants, centrates)** are generated. During the hydrolysis taking place in an anaerobic environment (e.g. digesters), many simple organic molecules (**volatile fatty acids, VFA**) are formed, which can be **easily utilized by denitrifiers**. These acids cannot be derived directly from the enzymatic breakdown of complex, large molecules (proteins, cellulose, etc.), but rather the metabolic products of the biomass undergoing the breakdown (acid formation). The volatile acid fraction is a part of rbCOD and contains molecules such as **acetic acid, propionic acid.** In general, it contains molecules with 2-6 carbon atoms (**C2-C6**) (Yuan et al. 2011). **The use of leachate to intensify cometabolism is an unexplored area.**

During their operation, AOP also provide a source of simple organic molecules, thus supporting heterotrophic cometabolism. By-products vary depending on the structure of the original compound: **aldehydes, alcohols, carboxylic acids, inorganic compounds** (Nawrocki et al. 2003). AOP has a dual effect. On the one hand, they destroy the structure of pharmaceutical molecules, thus making the molecules accessible to microorganisms by applying the appropriate dose. This provides an opportunity for biotransformation (Takács et al. 2022). On the other hand, rbCOD suitable for cometabolism is formed during the application of AOP. The effect of AOP on biodegradability is an area studied by a small group of researchers, but **the effect of AOP for intensifying cometabolism has not yet been addressed**.

Harmful by-products are also formed during the operation of AOP. Perhaps the most important is **H_2O_2**, from which oxygen is released during biotic (catalase enzyme) and abiotic decomposition. The H_2O_2 molecule by releasing O_2 causes errors in laboratory tests based on the measurement of oxygen concentration (Lee et al. 2011). So far, **this effect was only investigated for COD measurements, but for BOD and respiratory tests.**

II. Objectives

During my research, I examine the possibilities of **intensifying cometabolism in wastewater treatment**. To do this, I assess the **short-term and long-term effects** of this phenomenon in the biotransformation of pharmaceutical molecules. I map the short-term effects with **respiratory tests**, and the long-term ones with **BOD measurements**.

Cometabolism is a proven and extensively studied field for nitrifying bacteria, so I focus on another large group of microorganisms, the bacteria that consume organic molecules (**heterotrophs**). Among them, I give priority the less studied group in wastewater treatment, the **methylotrophic bacteria**, which exist in greater numbers in denitrifying systems fed with methanol as an external source.

For **heterotrophic cometabolism**, I mainly use simple organics (organic acids, alcohols) as substrates. Due to their **significant VFA content**, sidestreams from different points of the wastewater treatment system can support cometabolism. Therefore, I test the effect of several types of **sidestreams (supernatants, centrates, filtrates, decants)** on cometabolism. I also use pre-settled wastewater, which also has a significant VFA content, as a carbon source for the experiments. In the case of leachate and pre-settled wastewater, I use their distillate, which only contains volatile acid components.

In the case of 14 pharmaceutical compounds, the **prevalence of cometabolism** (the range of pharmaceuticals for which it works) was assess in wastewater treatment, and the **effectiveness of cometabolism** in the case of diclofenac (methylotrophic biofilm).

I investigate the **effect of AOP on the biodegradability of several pharmaceuticals** that were still missing from the literature (oxacillin, cloxacillin, tetracycline and chlortetracycline). For modeling I use 0,5; 1; 2; 4 kGy dose of γ-radiolysis. I test the **cometabolism-stimulating effect of AOP** in the case of sulfamethoxazole and diclofenac (cosubstrates), with the addition of several substrates (acetic acid, methanol, ethylene glycol). I assess **the effect of hydrogen peroxide formed during the application of AOP on BOD measurments and respiratory tests** in detail, since they can significantly influence the results of the tests.

III. Experimental methods

I investigated the **short-term effects** of cometabolism and AOP modeled with **γ–radiolysis** on the biological transformation of pharmaceuticals using **respiratory tests**. I checked the **long-term effects** with 5-day **biochemical oxygen demand (BOD_5) measurements**. I monitored the **quantitative change in the biomass of the inoculum during the respiratory tests and BOD measurements** by determining the Heterotrophic Plate Count (MSZ EN ISO 6222:2000).

The γ–radiolysis experiments were carried out with a Co-60 γ–radiation source of 1.8 PBq activity, model marked SLL–01, owned by Izotóp Intézet Kft., with 0.1 mM pharmaceutical solutions, using a dose of 0.5; 1; 2; 4 kGy, at room temperature. The hydrogen peroxide formed during the treatment was removed from the samples using manganese(IV) oxide (MnO_2).

I monitored the qualitative and quantitative changes in the organic matter content resulted by γ–radiolysis with a **chemical oxygen demand test** (**COD**, MSZ ISO 6060:1991) and **total organic carbon content (TOC)** measured by a LiquiTOC (Elementar) element analyzer. To set the appropriate concentration of activated sludge and biofilm used as an inoculating agent during the biological tests, I applied **total suspended solid content** (MSZ 260-3:1973) measurements. I assessed the community structure of the inoculants by **microscopic analysis (Zeiss Jenaval microscope)**. I used **high-performance liquid chromatography (HPLC)** to monitor pharmaceutical concentrations, the measurements were carried out by Bálint Analitika Mérnöki Kutató és Szolgáltató Kft. I measured the VFA concentration and composition of the sidestreams and pre-settled wastewater (primary clarifier effluent) using **gas chromatography** (**GC**, Agilent 6890N device).

For the minireactor experiments, the series of measurements was made in 1000 mL beakers.

The **change in antibiotic resistance due to γ-radiolysis** was investigated using an **agar diffusion test**, for which the *Staphylococcus aureus* strain (B.01755) was obtained from The Collection of National Institute of Agricultural and Industrial Microorganisms (NCAIM).

I performed the experiments with the following pharmaceuticals: erythromycin, oxacillin, trimethoprim, piperacillin, cloxacillin, oxytetracycline, chlortetracycline, doxycycline, sulfamethoxazole, diclofenac, ibuprofen, clofibric acid, carbamazepine. The following simple organic compounds were used as substrates (GS = growth substrate): methanol, ethanol, formic acid, acetic acid, propionic acid, butyric acid, valeric acid, caproic acid, lactic acid, ethylene glycol.

IV. New scientific results, thesis points

1. **I determined that the number of microorganisms used in the respiration tests (10^4 CFU cm^{-3}) exceeds the initial number of microorganisms in the BOI_5 measurement (10^2 CFU cm^{-3}) by approximately two orders of magnitude, and this does not change significantly during the 3-hour test. In contrast, at the end of the five-day BOI measurement, the number of microorganisms is four orders of magnitude higher (10^6 CFU cm^{-3}) than at the beginning.**

The starting Heterotrophic Plate Count (HPC) of the respiration tests (the number of heterotrophic aerobic bacteria growing at 22 °C) was 6.4 x 10^4 CFU cm^{-3} (72000 / 54000 / 66000 cm^{-3}; standard deviation 11.7%), which during the 3-hour tests did not change detectably 6.5 x 10^4 CFU cm^{-3} (69000 / 71000/ 56000 cm^{-3}; standard deviation 10.2%). The initial HPC of BOI_5 tests was 6.4 x 10^2 CFU cm^{-3} (650 / 690 / 580 cm^{-3}; standard deviation: 7.1%). During the 5-day tests, the bacteria grew on the consumed nutrients and the test ended with an HPC value 4.3 x 10^6 CFU cm^{-3} (3.1 x 10^6 / 3.9 x 10^6 / 5.9 x 10^6 cm^{-3}; standard deviation: 27.4%). *(Publication 1)*

2. **I proved that the H_2O_2 formed during γ-radiolysis distorts the BOD values through its toxicity and the liberated oxygen.**

H_2O_2 distorts the BOD value in two ways. On the one hand, the toxic compound increases the adaptation time required for the growth of more tolerant groups of bacteria. On the other hand, the maximum of the BOI value decreases because the oxygen released from the hydrogen peroxide results in a negative error as it counteracts the O_2 consumption. *(Publication 2)*

3. **I verified that in activated sludge technology, heterotrophic cometabolism significantly contributes to the biological degradation of sulfamethoxazole (SMX) and diclofenac (DCF).**

In our studies, we used active pharmaceutical ingredients sulfamethoxazole (SMX) and diclofenac (DCF). The evaluation is based on the quotient of cometabolic respiration and substrate respiration, which in case of a value greater than 1 confirms the functioning of cometabolism. Depending on the substrate used, this ratio ranged from 2.41 to 10.75 for sulfamethoxazole and 1.29 to 18.22 for diclofenac. *(Publication 3)*

4. **I proved that the efficiency of cometabolism in the case of sulfamethoxazole and diclofenac (as co-substrate) depends on the type of substrate.**

The ratio of cometabolic respiration to substrate respiration (expressed in OUR) varied depending on the substrate used. During experiments with sulfomethoxazole cosubstrate, this ratio was as follows: valeric acid (10.75) > acetic acid (9.95) > butyric acid (7.01) > caproic acid (4.90) > ethanol (4.87) > ethylene glycol (3.68) > lactic acid (3.39) > methanol (3.29) > propionic acid (2.87) > formic acid (2.41). For diclofenac: formic acid (18.22) > acetic acid (7.35) > ethylene glycol (6.43) > propionic acid (4.31) > valeric acid (2.67) > lactic acid (2.54) > caproic acid (2) .05) > butyric acid (1.97) > ethanol (1.82) > methanol (1.29). *(Publication 3)*

5. **I proved that in the case of cloxacillin, oxacillin, tetracycline, chlortetracillin and diclofenac, their biodegradability is greatly improved by AOP treatment (high-energy ionizing radiation, radiolysis).**

For all tested pharmaceuticals, a significant increase in respiration intensity (oxygen uptake rate, OUR) was achieved even with a dose of 0.5 kGy (Cloxacillin (CLX): 0.33; Oxacillin (OXA): 0.24; Tetracycline (TCN): 0.25; Chlortetracycline (CTC): 0.24 mg O_2 dm^{-3} $hour^{-1}$), then the OUR increased with the dose and reached the maximum at 4 kGy (CLX: 0.70; OXA: 0.53; TCN: 0.44; CTC: 0.69 mg O_2 dm^{-3} h^{-1}). In the case of DCF, the 0.5 kGy dose did not result in any change, but already at 1 kGy an increase in oxygen consumption of 0.14 mg O_2 dm^{-3} h^{-1} was measured. *(Publication 3, 4 and 5)*

6. **I proved that the biodegradability of sulfamethoxazole and diclofenac is more effective when radiolysis and cometabolism are used together than when the two methods are used separately.**

The efficiency of cometabolism increases due to irradiation. The increase in OUR (oxygen uptake rate) compared to the non-irradiated sample for a dose of 0.5 kGy, in the order of acetic acid, ethylene glycol, methanol; SMX: 0.42 – 0.88 - 2.24 mg O_2 dm^{-3} h^{-3} our^{-1}; DCF: 0.20 – 1,37 – 0,60 mg O_2 dm^{-3} $hour^{-1}$). In the case of SMX with methanol, the increase is not significant, so irradiation did not significantly increase the efficiency of cometabolism in all cases. *(Publication 3)*

V. Utilization possibilities of the results

During our research, we proved that cometabolism is probably a very widespread phenomenon in wastewater treatment technology. This phenomenon can be enhanced by internal sources, by sewage alone (mechanically treated) and sidestreams of various origins. Simple organic compounds added as external sources (e.g. methanol) are also effective means of intensification. γ-radiolysis, which is often used to model AOP, increased both the biodegradation of pharmaceutical molecules and the efficiency of cometabolism. Thus, it helped the biotransformation of pharmaceuticals in both metabolic and cometabolic pathways. The practical application of the method would significantly reduce the release of active pharmaceutical ingredients from sewage plants, and would also destroy bacteria.

Biologically, only a few micropollutants can be broken down only metabolically. In this case, these compounds can really be utilized by the bacteria (in the form of energy or incorporated as carbon). This is a rare phenomenon, not typical in the microorganism community of wastewater treatment systems. The molecular structure of most pharmaceuticals is probably accessible to bacteria through random enzyme reactions (cometabolism). Although this only means partial oxidation and not the complete mineralization of the molecule, the biological transformation of these complex molecules can begin.

Thus, we can remove micropollutants without major investments, if we are able to work with groups of bacteria that contribute to cometabolism (e.g. nitrifiers, methylotrophs). The more times an enzyme performs the same process, the higher the probability of random reactions (cometabolism). Nitrifying bacteria can be induced to cometabolism with a long residence time, and methylotrophic bacteria, which also contribute to denitrification, can be induced to cometabolism by adding methanol. However, activated sludge is generally able to do this by utilizing waters rich in simple organic acids (primary clarifier effluent, sidewaters).

The introduction of advanced oxidation processes can be beneficial from several points of view, despite the increased costs. They are able to destroy the structure of non-biodegradable or hard-to-degrade organic molecules to an extent dependent on the applied dose. The simple organic and inorganic molecules that are formed can already be used by bacteria, and they also induce cometabolism, because they serve as a driving force for the operation of enzymes that can randomly change the structure of pharmaceutical molecules.

VI. References

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VII. Publications related to theses

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