IOSUD - "Dunărea de Jos" University of Galati

Doctoral School of Mechanical and Industrial Engineering

DOCTORAL THESIS

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A research regarding the transport of industrial pollutants in aquatic ecosystems

PhD student,

Valentina - Andreea CĂLMUC

Scientific Supervisor,

Prof. univ. dr. ing. Puiu-Lucian GEORGESCU

Series I4 Industrial Engineering No. 98

GALATI

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President	**Prof. Ph.D. Eng. Eugen-Victor-Cristian RUSU** "Dunărea de Jos" University of Galați
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With great respect,

Ph.D. Valentina-Andreea Călmuc

Contents

Keywords: industrial pollutants, aquatic ecosystems, heavy metals, pharmaceutical compounds, lower Danube river basin, ichthyofauna, artificial intelligence, photodegradation

Introduction

The present doctoral thesis entitled "A research regarding the transport of industrial pollutants in aquatic ecosystems" addresses a state-of-the-art topic in the field of Industrial Engineering, which aims towards assessing the industry's impact on the aquatic environment using advanced analysis techniques.

Contamination of the aquatic environment is a serious consequence generated by the discharge of industrial pollutants. Water is one of the most valuable natural resources available to mankind and it is under constant threat. The fast population growth generated increased demands that could only be achieved through rapid industrialization [1]. At the same time, the industry has largely contributed to generating industrial wastes that can manifest toxic effects on the environment through contaminating water, air, and soil.

The present research uses a methodological and structural approach, different from the classical form. Before starting the actual research for the doctoral thesis, which focused more on the analysis of pharmaceutical compounds in the aquatic environment, I was directly involved in an extensive study that aimed to evaluate the presence of heavy metals in the main area of the hydrographic basin of the Lower Danube River, through the years of 2018 and 2019. Thus, the thesis structure also highlights my evolution from relatively simple experiments to integrated experimental systems using world-class complex analytical equipment.

Thus, in this doctoral thesis, two types of pollutant classes were studied, which are mainly generated from the industrial activities. In the first part of the thesis, the impact of the industry on the level of heavy metals contamination of the sediments collected from the lower basin of the Danube River was studied, since the studied area is exposed to important sources that have the potential for pollution with this type of contaminants. Further on, an important part of the doctoral thesis focuses on the assessment of the presence of some classes of pharmaceutical substances in the aquatic environment. This manifested as a consequence of the pharmaceutical industry being one of the fastest-growing sectors in the world economy, due to the high demand in the market. Through this fact, pharmaceutical derivatives are constituted into major consumer products and, as a consequence, become waste found in the environment [2].

All of the above arguments stand as the basis for writing the present doctoral thesis, which intends to contribute significantly to the need for continuous monitoring of aquatic ecosystems and the impact of industrial activities on the environment.

The main objectives of the thesis are as follows:

• Conducting a study regarding the transport of heavy metals in surface sediments collected from the riparian area of the industrial cities Brăila, Galați, and Tulcea;

• Assessing the impact of industrial activities on sediment pollution with heavy metals and determining their toxic potential using quality indices;

• Studying a class of emerging pollutants (pharmaceutical compounds) insufficiently investigated in the UNESCO World Heritage area (Danube Delta);

• Optimizing extraction methods and analysis of pharmaceutical compounds from environmental samples;

• Analysis of the transport of pharmaceutical substances in the water of the Danube River from the most complex area studied so far, which includes different types of surface aquatic ecosystems (fluvial, pre-Deltaic, and Deltaic);

• Evaluation of the temporal variation of the presence of pharmaceutical compounds in the water of the Danube River;

• Studying statistical relationships between different pharmaceutical compounds analyzed from water samples;

• Quantification of pharmaceutical substances in different fish species native to the Danube River basin;

• Realization of the first integrated water-sediment-fish study in the lower Danube River basin regarding the concentration levels of pharmaceutical compounds;

• Testing solutions for the degradation of some classes of pharmaceutical substances under the influence of UV-C radiation that can be adapted into treatment plant facilities;

The novelty of the doctoral thesis is highlighted by the complex approach of several original studies that focused on evaluating the impact of anthropogenic activities on the quality of aquatic ecosystems from the Lower Danube River Basin, among which are the following:

• The calculation of quality indices for the sediment samples was carried out for the first time in the monitoring sites selected in this thesis;

• Studying the transport and temporal variation of an insufficiently studied class of industrial pollutants (pharmaceutical compounds) in the Romanian sector of the Danube River;

• Studying the effects generated by the COVID-19 pandemic on the level of contamination of surface water with different classes of pharmaceutical substances in an extensive and complex area from the Lower Danube River Basin;

• Development of a predictive tool to improve the sustainability of the monitoring activity of pharmaceutical compounds in the water from the Lower Danube River Basin;

• The only research on the behavior and transport of some classes of pharmaceutical substances in the natural environment through the integrated approach of water-sediment-biota analysis;

• Innovative testing using an engineering method for the elimination of some pharmaceutical classes which involves reduced resource consumption;

The present doctoral thesis is structured in 5 chapters, an introduction, and a bibliography which was consulted to achieve the most complex approach to the presented studies.

The introduction of the thesis includes details related to the motivation for choosing the research topics addressed, the novelty that this thesis brings, and the main objectives that were the basis of its realization.

Chapter 1 - Environmental pollution with industrial pollutants. The state-of-the-art research summarizes the importance of monitoring the transport of industrial pollutants present in aquatic ecosystems and the general aspects of the studied contaminants. This chapter includes relevant studies in which these classes of industrial pollutants were studied.

Chapter 2 - A study on the transport of heavy metals in sediments from the surface layers of the lower basin of the Danube River. This second chapter also includes a study based on sediment samples collected throughout different seasons from the Danube River, to assess the impact of industrial activities on the level of contamination with heavy metals.

Chapter 3 - Studies on the presence of pharmaceutical compounds in aquatic ecosystems, including a various research which aimed at the identification and quantification of some classes of pharmaceutical compounds from biotic and abiotic samples collected from the natural aquatic ecosystem. The first study presents the spatio-temporal variation of the concentrations of pharmaceutical substances identified in the water samples collected from

the lower Danube River Basin and the statistical relationships manifested between them. Within the other sub-chapters, the ability of these contaminants to accumulate in sediments and fish species collected from the Danube River was approached.

In **Chapter 4** entitled Methods of degradation of pharmaceutical compounds, different laboratory experiments are presented that were based on testing a method of degradation of different classes of pharmaceutical substances that can be adapted to wastewater treatment plants with a minimum consumption of resources. This method involved the exposure to UV-C radiation of solutions with concentrations of pharmaceutical substances similar to those found in wastewaters.

Chapter 5 - Final conclusions, personal contributions, and future research directions summarize the most important results obtained from the studies which were the basis of the present doctoral thesis, through a major personal contribution. Also, in this chapter, the subsequent research topics that will be developed are presented, such as this doctoral thesis represents an important milestone for the research community.

Chapter 1. Environmental pollution with industrial pollutants. Current state of research

1.1 Types of industrial pollutants and the importance of monitoring them

The exponential growth of industrialization has led to environmental degradation [3]. The aquatic environment collects a wide variety of toxic industrial contaminants, including: heavy metals, solvents, fertilizers, pesticides, household products (detergents, paints), petroleum hydrocarbons, personal care products, pharmaceuticals for human and veterinary use, microplastics [4]. Lately, emphasis has been placed on the importance of monitoring emerging pollutants in aquatic ecosystems, including pharmaceutical compounds and microplastics, which have been the subject of several scientific papers that I authored or co-authored [5–7].

The monitoring of industrial pollutants in aquatic ecosystems is an important topic, as these have toxic potential on aquatic biota, but also on human health [8–9].

1.2 Pollution of the aquatic environment with heavy metals

Heavy metals are one of the classes of industrial pollutants that are found in the aquatic environment. Typically, 23 heavy metals have been identified in the environment that, in high concentrations, can become toxic and dangerous [10]. Among the most toxic metals found in the environment are the following: Cd, Ni, Zn, Pb, Cu.

1.2.1 Sources of heavy metal pollution

Among the main sources of heavy metal pollution in the aquatic environment: industry, municipal waste, wastewater, pesticides, fertilizers, fossil fuel burning, navigation activities, mining, storage spaces in the river's area of influence [11–13], cardiovascular diseases

1.2.2 Toxicity of heavy metals

In normal concentrations, some heavy metals, such as: Ni, Cu, Zn, are vital for the human body. However, when the maximum permissible concentrations are exceeded, heavy metals become toxic to the environment and human health, and can cause the following diseases:neuronal, hematological, and renal disorders [14], cardiovascular diseases, respiratory system disorders and genetic abnormalities [15,16].

1.3 Pollution of the aquatic environment with pharmaceuticals

The high consumption of drugs correlates with their presence in the aquatic environment, which is why it is very important to monitor this class of industrial pollutants. Statistics made worldwide in 2023 place the pharmaceutical industry on the 4th place in the top of the most financially profitable industries, which implies the existence of a high consumption of drugs [17]. Pharmaceutical compounds end up discharged into the aquatic environment through manufacture, consumption, excretion, but also through improper disposal

of expired or unused products. The category of emerging pollutants includes substances that present significant variations in behavior and toxicity and that involve the application of special techniques in the process of their treatment/remediation [18]. The pharmaceutical market comprises an enormous number of compounds, only in the European Union a number of approximately 3000 pharmaceutical compounds being frequently used, a number which is constantly increasing worldwide. Although pharmaceutical compounds are found at the level of trace amounts in the medium (ng/L), they have the potential to be toxic to flora and fauna due to their continuous discharge and the fact that they can interact with each other [19].

1.3.1 Sources of environmental pollution with pharmaceutical compounds

Pharmaceutical compounds and their metabolites are continuously released into the environment from point and diffuse sources. The main sources of contamination with emerging pollutants, implicitly also with pharmaceutical products, are: industrial wastewater, surface runoff from agricultural land and animal farms, leachate from landfills, domestic water from hospitals and homes. Discharges from industrial production also contribute to the contamination of the environment with pharmaceuticals due to the inadequate wastewater treatment process. Of all the sources of pollution of the aquatic environment with pharmaceutical substances, the one with the highest contribution is represented by urban domestic effluents, followed by effluents from hospitals and excretions from animal farms [20].

1.3.2 Risks and toxic effects of pharmaceuticals in the environment

The amount of drugs consumed, the physicochemical properties and ecotoxicity are important factors in the assessment of the environmental risks caused by pharmaceutical substances. Studies have shown that microorganisms, flora and fauna are affected by the presence of pharmaceutical residues in low concentrations. Due to the complicated behavior of different mixtures of pharmaceutical substances that are formed in the environment, pharmaceuticals can have harmful effects on organisms even when they are present in very low concentrations. Their continuous discharge into the aquatic environment can have a negative impact on living organisms and the environment. For example, it can change the behavior of fish, influencing their reproduction, feeding and aggression levels [18].

In a review study co-authored, the effects that different classes of pharmaceutical substances have on oxidative stress in fish were specified. For example, long-term exposure to antibiotics reduces antioxidant defense activity (catalase, glutadione), and contamination with anti-inflammatories showed a clear decrease in the activity of antioxidant enzymes in the fish species *Cyprinus carpio* [21].

1.3.3 Legislative regulations regarding pharmaceutical compounds detected in the environment

On the subject of environmental pollution by pharmaceutical compounds, Directive 2013/39/EU refers for the first time to the contamination of water and soil with pharmaceutical waste. At European level, there is a continuously updated watch list of pollutants with toxic potential. The pharmaceutical compounds that have been included in the latest version of the watch list under Article 8b of Directive 2008/105/EC are: clindamycin, ofloxacin, sulfamethoxazole, trimerprim, venlafaxine, o-desmethylvenlafaxine, metformin and its transformation product guanyluree [22].

Chapter 2. Study on the transport of heavy metals in sediments from the surface layers of the lower basin of the Danube River

2.1 Objectives of the study

The study conducted by Calmuc et. to, 2021 [23] assessed the transport of heavy metals in surface sediments (the surface layer of the riverbed) in the lower basin of the Danube River, the influence of anthropogenic activities on the level of heavy metal contamination in the sediment, as well as the potential risks to which the aquatic ecosystem is exposed. In order to achieve these objectives, various specific pollution indices important in the assessment of the quality of water bodies were used and tested, namely: Geo-accumulation Index (Igeo), Contamination Factor (CF), Pollution Load Index (PLI) and Potential Ecological Risk Index (RI).

2.2 Study area

15 sampling stations were selected to assess the transport of heavy metals in sediments and the level of sediment contamination with heavy metals (Figure 2.1) depending on the existing pollution sources located along the Danube River between km 180 and km 60. In this area, the Danube River crosses 3 major cities in Romania (Brăila, Galați and Tulcea) with a large number of inhabitants and significant industrial activity: (shipyards: Damen Galați, Vard Brăila, Vard Tulcea, Navrom Galați - naval transport company, Liberty Galați - cast iron and steel industry). Monitoring and evaluating the quality of surface sediments is important because this perimeter includes the pre-deltaic and deltaic area with an impact on the Danube Delta biosphere reserve, unique in Europe, and on the multitude of lakes and canals that host a wide variety of fauna and flora. Sediment samples were taken monthly during two different seasons, namely autumn 2018 and spring 2019.

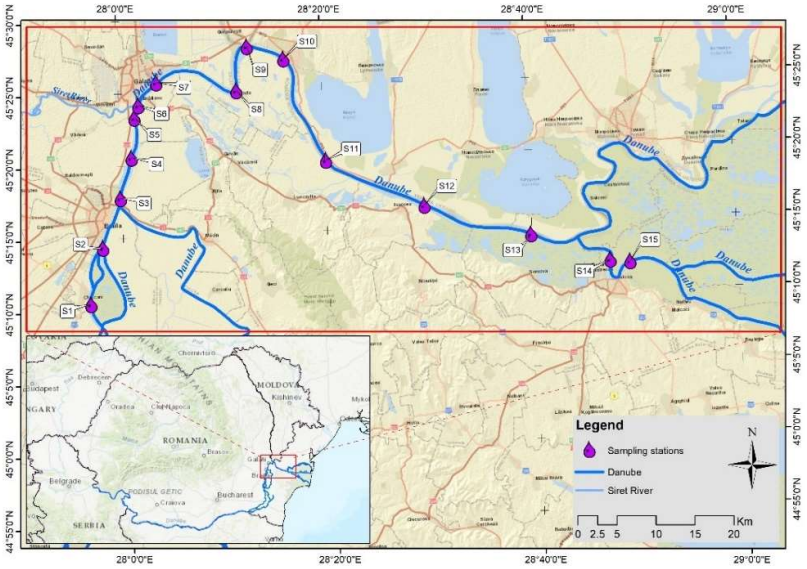


Figure 2.1 Sampling stations along the Lower Danube

The stations from which the sediment samples were taken were coded as follows (Figure 2.1):

S1 - Chiscani locality;
S2 - ferry crossingBrăila;
S9 - Prut;
S10 - Grindu locality;

S3 - ferry crossing1 Brăila;
S4 - insula Chici;
S5 - Danube Socket;
S6 - Siret;
S7 - Libertatea Restaurant;
S8 - Cat's Elbow;
S11 - Luncavița locality;
S12 - Isaccea locality;
S13 - Somova locality;
S14 - Vard Shipyard
S15 - Tulcea locality;

2.3 Materials and methods

The first 10 cm of sediment were taken from each station using a Van Veen dredger (KC Denmark A/S, Silkeborg, Denmark), the samples being preserved in polyethylene containers. In the preliminary step, the sediment samples were dried at 105 °C until they reached a constant mass, were mortised and sifted with a 125 μm sieve.

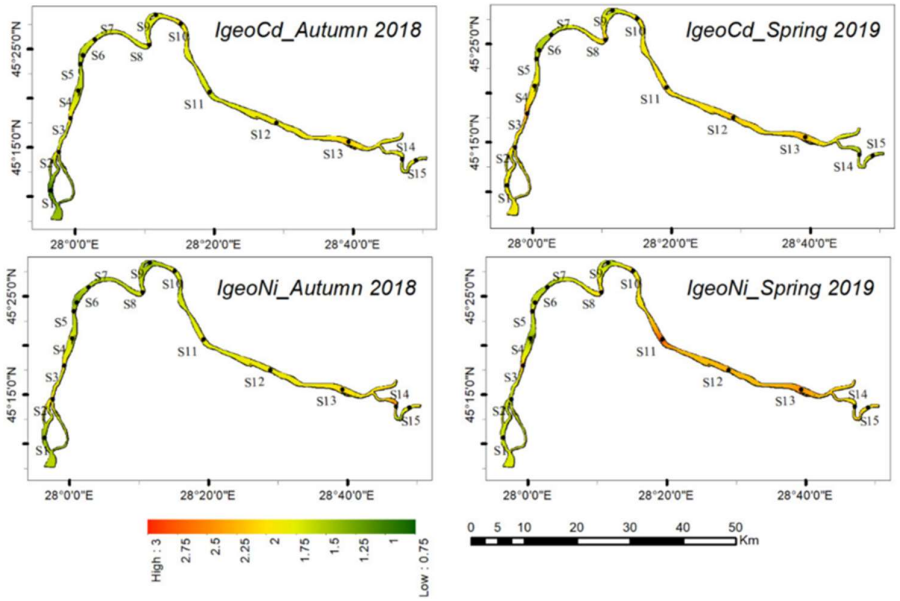
The mineralization of the samples was performed using the Anton Paar microwave digestion system. After the sample preparation stage, the concentrations of heavy metals (Pb, Cu, Cd, Zn, Ni) were determined in accordance with the SR EN ISO 17294–2, 2005 standard. The analysis was performed using the ICP-MS (Inductively Coupled Plasma Mass Spectrometry) Perkin Elmer Elan DRC-e equipment (PerkinElmer LAS (UK) Ltd, Seer Green, England, UK) provided by the National Institute for Research and Development "Danube Delta" – Tulcea [23].

In order to evaluate the transport of heavy metal concentrations in the sediment from one station to another, and their toxic potential, different indices were calculated and represented in the form of pollution distribution maps.

2.4 Results and discussions

2.4.1 Spatio-temporal distribution of the Geo-accumulation Index (Igeo)

Based on the Igeo values obtained in the autumn of 2018, a moderate to strong cadmium pollution can be observed in the stations: S3, S10 and S13 (Figure 2.2).



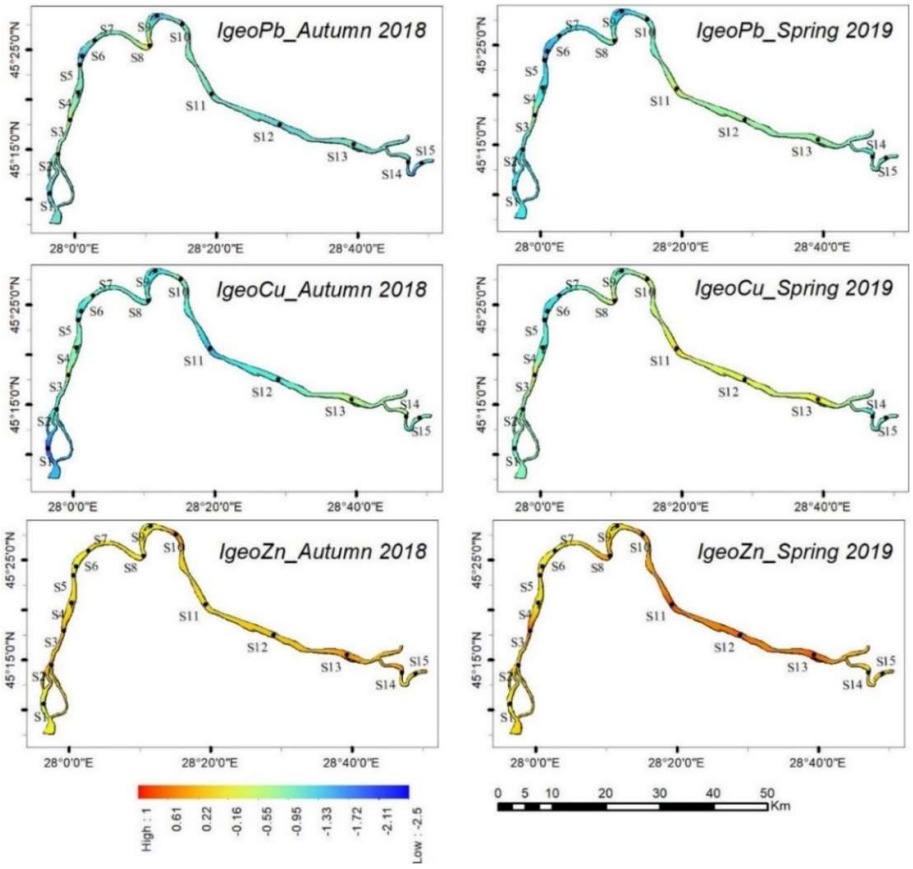


Figure 2.2 Spatio-temporal distribution of Igeo for Cd, Ni, Pb, Cu and Zn [23]

During the spring, the highest values of Igeo Cd (2 < Igeo <3) were recorded at stations S3, S8, S11, S12, S13. These monitoring stations are located near the ferry crossing (S3) and agricultural land (S8, S11, S12, S13). Usually, industry, fossil fuel combustion and agriculture (phosphorus fertilisers) are the main sources of environmental pollution with cadmium [24–27]. The Igeo values for Ni ranged from 1.07 (S6) to 2.54 (S14) in the fall of 2018 and 1.27 (S4)–2.92 (S11) in the spring of 2019. According to Igeo, Ni pollution ranges from Class 2 (moderately polluted) to Class 3 (moderately to heavily polluted). For this metal, the limit allowed according to Romanian Order 161/2006 (35 mg*kg^{-1}) was exceeded at stations S3, S11, S13, S14. In the monitored sector of the Lower Danube, the presence and persistence of nickel pollution can have several causes, such as shipping, industry, municipal and industrial waste [28]. The Igeo values of Pb were in the range of -1.78 – 0.34 in the autumn season and -2.00–1.03 during the spring and indicate the level of unpolluted sediment in most sampling stations, except for the S8 station, where the sediment was classified as "unpolluted to moderately polluted". In addition, the result of the Igeo calculation for the autumn season indicates that the values of the metal Cu for all sites were classified in the uncontaminated class (Igeo 0). On the other hand, two of the 15 stations (S3, S11) were unpolluted to moderately polluted with the metal Cu in the spring season. As for the Zn metal, the Igeo values ranged from -0.56 (S1) to 0.75 (S10) (in autumn 2018) and -0.46 (S7) –1.03 (S3) (in spring 2019).

2.4.2 Spatio-temporal distribution of the Pollution Load Index (PLI)

Analyzing the spatial distribution of PLI for the two monitored seasons (autumn 2018 and spring 2019), it can be seen that the PLI values ranged between 0.53 (S1) and 1.17 (S3) in the autumn months, which indicates the absence of heavy metal pollution (PLI < 1) in 73% of the monitored stations.

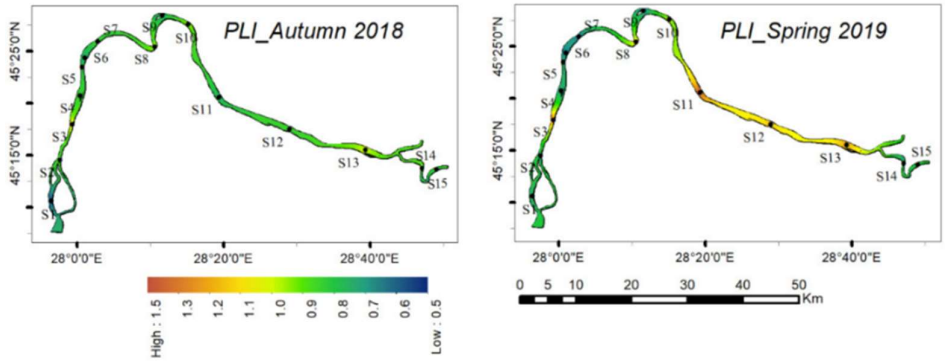


Figure 2.3 Spatio-temporal distribution of the PLI index [23]

In spring 2019, Figure 2.3 illustrates that PLI values ranged from 0.57 (S6) to 1.53 (S11), indicating the presence of heavy metal pollution of sediments, especially in monitoring stations S11–S13 (PLI > 1).

2.4.3 Spatio-temporal distribution of the Potential Ecological Risk Index (RI)

The RI index was calculated on the basis of the five heavy metals (Pb, Zn, Cd, Cu and Ni) and the results comprehensively reflect a low level of ecological risk for each element (ErMe≤ 40) as well as a low overall ecological risk for both monitored seasons. In addition, IR results in surface sediments ranged from 15.00 (S1) to 35.50 (S13) during the fall season and 17.61 (S6) to 45.96 (S3) during the spring season. However, it does not represent a high ecological risk in the sediments of the Danube River, due to the fact that the measured Cd values are below the limit allowed according to OM 161/2006.

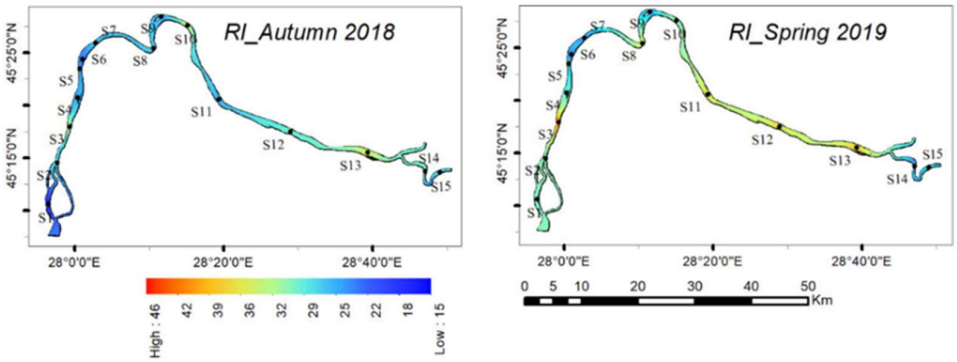


Figure 2.4 Spatial distribution of IR in autumn 2018 and spring 2019 [23]

Similar to the PLI index, the highest IR values were recorded during the spring season for stations S3 and S11–S13 (Figure 2.4).

Chapter 3. Studies on the presence of pharmaceutical compounds in aquatic ecosystems

3.1 General objectives of the study

Following the documentation conducted on the studies carried out on the transport of industrial pollutants in the lower basin of the Danube River on the territory of Romania, a gap was observed in terms of the class of emerging pollutants represented by pharmaceutical compounds. For this reason, I have chosen in this doctoral thesis to develop, in more detail, the topic regarding the transport of pharmaceutical compounds from one sampling station to another in the Danube water and their accumulation in aquatic components (Figure 3.1).

Also, in this study, different Machine Learning models were tested to try to obtain a predictive tool that is based on a support analytical framework in order to increase the sustainability of the monitoring activity of pharmaceutical compounds in the water of the lower Danube basin.

3.2 Study areas

The study areas are distributed over an average distance of about 370 km (Figure 3.1). As an analyzed area, the present thesis presents an extensive study on the presence of pharmaceutical compounds carried out on the Danube, including areas on which a strong anthropogenic impact (including industrial) is exerted.

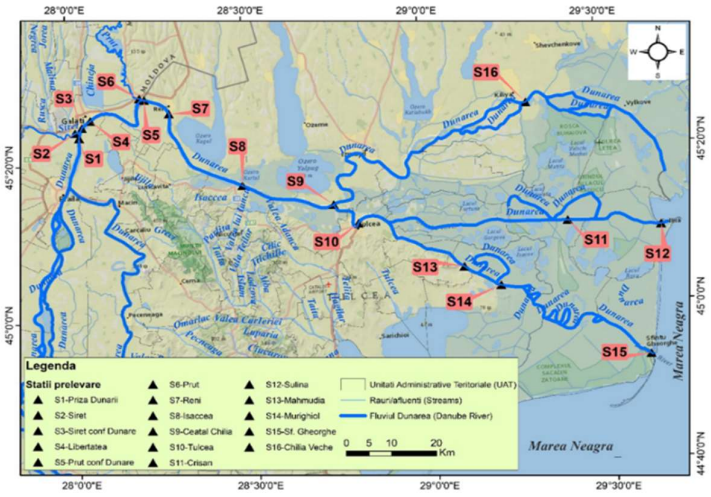


Figure 3.1 Water sampling stations in the lower Danube basin

3.3 Materials and methods

In environmental samples, pharmaceutical compounds are found in very low concentrations, of the ng/L order, most of the time below or very close to the detection limits of the techniques used to perform the analyses. For this reason, choosing the right methods/techniques for preparing samples and analyzing them, depending on their type, is very important in obtaining real results. In the present doctoral thesis, a manual sampler with a telescopic rod was used to collect water samples, and for sediment samples, a Van Veen dredger was used, which was handled by a crane installed on the REXDAN research vessel.

3.3.1 Extraction of pharmaceutical compounds from liquid samples

In the study presented in the doctoral thesis, the solid phase extraction method – SPE was used, using an automatic extraction system Dionex ™ AutoTrace ™ 280. The SPE cartridges used are Branchia C18, 500 mg/ 6 mL.

The steps of the method developed for the extraction of pharmaceutical compounds from surface water samples taken from the lower basin of the Danube River are as follows:

- Preliminary filtration of water samples;
- Acidification of samples up to pH 3 with glacial acetic acid;
- Conditioning of cartridges with 5 mL methanol;
- Conditioning of cartridges with 5 mL of ultrapure water;
- Drying the cartridges for 1 minute in the atmosphere of N2;
- Loading the 100 mL of each water sample into cartridges, with a flow rate of 10 mL/min;
- Washing the cartridges with 6 mL of ultra-pure water;
- Washing the cartridges with 6 mL methanol water mixture 20 %;
- Drying the cartridges for 5 minutes in the N_2 atmosphere (Figure 3.6);
- Elution of analytes with 6 mL methanol and collection of the extract;
- Concentration of the eluate by evaporation under a high purity nitrogen flux, at 42 °C;
- Re-dissolution of the eluate in 25 µL methanol and 225 µL water.

3.3.2 Extraction of pharmaceutical compounds from solid samples

To extract the pharmaceutical compounds from muscle tissue and roe, 5 g of sample was taken for analysis, 2 g of sample were used for the extraction of compounds from liver tissue and gills, and 4g of sample were weighed for sediment extraction.

Steps of QuEChERS extraction of pharmaceuticals from fish tissue and sediment:

- Weighing, shredding and homogenizing the samples;
- Adding solvents and standards to a 50 mL centrifuge tube: 1 mL ultrapure water and 10 mL acetonitrile, 50 µL formic acid and 50 µL internal standard mixture;
- Homogenization of the sample;
- Add the mixture of extraction salts (6 g magnesium sulphate anhydrous, 5 g sodium chloride, 1.5 g sodium citrate dihydrate and 0.75 g sodium citrate sesquihydrate) and stir the mixture intensively;
- Centrifugation of the mixture obtained at 4000 rpm for 5 minutes;
- Take-up of 5 mL of supernatant (acetonitrile) and solid-phase extract purification, dispersive method (d-SPE) containing 150 mg magnesium sulphate anhydrous, 50 mg PSA and 50 mg C18 + 15 mg graphitized activated carbon;
- Collection of the eluate in a 5 mL glass tube and its concentration in nitrogen stream at 42 °C;
- Re-dissolution of the extract with 400 µL ultra-pure H_2O and 100 µL methanol – fish tissue and 200 µL ultra-pure H_2O and 50 µL methanol – sediment;
- Filtration of the extract obtained using filters with a pore size of 0.2 µm.

3.3.3 Instrumental analysis techniques of environmental samples

- ***Liquid Chromatography Coupled with Mass Spectrometry***

Instrumental analysis often involves a chromatographic separation step to isolate the target analyte from chemicals in the matrix, followed by identification and confirmation of chemical species using mass spectrometry.

Pharmaceutical compounds (PhACs) are mostly polar and non-volatile and thermally stable, which makes it impossible to determine them directly by GC (Gas Chromatograph) without using derivatization. The coupling of liquid chromatography with the mass spectrometer combines the advantages of chromatography (high selectivity and separation efficiency) with the performance of mass spectrometry (structural and molar mass information, as well as increased selectivity, detectability).

The instrument I used for the analysis of the environmental samples that were the basis for the realization of an important part of the doctoral thesis is the Vanquish Flex Chromatograph Liquid coupled with the Orbitrap Exploris 120 high-resolution and exact mass mass spectrometer. This equipment is in the chromatography laboratory of the REXDAN Research Infrastructure of the Lower Danube University of Galati, where I hold the position of research assistant (Fig.3.2).

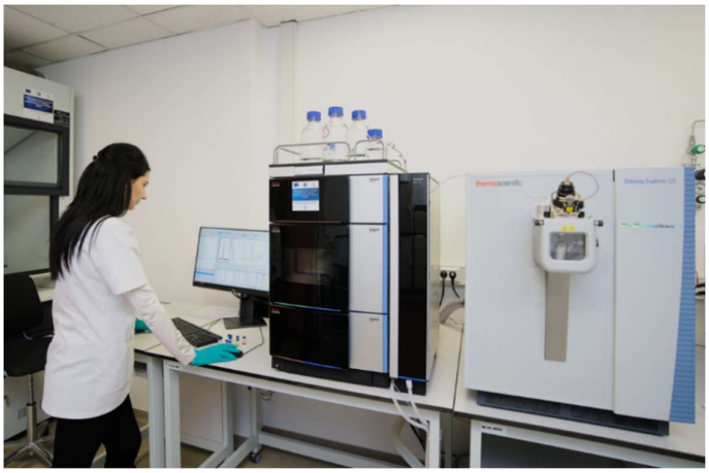


Figure 3.2 Vanquish Flex UHPLC Coupled with Orbitrap Exploris 120 High Resolution Accurate Mass Spectrometer (MS/MS) - Thermo Fisher Scientific (original photo)

Orbitrap HRAM technology provides mass accuracy at very low concentrations, allowing for accurate and selective detection of target analytes, and the entire mass range is m/z = 40-3000.

- **Scanning electron microscopy (SEM) technique**

Scanning electron microscopy is the most widely used method in obtaining information related to the morphological characteristics of a sample [29]. The principle of operation is based on scanning surfaces by using a beam of electrons. These electrons interact with the atoms of the sample to produce signals that provide information about the surface topography and composition of the sample [30].

Figure 3.2 of SEM TESCAN VEGA equipment (photo Georgiana Ghisman)

In the present doctoral thesis, in order to characterize from a morphological point of view different types of sediment taken from the lower basin of the Danube River, we used the SEM TESCAN VEGA equipment model (Fig. 3.2a), which is in the endowment of the Faculty of Engineering of the Lower Danube University of Galati and was acquired within the project Excellence and involvement in intelligent development based on research and innovation at the Lower Danube University of Galati (UDJG) – DINAMIC, 12PFE/30.12.2021.

3.3.4 Methods of qualitative and quantitative analysis of pharmaceutical compounds from environmental samples

The pharmaceutical substances analyzed in this doctoral thesis are: metformin, trimoprim, caffeine, sulfamethoxazole, clindamycin, carbamazepine, clarithromycin, ketoprofen and diclofenac.

Since determining this class of contaminants is time-consuming and very expensive, I chose to focus on the compounds considered to be the most problematic for the aquatic environment. The pharmaceutical substances clindamycin, sulfamethoxazole, trimoprim and metformin are on the European Priority Substances Watchlist, carbamazepine is considered one of the most persistent compounds in the environment, caffeine is frequently identified in the environment, and the anti-inflammatories ketoprofen and diclofenac have high toxic potential for aquatic organisms, with a significant increase in their consumption being observed in the last decade. The concept of doctorate is related to the present, but to a large extent to the future. The European Watchlist of Priority Substances will in the near future involve precision determinations and continuous monitoring with optimized procedures in high-performance laboratories. These are the arguments for the selection of classes of substances from the experimental list in this doctoral thesis.

The following are presented the parameters set in the instrument method of the liquid chromatograph and the mass spectrometer to ensure the achievement of the highest analytical performance in the separation, identification, confirmation and quantification of the compounds of interest.

UHPLC Parameters:

- Coloan compartment temperature: 40 °C;
- Duration of analysis: 20 minutes:
- Mobile debit phase: 0.4 mL/min;
- Mobile phases: A – ultrapure water with 0.1% formic acid; B- Methanol with formic acid 0.1%;

- Gradient Moving Phase:
  0-1 minutes: 90% A – 10% B;
  1-2,5 minutes: 90% A – 10% B;
  2,5-10 minutes: 60% A- 40% B;
  10 – 13 minutes: 0% A – 100% B;
  13 – 15 minutes: 0% A – 100% B;
  15- 20 minutes: 90% A – 10% B;
- Injection volume: 10 µl;

Parameters MS:

- Sursa de ioni HESI (Heated Electrospray);
- Debit sheath gas: 50;
- Gas flow: 10;
- Debit sweep gas: 1;
- Temperature of Ion Transfer Tube: 325°C;
- Vaporization temperature: 350 °C;
- HCD collision energy: different depending on the compound;
- Rezolution MS Orbitrap: 120 000;
- RF Lens: 70 %;
- Scan parameters: Full Scan și MS/MS;

The external mass and system calibration of the mass spectrometer was achieved by injecting calibration solutions in positive and negative operating mode before each series of analysis was performed. The separation of compounds in HPLC was facilitated by the use of an Accucore C18 chromatographic column (100 x 2.1 mm, 2.6 µm).

Data acquisition was performed for positive and negative ionization both in full-scan mode used for quantification of the compounds of interest and in MS/MS mode to confirm the presence of the compound in the sample. The data acquisition, processing and processing was carried out using the Chromeleon CSD software - Thermo Fisher Scientific.

For the quantitative analysis of the pharmaceutical compounds identified in the water samples, analytical standards produced by Sigma-Aldrich were used.

3.4 Experimental results and discussions on the presence of pharmaceutical compounds in the lower basin of the Danube River

3.4.1 Results of the application of the analysis methods

Following the application of the analysis methods, described above, all the pharmaceutical compounds that were analyzed in this doctoral thesis were identified. The results obtained after performing the UHPLC-MS/MS analyses are the following. Table 3.1 lists the molecular formulas by which the molecular weights were calculated, the ion masses obtained after ionization of the compounds in the positive mode and the retention times at which each analite was chosen.

Table 3.1 Pharmaceutical compounds analyzed with UHPLC - MS/MS

Compound	Formula	Adduct	m/z	Retention Time (min)
Metformin	$C_4H_{11}N_5$	+ H	130.1087	0.44
Trimetroprim	$C_{14}H_{18}N_4O_3$	+ H	291.1452	3.21
Caffeine	$C_8H_{10}N_4O_2$	+ H	195.0877	3.38
Sulfametoxazol	$C_{10}H_{11}N_3O_3S$	+ H	254.0594	3.69
Clindamycin	$C_{18}H_{33}ClN_2O_5S$	+ H	425.1871	4.91
Carbamazepina	$C_{15}H_{12}N_2O$	+ H	237.1022	5.72
Clarithromycin	$C_{38}H_{69}NO_{13}$	+ H	748.4842	6.50
Ketoprofen	$C_{16}H_{14}O_3$	+ H	255.1016	6.65
Diclofenac	$C_{14}H11Cl_2NO_2$	+ H	296.024	8.03

Figure 3.3 shows the chromatograms obtained for each pharmaceutical compound analyzed and the retention times at which they were eluted.

The first compound to elute is the drug metformin which had a retention time of 0.44. The other compounds analyzed were taken in the following order: trimoprim (3.21), caffeine (3.38), sulfamethoxazole (3.69), clindamycin (4.91), carbamazepine (5.72), clarithromycin (6.5), ketoprofen (6.65) and diclofenac 8.03 (Figure 3.21).

Figure 3.3 Full Scan chromatogram of identified compounds

The present doctoral thesis, as I have already mentioned, aims, among other things, at optimizing some procedures in order to transpose them to the level of repetitive monitoring to ensure minimum consumption and with human effort of the same order. Apart from obtaining efficient separations, with precise retention types and exact mass to the 5th decimal place, we have aimed, through successive attempts, to define separation conditions that would allow the

analysis of the entire set of compounds in a single injection, which complies with the optimal conditions defined above.

For each compound analyzed, in order to confirm its presence in the sample, at least one fragment specific to them was identified. The optimization of the fragmentation of the researched chemical species involves adapting the collision energies in order to obtain eloquent fragments, easily identifiable in MS. This is impossible with the constant assurance of a single collision energy value, therefore, in the experiment we managed to define optimal, "personalized" values for each compound through a judicious programming of the MS/MS system commands

3.4.2 Spatio-temporal distribution of pharmaceutical compounds in surface water samples

This subchapter describes the results obtained from the analysis of some classes of pharmaceutical substances from water samples taken from the Lower Danube Basin starting with the Siret tributary and ending with the stations located on the 3 arms of the Danube Delta. Water samples were taken in the summer seasons of 2021, 2022 and 2023.

The main purpose of this study is to analyze the transport of pharmaceutical compounds in the surface water of the lower basin of the Danube River. To better highlight this, the results were represented in the form of pollutant distribution maps.

Spatial distribution of Carbamazepine in the Lower Danube Basin in 2021-2023

For the pharmaceutical compound carbamazepine, in 2021, the highest concentration was recorded in station S10 (17.79 ng/L), in 2022 it was obtained in station S1 (10.42 ng/L), and in 2023 in station S4 (3.42 ng/L) (Fig. 3.4).

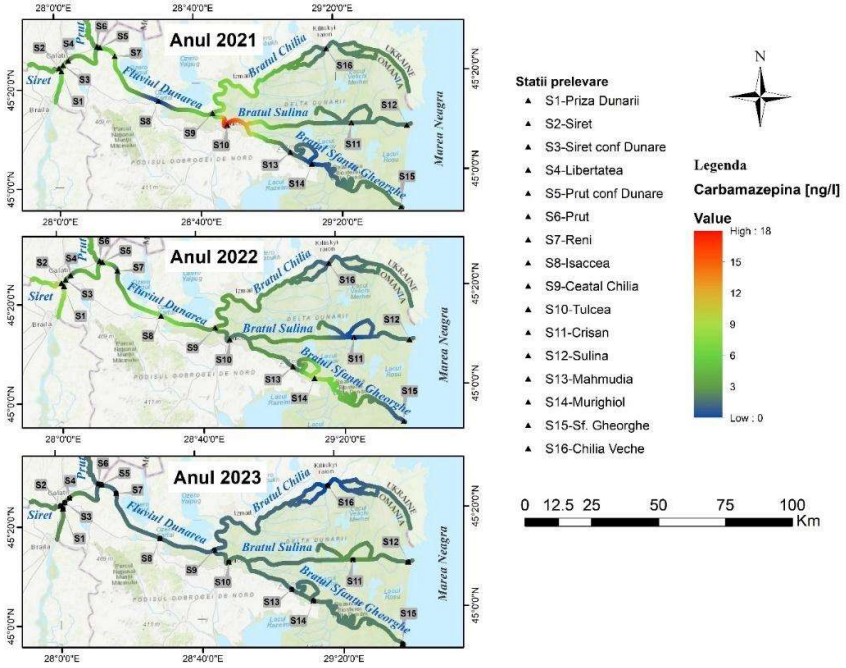


Figure 3.4 Spatio-temporal distribution of Carbamazepine in the Lower Danube Basin

This drug has been detected in almost all monitored stations and, according to the literature, carbamazepine is one of the most persistent pharmaceuticals in the environment [31]. In a study conducted by Chitescu, et al., in 2014, in Romania, on water samples taken from the Danube and its tributaries, carbamazepine was identified in all samples, and the highest concentration was 40 ng/L (Argeș River) [32].

Spatial distribution of Metformin in the Lower Danube Basin in 2021-2023

Over time, the use of metformin has increased greatly due to its efficiency in treating type 2 diabetes, cancer, and polycystic ovary syndrome [33]. Studies estimate that more than 451 million people worldwide suffer from diabetes, of which about 90% have type 2 diabetes [34].

In 2021, the values of metformin concentrations ranged from 0.56 ng/L (S16) to 3.54 ng/L (S10), in 2022, the values ranged from 0.34 ng/L (S9) to 2.08 ng/L (S5), and in 2023, the concentrations ranged from 1.13 ng/L (S3) to 2.31 ng/L (S4) (Fig.3.5). This compound was identified in most of the monitored stations, results that correlate with the literature where it is mentioned that metformin is the second pharmaceutical compound detected in the world. This is because metformin is on the list of the top 20 pharmaceutical compounds prescribed and produced worldwide [35].

At the same time, the increased frequency with which this compound was identified in water samples can be explained by the fact that the daily dose of administration is 2 g and is excreted in a proportion of approximately 70 % unchanged in the urine, and the rest in the faeces [36].

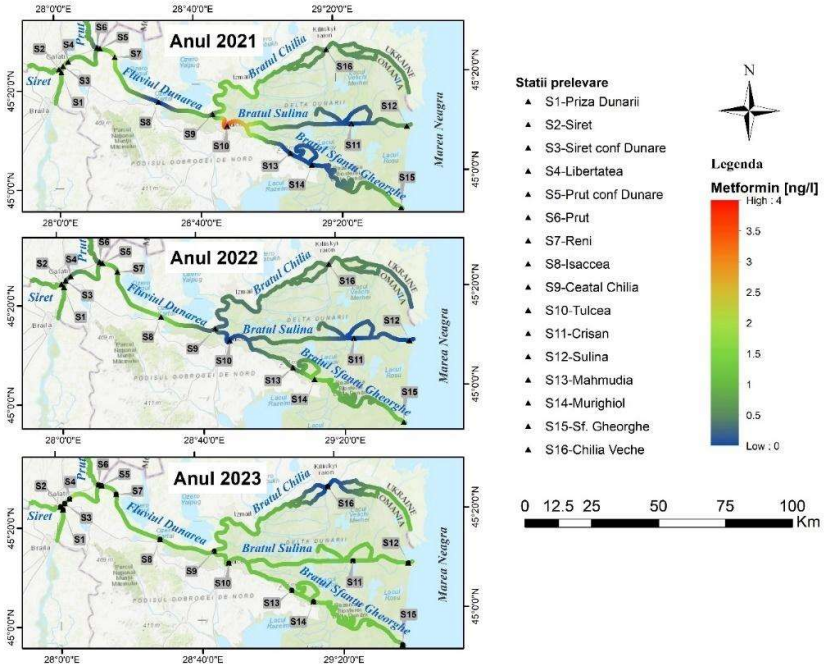


Figure 3.5 Spatio-temporal distribution of Metformin in the Lower Danube Basin

Spatial distribution of Caffeine in the Lower Danube Basin in 2021-2023

Caffeine is considered the most widely used pharmaceutical compound in the world, taking into account the multiple remedies for which this compound is used in mixture with other active ingredients [37].

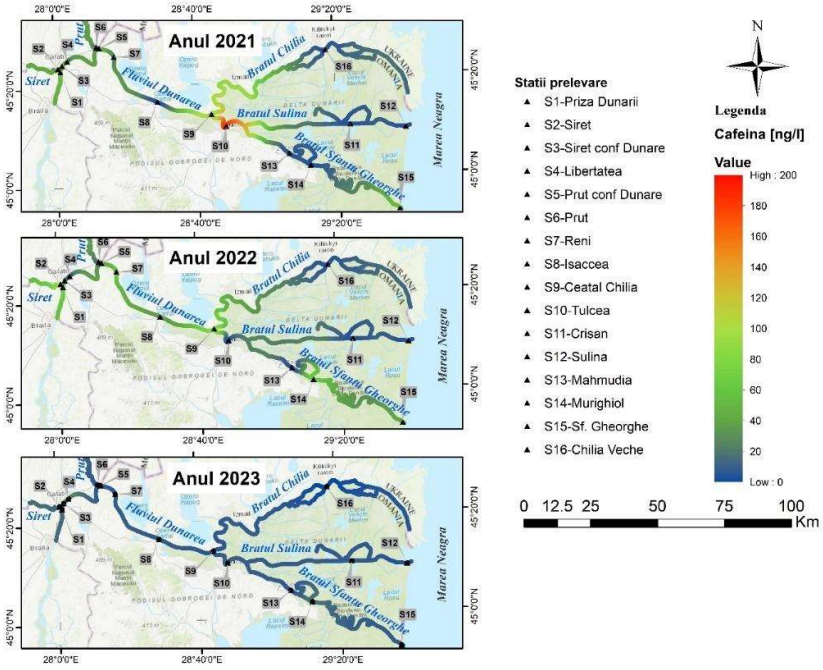


Figure 3.6 Spatio-temporal distribution of Caffeine in the Lower Danube Basin

The alkaloid caffeine was identified in all the water samples analyzed. In 2021, caffeine concentration values ranged from 3.22 ng/L (S14) to 184.15 ng/L (S10). In 2022, the caffeine concentration values were in the range of 3.52 ng/L (S10) - 118.52 ng/L (S2), while in 2023 the caffeine values were in the range of 3.79 ng/L (S12) - 29.25 ng/L (S4) (Fig.3.6).

Studies consider caffeine to be the most representative pharmaceutical compound due to its high abundance in the environment and classify it as an indicator of anthropogenic pollution that may indicate that the sources of surface water pollution are discharges from municipal treatment plants into effluents [38]. The presence of this pharmaceutical compound has also been noticed in areas that are outside the anthropogenic impact, such as Antarctica [37]. Caffeine residues are very stable in the environment, with a half-life of 100 to 240 days [39]. Also, this pshioactive stimulant is persistent in the environment due to its high water solubility (21.7 g/L) and low octanol-water partition coefficient (Kow= 0.01) [40].

Spatial distribution of Diclofenac in the Lower Danube Basin in 2021-2023

Figure 3.7 shows the spatial distribution of diclofenac for which values were obtained in 2021 only in stations S3, S9 and S10, the highest concentration being 5.81 ng/L (S3). In 2022, concentrations of diclofenac were recorded in stations S3, S6, S9, S14, the values being in the range of 4.91 ng/L and 6.45 ng/L. For water samples taken in 2023, diclofenac was quantified with a low frequency, only in stations S1, S4, S10 and S14, with concentrations in the range of 1.02 ng/L -1.91 ng/L.

According to literature, the presence of diclofenac in aquatic ecosystems can have toxic effects on aquatic biota. For example, in fish, diclofenac can cause kidney and gill disorders and histopathological changes in the liver [41].

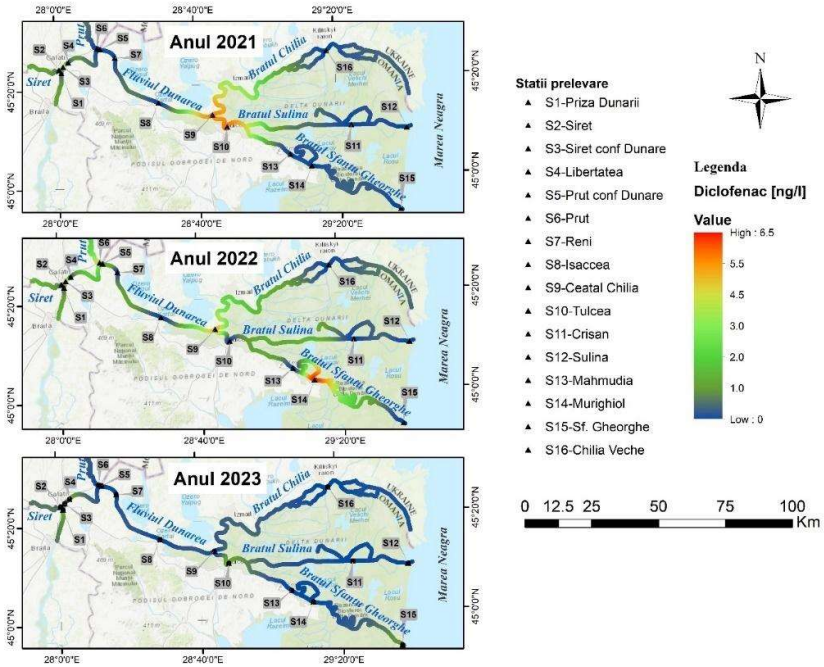


Figure 3.7 Spatio-temporal distribution of Diclofenac in the Lower Danube Basin

Spatial distribution of Ketoprofen in the Lower Danube Basin in 2021-2023

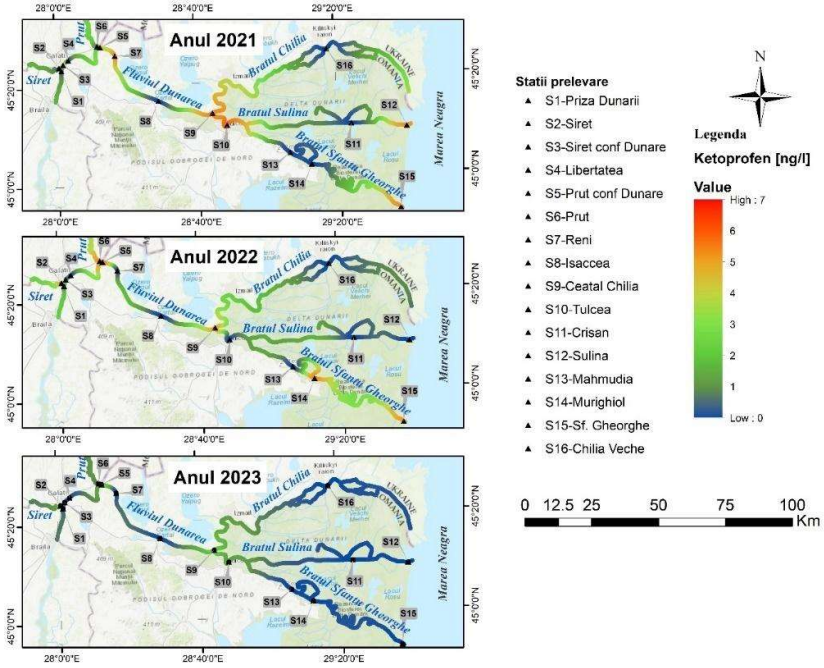


Figure 3.8 Spatio-temporal distribution of Ketoprofen in the Lower Danube Basin

Ketoprofen is considered the third most widely used non-steroidal anti-inflammatory in both human and veterinary medicine [42].

In 2021, Ketoprofen concentration values ranged from 5.14 ng/L to 5.77 ng/L and were recorded in 7 of the 16 sampling stations. In 2022, the concentrations were in the range of 5.00 ng/L - 7.03 ng/L, and in 2023 the results were in the range of 1.24 ng/L - 3.15 ng/L and were identified with a low frequency, in only 4 stations (S2, S5, S9, S10). The low frequency with which this compound was detected may be due to the fact that it has a low persistence in the environment, its half-life being in the order of hours [43]. However, toxicity studies have shown that this compound has toxic effects on living organisms even in low concentrations [19].

Spatial distribution of the Trimetroprim in the Lower Danube Basin in 2021-2023

For the pharmaceutical compound trimoprim (Figure 3.9), in 2021, values were recorded in the range of 0.20 ng/L - 9.17 ng/L. In 2022, trimoprim concentrations ranged from 0.2 ng/L to 10.09 ng/L, and in 2023 the values ranged from 0.25 ng/L to 4.12 ng/L. In all three monitored years, this compound has been identified with a frequency of approximately 50 %.

The pandemic period 2020-2021 and the post-pandemic period 2022 recorded, in addition to the use of substances with antiviral effect, an increase in the consumption of antibiotics. It can be seen that apart from the significant increases in the concentration of the trimoprimer at the mouth of the Siret (S1, S2) along the Danube in all other stations, its concentrations are low. One of the causes could be that trimoprimer has a nephrotoxic effect [45] known reason why it was eliminated from the treatment of bacterial infections associated with COVID 19.

The presence of antibiotics in the aquatic ecosystem can cause the growth of bacteria, algae and aquatic plants to slow down [45].

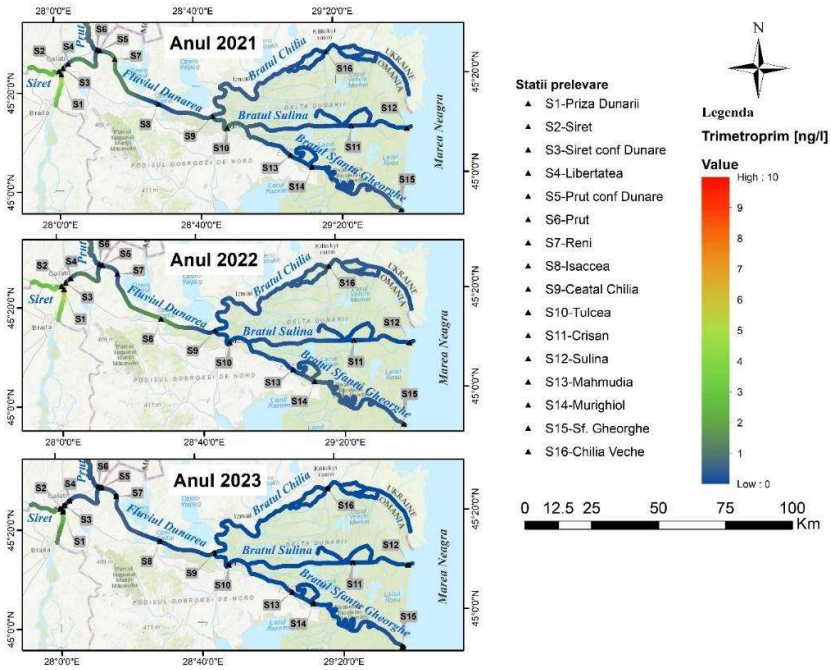


Figure 3.9 Spatio-temporal distribution of the Trimetroprim in the Lower Danube Basin

Spatial distribution of Clarithromycin in the Lower Danube Basin in 2021-2023

In 2021, concentrations were obtained for the antibiotic clarithromycin only in two sampling stations: S7 (5.41 ng/L) and S10 (5.41 ng/L). In 2022, values were recorded in stations S5 (5.50 ng/L) and S7 (5.93 ng/L), and in 2023 results were obtained in the range of 2.07 ng/L - 2.17 ng/L (Fig. 3.10).

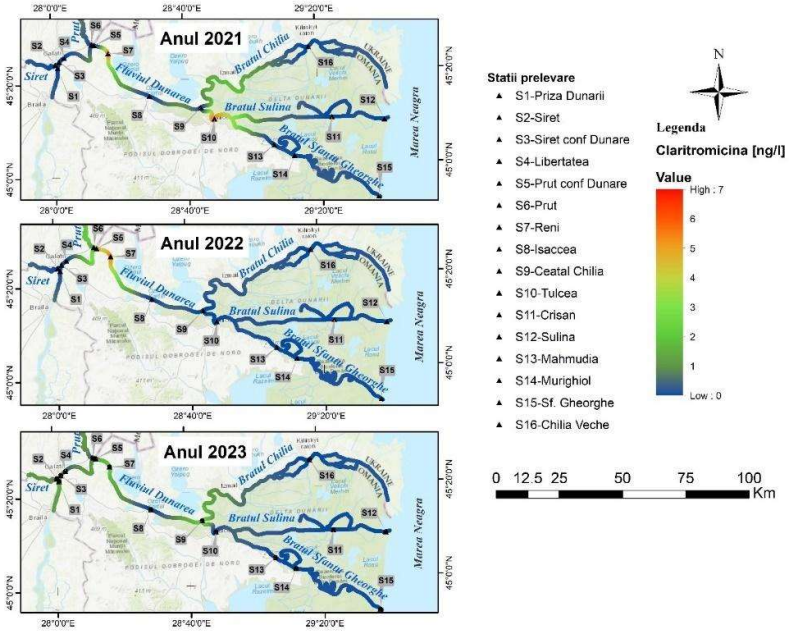


Figure 3.10 Spatio-temporal distribution of Clarithromycin in the Lower Danube Basin

Also, in the case of clarithromycin, important increases are observed during the pandemic period in the S9, S10 area. A correlation can be made with medical practice that considers the macrolide clarithromycin as potentially useful in preventing secondary infections of covid-19 through its antimicrobial and antiviral effect [46].

The low frequency of detection of this compound in the aquatic environment may be due to the fact that only 40% of the clarithromycin consumed is excreted as the base compound, the remaining 60% being eliminated as metabolites [47].

Spatial distribution of Clindamycin in the Lower Danube Basin in 2021-2023

In 2021, the pharmaceutical compound clindamycin was detected in 8 of the 16 monitored stations, and the concentrations ranged from 3.32 ng/L to 3.75 ng/L. In 2022, this antibiotic was identified in the following sampling stations: S1, S5, S7, S8, S9, S14, S15, the concentration values being in the range: 3.22 ng/L (S9) - 3.68 ng/L (S5). For water samples taken in 2023, clindamycin concentration values were in the range of 2.12 ng/L (S15) - 3.12 ng/L (S7).

In the case of clindamycin, the average frequency of its identification in water samples taken in the 3 years monitored was approximately 42%, with the lowest values recorded in 2023 (Figure 3.11).

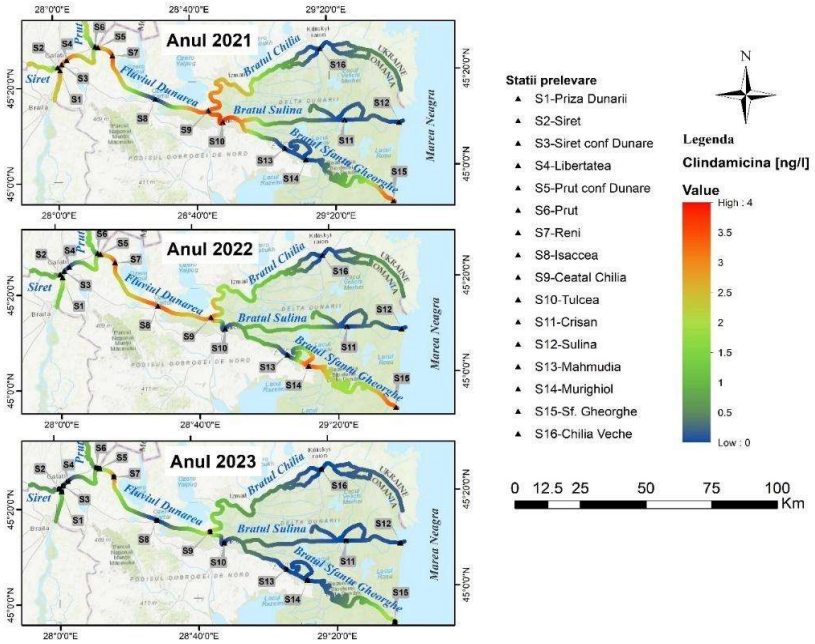


Figure 3.11 Spatio-temporal distribution of Clindamycin in the Lower Danube Basin

Spatial distribution of Sulfamethoxazole in the Lower Danube Basin in 2021-2023

In 2021, sulfamethoxazole was detected in 10 of the 16 monitored stations, with concentrations ranging in the range of 3.44 ng/L - 4.57 ng/L, while in 2022, sulfamethoxazole concentrations were in the range of 3.10 ng/L (S9) - 5.97 ng/L (S14). For 2023, values were recorded in the range of 1.22 ng/L – 3.25 ng/L. The highest values of this compound were recorded in sampling stations located near urban agglomerations (Galati, Isaccea, Tulcea, Murighiol) (Figure 3.12).

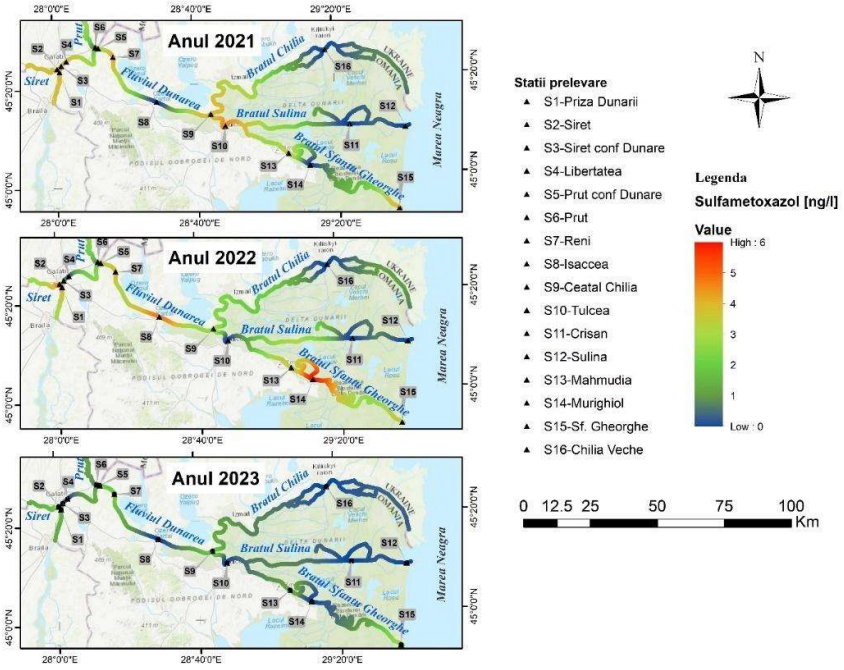


Figure 3.12 Spatio-temporal distribution of Sulfamethoxazole in the Lower Danube Basin

Compared to the other antibiotics, sulfamethoxazole was identified most often, with the average frequency of its detection in water samples taken in the 3 years analysed being 56 %. These results also correlate with the literature where it is mentioned that sulfamethoxazole is one of the most frequently identified antibiotics in aquatic environments [48].

Sulfamethoxazole has clinical effects and proves effective in reducing the immunodeficiency diagnosed in viral infections, which leads to the conclusion that its presence in the analyzed samples is due to consumption associated with COVID 19 [49].

3.4.3 Accumulation of pharmaceutical compounds in Danube sediments

In order to study the behavior of substances in the aquatic ecosystem, sediment samples taken from different stations located in the lower Danube basin were also analyzed, as follows:

- S1 – Prut-Danube confluence;
- S2 - Cat's Elbow;
- S3 - loc. Reni;
- S4 - loc. Isaccea;
- S5 - loc. Somova;
- S6 - loc. Tulcea;
- S7 - loc. Mahmudia;
- S8 - loc. Murighiol;
- S9 - loc. Sf. Gheorghe.

Table 3.2 shows the spatial distribution of pharmaceutical compounds in sediment samples taken from the lower basin of the Danube River, after chromatographic analysis associated with mass spectrometry (Fig. 3.12). From the results obtained, it can be seen that, of all the compounds investigated, the following three were identified: carbamazepine, metformin and caffeine. The most commonly identified compound is the alkaloid caffeine, which was found in all the samples analyzed, in concentrations ranging from 0.6 ng/g to 15.1 ng/g. Studies indicate that for caffeine, the average per capita consumption is 70 mg/day. For this reason, caffeine is considered to be one of the most ubiquitous active pharmaceutical compounds in the environment, a fact also demonstrated in this doctoral thesis [50].

Table 3.2 shows a selection of the concentrations identified in the sediments, since for the other species analysed (trimoprim, sulfamethoxazole, clindamycin, clarithromycin, ketoprofen and diclofenac) traces present in the sediments could not be detected.

Table 3.2 Spatial distribution of pharmaceutical compounds in sediment

Sampling station / **Compounds**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**
Carbamazepine (ng/g)	ND	0.5	ND	ND	0.7	ND	ND	0.9	ND
Metformin (ng/g)	ND	ND	ND	ND	0,2	ND	ND	4.3	0.5
Caffeine (ng/g)	2.4	1.6	0.6	0.8	5	0.6	1.1	15.1	1.2

ND – Undetected

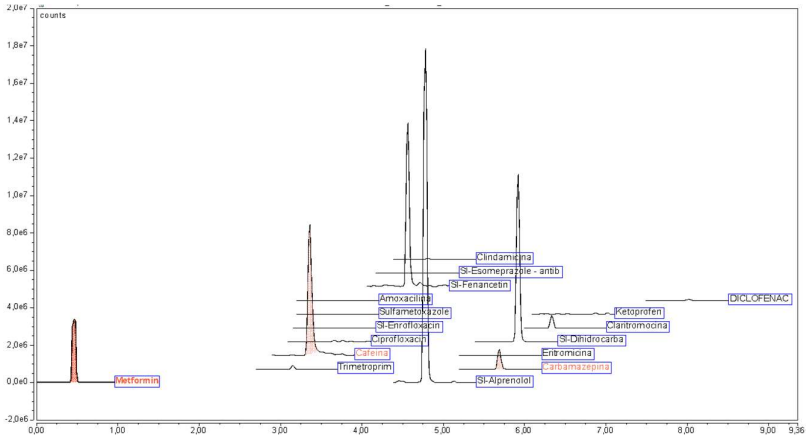


Figure 3.13 Chromatogram Pharmaceutical Compounds Detected in S8 Sediment Sample

According to the literature, most pharmaceutical compounds generally show low affinity for solids. For example, experiments carried out with the pharmaceutical substances diclofenac, ketoprofen, sulfamethoxazole, carbamazepine, have shown that these compounds have low absorption in sediment and soil [51]. Another study highlighted the fact that for most pharmaceutical compounds, including the substances analyzed in the present doctoral thesis, such as: trimoprim, sulfamethoxazole, erythromycin, carbamazepine, diclofenac, the absorption coefficients (distribution) Kd are relatively low, which indicates a reduced absorption capacity in sediments and a high probability of their identification in the aqueous phase [52]. For the pharmaceutical compound metformin, studies point out that its high solubility and low Kow values (octanol-water partition coefficient) explain the higher distribution in the liquid phase, which leads to its identification in water samples and less in sediments [35].

Fine sediment – S8 Coarse sediment - S3

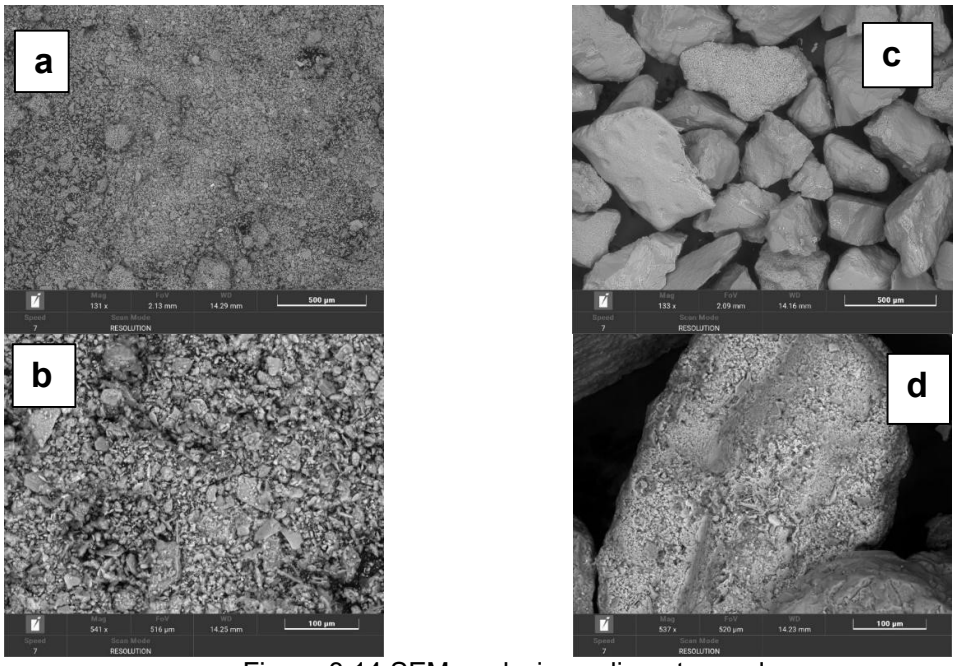


Figure 3.14 SEM analysis, sediment samples

Figure 3.14 **a** shows the image obtained for sample S8 with a magnification of 131 times, and Figure 3.14 **b** shows the same sample that was magnified 541 times. For sample S3, with the largest grain, the image obtained from the 133-fold magnification is represented in Figure 3.14 **c,** while in Figure 3.14 **d** the image obtained for the same sample is magnified 537-fold.

In order to see if the morphological structure of the sediments influences the degree of accumulation of pharmaceutical compounds, SEM (Scanning Electron Microscopy) analyses were performed on two different types of sediment samples, with the finest grain and the largest grain size. In Figures 3.14 **b** and 3.14 **d** and the difference in granulation between the two types of sediment samples analyzed is observed, the sediment sample taken from the S8 station has the smallest grain, while the sample from the S3 station has a much higher granulation. From the results obtained from the determination of the pharmaceutical substances from the sediment samples, it is found that in the S8 station, the station with the smallest granulation, the most compounds were identified from all those investigated and in the highest concentration compared to the other stations in which they were identified. In the sample with the largest grain, taken from the S3 station, only caffeine in the lowest concentration was identified. Studies show that fine sediment particles (<63 μm) are the most chemically active and effectively retain nutrients, metals and organic contaminants [53].

3.4.4 Accumulation of pharmaceutical compounds in fish tissue

It is known that the presence of pharmaceutical compounds in the aquatic environment, even in low concentrations, can have toxic effects on aquatic biota. Also, some studies report that, under certain conditions, these industrial pollutants can accumulate in aquatic organisms. There are only few studies reported in literature focusing on fish species collected from the natural environment, as to assess the degree of accumulation of pharmaceuticals in fish tissue. So far, no complex studies have been carried out on the Danube River that would include data on the property of some pharmaceutical classes found in water to accumulate in ichthyofauna. Most studies that followed the accumulation capacity of pharmaceutical substances in aquatic biota were carried out under laboratory conditions, in which pharmaceutical substances were present in the experimental environment in concentrations much higher than the real ones.

In this subchapter, we have followed the tendency of the pharmaceutical compounds studied in this doctoral thesis to accumulate in the organs of different fish species that have the Danube River as their natural habitat. Thus, six species of fish were analyzed, for which the presence of certain classes of pharmaceutical substances in organs was investigated, such as: muscles, gills, liver and roe.

The fish species under analysis were:

- *Alosa immaculata;*
- *Carassius gibelio;*
- *Hypophthalmichthys molitrix*;
- *Perca fluviatilis;*
- *Abramis bream;*
- *Vimba Vimba*;

- **Analysis of pharmaceutical compounds from *Alosa immaculata***

The mackarel is a migratory fish species with a high economic value, due to the large quantities present in the Danube, Danube Delta and the Black Sea. Its meat also has a high

nutritional value [54]. However, studies show that the abundance of this species is constantly decreasing due to habitat destruction, uncontrolled exploitation, the presence of invasive species, pollution of the aquatic environment and climate change. According to the European Habitats Directive Annex II and V, the conservation status of the *Alosa immaculata* in the Danube-Black Sea River Basin is classified as unfavorable and inadequate [55]. Moreover, this species is included on the IUCN (International Union for Conservation of Nature) Red List as a vulnerable species, with a population trend decreasing under anthropogenic pressure [56]. In the present study, 3 specimens of mackarel were analyzed for which the presence of pharmaceutical compounds in muscle tissue, liver tissue, gills and roe was investigated in order to follow the tendency of their accumulation in the organs of fish.

Table 3.3 Pharmaceutical compounds investigated in *Alosa immaculata*

Compounds pharmaceuticals analyzed		*Fish specimens analyzed*		
	Fish organs	*Alosa immaculata_1*	*Alosa immaculata_2*	*Alosa immaculata_3*
Caffeine (ng/g)	Muscular tissue	6.31	8.80	8.14
	Hepatic tissue	6.65	8.77	4.77
	Gills	44.29	7.75	4.68
	Spawn	6.08	5.85	5.34
Clindamycin (ng/g)	Muscular tissue	0.3	ND	ND
	Hepatic tissue	ND	ND	ND
	Gills	ND	ND	ND
	Spawn	ND	ND	ND
Clarithromycin (ng/g)	Muscular tissue	1.9	ND	ND
	Hepatic tissue	ND	ND	ND
	Gills	ND	ND	ND
	Spawn	ND	ND	ND
Carbamazepina (ng/g)	Muscular tissue	0.24	0.25	0.24
	Hepatic tissue	ND	ND	ND
	Gills	ND	ND	ND
	Spawn	0.34	0.83	0.99

ND - Undetected

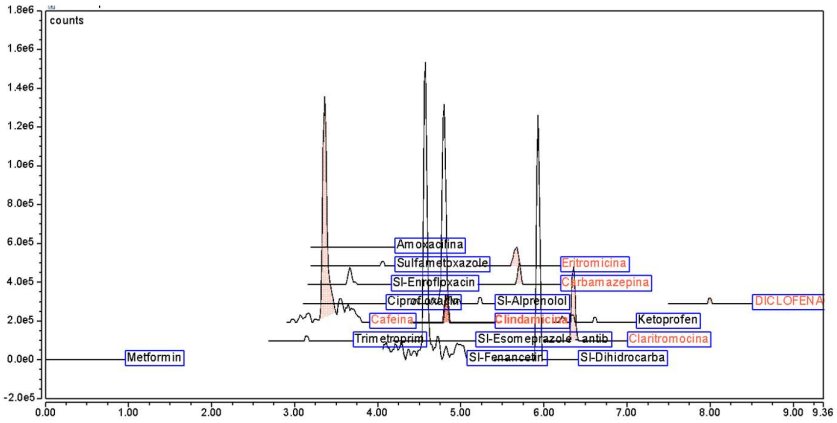


Figure 3.15 Chromatogram of pharmaceutical compounds identified in muscle tissue *Alosa immaculata_1*

The results obtained from the analysis of fish tissue samples from the *Alosa immaculata* species highlighted the presence of caffeine in all 3 specimens of the analyzed fish and in all 4 types of organs investigated. The caffeine concentration values were in the range of 4.68 ng/g – 44.29 ng/g, the highest concentration was recorded in the gills extracted from the specimen that had the highest weight.

Also, traces of carbamazepine were also observed in the muscle tissues prevalated from the 3 fish in concentrations ranging from 0.34 ng/g - 0.99 ng/g. In the muscle tissue from *the Alosa immaculata_1* specimen, most pharmaceutical compounds were found, namely: caffeine, clindamycin, clarithromycin, carbamazepine and diclofenac. Figure 3.15 shows the chromatograms of the pharmaceutical compounds identified in the muscle tissue from the mackarel_1 specimen and the internal standards added to the sample to quantify them. The specimen of mackarel in which the most pharmaceutical compounds were identified is the one that had the highest weight and length compared to the other 2 fish analyzed.

- **Analysis of pharmaceutical compounds in *Carassius gibelio***

Carassius gibelio - The Prussian carp is considered to be an abundant species in the Danube River with a high importance in commercial fishing [57]. In Romania, the carp is considered to be the most consumed local freshwater fish [58].

To identify the presence of pharmaceutical compounds in the species *Carassius gibelio,* 3 fish specimens were analyzed and extractions were made from muscle tissue, liver tissue, gills and spawn. Subsequently, the samples were injected into the UHPLC-MS/MS equipment and the pharmaceutical compounds studied in the present doctoral thesis were searched. Of all the compounds investigated, caffeine was the one that was found in all the organs analyzed in the 3 fish, with values in the range of 0.56 ng/g -3.97ng/g. The highest concentrations were recorded in the gills. Since gills are in direct contact with the aquatic environment, they can reflect its level of contamination [59].

Table 3.4 Pharmaceutical compounds investigated in Carassius gibelio

Compounds pharmaceuticals analyzed		*Fish specimens analyzed*		
	Fish organs	***Carassius gibelio _1***	***Carassius gibelio _2***	***Carassius gibelio_3***
Caffeine (ng/g)	Muscular tissue	1.04	1.28	0.81
	Hepatic tissue	1.62	1.88	1.31
	Gills	1.58	3.97	1.72
	Spawn	0.59	0.53	1.20

ND – Undetected

Taking into account the fact that this species is used for human consumption, most importantly in the Danube area, and the fact that there are important similarities between the mechanisms of affectation/accumulation of pollutants in the tissues of the species and in some of the human tissues (intestine and kidneys), the present study constitutes an important research base that can be extended to the effect on human health, especially for the inhabitants of the areas adjacent to the Danube, for whom carp is sometimes considered a delicacy.

- **Analysis of pharmaceutical compounds in *Hypophthalmichthys molitrixn***

Hypophthalmichthys molitrix is one of the most commonly farmed freshwater fish species worldwide due to its low production costs in aquaculture, its high availability, and nutritional value. This species is rich in protein, polyunsaturated fatty acids, micronutrients and fat-soluble vitamins.

Table 3.5 Pharmaceutical compounds investigated in Hypophthalmichthys molitrix

Compounds pharmaceutic als analyzed	Fish organs	*Fish specimens analyzed*		
		Hypophthalmichthys molitrix _1	***Hypophthalmichthys molitrix _2***	***Hypophthalmichthys molitrix _3***
Caffeine (ng/g)	Muscular tissue	5.31	2.78	2.91
	Hepatic tissue	1.27	1	3.51
	Gills	3.97	3.30	4.61

ND – Undetected

The analysis carried out on specimens belonging to the *species Hypophthalmichthys molitrix* showed that, out of all the pharmaceutical substances investigated in the analysed organs, only caffeine was identified. Table 3.5 shows that low concentrations of caffeine were measured in the muscle tissue, liver tissue and gills taken from the three fish analyzed.
In muscle tissue, caffeine concentration values ranged from 2.78 ng/g to 5.31 ng/g, in liver tissue concentrations ranged from 1 ng/g to 3.51 ng/g, and in gills values ranged from 3.30 ng/g to 4.61 ng/g. In general, it can be seen that caffeine accumulates in the gills in a greater proportion compared to the other organs. This may be due to the fact that the gills have the role of filtering water, thus also favoring the accumulation of compounds in these organs.

- **Analysis of pharmaceutical compounds from *Perca fluviatilis***

Perca fluviatilis is a predatory fish native to most of Europe and northern Asia. Fish belonging to this species have high nutritional value and a low fat content of 1%, which makes them suitable for inclusion in the diet [60,61].

Table 3.6 shows the compounds that have been investigated in the organs of fish belonging to the species *Perca fluviatilis* and the values obtained for these compounds that were identified in the samples. The experimental results demonstrated the presence of caffeine in the muscle tissue of the three perch specimens, in concentrations ranging from 1.73 ng/g to 3.57 ng/g. For this species, the highest concentrations of caffeine were recorded in liver tissue, the values being in the range of 1.35 ng/g – 7.41 ng/g. The liver is considered an important environmental indicator due to its role in the accumulation and transfer of pollutants [61]. In the roe samples collected from the 3 perch specimens, the presence of caffeine was observed in concentrations ranging from 0.75 ng/g to 2.47 ng/g. In muscle tissue collected from the specimen *Perca fluviatilis_2* a concentration of 0.4 ng/g was identified for the compound diclofenac. For the other compounds that have been investigated in biotic tissues taken from specimens belonging to the *Perca fluviatilis* no values were obtained. This is because some pharmaceuticals are found in very low concentrations in surface water samples and with a low frequency. Moreover, this class of industrial pollutants exhibits low affinity for

solid samples, compared to other pollutants such as heavy metals, pesticides, perfluoroalkylated compounds.

Table 3.6 Pharmaceutical compounds investigated in Perca fluviatilis

Compounds pharmaceuticals analyzed	*Fish specimens analyzed*			
	Fish organs	*Perca fluviatilis _1*	*Perca fluviatilis _2*	*Perca fluviatilis _3*
Caffeine (ng/g)	Muscular tissue	3.57	2.98	1.73
	Hepatic tissue	7.41	5.78	1.35
	Gills	ND	4.01	ND
	Spawn	0.75	1.44	2.47

ND – Undetected

- **Analysis of pharmaceutical compounds in *Abramis brama***

Abramis brama known is a freshwater benthic species, widespread in the Danube River, with an abundance of 16% of total catches and frequently used in human food [62]. This species is known to be one of the most commercially valuable [63].

This species is often used as a bioindicator of the aquatic environment due to its high abundance and high potential for the accumulation of pollutants. To date, studies have been reported in the literature demonstrating the ability of this species to accumulate heavy metals [62,64]. The present doctoral thesis aimed to evaluate the degree of accumulation in fish of the species *Abramis bellows* for different classes of pharmaceutical compounds identified in water samples taken from the Danube River. The results obtained demonstrate the presence of caffeine in the organs collected from the bream specimens taken in the analysis. The caffeine concentration values were in the range of 2.98 ng/g – 13.94 ng/g.

Studies show that the alkaloid caffeine has been detected in aquatic organisms due to its ability to bioaccumulate in tissues after long-term exposure to contaminated water [50]. Caffeine concentrations have been identified in fish species collected from the Red Sea, such as: *Gerres oyena, Chanos chanos, Lethrinus nebulosus, Lethrinus nebulosus, Oreochromis niloticus,* but also in marine microalgae [66]. In a study whose main objective was the analysis of different specimens of carp fish collected from a river in Spain that is directly contaminated with wastewater from the treatment plant, it was observed that in 9 out of the 10 fish analyzed, caffeine was identified in concentrations between 0.44 ng/g and 24.5 ng/g [66].

Table 3.7 Pharmaceutical Compounds Investigated in Abramis Brama

Compounds pharmaceuticals analyzed	*Fish specimens analyzed*		
	Fish organs	***Abramis brama _1***	***Abramis brama _2***
Caffeine (ng/g)	Muscular tissue	5.79	12.37
	Hepatic tissue	8.37	ND
	Gills	5.16	3.02
	Spawn	13.94	2.98

ND – Undetected

For the other compounds analyzed, no accumulation trends were observed in the organs of fish belonging to the species *Abramis brama*, which is a positive aspect for fish consumers in the natural aquatic environment.

- **Analysis of pharmaceutical compounds in *Vimba vimba***

Vimba vimba known is a small, anadromous bentophagous species of the Carp family that in the natural environment is highly sensitive to habitat changes and pollution [67,68]. It is known as one of the species of European ichthyofauna that is endangered, in particular, due to overfishing [69].

In the present doctoral thesis, there was also followed the degree of accumulation of some classes of pharmaceutical substances in specimens of fish belonging to the species *Vimba vimba* collected from the Danube River. From the results obtained, it can be seen that caffeine is also the one that was detected in all the analyzed organs. For the *Vimba vimba _1 specimen,* the caffeine values were in the range of 16.69 ng/g - 22.55 ng/g, the highest concentration was obtained in the gills. In the second vimba vimba, lower concentrations of caffeine were identified compared to the first, the values being in the range of 2.16 ng/g -11.24 ng/g.

Table 3.8 Pharmaceutical compounds investigated in Vimba vimba

Compounds Pharmaceuticals analyzed	*Fish specimens analyzed*		
	Fish organs	*Vimba vimba _1*	*Vimba vimba 2*
Caffeine (ng/g)	Muscular tissue	16.69	10.17
	Hepatic tissue	19,91	11.24
	Gills	22.55	2.16

ND – Undetected

From Table 3.8 it can be seen that for the specimens of the species *Vimba vimba* apart from caffeine, no other pharmaceutical compounds were detected from the list of those studied. The octanol/water partition coefficient of each pharmaceutical compound analyzed confirms that the potential for their bioaccumulation and bioconcentration in biota is low.

In the literature, there are no studies that follow the analysis of this type of industrial pollutants in the species *Vimba vimba.* However, in a study in which I am a co-author, it was found that this species of fish has the ability to accumulate heavy metals existing in the natural aquatic environment, especially Hg, for which high values (0.28 mg/kg) were obtained [64].

3.4.5 Predictive tool based on a supporting analytical framework in order to increase the sustainability of the monitoring activity of industrial pollutants in the water of the lower Danube basin

The present study aims to achieve a complex predictive framework, based on multiple mathematical algorithms, in order to elucidate complex relationships between various pharmaceutical compounds in water, thus providing the necessary basis for the further development of virtual sensors in order to increase the sustainability of monitoring activities at the level of the lower Danube basin.

The absolute novelty of the study is supported, in particular, by the structure and composition of the database, which consists of a number of 9 emerging pharmaceutical compounds that are on the priority list at the level of European and international decision-making bodies, as potentially added to the lists of chemical species in the Water Framework Directive. It is worth mentioning that the very identification and quantification of these products was a challenge, especially from the point of view of analytical requirements and strategies for optimizing the procedures used. The compounds in question were quantified with a different frequency, which generated a varied number of inputs at the level of each parameter, a challenging scenario in the context of the use of Artificial Intelligence (AI) algorithms.

In order to achieve the analytical framework, AI-supervised and unsupervised techniques were tested. The unsupervised models aim to identify the mode of action of the algorithm and their purpose is to record all types of results, analyzing, as a whole, all the existing relationships at the level of the inputs. In this case, a series of unsupervised learning algorithms can be identified, which can be used to cluster the database into groups of data with the same statistical behavior. Given this, the Principal Component Analysis (PCA) was taken into account, which was preceded by the correlative and statistical analysis.

Supervised learning is generated by a series of algorithms capable of identifying relationships between multiple predictors, also called independent variables, and a dependent variable called a predicted variable associated with the virtual sensor to be developed. Thus, in the present study, a series of 12 supervised learning models were tested, as follows:

- Linear Regression;
- Ridge;
- RANSAC Regressor;
- Voting Regressor;
- Stacking Regressor;
- Support vector Regression – SVR;
- XGBoost Regressor -XGB;
- AdaBoost Regressor;
- RandomForest Regressor;
- Decision Tree Regressor;
- Gaussian Process Regressor;
- Linear GAM;

The accuracy metrics of the above-mentioned models are as follows: mean squared error, root mean squared error, R-squared, Adjusted R-squared.

- ***Correlative and statistical analysis***

The graph has rectangles in the abscissa and ordered next to each chemical species, the degree of correlation being described by the legend, the minimum value being -1, and the maximum value +1.

From the analysis of the correlation matrix made between the 9 compounds, a strongly positive correlation between ketoprofen and clindamycin is observed, as well as positive correlations (correlation coefficient >0.5) between carbamazepine and metformin, sulfamethoxazole and clarithromycin.

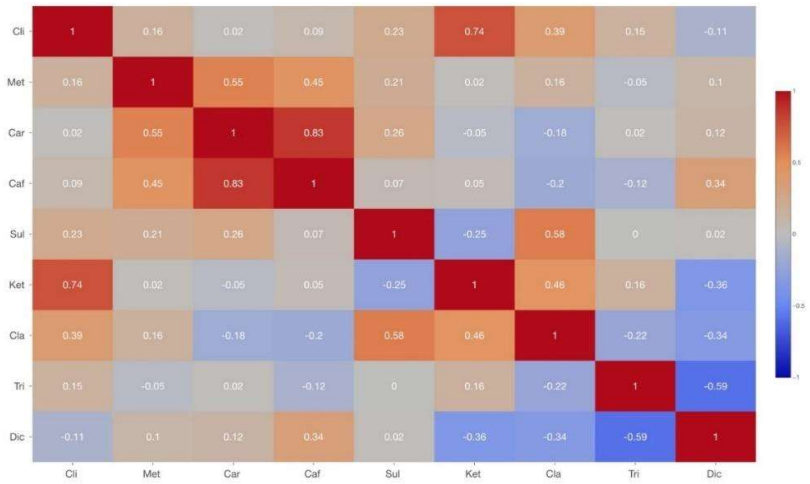


Figure 3.16 Matrix of correlations of pharmaceutical compounds

The only negative correlation with a coefficient greater than 0.5 is recorded between trimoprim and diclofenac (Figure 3.16).

➢ ***Unsupervised Artificial Intelligence Techniques***

- PCA Analysis of Pharmaceutical Compounds

The PCA analysis of pharmaceutical compounds highlights 7 distinct components at the database level.

Thus, caffeine, carbamazepine and metformin are the main compounds within the first component, which explain more than 80% of the variation in the component. Within the second component, clindamycin and ketoprofen explain more than 90% of the variability, while for component 3, trimester is the central component (Figure 3.17).

	Components							
Variables	PC1	PC2	PC3	PC4	PC5	PC6	PC7	Total
Caf	0.26	0.01	0.04	0.14	0.13	0.24	0.17	1.00
Car	0.30	0.03	0.00	0.07	0.03	0.13	0.44	1.00
Cli	0.08	0.34	0.00	0.10	0.06	0.27	0.15	1.00
Ket	0.01	0.48	0.00	0.01	0.21	0.24	0.06	1.00
Met	0.29	0.00	0.00	0.02	0.42	0.11	0.16	1.00
Sul	0.06	0.10	0.17	0.52	0.15	0.00	0.01	1.00
Tri	0.00	0.04	0.78	0.15	0.01	0.01	0.01	1.00

Figure 3.17 Classification of the database by components

The first two components explain more than 65% of the variability of the entire dataset (Fig. 3.18). There are close relationships of variation between metformin and caffeine, these being related to component 1 (PC1), as well as between trimyreb and ketoprofen, these conditioning to a good extent the variation of PC2.

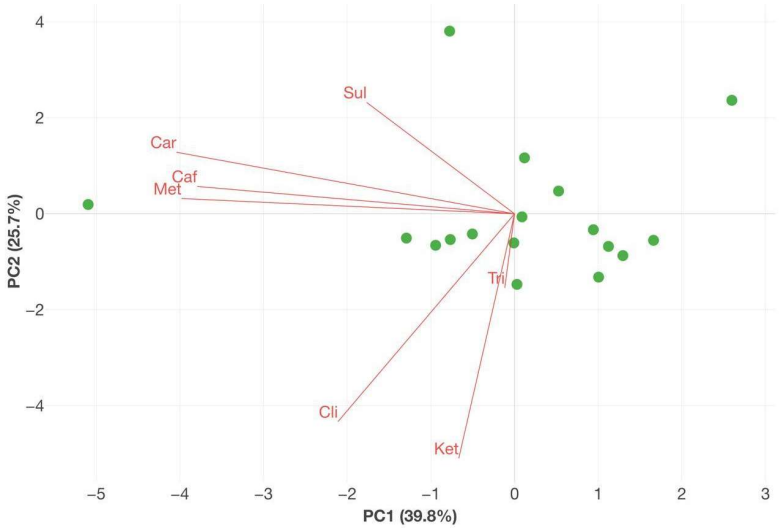


Figure 3.18 PCA Analysis of Pharmaceutical Compounds

- **Supervised modeling – Machine Learning**

- ***Component 1 of the analytical framework***

  - ***Caffeine Prediction Model***

The Machine Learning algorithms presented above were used to generate predictive models that aim to predict the concentration of caffeine according to carbamazepine and metformin.

Taking into account the algorithms with high interpretability in terms of feature importance values, we can say that carbamazepine is the main predictor for determining the concentration of caffeine in water, a fact confirmed by both AdaBoost Regressor and Decision Tree Regressor, XGBoost Regressor -XGB.

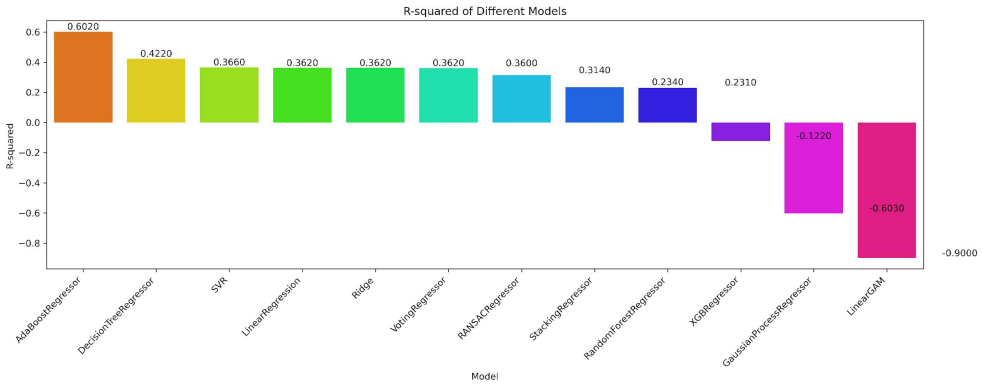


Figure 3.19 R-squared obtained for caffeine prediction – component 1

Thus, analyzing the accuracy metrics of the tested models (Figure 3.19) it can be seen that the AdaBoost Regressor and Decision Tree Regressor algorithms record the best prediction performance, while the Gaussian Process Regressor and Linear GAM indicators have negative R-sq values.

Table 3.9 *Feature importance values associated with machine learning algorithms with high interpretability for caffeine prediction*

Feature	RandomForest	AdaBoost	DecisionTree	XGBoost
Carbamazepine	0.843	**0.634**	**0.893**	0.953
Metformin	0.157	0.366	0.107	0.047

There is a high similarity of the feature importance results when using Decision Tree Regressor and RandomForest Regressor (Table 3.9). We can conclude that Decision Tree Regressor offers superior accuracy and is the only superior accuracy model validated by another similar model, namely RandomForest Regressor.

- ***Component 2 of the analytical framework***

  - ***Carbamazepine prediction model***

The Machine Learning algorithms presented above were used to generate predictive models that aim to predict the concentration of carbamazepine according to caffeine, metformin, trimoprim, clindamycin and sulfamethoxazole.

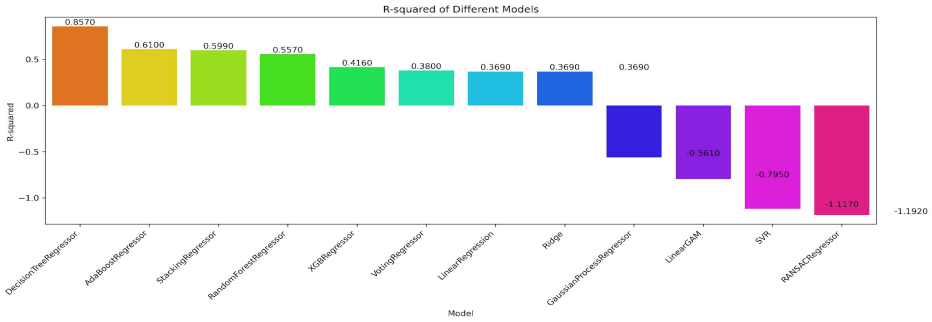


Figure 3.20 R-squared obtained for the prediction of carbamazepine - component 2

Contrary to the situation encountered in component 1, for carbamazepine modeling, in component 2 the diversification of the number of predictors induces an increase in the accuracy metrics of the prediction models. Thus, the best results are recorded next to Stacking Regressor; AdaBoost Regressor; RandomForest Regressor, Decision Tree Regressor. Of these algorithms, 3 are ranked in the area of those with high interpretability, 2 being similar in terms of feature importance ranking.

Caffeine turns out to be the most important predictor for predicting carbamazepine according to RandomForest Regressor and Decision Tree Regressor followed by metformin. Thus, the result recorded in component 1 is validated and it is concluded that a more diversified analytical framework (high number of predictors) can enhance the accuracy of the prediction metrics for this compound (Figure 3.20).

  - ***Sulfamethoxazole Prediction Model***

The Machine Learning algorithms presented above were used to generate predictive models that aim to predict the concentration of sulfamethoxazole according to caffeine, metformin, trimoprim, clindamycin and carbamazepine.

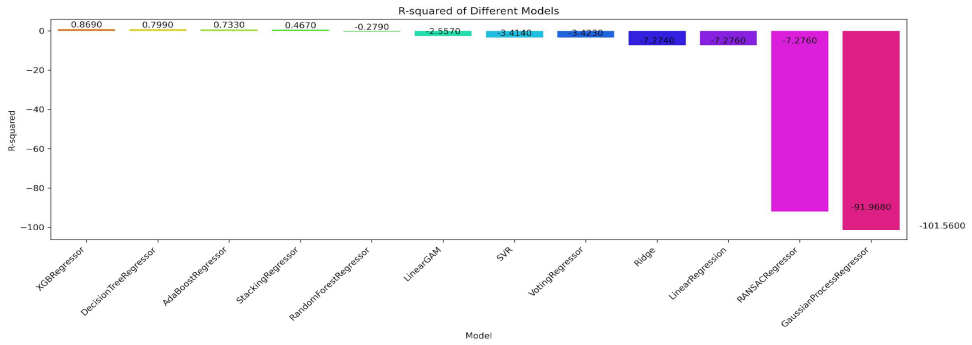


Figure 3.21 R-squared obtained for the prediction of sulfamethoxazole - component 2

For the modeling of sulfamethoxazole, in component 2 the diversification of the number of predictors induces an increase in the accuracy metrics of the prediction models. Thus, the best results are recorded next to Stacking Regressor; AdaBoost Regressor; RandomForest Regressor, Decision Tree Regressor. Of these algorithms, 3 are classified in the area of those with high interpretability. Carbamazepine, followed by trimoprim and clindamycin prove to be the most important predictors for the prediction of sulfamethoxazole according to the AdaBoost Regressor; RandomForest Regressor, Decision Tree Regressor, and XGBoost Regressor - XGB. Thus, the prediction model represents an effective support tool for the prediction of sulfamethoxazole, based on the variation behavior of the 5 predictors previously recorded (Fig. 3.21).

- ***Component 3 of the analytical framework***

  - ***Prediction model of antiepileptics***

The grouping of the parameters by classes of pharmaceutical compounds was made for two reasons, the first reason generated by the need to overcome the limiting factor related to the low number of inputs associated with each compound and the second reason that is triggered by the assumed methodological design, that of validating the results obtained in the compound modeling scenarios (component 1 and component 2), using drug class modelling scenarios.

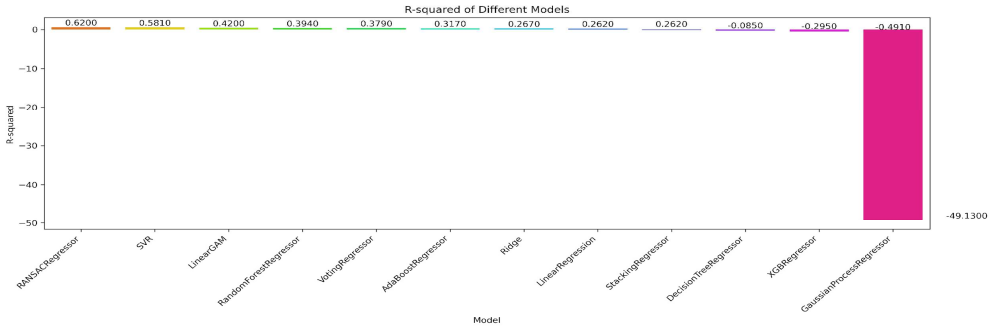


Figure 3.22 R-squared obtained for the prediction of antiepileptics - component 3

The metrics of the antiepileptic prediction model according to alkaloids and antibiotics are superior to those associated with the carbamazepine prediction model within component 1. Thus, RANSAC Regressor and Support vector Regression – SVR record the highest values for R-sq, while among the algorithms with high interpretability, AdaBoost Regressor,

RandomForest Regressor are the most accurate in prediction. In conclusion, analyzing the feature importance values of the last 2 shows a high similarity of them, thus highlighting the high influence of antibiotics on antiepileptic drugs.

Table 3.10 *Feature importance values associated with machine learning algorithms with high interpretability for the prediction of antiepileptics*

Feature	RandomForest	AdaBoost	DecisionTree	XGBoost
Alkaloids	0.383	0.398	0.344	0.168
Antibiotics	0.617	0.602	0.656	0.832

The results of the prediction partially confirm the value of the feature importance of caffeine, respectively the class of alkaloids, over carbamazepine, respectively of the group of antiepileptics and, at the same time, offer new perspectives for the development of the predictive framework in the direction of antibiotics as main predictors (Fig.3.22).

Alkaloid prediction model

The algorithm for predicting alkaloids according to antiepileptics and antibiotics reveals good performance in the RANSAC Regressor, Stacking Regressor Support vector Regression – SVR, Linear GAM, as well as XGBoost Regressor -XGB, AdaBoost Regressor, RandomForest Regressor among the models with high interpretability (Fig. 3.23).

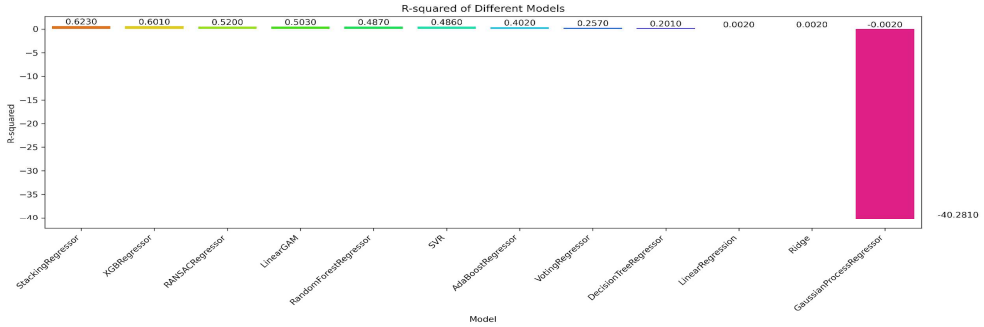


Figure 3.23 R-squared obtained for alkaloid prediction - component 3

Table 3.11 *Feature importance values associated with machine learning algorithms with high interpretability for alkaloid prediction*

Feature	RandomForest	AdaBoost	DecisionTree	XGBoost
Antiepileptics	**0.657**	0.514	0.650	**0.577**
Antibiotics	0.343	0.486	0.350	0.423

A concordance of the feature importance value associated with the 2 classes of antiepitheliptic and antibiotic predictors is observed when the last mentioned algorithms are taken into account. Thus, antiepileptics are considered the main predictor for alkaloids, confirming the hypothesis demonstrated in component 1, according to which carbamazepine is the main predictor for caffeine (Table 3.11).

Chapter 4. Methods of degradation of pharmaceutical compounds

4.1 Objectives of the study

In the previous chapters, the presence of persistent pharmaceutical substances has been demonstrated, especially in water, which can affect the quality of surface aquatic ecosystems, biota and, directly, human health, taking into account that on the territory of Romania there are over 6 million people whose main source of drinking water is the water of the Danube.

The previous chapters had as objectives, in the first part, the optimization of monitoring methods and their application on environmental samples. Another objective of the doctoral thesis in industrial engineering is to find practical solutions that allow the reduction or elimination of the pharmaceutical substances highlighted in the previous chapters. A principle that was the basis for the choice of the wastewater treatment method is related to the sustainability of the processes, respectively to their economic affordability, with a reasonable cost-benefit ratio that does not raise the operating costs above certain bearable limits. Apart from these considerations, the selection of treatment methods took into account the possible multifunctionality, i.e. the ability of the methods to ensure the elimination of potentially toxic microorganisms.

4.2 Experimental description

Most water treatment plants use, in water disinfection, chemical oxidants, such as chlorine, which implies a number of disadvantages, among which, the most important is the obtaining of disinfection by-products with very high toxicity for the environment and human health. For this reason, lately, special attention has been paid to the development of advanced processes that do not involve affecting the environment and human health.

Advanced wastewater treatment plants use the application of UV-C lamps as an effective method of disinfection. For this reason, the present study aims to capitalize on this technology by researching its ability to achieve photolysis of different classes of pharmaceutical substances [70]. In view of the above, the method that corresponds to the conditions described above involves the exposure of classes of pharmaceutical substances found in wastewater to UV-C radiation with a wavelength of 254 nm, a method that has the ability to react both on chemical species with multiple covalent bonds and on microorganisms.

The compounds subjected to photolysis were: sulfamethoxazole, carbamazepine, ketoprofen, diclofenac, trimoprim, claritomycin, ciprofloxacin, clindamycin, caffeine and metformin. For each compound, individual standard solutions of 100 ng/mL concentration were prepared, this concentration was chosen to be close to the actual concentrations in which these compounds are most often found in wastewater. The resulting solutions were exposed to a UV-C lamp with a power of 20 W over the following time intervals: 0 minutes, 5 minutes, 15 minutes, 25 minutes, 45 minutes, 60 minutes and 90 minutes. In literature, there are similar experiments, but with values of chemical concentrations 1000 times higher than those in real wastewater, which leads to uncertainties about the reproducibility of experiments in real conditions. Taking into account the multitude of industrial chemical pollutants containing multi-link systems (chromophores) sensitive to UV-C attack, the experiments meet the conditions for transfer to wastewater treatment plants with minimal technological costs.

4.3 Results and discussions

- ***Degradation of Sulfamethoxazole under the action of UV-C radiation***

The degradation experiment of the pharmaceutical compound sulfamethoxazole consisted of exposing a solution of 100 ng/mL concentration to UV-C radiation at the following time intervals: T0 - sample without exposure to UV-C radiation, T1 - exposure for 5 minutes to UV-C radiation, T2 - exposure for 15 minutes to UV-C radiation, T3 - exposure for 25 minutes to UV-C radiation, T4 - exposure for 45 minutes to UV-C radiation, T5 - exposure for 60 minutes to UV-C radiation and T9 - exposure for 90 minutes to UV-C radiation (Fig.4.1).

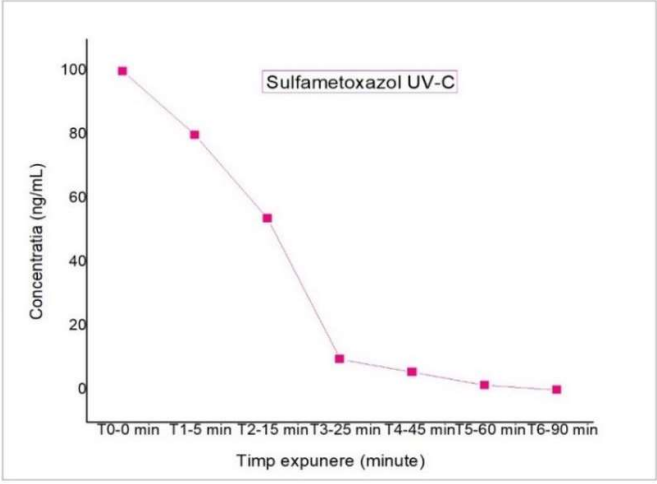


Figure 4.1 Variation of Sulfamethoxazole concentration according to UV-C exposure time

For this compound, a decrease in the concentration of sulfamethoxazole is observed, which is directly proportional to the time of exposure to UV-C radiation. After 5 minutes of lamp exposure, the concentration of sulfamethoxazole decreased by 20.16%, after 15 minutes the concentration decreased from 79.94 ng/mL to 53.83 ng/mL. A significant decrease in the concentration of sulfamethoxazole was observed after exposing the sample for 25 minutes to UV-C radiation, when the concentration decreased by approximately 90%. After a 90-minute exposure to UV-C radiation, the sulfamethoxazole compound was completely degraded.

In the experiment carried out in the present doctoral thesis on the sulfamethoxazole solution of low concentration of 100 ng/mL, the appearance of an isomer of sulfamethoxazole at retention time 1,6 was observed with exposure to UV-C radiation, which is represented by the main degradation product of this compound, namely, 4-amino-N-(5-methyloxazole-2-yl) benzensulfonamide - $C_{10}H_{11}N_3O_3S$ which was also reported in literature. The secondary degradation products mentioned in the specialized studies were not identified in the present study, they are below the detection limit of the LC-MS equipment. This may highlight the fact that for low concentrations of sulfamethoxazole, in the ng/mL order, the concentration of this compound and its degradation products decreases significantly, following photolysis, below the detection limit of high-performance and high-resolution equipment.

- ***Degradation of Diclofenac under the action of UV-C radiation***

For the photolysis of the diclofenac compound, the same experimental conditions were observed as in the case of the sulfamethoxazole compound. In Figure 4.2 it can be seen that

after 5 minutes of exposure to UV-C radiation the concentration of diclofenac decreased by 16.94%, and after 15 minutes there was a significant decrease of about 62.8% compared to T0. After 25 minutes of exposure to UV-C radiation, the concentration of diclofenac reached 14.91 ng/mL, and after 45 minutes the concentration of 1.09 ng/mL was obtained. Exposure to more than 60 minutes completely degraded the compound diclofenac, as can be seen in Figure 4.2.

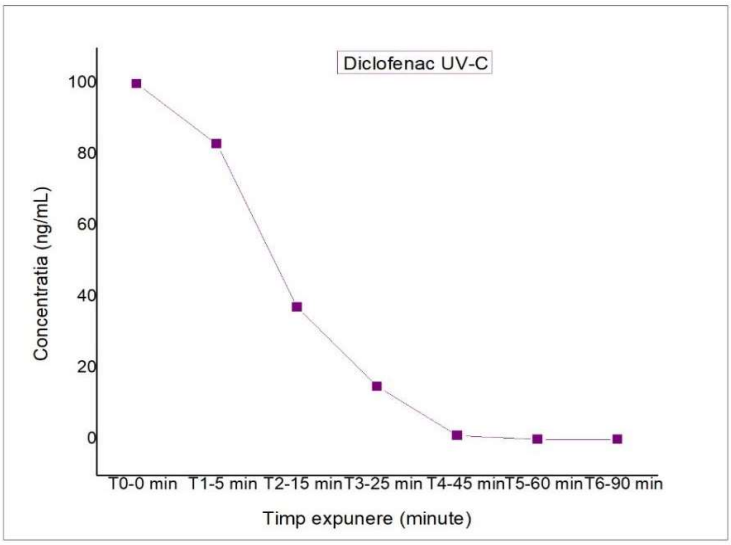


Figure 4.2 Variation of Diclofenac concentration according to UV-C exposure time

In the specialized studies carried out on the photolysis of diclofenac in concentrations of the order mg/L, different degradation products were obtained. The primary degradation product of diclofenac obtained from exposure to UV-C radiation is 2-(8-chloro-9H-carbazole-1-yl) acetic acid - $C_{14}H_{10}ClNO_2$ (m/z= 260.04783) which undergoes a subsequent transformation into the acetic acid (8-hydroxy-9H-carbazol-1-yl) - $C_{14}H_{11}NO_3$ (m/z=242.08169) [71,72]. In the present doctoral thesis, at the retention time 7.54, after exposure of diclofenac for 5 minutes to UV-C radiation, the appearance of its primary degradation product ($C_{14}H_{10}ClNO_2$, m/z= 260.04783) was observed, which was also identified in the samples exposed for 15, 25 and 45 minutes. Also, the secondary degradation product $C_{14}H_{11}NO_3$ (m/z= 242.08172) was identified at retention time 6, in samples exposed for 5, 15, 25, 45 and 60 minutes with a decrease in area intensity with increasing exposure time. In the sample, which was degraded for 90 minutes, neither of the two degradation products of diclofenac mentioned above was identified.

- ***Degradation of Ketoprofen under the action of UV-C radiation***

The degradation of the non-steroidal anti-inflammatory Ketoprofen under the action of UV-C radiation proved to be very effective even after the first 5 minutes of exposure. The initial ketoprofen concentration of 100 ng/mL decreased significantly by approximately 90.49% after being subjected to photolysis for 5 minutes. After a 15-minute exposure of ketoprofen solution to UV-C radiation, this compound was no longer identified in the sample, which was also observed in solutions that were exposed for 15, 25 and 45 minutes (Fig. 4.3).

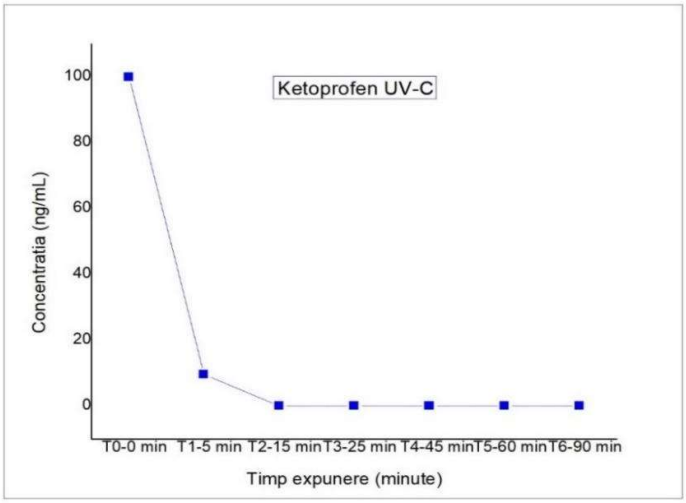


Figure 4.3 Variation of Ketoprofen concentration according to UV-C exposure time

The efficiency of the process of direct degradation of ketoprofen from UV-C radiation is given by the presence of the benzene ring that is in its structure. Direct photolysis of ketoprofen favors the breakdown of the hydroxyl group linked to the carbonyl group that leads to the formation of •OH radicals [73]. In a study carried out on the degradation of ketoprofen in a solution with a concentration of 1 mg/L under the action of UV-C radiation, the following degradation products were obtained: 2-(3-(carboxyoxomethyl)-phenyl) propanoic acid - $C_{11}H_{10}O_5$ (m/z=223), acid 2-(3-(carboxi(hidroxi)-metil)fenil) propanoic - $C_{11}H_{12}O_5$ (m/z=225) [74]. In the present study, the degradation products mentioned in the literature were not identified, which may be due to the low concentration of ketoprofen subjected to degradation (100 ng/mL), and the degradation products being, most likely, below the detection limit of the equipment.

- ***Degradation of Ciprofloxacin under the action of UV-C radiation***

Another antibiotic subjected to photolysis is Ciprofloxacin which has been exposed to UV-C radiation under the same experimental conditions as the other compounds described above.

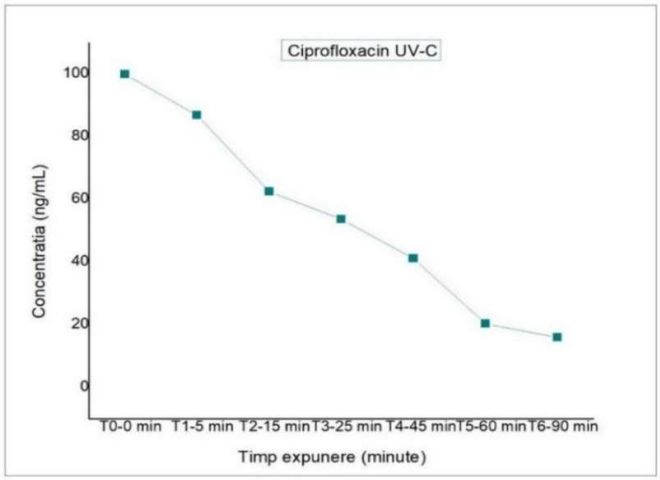


Figure 4.4 Variation of Ciprofloxacin concentration according to UV-C exposure time

From graph 4.4 there is a decrease in the initial concentration by 13.08%, even after 5 minutes of exposure of the sample to UV-C radiation. After 15 minutes of degradation, the concentration of ciprofloxacin decreased by approximately 46% and after 25 minutes the concentration halved. Exposure of this pharmaceutical compound at 45 and 60 minutes led to its degradation by 59% and 79%, respectively. The results obtained showed that, even after 90 minutes, its total degradation was not achieved, the concentration measured in the last sample being 15.89 ng/mL. After the analysis of the samples subjected to photolysis was performed and the decrease in the concentration of the compound ciprofloxacin was observed, its degradation products were searched in the mass spectrum. At the retention time of 3.1 minutes, the main degradation product was identified, which has m/z = 330.14538 and for which an increase in the intensity of the area was observed with the increase in the exposure time to UV-C radiation.

Another degradation product of ciprofloxacin is the compound with the molecular formula $C_{17}H_{17}N_3O_5$ and m/z = 344.12465 which was identified at retention time 3.9 minutes and for which the highest intensity of area was observed in the sample that was degraded for 90 minutes.

For the other pharmaceutical compounds subjected to direct photolysis with UV-C, their degradation was not observed.

Chapter 5. Final conclusions, personal contributions and future research directions

The results presented in this doctoral thesis were obtained following several studies that had an important personal contribution in generating the following relevant conclusions aimed at highlighting the importance of studying the transport of industrial pollutants in aquatic ecosystems:

- industrial activities have an important contribution to the contamination of sediments with heavy metals;
- assessing the level of heavy metal contamination of sediments in the Danube river basin, using quality indices, has proven to be an effective and internationally appreciated method;
- the geo-accumulation index and the contamination factor calculated for each metal analysed indicated the existence of sediment contamination in particular with the heavy metals Ni and Cd;
- the Potential Ecological Risk Index have moderate values and indicate the existence of a low ecological impact for sediment samples taken and analysed from the lower sector of the Danube River;
- the studies and scientific researches whose results are mentioned in this thesis fill in some gaps (geographical and temporal) regarding the monitoring in the Danube basin on the territory of Romania of an important class of emerging pollutants, represented by pharmaceutical compounds;
- the pharmaceutical industry, through the important production of products for human and animal use, has a major contribution to the presence of pharmaceutical compounds in aquatic ecosystems;
- the identification of pharmaceutical substances, such as: metformin, caffeine, carbamazepine, trimoprim, sulfamethoxazole, clindamycin, clarithromycin, diclofenac and ketoprofen in surface water samples taken from the lower Danube basin has paved the way for new medium and long-term studies to complement the priority lists that will be in the normative documents at national and European level;
- pharmaceutical pollutants have both industrial origin and origin resulting from quantities of unmetabolised substances; as a result, the wastewater discharged from municipal wastewater treatment plants has a major contribution to the contamination of the Danube with pharmaceutical substances; it is clear that there is a need to adapt wastewater treatment techniques to the presence of new classes of pollutants, in particular those for pharmaceutical use;
- experiments carried out in order to determine the photosensitivity of some classes of pharmaceutical substances have led to conclusions regarding the possibility of optimizing wastewater treatment involving UV-C radiation in possible combination with other physical methods or with the presence of strong oxidants, producers of free radicals that do not affect the quality of water after treatment;
- the analysis of pharmaceuticals from sediment samples collected from the Danube River demonstrated the existence of a low affinity of this class of industrial pollutants for the solid phase of the aquatic environment;

- the morphological analysis of the sediments showed that the sediments with the smallest granulation have the superior absorption capacity of pharmaceutical compounds compared to those with large granulations;
- research carried out on the accumulation of pharmaceutical compounds in the tissues of different species of fish collected from the natural ecosystem of the Danube has revealed the existence of chemical species in certain organs; It is noted that for some chemical species, the concentrations of medicinal substances are below the detection level, which does not eliminate the risk posed by them, but leads to the need for further studies in this regard.
- the chemical species identified as having the highest frequency in ichthyofauna is caffeine; the species *Alosa immaculata* (Mackarel) is the one that has accumulated the most pharmaceutical compounds of those analyzed in the present study; it is necessary to deepen the studies on migratory species in the Danube River;
- The use of Machine Learning algorithms has shown promise in predicting pharmaceutical compounds and classes of pharmaceutical substances. However, the database used presented a series of limitations highlighted both dimensionally and qualitatively. In this context, a series of guidelines have been identified regarding the optimal algorithms that can be used to obtain possible satisfactory prediction metrics, under supportive, favorable conditions.

Future research directions

As research in the pharmaceutical industry is constantly developing, and pharmaceutical compounds are found in a very wide variety on the consumer market, it is necessary to carry out new studies that follow their transport and behavior in the aquatic environment, such as:

- extension of monitoring to other classes of pharmaceutical compounds reported in the literature as having toxic potential on the aquatic environment;
- developing the database by expanding and diversifying in order to maximize the prediction performance associated with the support analytical framework initiated and developed in this study;
- providing new concepts and technologies for municipal wastewater treatment, including experiments involving testing different methods of degradation of pharmaceutical compounds by combining UV-C radiation with other oxidants;
- study of compounds resulting from the application of several wastewater treatment methods, including products resulting from the interference of pharmaceuticals with commonly used oxidants, e.g. chlorine and its derivatives;
- geographical extension of research throughout the Romanian Danube area, as well as upstream of it through the efficient use of the REXDAN Infrastructure, including the research vessel;
- in order to define the accumulation of pharmaceutical substances in ichthyofauna in a controlled manner, in the next period at the level of the team we will proceed to set up a natural laboratory (pond) that will be populated with fish (juvenile) from areas independent of the Danube, and the accumulation of pharmaceutical substances will be periodically monitored on chipped specimens, the pond having water exclusively from the Danube;

- the research initiated by this doctoral thesis will be continued with the introduction of pharmaceutical substances in WQI impact factors, in an integrated form that will allow the extension of the way in which this index reflects, globally, the quality of aquatic ecosystems;
- Taking into account the fact that the Danube is a main or secondary source for drinking water for 6 million consumers, one direction of development will be to predict the impact that the accumulation of pharmaceutical substances can have on human health.

Personal contributions
WOS: H – Index = 6
Google Academic: H – Index = 7

List of projects implemented during the doctoral period

1. POC project "Integrated system for complex environmental research and monitoring in the area of the Danube river REXDAN", SMIS code 127065;
2. POIM project Improvement of hydrological conditions in the natural aquatic habitats of the Danube Delta Biosphere Reserve for the conservation of biodiversity and fishery resources - Gorgova-Uzlina, Roșu-Puiu lake complexes", POIM 120890;
3. POIM project "Improvement of hydrological conditions in the natural aquatic habitats of the RBDD for the conservation of biodiversity and fishery resources - Dunăvăț-Dranov, Razim-Sinoie lake complexes, Sinoie-Istria-Nuntași area";
4. POIM project, "Improvement of hydrological conditions in the natural aquatic habitats of the RBDD for the conservation of biodiversity and fishery resources - Șontea-Furtună, Matița-Merhei, Somova Parcheș lake complexes" SMIS Code 2014+ 120889;
5. POIM project "Revision of the management plan and RBDD regulation" MYSMIS Code 2014+ 123322
6. Proiect „Advanced nanotechnology based approaches to waste water purification form organic pollutants and their monitoring in water bodies”, 2SOFT/1.2/139;
7. Proiectul „HORIZON-MISS-2021-OCEAN-02, DANUBE REGION WATER LIGHTHOUSE ACTION”, Project: 101094070 — DALIA;
8. Proiectul PNRR „Integrated research and sustainable solutions to protect and restore Lower Danube Basin and coastal Black Sea ecosystems” ResPonSE 760010/30.12.2022;
9. HORIZON project "Restoration of wetland complexes as life supporting systems in the Danube Basin (Restore4Life)" financed from non-reimbursable European funds, financing contract no. 101112736/01.06.2023.
10. Proiect: 101156533 „Innovative sediment management framework for a SUstainNable DANube black SEa system” (SUNDANSE)— HORIZON-MISS-2023-OCEAN-01
11. Contract with third parties with the socio-economic environment - Analysis of heavy metal concentrations in soil samples using the ICP-MS technique - Project director;
12. Contract with third parties with the socio-economic environment - Analysis of pharmaceutical substances from 18 water samples and 16 sediment samples" - financing contract no. 820/27.03.2024 - Project Director;
13. Participation in training internship – In May 2022 I participated in a training internship at the INCDO-INOE 2000 institute, ICIA Analytical Instrumentation Research Institute Branch.

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4. Calmuc, M., **Calmuc, V**., Arseni, M., Topa, C., Timofti, M., Georgescu, L.P., Iticescu, C., 2020. A Comparative Approach to a Series of Physico-Chemical Quality Indices Used in Assessing Water Quality in the Lower Danube. Water 12, 3239. https://doi.org/10.3390/w12113239 (IF =2.544).;
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9. Alina Antache, **Valentina Calmuc**, Stefan-Mihai Petrea, Ira-Adeline Simionov, Madalina Calmuc, Aurelia Nica, Dragos Cristea, Mihaela Neculita, The influence of pharmaceutical residues from surface waters on fish oxidative stress: A review, Scientific Papers. Series E. Land Reclamation, Earth Observation & Surveying, Environmental Engineering, ISSN 2285-6064 IF =0.4.
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2. **Călmuc V**, Călmuc Mădălina, Georgescu P.L., Iticescu C., Timofti M.,ArseniM., ȚopaC., Roșu,A.,. Spatial distribution of heavy metals in the Danube surface sediment near the Galati City. Tehnomus Journal, 2019, pp. 66-70;
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1. **Valentina Andreea Calmuc**, Madalina Călmuc , Maxim Arseni, Cătălina Iticescu, Puiu-Lucian Georgescu, Detection of pharmaceuticals in fish species from the Danube River, SCDS-UDJG 2024 The 12th Edition, GALAŢI,6th-7th of June 2024.
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9. Alina ANTACHE, **Valentina Calmuc**, Stefan-Mihai Petrea, Ira-Adeline Simionov, Madalina Calmuc, Aurelia Nica, Dragos Cristea, Mihaela Neculita, The influence of pharmaceutical residues from surface waters on fish oxidative stress: A review, The International Conference Agriculture For Life, Life For Agriculture, iunie 2022;
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19. **Valentina Andreea Calmuc**, Madalina Călmuc, Maxim Arseni , Catalina Iticescu, Lucian P. Georgescu, *Preliminary statistical relationship between heavy metals in Lower Danube sediment,* (poster presentation), Conferința Școlilor Doctorale: 8th Edition of SCDS-UDJG, Galați, 18-19 June, 2020
20. Madalina Călmuc, **Valentina Andreea Calmuc**, Maxim Arseni, Lucian P. Georgescu, Catalina Iticescu, *Assessment of nutrients and oxygen regime in the Lower Danube water,*(poster presentation), Conferința Școlilor Doctorale: 8th Edition of SCDS-UDJG, Galați, 18-19 June, 2020.
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22. **Călmuc Valentina-Andreea,** Călmuc Mădălina, Georgescu P. Lucian, Iticescu Catalina, Timofti Mihaela, Arseni Maxim, Țopa Cătălina, Roșu Adrian. *Spatial distribution of heavy metals in the Danube surface sediment near the Galati City.* The 20th Edition of the International Conference Tehnomus New Technologies And Products In Machine Manufacturing Technologies, Section 3 Environmental engineering and sustainable development, Oral presentation no. 3, 2019, http://www.tehnomus.usv.ro/
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1. **Valentina Calmuc**, C. L. Chițescu, M. Călmuc, C. Iticescu, L. P. Georgescu,

Applications of Liquid Chromatography Coupled with Mass Spectrometry in the Analysis of Pharmaceutical Compounds in Water Samples, WORKSHOP Modern Approaches to Feedback between Environmental Processes and Climate Change 6-9 July 2022, GALAȚI;

2. Madalina Calmuc, **Valentina Calmuc**, Maxim. Arseni, Adrian. Roșu, Lucian. Puiu. Georgescu, Catalina. Iticescu, Using Microscope-Coupled FTIR Spectrometry (Micro-FTIR) in the Identification of Microplastics in Danube Water, WORKSHOP Modern Approaches to Feedback between Environmental Processes and Climate Change 6-9 July 2022, GALAȚI.

Published Patents

1. I contributed to the development of the patent no. 134991 - **Method and apparatus for collecting microplastics from rivers and lakes.**

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