THESIS

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Analytical and ecotoxicological studies of carbamazepine pharmaceutically active compound

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Table of Contents

1. Introduction and objectives

Environmental pollutants enter the environment partly as a result of human activity, which poses a threat to living organisms. Their harmful effects are determined by three factors: chemical properties, concentration and persistence. Pollutants that are present in the environment in very low concentrations (ng/L) are called micropollutants. These may include organic and inorganic micropollutants. Drug residues belong to the group of organic micropollutants. In general, medicines are only partly utilized in the body, and the remaining part leaves the body in form of the urine or faeces (József *et al.*, 2023). Organic pollutants generated frequently flow from households to the environment *via* the sewage system; for instance, taken or disposed of medications, cosmetics, oil, and other chemicals are likely to find their way into the sewage system (Bahlmann *et al.*, 2009). Pharmaceutically active compounds (PhACs) are emerging environmental issues due to their presence in the aquatic environment and potential for impacts on wildlife and humans. Worldwide average *per capita* consumption of pharmaceuticals per year is estimated to be about 15 g. One of the most frequently reported pharmaceuticals in surface water is carbamazepine (CBZ). This is a high-selling drug (61 tons prescribed in Germany in 2006) (József *et al.*, 2023). CBZ is an antiepileptic drug with an annual consumption rate of about 2235000 pounds worldwide (Abdelhafidh *et al.*, 2018).

CBZ has been classified as a high-risk compound for aquatic systems; it has a negative impact on aquatic life, including algae, invertebrates, and fish. Even though CBZ was not anticipated to cause an immediate toxic impact on aquatic organisms (EC_{50}: 4.5–383.5 mg/L), it may cause long-term adverse effects in the aquatic environment (Jos *et al.*, 2003). As the key component of trophic chain of water ecosystems microalgae are recognized as perspective objects to assess the condition of water environment due to their small size, short generation time, and high sensitivity to pollutions. Moreover, the chlorophyll fluorescence technology of microalgae makes it possible to greatly assess the ecological risk of water contaminants. It enables fast, precise, and contactless measurements of the photosynthesis efficiency of microalgae cells. In most prior studies, the researchers merely investigated the effects of the growth inhibition of CBZ only on microalgae (Wu *et al.*, 2022a). *Chlorella vulgaris* is a typical phytoplankton in freshwater ecosystems and often used as a toxicity testing biota and evaluation. Furthermore, the growth and the photosynthesis activity of *C. vulgaris* decreased with the increase in CBZ concentration (Wu *et al.*, 2022a).

A fast and inexpensive analytical method for the quantitation of CBZ in wastewater and surface waters is needed due to the assessment or measure must be taken as soon as possible when the samples are collected. At present, the common analytical procedure for determination of CBZ in aquatic samples is liquid chromatography coupled with single or tandem mass

spectrometry (LC–MS/MS). The limit of quantitation (LOQ) highly depends on the matrix and the sample preconcentration. LOQs between 1 and 50 ng/L are usually reported, these methods are time-consuming and expensive and require costly instrumentation and dedicated personnel. To determine CBZ in the aquatic environment, the enzyme-linked immunosorbent assay (ELISA) has been applied; its potential to perform in low concentrations of pharmaceutically active ingredient(s) in water (Jaria *et al.*, 2020).

The target compound of this study is the pharmaceutically active compound, CBZ, that a surface water pollutant. The specific objectives are:

- Investigating the ecotoxicological effect of CBZ on green algae (*Chlorella vulgaris*) through alga growth inhibition test;
- Monitoring of CBZ in freshwater samples from the Lake Balaton and the Danube River with application of enzyme-linked fluorescent immunoassay (ELFIA);

2. Literature review

The increasing chemical pollution of aquatic environments is a growing concern and is of global relevance. A large number of organic chemicals are termed “micropollutants” due to their low concentration, and long-term exposure to micropollutants may pose considerable risks significantly increased in the last few decades. Currently, over 350000 different compounds and mixtures of chemicals are recognized for use and manufacturing (Wang *et al.*, 2020).

2.1. Micropollutants in surface water

Organic micropollutants, including pharmaceuticals, personal care products, and nanomaterials, as the size of the global chemical industry exceeded five trillion dollars in 2017, and it is expected to double by 2030 according to (UN Environment Program, 2019) Pharmaceutical medication concentrations are dispersed throughout environmental matrices. Despite significant progress in wastewater treatment, these compounds still have low removal efficiency. Some or all of them wind up in the treated effluent that is released into surface waters, groundwater, and marine waters, among other receiving water bodies. Indeed, across a range of environmental water samples, pharmaceutically active compounds (PhACs) have been detected at amounts ranging from a few ng/L to several µg/L (Álvarez-Muñoz *et al.*, 2015; Fatta-Kassinos *et al.*, 2011).

In Germany, it was estimated that amounts of up to 16000 tons of pharmaceuticals were disposed of each year from human medical care and 60-80% of those disposed drugs were either flushed down the toilets or disposed of with normal household waste (Scheytt *et al.*, 2006). For example, the antiepileptics (CBZ) were the most dominant of all investigated drugs concentration varied between 155 and 195 ng/L (Drewes *et al.*, 2002); as well as in Berlin, Heberer reports CBZ concentration up to 1075 ng/L in surface water samples (Heberer, 2002).

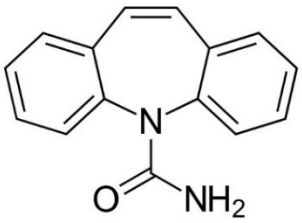
PhAC concentration has been increasing in the surface water and it can be a risk factor of drinking water quality because of the poor filtration capacity of the river bank (Branchet *et al.*, 2019). A previous analysis of the screening of 111 PhACs in one section of the Danube implemented riverside screening in Hungary. Overall, 107 samples collected from the Danube were compared with 90 samples collected from drinking water wells during the five time periods of sampling. In the course of the study, 52 PhACs were identified in the river water, and 10 active compounds were identified in over 80% of the samples. The use of the NSAID test-kit showed that among the active substances the highest frequency of 87.9% was of DCL while naproxen accounted for 5.6%. Thirty-two compounds were found in the samples taken from the drinking water wells in the heptic concentration levels. Compared with the results with

the testing of the rivers, DCL was detected in well water in only 1.1%. CBZ was detected in 106 samples (99.1%) with average concentration of 77.2 ng/L. In the assessment of the riverside filtration, out of all the 22 active analytes detected to be present in the water and highlighted in this paper, fifteen of them were almost wiped out by the filtration from the freshwater riverside. Nevertheless, the effectiveness of the process does not mean that a certain active compound has been quantitatively and qualitatively removed because it stays in the environment in the form of various metabolites which can be identified even after several years (Kondor *et al.*, 2020).

2.2. Carbamazepine (CBZ)

The presence of CBZ (Figure 1) is an antiepileptic medication utilized for managing epilepsy and neuropathic conditions and psychiatric disorder (Ambrósio *et al.*, 2002). CBZ was first synthesized in 1960 and is widely used. According to the global consumption volume of 1014 tons per year and predicted to be increasing in each year (Zhang *et al.*, 2008). Even though, the CBZ is almost entirely metabolized in humans, it is mainly excreted as hydroxylated and conjugated metabolites, with minimal amounts eliminated unchanged, less than 2%. However, the continuous release to the environment, the low removal rate of wastewater (<45%) and sludge (<10%) treatment process (Bueno *et al.*, 2012; Clara *et al.*, 2004; Verlicchi *et al.*, 2012; Zhang *et al.*, 2008).

Figure 1. Chemical structure of carbamazepine
(*source: Zhang* et al*., 2008*)



Physical and chemical properties of CBZ (Zhang *et al.*, 2008)

Chemical formula: $C_{15}H_{12}N_2O$

CAS No. 298-46-4

Therapeutic class: Antiepileptic

Molecular weight (g/mol): 236.3

Water Solubility: 17.7 mg/L (25°C)

Log*P* (octanol-water): 2.45

Amount in Europe in 1999: 268.1 tons

Excretion: 72% in urine and 28% in faeces

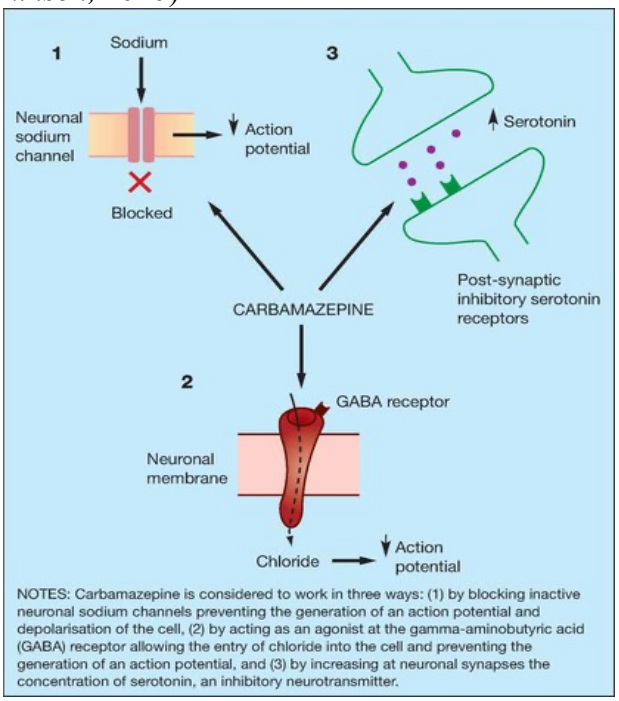
Metabolites in urine: CBZ-epoxide, CBZ-diol, CBZ-acridan, 2-OH-CBZ, 3-OH-CBZ.

A given drug can be sold under different brand names, further obscuring the data. For example, CBZ has the following brands: Biston, Calepsin, Carbatrol, Epitol, Equetro, Finlepsin, Sirtal, Stazepine, Tegretol, Telesmin, Timonil, etc. Furthermore, international trading and repacking complicate tracking the market distribution of drugs. Global CBZ consumption was estimated to have increased from 742 tonnes in 1995 to 1214 tonnes in 2015 (Oldenkamp *et al.*, 2019). As annual consumed volumes of CBZ in some countries, for example in Finland, 4.6 tons in 2005, the dose per capita (DPC) is 920 mg (Vieno *et al.*, 2007), and in Czech Republic 3450 kg in 2020.

CBZ, a sodium channel protein inhibitor, has the properties to treat convulsions. Blom discovered it is potential for treating paroxysmal trigeminal neuralgia in 1962. CBZ is absorbed gradually from the gastrointestinal system within a timeframe of four to twelve hours. It strongly induces cytochrome P-450 (CYP450), which regulates the metabolism of the drug itself and results in numerous significant clinically relevant interactions. CBZ is metabolized in the liver by an isoform of CYP450 (isoform3A4), The active metabolite CBZ epoxide is produced. This active metabolite is primarily eliminated through urine following further degradation via oxidative processes, conjugation, or hydroxylation.

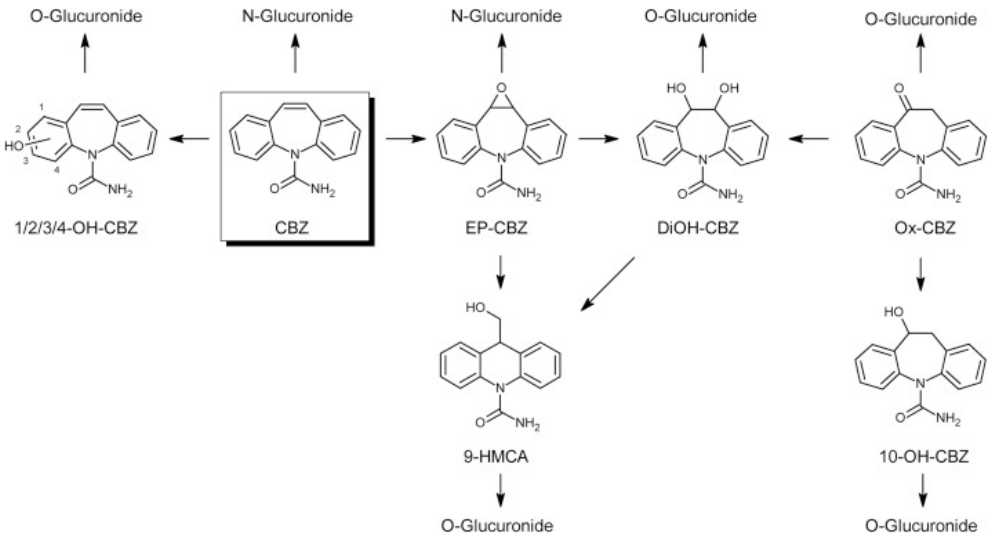
CBZ, also called 5H-dibenz[b,f]azepine-5-carboxamide, is an iminostilbene. Three mechanisms of action have been identified, each of which can be associated with the clinical effects of CBZ in different conditions (Figure 2). The main mechanism of action involves the use-dependent blocking of inactivated neuronal sodium channels, which prevents their opening and thus inhibits the repetitive firing of neurons during a seizure. This also decreases excessive pain signals from damaged sensory nerves. The drug has minimal effects on normal neuronal transmission frequencies because a smaller percentage of sodium channels are in an inactivated state. CBZ functions as a GABA agonist, promoting the activation of the GABA receptor, leading to a calming effect, as well as anxiety-reducing and muscle-relaxing effects. This mechanism of action is thought to be advantageous in the treatment of bipolar disorder (Buescher, 2006).

Figure 2. Mode of action of carbamazepine
(*source: Harkin & Hopkinson, 2010*)



DiOH-CBZ, 2-OH-CBZ, 3-OH-CBZ, EP-CBZ as well as Ox-CBZ and 10-OH-CBZ have been detected in wastewater in various countries (Figure 3). In some cases, the concentrations of the metabolites even exceeded the concentration of the parent compound, e.g., DIOH-CBZ and CBZ The influent wastewater in Germany contained concentrations of 3.7 and 2.0 µg/L, respectively (Bahlmann *et al.*, 2014).

Figure 3. Metabolism of carbamazepine
(*source: Bahlmann* et al.*, 2014*)



In study of Gayford & Redpath, the only indication for CBZ treatment was chronic muscle pain (Gayford & Redpath, 1969). With a mean duration of CBZ intake of 13.4 ± 22 months (range 0–120 months), the average daily dosage was 253.7 ± 138.3 mg (range 100–900 mg). it was observed continuation in 17%, and 15% did not report any side effects. Seven percent of patients received a CBZ prescription but decided not to take it. Forty-four percent stopped taking CBZ due to side effects. The most frequently reported side effects that led to the discontinuation of the drug included elevated liver enzymes, nausea, and fatigue (with potential for double entries). Other reasons for halting CBZ use were a lack of improvement in muscle pain (11%) and interactions with birth control and pregnancy (2%). Additionally, in 33% of cases, no follow-up data was available, including information on the overall duration of medication.

2.3. Carbamazepine in aquatic ecosystems

After consumption, it is estimated that up to 10% of CBZ is expelled from the human body. Recent studies this range has been reported to be from a few tens to several thousands of ng/L CBZ in municipal wastewater (Kasprzyk-Hordern *et al.*, 2009; Luo *et al.*, 2014). Most of the conventional waste water treatment plants (WWTPs) are capable of deleting only a minimal percentage of CBZ which is below 10% (Wijekoon *et al.*, 2013). Therefore, the effluent of the treatment plant serves as the major route through which the compound CBZ gets into the surface and groundwater sources. Table 1. presents the occurrence of CBZ in WWTP effluent, surface water and groundwater in Canada, Germany, Japan, South Korea, Taiwan, the UK and the USA (Hai *et al*., 2018; Stuart *et al.*, 2012) (Table 1). In general, it has been found that the concentration level of CBZ was comparatively higher in the effluents of WWTP rather than the surface water since diluted samples and natural decay could lower the concentration of pollutants.

Table 1. Occurrence of carbamazepine in effluent of WWTPs, in surface water and in ground water in different countries
(*source: Hai* et al*., 2018)*.

Country	WWTP* effluent		Surface water	Groundwater
	No. of WWTPs	concentration (ng/mL)	concentration (ng/mL)	concentration (ng/mL)
Canada	7	33–426	0.7–126	10–49
Germany	5	1075–6300	81–1100	1–100
Japan	20	81–86	0.1–34.7	1.64–97
South Korea	11	73–729	6–61	NA**
Taiwan	4	290–960	0.5–120	NA
UK	3	152–4596	9–327	425–3600
USA	16	33–270	2–172	1.5–42

* waste water treatment plant

** not available

It can be postulated that the most probable mechanism of CBZ contamination of groundwater is bank infiltration of the WWTP effluent (Lapworth *et al.*, 2012). Also, leachate from the landfill and effluents from combined sewers pollute the groundwater. In this section, the factors associated with the presence of PhACs such as CBZ in raw wastewater, WWTP effluent and fresh water sources shall be analysed and discussed (Stuart *et al.*, 2012). Twenty-nine samples were collected from the largest shallow freshwater lake in China to determine residual concentration and ecological risk of CBZ to aquatic life. Concentrations of the active compound in the samples fell within the range of 3.30-128.20 ng/L. The major source of the CBZ in the environment may therefore be attributed to anthropogenic influence (Wu *et al.*, 2022b).

Noticeably, the major contribution of CBZ to the surface water is as a result of the wastewater treated by WWTPs. When released to water bodies, processes such as photo degradation, biodegradation under aerobic conditions, adsorption to the bottom sediment and dilution in surface water contribute to the reduction of CBZ concentration. However, in-stream attenuation rate of specific PhACs depends on the characteristics of the compound as well as the site-specific factors. For instance, Kunkel & Radke compared the attenuation rates of 10 pharmaceuticals including CBZ in river water and found that the variability was normally explained by the physicochemical characteristics of the substances (Kunkel & Radke 2012). In a similar study that examined the effect of attenuation rate and the physicochemical characteristics of 225 micropollutants, the obtained high attenuation rate only applied to those compounds that have a medium to relatively low volatility (-4<logKow<-2) coupled with

significant hydrophilicity (0< logKow < 4.5). This is because these micropollutants are better exposed to in-stream biotic (e.g., biotransformation) and abiotic (e.g., photolysis) attenuation processes compared to hydrophobic micropollutants that get adsorbed onto river sediments. Given that the log Kow value for CBZ lies in the range of 0-4.5, it was hypothesized that its concentration would be removed via in-stream attenuation processes.

In a study in February 2022, sampling sites for 1052 samples from 104 countries were examined, including 258 river water samples. A total of 61 drug residues were identified in the study. In Hungary, samples were taken from eight different sites, revealing that the most frequently detected active substances were CBZ, metformin, and caffeine, all of which appeared in over half of the investigated locations. CBZ was found in the water samples at an average concentration of 116.35 ng/L in the Danube. In 25.7% of the sampling sites, the level of at least one active substance exceeded the safe limit for aquatic organisms (Pot *et al.*, 2022). Thus, not only big river systems but also a small creek or a stream are indispensable but rather sensitive links in the matrices of biogeographical connectors. A Hungarian research group studied the second-order water bodies in the Budapest conurbation, including the Danube, for two years regarding their pollution with pharmaceuticals and analysed 141 water and sediment samples. In the case of the study in 2017 and 2018, water samples were collected from twenty-six small streams at 75 sampling points. In total, 111 PhACs were analysed in water and sediment of streams, among which 81 PhACs were identified in the water samples, and 62 types of drug residues were detected in sediment samples. Among the compounds identified, the one present more often pharmacologically active was CBZ, which was detected in 91.5% of all analysed samples. In this case, CBZ sediments' maximum concentration was determined to be 395.9 ng/g. Concerning DCL, a non-steroidal anti-inflammatory agent, the maximum level achieved 2070 ng/L, while it was found more than 100 ng/L during analysis of 39 other samples (Kondor *et al.*, 2022).

2.4. Carbamazepine in soil

Regarding the contamination report, the half-life of CBZ in the soil is between 462 to 533 days. The initial cause of the terrestrial environment contamination of CBZ occurs via effluents used as a source of reclaimed wastewater and the application of biosolids for use as a soil amendment (Löffler *et al.*, 2005; Ternes 1998). Even though CBZ is able to decrease but they are still difficult to eliminate from the environment and is highly variable depending on the region, season, number of consumer demand for medication. For example, CBZ is reported to occur in wastewater treatment plant effluent, with concentrations ranging from 0.24 to 2.10 µg/L, in biosolid concentrations 7.8 to 258 µg/kg, and in soil concentrations from 0.0065 to 7.5

µg/kg (Durán-Alvarez *et al.*, 2009; Spongberg & Witter, 2008; Ternes 1998). due to the long half-life of more than 365 days, it raised concerns about bioaccumulation in terrestrial systems.

Table 2. Carbamazepine concentrations (ng/g) with respect to depth and land uses (*source: Walker* et al*., 2012*).

Depth (cm)	Site		
	Forested	**Grassed**	**Cropped**
1	4.92 ± 1.23	2.91 ± 1.22	1.98 ± 0.19
7	4.58 ± 1.82	2.15 ± 1.10	1.23 ± 0.49
15	1.54 ± 1.43	1.14 ± 0.41	0.89 ± 0.22
30	0.23	0.42	0.46
60	ND	0.27	ND*
119	0.51	ND	ND

*ND: not determined because the content was below the detection limit

Table 2 summarises the concentration of CBZ in soils under different land uses: cropped, grassed and forested. This research indicates that soil sorption of CBZ is strongly dependent on soil organic carbon. The CBZ average concentration of 4.92 ± 1.23 ng/g at the surface of forest soil exhibited a significantly higher concentration compared to cropped and grassed areas. As the depth beneath the soil surface exceeded 15 cm, the concentration of CBZ diminished to below 0.5 ng/g across all land uses, with detections being erratic. The predominant concentration of CBZ in the soils was found in the O (forested) and A horizons, situated within the upper 30 cm of the soil profile. Reduced concentrations of CBZ were noted at and beneath the 30-cm depth, where the soils began transitioning to greater clay content and reduced organic carbon B horizons. The sole anomaly in this trend was observed in a sample from the forested region, where CBZ was detected at a depth of 120 cm but absent at 60 cm. This inconsistency may have resulted from the preferred flow through root canals in the forested region. concentrations of organic carbon in comparison to soils from grassed and cultivated regions. CBZ concentrations exhibited a strong positive connection with the quantity of organic carbon in the soil. The sorption of CBZ in soil is significantly influenced by soil organic carbon content (Walker *et al.*, 2012). These findings align with sorption experiments conducted by (Chefetz *et al.*, 2008; Williams *et al.*, 2006)

2.5. Removal efficiency of carbamazepine

The removal efficiency of CBZ is less than 10%, depending on the operation conditions of WWTPs (e.g. anoxic-oxic ratios, acidic conditions and sunlight irradiation conditions).

Different methods can be used in wastewater treatment processes in order to remove CBZ and others PhACs. One of the basic methods is chlorination. Bahlmann *et al.* discovered that CBZ and its epoxy metabolites are less removed from wastewater of slightly above 5% and 30%, respectively after chlorination yielding to almost unaltered concentration after treatment (Bahlmann *et al.*, 2014). Thus, the removal of CBZ method metabolites by chlorination is more efficient than that of the parent CBZ. Although the presence of PhACs like CBZ does not pose an immediate threat to aquatic ecosystems or human health, we still require an effective removal technology for the safety of water reuse and drinking water treatment. These are some removal technologies that are quite capability in water treatment plant for instance: activated carbon adsorption, the conventional active sludge, white-rot fungi and their extracellular enzymes and nanofiltration and reverse osmosis membranes.

2.5.1. Biological treatment technology for carbamazepine removal

The conventional activated sludge (CAS) process, microorganisms produce energy in utilizing bulk organics which is present in wastewater as a primary substrate. Since some PhACs such as antibiotics can be toxic to microorganisms and can inhibit their growth. The CAS process involves the application of microorganisms for the degradation of pollutants (Zhang *et al.*, 2008). The integration of the CAS process like a microfiltration (MF) membrane with an ultrafiltration (UF) to increase the effective solid-liquid separation (Hai *et al.*, 2005; Radjenović *et al.*, 2009). Since CBZ is moderately hydrophobic, its removal via sorption onto activated sludge has been reported to range between 5 to 20% only (Zhang *et al.*, 2008). In different research, CBZ degradation was enhanced by the combination of activated sludge and gamma irradiation together in two steps treatment, highly up to 99.8% CBZ was removed and 79.3% mineralization when 800 Gy irradiation was utilised.

Another alternative to bacterial-dominated activated sludge process White-rot fungi and their extracellular enzymes (i.e., laccase and manganese peroxidase) for treatment cycle of 48 hours showed 60% elimination (Wang & Wang, 2017). The extracellular enzymes from WRF species have been reported previously in the degradation of PhACs such as CBZ in batches as well as in continuous-flow enzymatic bioreactor systems. Metabolism of PhACs by extracellular enzymes like laccase is attributed to the ability of this enzyme to accept and donate one electron to the target pollutant molecules. Like the activated sludge-based treatment process, the extent of degradation by the enzyme is also influenced by the molecular properties of the PhACs but its degradation has been reported only between 5-15% (Nguyen *et al.*, 2015).

2.5.2. Physicochemical treatment technology for carbamazepine removal

The adsorption process using activated carbon (AC) due to high porosity, surface area, and affinity to interact with a wide range of compounds; finally, it retains particles on its surface. As the tertiary treatment process for colour and odour removal in drinking water is primarily used granular active carbon (GAC) and powdered active carbon (PAC) (Hai *et al.*, 2018). Efficient removal of PhACs has been achieved by granular active carbon (GAC) from previous studies because it can effectively adsorb pollutants with different shapes and sizes like larger pore size in this case (Choi *et al.*, 2008; Rossner *et al.*, 2009). Regarding to the survey on the available literature suggests that both powdered active carbon and granular active carbon are effective in CBZ removal. Notably that under powdered active carbon, the removal effectiveness of over 90% has been reported even though the low numbers of carbon doses were used (Rizzo *et al.*, 2019).

In connection with the membrane filtration in wastewater treatment, it was observed that the pollutant removal depends on the efficiency of a pressure-driven physical barrier in preventing the passage of the target compounds and thus in effect, separating them from the influent (Rizzo *et al.*, 2019). Based on the scale used in wastewater treatment plants and water reclamation, the two most frequently used kinds of membrane technologies are nanofiltration (NF) and reverse osmosis (RO). Both of them are two types of the membrane techniques that incorporate the use of pressure to drive a separation of the dissolved substances. they use semi permeable membranes mainly to achieve dissolved impurities removal. NF and RO have also been investigated for treating PhACs contaminated secondary treated WW and FW obtaining very good quality effluent. From the previous studies it evident that both NF and RO membranes have ability to retain CBZ with removal efficiency of greater than 95% (Taheran *et al.*, 2016). summarized literature data concerning CBZ removal by NF and RO processes, which concluded that the last efficiency was highly influenced by the MWCOs of the membranes and fouling (Hai *et al.*, 2018).

2.6. Ecotoxicological effects of carbamazepine

Aquatic biota is chronically exposed to multiple exogenous substances. Several numbers of those compounds are suspected of impacting living organisms and human health. Many of the past studies on aquatic organisms have focused on toxicity assessment of single pharmaceuticals; most of the bioassays currently used investigate short-time exposure (72 hours or 96 hours) at low concentrations. The ecotoxicology of CBZ is poorly documented, especially its possible effect on aquatic organisms such as green algae *Desmodesmus communis* or

Desmodesmus subspicatus (Cleuvers, 2003; Desbiolles *et al.*, 2020). Based on the results of the study and present European legislation concerning the classification and labelling of chemicals 92/32/EEC, the authors qualified CBZ as "R52/53, Harmful to aquatic organisms and may cause long-term toxic effects to aquatic life."(Jos *et al.*, 2003).

Since CBZ is frequently identified in water systems, it is necessary to assess impacts of this chemical. Table 3 lists several investigations on the aquatic ecotoxicity of CBZ. From estimation a respiratory quotient value of 4.69 for CBZ in the experiment which points to the possible danger of the compound to aquatic life. On the other hand, the other experimental work has demonstrated that CBZ may not be lethal in the short term. For example, investigated the toxic effects of CBZ on bacteria, algae, microcrustacean invertebrates and fish. On the tested organisms, it was ascertained to possess a relatively low acute toxicity on the acute ecotoxicity (Ferrari *et al.*, 2003).

Table 3. Ecotoxicological effects of carbamazepine on certain aquatic organisms (*source: Hai* et al.*, 2018*)

Species	**LC_{50} (mg/L)**	**EC_{50} (mg/L)**	**NOEC (mg/L)**	**LOEC (mg/L)**	**Obs.dur (Days)**	**Critical effective**
Ceriodaphnia dubia (water flea)		77.7			2	intoxication (immobile)
Chironomus riparius (Midge)	>4				1	mortality
Chironomus tentans (midge)	47.3				10	mortality
Chironomus tentans (midge)		9.5			10	growth
Danio rerio (zebra danio)		52.452			3	developmental effects
Danio rerio (zebra danio)	>=245				3	mortality
Danio rerio (zebra danio)		86.5			3	mortality
Daphnia magna (water flea)		13.8			2	intoxication (immobile)
Daphnia magna (water flea)	111				2	mortality
Daphnia magna (water flea)		76.3			4	intoxication (immobile)
Hyalella Azteca (amphipod)	9.9				10	mortality
Hyalella azteca (amphipod)		15			10	mortality

Ceriodaphnia dubia (water flea)			0.1967		7	reproduction inhibition
Danio rerio (zebra danio)			30.6		3	developmental effects
Oncorhynchus mykiss (rainbow trout)			19.9		4	morphology (organ weight)
Brachionus calyciflorus (lotifer)			0.377		2	reproduction inhibition
Oryzias latipes (medaka)				6.15	5	feeding behavior
Brachionus calyciflorus (lotifer)				0.754	2	survival
Hydra attenuate (amphipod)				5	4	morphology (abnormal)
*Ceriodaphnia dubia (*water flea)				0.2646	7	reproduction
Chlorella vulgaris (green alga)		36.622			2	population (abundance)
Chlorella pyrenoidosa (green alga)		565.57			5	population growth rate
Chlorella pyrenoidosa (green alga)		10.14			15	population growth rate
Raphidocelis subcapitata (green alga)			>100		3	population (abundance)
Desmodesmus subspicatus (green alga)		74			3	inhibition of average growth rate
Raphidocelis subcapitata (green alga)		89			4	growth inhibition

Long-term chronic effects of exposure to various pharmaceuticals may include endocrine disruption, antibiotic resistance induction in human pathogens, genotoxicity, carcinogenicity, allergy, reproductive or developmental disorders (Corcoran *et al.*, 2010; Nash *et al.*, 2004). However, there are only few published papers linking the long-term impacts of CBZ residues in water for human health hazards. Conducted hitherto risk assessment exercise also reveal that the very low concentrations of CBZ reported to be present in drinking water does not pose any health risk to humans. But monitoring must still remain close due to the documented effects that CBZ has on the human body medicinally. CBZ has been identified as the key drug responsible for causing the Stevens–Johnson syndrome and its associated disease toxic epidermal necrolysis to the Southeast Asian countries due to it is taking medicinally (Chen *et al.*, 2011). These are two types of a fulminant skin disease that has a mortality rate of 30 %

in general, In this disease, necrosis of the cellular structures results in separation between the epidermis and dermis (Pereira *et al.*, 2007). Atkinson *et al.* also observed increased fetal losses and congenital malformations in the women who were taking CBZ while in pregnancy. (Atkinson *et al.*, 2007) melting, because the residue of CBZ in drinking water may lead to embryo and infant via intrauterine exposure or breast-feeding, the concerns about the presence of CBZ in Groundwater, and drinking water remain systematic risk assessment studies.

2.7. Analytical methods for carbamazepine detection

As new analytical methods are constantly developed, the micropollutant compounds detected in source water continues to expand. Among these compounds, some of them are classified as emerging pollutants, which are groups of compounds that are still the object of research and for which most of the information regarding the potential impact on human and aquatic life is still missing. They include pharmaceuticals, hormones, perfluorinated compounds (PFCs), corrosion inhibitors, algal toxins, or pesticide transformation products (Greskowiak *et al.*, 2017).

If the analysis of CBZ in wastewater (WW) and wates water sludge (WWS) is a challenge; this is based on of the fact that the contaminant is usually present at very low concentrations in the matrix, and the matrix itself is quite complex. A successful quantification of CBZ in WW and WWS requires exhaustive extraction, effective clean-up, and sensitive detection. In most cases, extraction must be done prior to quantification of CBZ by various analytical methods. (Rudel *et al.*, 1998) reported that liquid-liquid extraction (LLE), Solid-phase extraction (SPE) and solid-phase microextraction (SPME) techniques have been widely applied to extract the CBZ present in the WW and the mentioned techniques have been described by other researchers such as (García *et al.*, 2009; Gasser *et al.*, 2011; Mohapatra *et al.*, 2012). Among all the extraction methods, SPE has the advantages as: slightly less soluble material being employed, easier to perform operation, and a high degree of repeatability (Lavén *et al.*, 2009). However, recently (Yu *et al.*, 2012) investigated the determination of CBZ in municipal wastewater by using UHPLC–MS/MS without using the SPE sample preparation step. Non-SPE pre-concentrated influent and effluent samples exhibited a noticeable ion enhancement of CBZ and it was not enough information to prove on this experiment.

Various analytical techniques have been reported for the determination of CBZ in WW and WWS samples, and more use chromatography techniques, including gas chromatography complementary with mass spectrometry or with tandem mass spectrometry. The separation techniques used are gas chromatography (GC) and liquid chromatography (LC) while for detection, mass spectrometry (MS) is the most used technique. However, because of the low

volatility of CBZ (polar PhACs), the GC–MS analysis needs derivatization, which makes sample preparation tedious and time-consuming and also exposes the samples to contamination and errors. Further, CBZ is thermolabile and undergoes degradation during GC analysis; CBZ forms iminostilbene as the degradation product (Ternes, 2001). In contrast, the employment of high-performance liquid chromatography (HPLC) with several detectors, for example, UV, fluorescence and electrochemical detector just in need a simple and rapid pre-treatment processes used HPLC to detect the CBZ (5000 μg/L) existing in hospital WW. However, to accomplish the quantitation limits in the lower ng/L range (or below) of CBZ in the environmental matrices such as wastewaters and wastewater sludge, LC-MS/MS or LC-electrospray ionization tandem MS (LC-ESI-MS/MS) are crucial (Ternes, 2001). Table 4 presents the LOD values of different analytical procedures with different clean-up/preconcentration methods.

Table 4. Limit of detection values of different analytical procedures applied in carbazepamine quantification (*source: detailed in the footnotes*)

Country	**sample type**	**clean-up/pre-concentration**	**analytical methods**	**limit of detection**
Canada	surface water	SPE	LC-MS/MS	2 ng/L [1]
	wastewater	SPE	GC-MS/MS	18 ng/L [2]
Austria	wastewater	SPE	LC-MS/MS	10 ng/L [3]
Germany	wastewater and surfacewater	SPE	HPLC-MS/MS	10 ng/L [4]
	wastewater	SPE	LC-MS/MS	0.0001 μg/L [5]
	wastewater and surfacewater	SPE	LC-MS/MS	0.01 μg/L [6]
France	wastewater	SPE	HPLC-MS/MS	0.02 μg/L [7]
	wastewater	SPE	LC-MS	2.4 ng/L [8]
Italy	wastewater and surfacewater	SPE	HPLC-MS/MS	<1 ng/L [9]
Switzerland	surface water	SPE	GC-MS	1.5 ng/L [10]
Norway	wastewater and surfacewater	SPE	HPLC-MS/MS	1 ng/L [11]
Israel	wastewater	SPE	GC-MS and LC-MS/MS	100 ng/L and 0.1 ng/L [12]
Republic of Korea	surface water	SPE	LC-MS/MS	1.0 ng/L [13]
Taiwan	wastewater and surfacewater	SPE	LC-MS/MS	0.2 ng/L [14]
Germany	surface water	Paper filtered	ELISA	0.024 μg/L

[1] Daneshavar *et al.*, 2012; [2] Gagnon & Lajenuesse, 2012; [3] Clara *et al.*, 2004; [4] Scheurer *et al.*, 2011; [5] Gebhart & Schröder, 2007; [6] Zuehlke *et al.*, 2004; [7] Martin Ruel *et al.*, 2012; [8] Leclercq *et al.*, 2008; [9] Castiglioni et al., 2005; [10] Tixier *et al.*, 2003; [11] Langford & Thomas, 2011; [12] Gasser *et al.*, 2011; [13] Kim et al., 2007; [14] Chen et al., 2008

Screening for sources of antibiotic residues in meat, milk, surface and groundwater, wastewater, soil, and manure, is carried out using traditional methods such as Enzyme-Linked immunosorbent assay (ELISA) technique (Aga *et al.*, 2003; Barber *et al.*, 2009; Bradley *et al.*, 2014; Černoch *et al.*, 2012; Galarini *et al.*, 2014; Kandimalla *et al.*, 2007; Kumar *et al.*, 2004). ELISA techniques can be used during analysis if the contents of the sample are structurally similar antibiotic mixtures (Aga *et al.*, 2016). The compounds with similar structures are difficult to differentiate with immunoassays, therefore, liquid chromatography mass spectrometry (LC/MS) or liquid chromatography with tandem mass spectrometry (LC-MS/MS) techniques are used for detection and quantification of structurally similar compounds.

Pharmaceuticals exist in the environment in very small concentrations, rendering direct chromographic analysis of the samples near impossible and, hence, the need for sample concentrate. The pre-concentration step is not only crucial for enabling detection of pharmaceuticals at low concentrations but also demasks matrix effect during liquid chromatography- mass spectrometry (LC-MS) analysis. A review of the sample preparation methods reveals the following advantages where SPE is used as the preferred method; The amount of organic solvent used in loading and rinsing the column is small, SPE requires a short period of time to prepare the sample, SPE can pre-concentrate the sample, it is easier to isolate target compounds in the sample, SPE affords sample reproducibility and finally, SPE allows for optimisation and sample clean-up in different matrix (Vumazonke *et al.*, 2020).

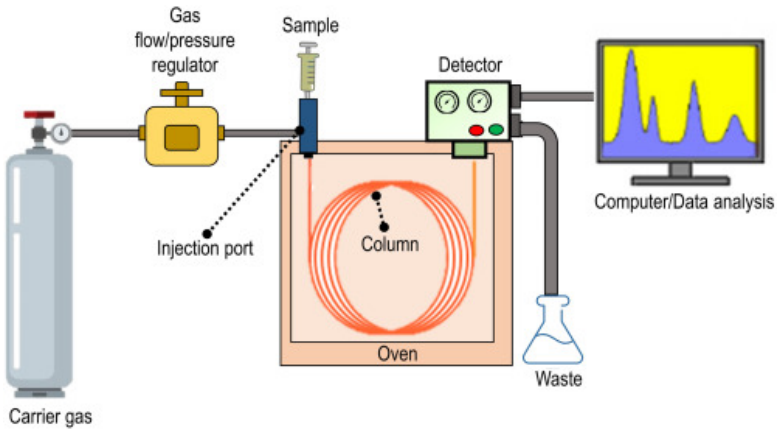
However, because of costs that are involved when using LC/MS or the LC-MS/MS methods, they cannot be applied to routine analysis of the pharmaceuticals in environmental samples; hence, ELISA methods are employed. One of the most important reasons of frequently analysing the antibiotics in environmental samples is due to the obtaining information about antibiotics in the environment as well as their concentration and probability of causing negative effects to the environment. ELISA has the advantage of being able to screen a number of samples at once within a shorter time span and at less cost. In ELISA, the antibodies are typically high specific to one analyte, but they are class-specific and consequently they demonstrate a high cross-reactivity to other structurally related compound (Vumazonke *et al.*, 2020).

2.7.1. Gas chromatography

Gas chromatography (GC) is one of the widely known chromatography practically used in analytical chemistry for separating and determining compounds that can be vaporised without decomposing (Figure 4). The mobile phase in the context of GC may be termed as a carrier gas, which the common ones being helium, or an inert mobile phase could be any unreactive,

uncharged gas, for instance, nitrogen. The stationary phase may be liquid or polymer, coated on an unimportant solid substrate and located inside a glass or metal tube called the column. The gaseous compounds under consideration come into contact with the walls of the column and, consequently, with a phase that is immobilized. This leads to each compound being parted at a given time, the retention time of the compound (Cocks *et al.*, 1981; Kumps & Mardens, 1980; Ranise *et al.*, 1981; Riva *et al.*, 1981).

Figure 4. Scheme of a gas chromatogram
(*source: Arruda* et al.*, 2023*)



GC has a lot helped human beings to be wise in that in agriculture and industries, one has to be very careful when handling these chemicals so as not to affect us or our environment. Thus, GC is an often-used method as one of the essential components of the research and industrial laboratories to perform qualitative and quantitative analysis of mixtures. Likewise, GC has also been used at some of the environmental and forensic labs at GC due to its ability to factor in the detection of traces. Many types of tests can be analysed until the compounds are thermally stable enough and have moderate volatility.

The principles of separation in chromatography rely on the differential retardation of mixture components as they move through a column with a more permeable carrier gas, typically helium or nitrogen. The column may be made of steel or glass and packed with glass or ceramic beads coated in a non-volatile liquid for gas-liquid chromatography (GLC). Without the liquid layer, it's known as gas-solid chromatography (GSC). As the sample is injected, its molecules distribute between the gas and liquid phases. Molecules in the gas phase move down the column, while some may dissolve in the liquid phase. Components with higher volatility will be more present in the gas phase and exit the column more quickly, allowing for their separation at different times (Al-Bukhaiti *et al.*, 2017). A simple, accurate, and sensitive microextraction by packed sorbent-gas chromatography-mass spectrometry method has been

developed by (Rani & Malik, 2012) for the determination of the concentration of four AS such as OXC, CBZ, PHT and ALP in human plasma and urine samples as a routine tool in clinical pharmacokinetic profiles. Caffeine was used internal standard for the electron ionization mode. In the present study, a novel pre-treatment process for biological sample, micro extraction in packed syringe technique using C18 material as the packing material provided satisfactory pre concentration recoveries varying between 69.92-99.38%, RSD <4.7% and selectivity. Alv Kernel Linearity was obtained in the concentration range of 0.1–500 ng/mL for these drugs and the limits of detection (LOD) ranged from 0.0018 ng/mL to 0.0036 ng/mL. In validation, the method was successfully applied to some plasma samples from patients receiving therapy with one or more of these drugs. Using the present method, it was possible to analyse these drugs in the real urine and plasma samples of the epileptic patients.

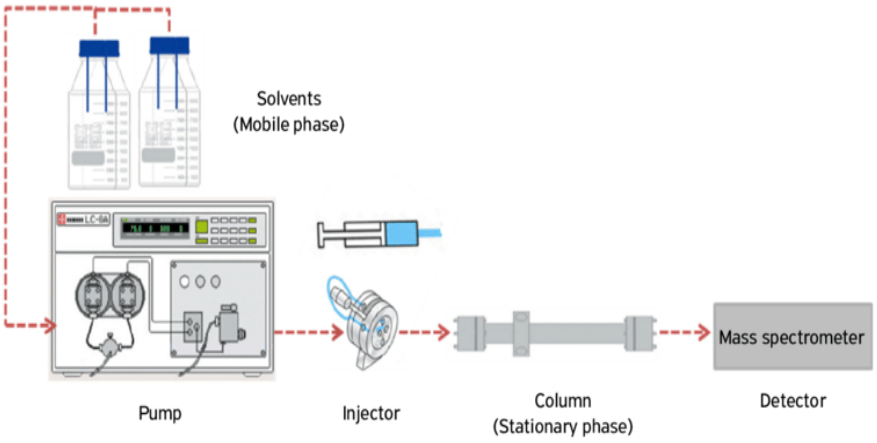
2.7.2. Liquid chromatography

Liquid chromatography is a technique used to separate a sample into its individual parts (Figure 5). This separation occurs based on the interactions of the sample with the mobile and stationary phases. Because there are many stationary/mobile phase combinations that can be employed when separating a mixture, there are several different types of chromatography that are classified based on the physical states of those phases. Liquid-solid column chromatography, the most popular chromatography technique and the one discussed here, features a liquid mobile phase which slowly filters down through the solid stationary phase, bringing the separated components with it. components within a mixture are separated in a column based on each component's affinity for the mobile phase. So, if the components are of different polarities and a mobile phase of a distinct polarity is passed through the column, one component will migrate through the column faster than the other. Because molecules of the same compound will generally move in groups, the compounds are separated into distinct bands within the column. If the components being separated are coloured, their corresponding bands can be seen. Otherwise as in high performance liquid chromatography (HPLC), the presence of the bands is detected using other instrumental analysis techniques such as UV-VIS spectroscopy1. The following figure shows the migration of two components within a mixture: In the first step, the mixture of components sits atop the wet column. As the mobile phase passes through the column, the two components begin to separate into bands. In this example, the red component has a stronger affinity for the mobile phase while the blue component remains relatively fixed in the stationary phase. As each component is eluted from the column, each can be collected separately and analysed by whatever method is favoured. The relative polarities of these two compounds are determined based on the polarities of the stationary and mobile

phases. If this experiment were done as normal phase chromatography, the red component would be less polar than the blue component. On the other hand, this result yielded from reverse phase chromatography would show that the red component is more polar than the blue component (Breton *et al.*, 2005).

LC–MS/MS is a linked technique, bringing together the elements of HPLC and MS. The advantages of LC–MS/MS are related to the method selectivity and sensitivity, which are high speed, low detection limits, generation of structural information, minimal sample treatment and the possibility to include a list of different polarities of the analytes. Recent methods identified for analysis of CBZ in the aquatic environment include liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) and liquid chromatography with quadrupole time of flight mass spectrometry (LC-Q-TOF MS) (Stolker *et al.*, 2004).

Figure 5. Scheme of a liquid chromatogram
(*source: Ardrey, 2003*)



High-performance liquid chromatography

Modern high-performance liquid chromatography or HPLC has its roots in this separation, the first form of liquid chromatography. The chromatographic process has been significantly improved over the last hundred years, yielding greater separation efficiency, versatility and speed. HPLC is a chromatographic technique used to separate the components in a mixture, to identify each component, and to quantify each component. In general, the method involves a liquid sample being passed over a solid adsorbent material packed into a column using a flow of liquid solvent. Each analyte in the sample interacts slightly differently with the adsorbent material, thus retarding the flow of the analytes. If the interaction is weak, the analytes flow off the column in a short amount of time, and if the interaction is strong, then the elution time is long (Swadesh, 2000). HPLC can be used in both qualitative and quantitative applications, that is for both compound identification and quantification. Normal phase HPLC is only rarely used now, almost all HPLC separation can be performed in reverse phase.

HPLC principle (Bélanger *et al.*, 1997):

- This process occurs in a separation system between a stationary and a mobile phase.
- The stationary phase can be thought of as a solid with small particles with through-holes in a stationery called the separation column.
- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- Through a valve linked to a sample loop, which can be a small stainless-steel tube or capillary, the sample is introduced into the flow of the mobile phase from the pump to the separation column with the help of a syringe.
- The individual components of the sample subsequently migrate through the column at varying rates, as they are retained to different extents due to their interactions with the stationary phase.
- Once they exit the column, the substances are detected by an appropriate detector and transmitted as signals to the HPLC software on the computer.
- At the conclusion of this process, a chromatogram is generated within the software. This chromatogram facilitates the identification and quantification of the various substances.

HPLC methods for the determination of CBZ-related impurities were reported in USP, EP, BP, and IP. A number of HPLC methods for simultaneous determination of CBZ and its metabolites in plasma have been published, using pre-treatment techniques such as liquid-liquid extraction, Solid phase extraction, deproteinization, stir bar-sorptive extraction, fluorescence polarization immunoassay, chemiluminescence, spectrophotometry, spectrofluorimetric method, FT-Raman spectroscopy, and planar chromatography (Datar, 2015).

2.7.3. Enzyme-Linked Immunosorbent Assay

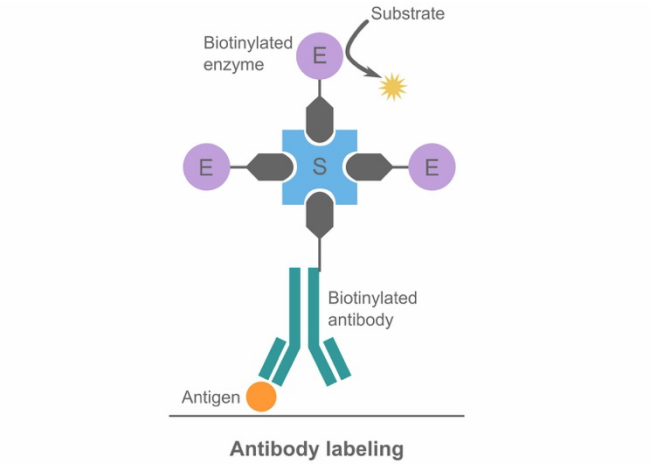
Enzyme-linked immunosorbent assay (ELISA) applies the fundamental immunological idea where an antigen will bind to its corresponding antibody for the detection of tiny portions of antigens such as protein, peptide, hormones, or antibodies in a fluid sample. Enzyme immunosorbent assay (EIA) and Enzyme-linked immunosorbent assay (ELISA) depend on enzyme labelled antigens and antibodies to identify the biological molecules, the most often applied enzymes being alkaline phosphatase and glucose oxidase. The antigen in fluid phase is immobilized, typically on to 96-well microtiter plates or any other suitable surfaces. The antigen is permitted to react with a particular antibody and the reaction is then identified by a second antibody tagged with an enzyme. A chromogenic substrate, when added to the enzyme, catalyses a reaction that results in visible colour change or fluorescence of the antigen. Thus,

colorimetric reading can be measured either quantitatively or qualitatively. Fluorogenic substrates are more sensitive than chromogenic substrates and provide the opportunity to quantify the levels of antigen concentrations in the sample (Gan & Patel, 2013).

Indeed, aquatic organisms can be used as indicators of pharmaceutical pollution in natural waters, and ELISA may be a rapid and cost-effective technique to support such studies. The main advantage of immunoassays, more specifically ELISA, is that they present high sensitivity and specificity, and generally require straightforward protocols together with inexpensive and simple sampling procedure; Furthermore, ELISA can be used as a rapid screening method enabling testing a large number of samples at low cost and with little operator training (Jaria *et al.*, 2020).

Immunoassays are based on the specific interactions between an antibody and an antigen (the analyte). Additionally, tracers (compounds that are analogue to the antibody or the antigen, which might be radioisotopes, fluorophores, chemiluminophores, or enzymes) are used to detect the compound of interest. In the case of ELISA, this detection relies on the use of an enzyme-labelled antibody or enzyme-labelled antigen that functions as a tracer or conjugate. In ELISA, one of the reagents is bound to a solid phase, generally a 96-well microtiter plate, which allows the analysis of several samples at one time (Jaria *et al.*, 2020). Enzyme-Linked Immunosorbent Assay (ELISA) is the most frequently applied and highly sensitive assay technique for measuring the concentration or detecting and identifying biomolecules through the use of the antibody as the recognition molecule. Enhancing ELISA is very important in the detection of disease-causing agents, more so at the early stages of disease. Biotinylated antibody and another product called streptavidin-conjugated horse radish peroxide, known as streptavidin-HRP, are combined with ELISA in order to improve the detectability of diverse targets. Streptavidin is widely used as a linker in biotechnology because the scheme with the wt-streptavidin demonstrated the highest stability clearly under the physiologically more appropriate conditions (Kd ∼ 10−15, pH 3–13) (Lakshmipriya *et al.*, 2016). Enhancement of signals can be achieved through the use of labelled streptavidin to detect a biotinylated antibody (primary or secondary antibody) (Figure 6). Each antibody can possess multiple biotins in its structure and these multiple biotins can attach multiple streptavidin. Such multiple probe molecules signify that there is more than one that can either catalyse the detection substrate to the final product or cause a higher fluorescent emission to increase the signal intensity and sensitivity. Enzyme-conjugated antibodies or antigens: The antibodies or antigens required will depend on the analyte. To label the antibodies or antigens with an enzyme, a streptavidin-biotin bridge is generally used to link the antibodies or antigens to the detection enzyme. The two most common enzymes used are horse radish peroxidase (HRP) and alkaline phosphatase (Mészáros, 2022).

Figure 6. A streptavidin-biotin bridge
(*source: Mészáros, 2022*).



ELISA types:

There are several types of ELISA, namely direct, indirect, and competitive (Figure 7). In the immobilization stage the antigen can be attached directly to the plate or indirectly to the plate, by binding with antibodies that have been attached to the bottom of the plate. Furthermore, the antigen is detected directly with the primary antibody bound to the enzyme or the antigen is detected indirectly with the secondary antibody that is bound to the primary antibody and the enzyme. Enzyme that are generally bound to antibodies include alkaline phosphate (AP) or horseradish peroxidase (HRP) (Jaria *et al.*, 2020).

- Direct ELISA (antigen immobilized, screening antibody)
- Indirect ELISA (antigen-coated plate, screening antigen/antibody)
- Sandwich ELISA (antibody immobilized, screening antigen)
- Competitive ELISA (screening antibody)

a) Direct ELISA

The basic steps of direct and indirect ELISAs are almost similar starting by coating of the antigens to the ELISA plates. The first binding step is addition of antigens to the plates, and then it can be incubated at 37°C for about one hour or the plates can be stored at 4°C for overnight. After incubation is done, then the next thing is to wash the plates with potential unbound antigens away. This second step is excellent since it can clear up any non-specific antibodies to the plate thereby wiping out wrong positives. After that a buffer is added and the plate is washed again and the selected enzyme-conjugated primary detection antibody is added (Kohl & Ascoli, 2017).

In a direct ELISA the first antibody will bind to the protein of interest and form a complex of enzyme labelled. After this, the plate is washed again so that it clears all the excess non-binding antibodies. Substrate: If an enzyme such as AP is incubated with the plate, then the plate emits a particular colour of light, which is HRP. Sample colour change may be attributed to the cleavage of phosphate groups of the substrate by AP or oxidation of the substrates by HRP. HSO is that none of the secondary antibodies cross-react, this method takes lesser time than indirect ELISA due to reduced steps. Among the disadvantages experienced while using the method, they include the following: the method is inferior to other ELISA methods: cost of the reaction used (Kohl & Ascoli, 2017).

b) Indirect ELISA

The procedure of the indirect ELISA is also the same as in the direct ELISA, although sometimes an extra wash step is performed, and the types of antibodies that are applied when the buffer is removed are different. Indirect ELISA requires two antibodies: an initial reliance antibody that binds to the protein of interest and an additional enzyme-tagged antibody that binds to the primary antibody. The first layer is placed on the right section; the layer is washed; the second layer is placed; and the last incubation is done. Subsequent procedures are similar to the procedures followed in the direct ELISA, namely the washing, addition of substrate, and the change of colour. Here, it is also critical while discussing the technique of the indirect ELISA that this test provides a higher sensitivity than the direct ELISA test. It is also less expensive, and multiple applications of the technique can be made because of the many potentially useful primary antibodies that may be used. The only downside of this major type of ELISA is that secondary detection antibiotics can cross-react (Alhajj *et al.*, 2024; Kohl & Ascoli, 2017).

c) Sandwich ELISA

In contrast to direct and indirect ELISA, in sandwich ELISA the test begins with a capture antibody that binds to the wall of the well. It is called a sandwich because the antigens of the substance in question fill the middle of two antibodies: capture and detection. Next day, the capture antibodies are pipetted into the plates and the plates are covered and left to incubate overnight in 4°C. It begins by after the coating step the plates are washed with PBS after which the wells are buffered/blocked with BSA. The numbers of buffer washes completed at ambient temperature are accompanied by 1–2 minutes of 1–2 hours. Last step is PBS wash is then done and the antigen is added in the dish (Alhajj *et al.*, 2024).

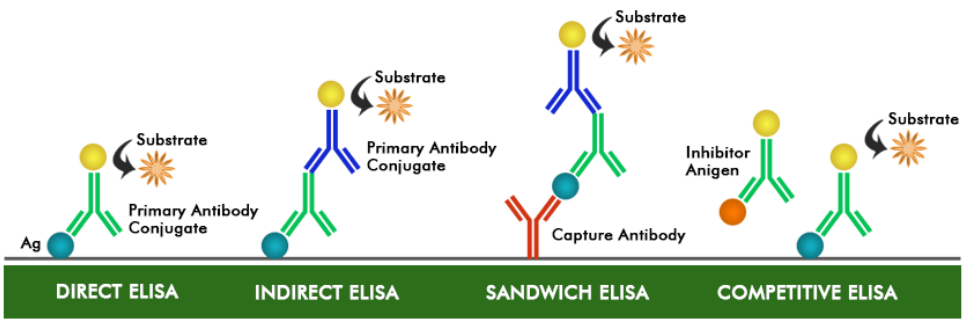
The specific antigen is finally pipetted onto the plates to allow it to get attached to the capture antibody to be included for 90 min at 37°C. The plate is rewashed and the primary

detection antibody is added to the plate and incubated for 1 to 2 hrs at room temperature After incubation the plate is washed with buffer. Then the secondary enzyme-conjugated antibody is added and left for 1 to 2 hours The reaction is stopped, pH 8. The plate is rewashed and the substrate added so that it turns a particular colour. The sandwich ELISA claims the upper hand over all some of the other types of ELISA with regard to the sensitivity it obtains. The major disadvantage of this type of ELISA is time and cost and the need for 'matched pair' (divalent multivalent antigen) and secondary antibodies (Alhajj *et al.*, 2024).

d) Competitive ELISA

The highly sensitive ELISA tests are designed to search for negativity of an antibody concerning antigens in test serum. This type of ELISA utilizes two specific antibodies: The one that forms a complex with the enzyme and an immunosorbent and another antibody in the test serum if the serum is positive. The two different antibodies will be added into the wells which will let use antigens to compete for binding sites. The presence of a colour change consequently implies a negative test because the enzyme conjugate gets bonded to antigen only (and not the test serum antibodies). The absence of colour significance is desirable, and the presence of the antibodies within the test serum. With small molecules including lipids, steroids or peptides, small molecules that cannot be immobilized easily on the solid phase and for target analytes that may contain only one epitope, competitive ELISA are the option of choice (Alhajj *et al.*, 2024; Tian *et al.*, 2012). They are usually less complex and time-consuming as most other ELISA formats due to the fact that there is usually only one incubation step. In another word, in a competitive ELISA, a native target analyte in biological samples will come into competition with an enzyme-labelled analyte or tracer for a finite amount of specific antibodies that are bound to the plate. The signal intensity is positively related to the amount of tracer bound to the antibody binding sites, and thus, negatively related to the concentration of sample target analyte (Alhajj *et al.*, 2024).

Figure 7. Different types of the enzyme-linked immunosorbent assay (ELISA) (*source:* www.geeksforgeeks.org)



3. Material and method

3.1. Monitoring of carbamazepine in the Lake Balaton and in The Danube

3.1.1. Sampling

For the monitoring study, water samples were collected from the Danube River and the Lake Balaton in Hungary. To determine the concentration of CBZ in the surface water samples, 8 samples and 2x10 samples from the Lake Balaton and the Danube River were collected respectively. The Lake Balaton was sampled on 25th September 2024, while the Danube River during and after the flood period on 2nd October 2024 and 9th October 2024, respectively (Table 5, Table 6). Samples were collected in dark brown glass bottles with a volume of 1000 mL and were kept at 4°C until the analytical measurement. Before the measurement, all samples were filtered with filter paper.

Table 5. Name and GPS coordinates of the sampling sites and the date of sampling procedure at the Lake Balaton.
(*source: own work*)

Name of the sampling site	GPS coordinates of the sampling site	date of sampling
Balatonvilágos	46.979393; 18.162955	25th September 2024
Zamárdi	46.888418; 17.955255	25th September 2024
Balatonlelle	46.789966; 17.687248	25th September 2024
Balatonfenyves	46.716051; 17.482776	25th September 2024
Keszthely	46.749110; 17.246045	25th September 2024
Szigliget	46.785064; 17.436729	25th September 2024
Révfülöp	46.826260; 17.631550	25th September 2024
Tihany-Sajkod	46.908717; 17.849444	25th September 2024

Table 6. Name and GPS coordinates of the sampling sites and the date of sampling procedure at the Danube River
(*source: own work*)

Name of the sampling site	GPS coordinates of the sampling site	date of sampling
Nagybajcs	47.7668050; 17.6945341	2nd October 2024 9th October 2024
Komárom	47.753992; 18.069249	2nd October 2024 9th October 2024
Esztergom	47.795750; 18.732837	2nd October 2024 9th October 2024
Szentendre	47.665235; 19.079307	2nd October 2024 9th October 2024
Budapest, Zöldsziget	47.480520; 19.058501	2nd October 2024 9th October 2024
Dunaújváros	46.956882; 18.956055	2nd October 2024 9th October 2024
Dunaföldvár	46.809236; 18.930911	2nd October 2024 9th October 2024
Paks	46.618546; 18.859854	2nd October 2024 9th October 2024
Baja	46.193724; 18.923801	2nd October 2024 9th October 2024
Mohács	46.028987; 18.685941	2nd October 2024 9th October 2024

3.2.2. Analytical method applied in the monitoring study

Determination of carbamazepine active compound in surface water samples collected from the Lake Balaton and the Danube River were performed by enzyme-linked immunofluorescent immunoassay (ELFIA) developed in the frame of the Aquafluosense project (NVKP_16-1-2016-0049). The ELFIA kit developed for quantification of CBZ in water matrix contains:

- tracer containing carbamazepine-HRPO (horseradish peroxidase), ready to use
- antiserum have to be diluted with 8 mL dH_2O
- CBZ standards, 7x0.5 mL, ready to use, cc: 0, 0,05, 0,15, 0,6, 2, 8, 25 ng/mL
- microtiter plate coated with streptavidin
- washing buffer concentrate, 20 mL, have to be diluted with 600 mL dH_2O
- TMB (3,3',5,5'-tetramethylbenzidine) substrate, ready to use
- STOP reagent, ready to use

The ELFIA method applied in this study is a competitive form of the immunoassay, where free CBZ in the sample or in the standard solution compete with CBZ-HRP conjugate (tracer) for the binding sites of the CBZ-specific primary antibody. The more CBZ in the samples or standard solution, the less CBZ-HRP conjugate can occupy the

binding sites. As a result, the more CBZ in the samples or standard solution, the less intensity of the analytical signal detected. In this case, the calibration curve is the reverse 4-parametric sigmoid curve fitting by the Rodbard equation (Rodbard, 1974):

$$y = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} + A_2$$

A_1—the upper plateau describing the background of the method where the maximum signal can be obtained; here, there is no inhibition in the binding of the glyphosate analogue immobilized onto the microplate surface and the specific antibody

A_2—the lower plateau regarding the minimum signal at an infinite dose, where full inhibition is detected

x_0—the point of inflection, meaning the concentration where 50% of inhibition appears (IC_{50})

p—Hill's slope of the curve, which is related to the steepness of the curve at this point and characterizes the sensitivity of the analytical method

Self-prepared standards solutions were applied in the immunoassay, for this 1 mg/mL stock solution of CBZ was prepared in methanol. From the stock solution standard solutions of 0,05, 0,15, 0,6, 2, 8, 25 and 50 ng/mL were diluted with PBS buffer (137 mM NaCl, 2.7 KCl, 10 mM $Na_2HPO_4 \times 2H_2O$, pH = 7.4). As we applied fluorescence determination as analytical signal, instead of TMB substrate, QuantaRed Enhanced Chemifluorescent HRP Substrate Kit was applied (content of the working solution: QuantaRed ADHP concentrate, QuantaRed Enhancer Solution, and QuantaRed stable Peroxide in 1:50:50 (*v*/*v*) proportion). The kit contains 10-acetyl-3,7-dihydroxyphenoxazine (ADHP), a nonfluorescent compound that is dehydrogenated (oxidized) by HRP to resorufin, a highly fluorescent reaction product.

The ELIFA procedure is the following:

- addition of 20 μL of standards/control/sample (in triplicates)
- addition of 50 μL tracer to each well
- addition of 50 μL of the antiserum to each well
- covering the plate with parafilm, mixing the content and incubation at room temperature for 2 hours on microplate shaker
- vigorously shaking of the whole content of the wells into a waste container, and washing of the wells 6 times with 1X wash solution (250 μL/well)
- addition of 200 μL of the QuantaRed working solution to each well

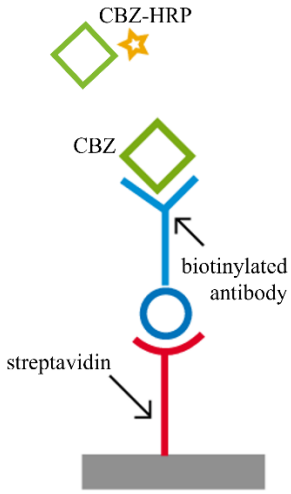
- addition of 10 µL of QuantaRed STOP solution to each well
- reading of fluorescence at 593 nm (excitation at 531 nm)

Fluorescent analytical signal was read by SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA) (Figure 8).

Figure 8. SpectraMax iD3 Multi-Mode Microplate Reader
(*source: own photo*)

The scheme of the immunocomplex developed in this ELFIA system is presented on Figure 9. The LOD and LOQ values of the ELFIA method are 0.02 ng/mL and 0.03 ng/mL, respectively.

Figure 9. Scheme of the immunocomplex developed in the ELFIA procedure applied in this study. CBZ: carbamazepine, CBZ-HRP: carbamazepine-horseradish peroxidase conjugate (tracer), biotinylated antibody: biotinylated CBZ-specific primary antibody. (*source: own figure*)



3.2. Ecotoxicological evaluation of carbamazepine on *Chlorella vulgaris* green alga species

3.2.1. Characterization of *Chlorella vulgaris* and the culturing conditions

During the ecotoxicological testing *Chlorella vulgaris* (*Chlorella vulgaris* Beijerinck - CCAP 211/11B) was used as testorganism. It was obtained from the Scottish Culture Collection of Algae and Protozoa. *C. vulgaris* is a freshwater eukaryotic green microalga. The batch culture of *C. vulgaris* was maintained in liquid nutrient solutions of specified composition (Zehnder-8 [Z8]) (pH = 6-7). Nutrient solutions are prepared in distilled water from stock solutions of various salts and trace elements (Figure 10, Table 7). Cultures were maintained under continuous lighting at 23°C and illuminated in a 14:10 light/dark period with the use of cool-white fluorescence tubes (15 µmol/m^2/s) according to OECD 201 protocol (OECD 2006). The cultures are maintained in 300 mL conical flasks, which were sterilized in an autoclave before use (20 minutes, 121°C, Boeco BTE-23D, Boeco Germany, Hamburg, Germany). The volume of the culturing media (Z8) in the culture vessels is about 100 mL, which are closed with cotton wool plugs and aluminium foil to protect the cultures from external sources of contamination (Figure 11). The algae cells were checked every week via microscopic to choose the best cultures to continue the culturing process.

Figure 10. The applied stock solution during the preparation of Z8 nutrient media (source: own photo).

Figure 11. The applied sterilized Erlenmeyer flasks containing the fresh nutrient media inoculated with algae cells
(*source: own photo*).

In the algae growth inhibition tests, it is a requirement that the algae culture used be in an exponential growth phase during the entire duration of the experiment (72 hrs), therefore the cultures were inoculated on a weekly basis under sterile conditions with the use of a laminar cabinet (Laminar AirFlow BL 1200-F, Radel & Hahn Zrt, Debrecen), avoiding the possible contamination (Figure 12). During algae culturing, the amount of inoculum (volume of algae suspension based on algae cell concentration) is selected so that the concentration of cells in the freshly inoculated culture is approximately 100 times lower. The algae culture in the new media enters an exponential growth stage in about 11 days. The cell density is checked by Bürker chamber cell counting and the measurement of optical density (OD).

Figure 12. The laminar cabinet applied during algae culturing (*source: own photo*).

During culturing, the glassware, nutrient solution and cotton plugs used are sterilized in an autoclave before use and the quality of the original cultures is checked under a microscope (presence of other algae species/protozoa, aggregation, abnormal cell forms).

During the investigation of the phytotoxic effects of CBZ on algae, application of *C. vulgaris* is advantageous due to its sensitivity to changes in water quality. *C. vulgaris* is a typical phytoplankton in freshwater ecosystems and often used as a test organism during ecotoxicological assessments (Wu *et al.,* 2022a). Furthermore, the selected algae strains can be easily cultured under laboratory conditions and can be characterized by a fast reproduction and life cycle.

Table 7. The preparation and composition of Zehnder 8 nutrient media for green algae culturing
(*source: Z8 Medium. Available online: https://www-cyanosite.bio.purdue.edu/media/table/Z8.html.*)

Firstly, four stock solutions had to be prepared according to the following compositions
1. **Stock solution:** 300 cm^3 final volume with distilled water
46.7 g sodium nitrate ($NaNO_3$)
5.9 g calcium nitrate ($Ca(NO_3)_2x4\ H_2O$)
2.5 g magnesium sulphate ($MgSO_4x7\ H_2O$)
2. **Stock solution:** 300 cm^3 final volume with distilled water
9.3 g dipotassium phosphate (K_2HPO_4)
6.3 g sodium carbonate (Na_2CO_3)
3. **Stock solution:** 500 cm^3 final volume with distilled water
5 cm^3 (Component A) + 5 cm^3 (Component B) + 490 cm^3 distilled water
Component A: 150 cm^3 of distilled water with
1.3515 g ($FeCl_3x2H_2O$) + 1.5 cm^3 concentrated hydrochloric acid
Component B: 150 cm^3 of distilled water with
2.1915 g (Na_2-EDTAxH_2O)
4. **Stock solution:** 500 cm^3 final volume with distilled water
0.025 g Sodium metasilicate nonhydrate ($Na_2SiO_4x9H_2O$)
1.550 g boric acid (H_3BO_3)
1.115 g manganese (II) chloride tetrahydrate ($MnCl_2x4H_2O$)
0.044 g ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}x4H_2O$)
0.0596 g potassium bromide (KBr)
0.0415 g potassium iodide (KI)
0.1435 g zinc sulphate heptahydrate ($ZnSO_4x7H_2O$)
0,073 g cobalt (II) nitrate hexahydrate ($CO(NO_3)_2x6H_2O$)
0.0625 g copper sulfate pentahydrate ($CuSO_4x5H_2O$)
0.237 g aluminium sulphate ($Al_2(SO_4)_3$)
0.025 g lithium chloride, monohydrate ($LiClxH_2O$)
Preparation of Zehnder 8 nutrient media: 1000 cm^3 distilled water
- **Stock1**: 3 mL
- **Stock2**:1 mL
- **Stock3**: 10 mL
- **Stock4**: 0.08 mL

The procedure of algae culturing:

1. Diluting 100 mL from the z-8 media that we have prepared with 900 mL distilled water for 2 bottles of stock.
2. Preparing an Erlenmeyer flask with a cotton tip covering and foil for the outer to be sterilized at 160°C for 45 minutes.
3. Before being used for culturing, the stock must be sterilized for 2 hours at 120°C in a sterilization machine.
4. The important thing in preparing a laminar box is a place where we have to do culturing by wiping alcohol around inside the box, turning the device up, and using UV light for about 3-5 minutes to sterilize and turn UV off.
5. Preparing a stock, 3 replicates (Erlenmeyer flasks), autopipettes, tips, glass slides, and waste glass.
6. Always wear gloves when working in the laminar box.
7. Using autopipette to get algae on a glass plate (slide) for checking cells on microscopic. In three replicates, 100 mL of stock and 5 mL of algae were filled by automate pipettes (the same volume for all three green algae types).
8. stirring (continuous, 100 rpm) were ensured in a shaking incubator (Witeg WIS-10RL, Wertheim, Germany).
9. A weekly transfer is required with the best algae cells that have been checked by microscopic every week (an amount of "old" culture is transferred with sterile pipettes into a flask of fresh medium so that with the fast-growing species, the initial concentration is about 100 times smaller than in the old culture).

3.2.2. Algae growth inhibition test

Growth inhibition tests were carried out in 300 mL conical flasks under appropriate ambient conditions [T = 23°C, continuous and uniform illumination with cool white light (7760 ± 1100 lux, continuous shaking]. Z8 nutrient was used for the preparation of test media. A three-member dilution series was prepared from the stock solution of the test substance by half-dilution. Due to the poor water solubility of CBZ, the CBZ stock solution was prepared it as follows: 50 mg CBZ was dissolved in 1 mL methanol. 50 mL of test solution for each replica and 50 mL of Z8 nutrient medium for the control groups were weighed into the test vessels. Since we had to use solvent during the preparation of the stock solution, additional solvent controls were included. The effects of the test concentrations and the control conditions were investigated in 3 replicates, and a fourth replica was set, which was used as a measurement blind during the tests. The tested

concentrations of CBZ were as follows: 100, 50, 25 mg/L, while the quantity of the solvent was 2 mL, 1 mL, and 500 µL, respectively.

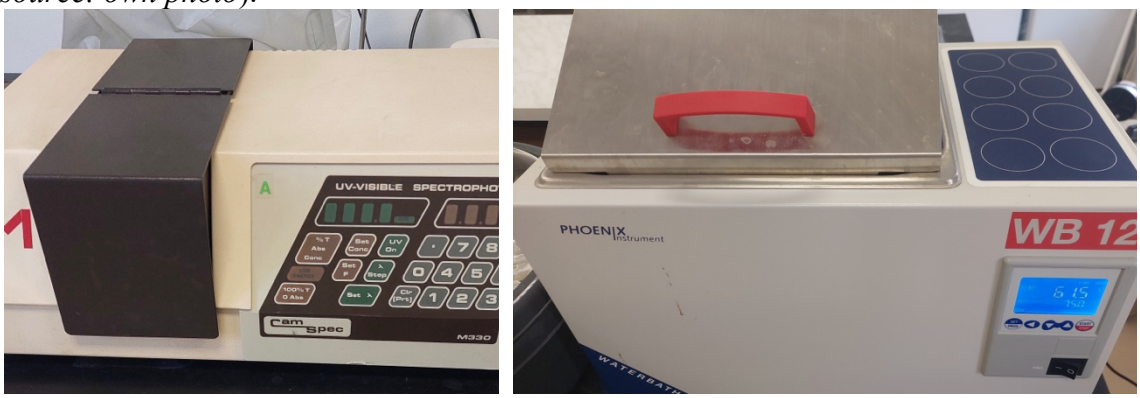
After the addition of the test solutions in the test vessels, algae cells were added to each flask, except for the measuring blanks, to ensure the same initial concentration of biomass (algae density) in all flasks and to ensure exponential growth of biomass throughout the exposure time (72 hrs). The initial biomass concentration was about $4x10^4$ cells/mL, which exceeds the cell concentration range recommended by the OECD, but the method determining the surrogate parameter (optical density) studied is able to determine this concentration range with high reliability and in a highly reproducible way, with the fulfilment of the conditions for exponential growth during the entire exposure time. After the addition of the algae cells, the flasks hours covered with filter paper were incubated for 72 hours. During the tests, continuous and uniform cool-white illumination (7760 ± 1100 lux, controlled temperature (23°C) and stirring (continuous shaking, 100 rpm) were ensured in an incubator (Witeg WIS-10RL, Wertheim, Germany). The tested end point of the study was the inhibition of algae growth. At the end of the experiment, the amount of algae biomass was determined in each control and treated group.

The most commonly used parameters for the quantification of algae biomass are cell count and optical density. The optical density of the test solutions was determined with the use of a spectrophotometer (UV/VIS Camspec single beam M330, Camspec, Crawley, United Kingdom) at 750 nm (Figure 13a), also in 3 replicates for each sample, with the dilution of the samples if needed. The correlation between cell counting and the measurement of optical density was previously assessed and proved to be very high ($R^2 > 0.998$). In addition to the measurement of optical density, the effects of CBZ on algae growth were also evaluated based on chlorophyll-a content of the samples. The chlorophyll-a content of the samples was determined spectrophotometrically after alcohol extraction, also in 3 replicates (ISO 1992). The first step of chlorophyll-a determination was the centrifugation of samples (50 ml algae suspension, M) using Rotina 46R (Hettich Zentrifugen, Tuttlingen, Germany) for 25 minutes at 3700 rpm. After 20 mL of 96% ethanol (m) was added to the settled algae cells, and the samples were placed in a water bath at 75°C for 15 minutes (Figure 13b). Then the centrifuge tubes were cooled back to room temperature and centrifuged for 20 minutes at 4700 rpm. The absorbance of the samples was determined at three different wavelengths: 750 (degree of turbidity), 666 (first measurement wavelength of chlorophyll-a) and 653 nm (second measuring wavelength of chlorophyll-a). To determine the chlorophyll-a content [µg/L], Felföldy's formula was used (Felföldy, 1987):

$$C_a = [((17{,}12*(E_{666}-E_{750})-8{,}68*(E_{653}-E_{750}))*m*10^3]/M$$

During the algae growth inhibition test, in the control groups, the mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) was below 35%. Over the entire duration of the experiment, the coefficient of variation of average specific growth rates did not exceed 7% in the parallel control groups and the amount of biomass in the control increased more than 16-fold after 72 hours, thus the performed test was considered valid. Based on the results of the measurements (optical density and chlorophyll-a content), inhibition percentages were calculated considering the effects observed in the corresponding solvent controls.

Figure 13. (A) Camspec M330 UV-Visible Spectrophotometer and (B) Phoenix instrument Water bath WB-12
(*source: own photo*).



(A) (B)

3.6. Data analysis

Concentration of CBZ determined by ELFIA in the surface water samples from the Lake Balaton and the Danube River were expressed as mean ± standard deviation, as well as the OD values and the inhibition data from the acute ecotoxicological assay on *C. vulgaris*. The 4-parametric calibration curves in ELFIA measurements were drawn by OriginPro 8.5 software (OriginLab Corporation, Northampton, MA, USA).

4. Results and discussion

4.1. Monitoring of carbamazepine in the Lake Balaton and the Danube River

Surface water samples were collected at 8 and 10 sampling points from the Lake Balaton and from the Danube, respectively. Samples from the Lake Balaton were collected on 25th September 2024, while samples from the Danube were collected during and after the flood period on 2nd October 2024 and 9th October 2024, respectively. Samples were measured without any further dilution.

For the Lake Balaton, concentration of CBZ was above the LOD value (0.02 ng/mL) and above the LOQ value (0.03 ng/mL) for all samples (frequency of occurrence: 100%). The highest concentration was determined at the Révfülöp sampling site (4.77 ± 0.20 ng/mL), while the lowest concentration was measured at Zamárdi (0.03 ±0.00 ng/mL). Concentrations measured at the sampling sites are summarized in Table 8.

Table 8. Concentration of carbamazepine determined by enzyme-linked fluorescent immunoassay in water samples collected in the Lake Balaton.
(*source: own work*)

Name of the sampling site	GPS coordinates of the sampling site	carbamazepine concentration (ng/mL)
Balatonvilágos	46.979393; 18.162955	0.10 ± 0.01
Zamárdi	46.888418; 17.955255	0.03 ± 0.00
Balatonlelle	46.789966; 17.687248	0.09 ± 0.01
Balatonfenyves	46.716051; 17.482776	0.05 ± 0.01
Keszthely	46.749110; 17.246045	0.06 ± 0.01
Szigliget	46.785064; 17.436729	0.08 ± 0.01
Révfülöp	46.826260; 17.631550	4.77 ± 0.20
Tihany-Sajkod	46.908717; 17.849444	0.29 ± 0.01

In Hungary a similar, systematic monitoring was performed in 2017-2018 at the Lake Balaton (Maasz et al., 2019). Samples were collected from the Zala River, the Lake Balaton Minor and the Lake Balaton. The frequency of occurrence for CBZ was 100%, the average concentration was in the range of 0.0532 – 0.1731 ng/mL among months investigated. Although the study indicated, that the main origin of PhAC contamination is the WWTPs, however the mass tourism-induced PhAC contamination was also detectable. Nowadays, >40 WWTPs can be found in the catchment of Lake Balaton. Their capacity varies between 2 and 50,000 m^3/day. The largest WWTP is situated to the city of

Zalaegerszeg, where the highest concentration was detected (0.804 ng/mL). CBZ is frequently appeared in surface waters of Europe, reported median concentration is 75 ng/mL, 70 ng/mL, 78 ng/mL, 25 ng/mL and 30 ng/mL in Austria, Finland, France, Germany and Switzerland, respectively (Lindholm.Letho et al., 2015; Vieno et al., 2006; Ternes et al., 2004).

For the Danube River, average concentrations of CBZ on the first sampling date (2nd October 2024) during the flood period were determined mostly around the LOQ value (0.03 ng/mL). Only CBZ content of sample from Dunaújváros was below the LOD value (0.02 ng/mL). Second sampling date (9th October 2024) was after the flood period. Although, on the second sampling date CBZ concentration was below the LOD value at 3 sampling sites (Nagybajcs, Komárom, Baja), the concentration at remaining sites were 1.59 – 29.37 times higher compared to the first sampling date (Table 9). Results indicate, that the flood mostly diluted the concentration of CBZ in the Danube River.

Table 9. Concentration of carbamazepine determined by enzyme-linked fluorescent immunoassay in water samples collected in the Danube River.
(*source: own work*)

Name of the sampling site	GPS coordinates of the sampling site	carbamazepine concentration (ng/mL)
Nagybajcs	47.7668050; 17.6945341	1st: 0.07 ± 0.01 2nd: <LOD
Komárom	47.753992; 18.069249	1st: 0.06 ± 0.02 2nd: <LOD
Esztergom	47.795750; 18.732837	1st: 0.03 ± 0.01 2nd: 0.95 ± 0.06
Szentendre	47.665235; 19.079307	1st: 0.04 ± 0.02 2nd: 0.57 ± 0.08
Budapest, Zöldsziget	47.480520; 19.058501	1st: 0.04 ± 0.01 2nd: 0.51 ± 0.08
Dunaújváros	46.956882; 18.956055	1st: <LOD 2nd: 0.07 ± 0.01
Dunaföldvár	46.809236; 18.930911	1st: 0.06 ± 0.01 2nd: 0.22 ± 0.07
Paks	46.618546; 18.859854	1st: 0.04 ± 0.02 2nd: 0.05 ± 0.01
Baja	46.193724; 18.923801	1st: 0.05 ± 0.01 2nd: <LOD
Mohács	46.028987; 18.685941	1st: 0.04 ± 0.02 2nd: 0.06 ± 0.00

A systematic monitoring was performed between 2017 and 2019 in the Danube River, where 111 PhACs were quantified. Frequency of occurrence was 99.1% with the mean concentration of 0.0772 ± 0.06 ng/mL CBZ. The maximum concentration was found to be 0.498 ng/mL, that is half of the highest concentration detected in this study at

Esztergom (0.95 ± 0.06 ng/mL) (Kondor et al., 2020). The Danube River, stretching 2780 kilometers, flows through 10 countries and 4 capitals before emptying into the Black Sea via the Danube Delta. In the Hungarian section of the river, approximately 7.2 cubic meters per second of treated wastewater is released into the Danube, accounting for less than 1% of the river's total flow. Three major wastewater treatment plants (WWTPs) handle 80% of this discharge, releasing a combined total of about 500000 cubic meters of treated wastewater per day into the river. The North-Pest WWTP has an average discharge capacity of 180000 cubic meters per day, the Central WWTP can handle up to 250,000 cubic meters per day, and the South-Pest WWTP, which discharges treated wastewater into the Ráckeve-Soroksár branch of the Danube, has a capacity of 80000 cubic meters per day. Additionally, treated wastewater enters the river from 15 larger WWTP effluents (accounting for more than 70% of the total discharge), as well as from over 100 smaller WWTPs or communal wastewater disposal systems (Middle Danube Valley Water Management Directorate 2010, 2016).

4.2. Ecotoxicological effects of carbamazepine on *Chlorella vulgaris*

Growth inhibition by carbamazepine on *C. vulgaris* was determined by measurement of OD and chlorophyll-a content. A 16x growth in the untreated control is required for a valid assay. In the experiments this requirement always fulfilled, thus all assay declared as valid. The initial OD was in all replicates 0.03 that refers to the cell number of $4x10^4$ cell/mL. After 72 hrs the average OD value in the untreated control groups was 1.723 ± 0.214. As we tested 3 concentrations of CBZ (25, 50, 100 mg/L) we did not calculate EC_{50} values neither for OD or chlorophyll-a content (Table 10). 100 mg/L is the recommended concentration for limit test in the OECD guideline, thus higher concentration was not investigated. Besides, with the 100 mg/L the maximum solubility of CBZ was reached. Thus, growth inhibition is presented for OD and chlorophyll-a content (Table 11).

Table 10. Effects of carbamazepine (CBZ) on biomass of *Chlorella vulgaris* determined by OD and chlorophyll-a content.
(*source: own work*)

treated group	OD	Chlorophyll-a content (µg/L)
CBZ 25 mg/L	1.110 ± 0.338	10375.5 ± 1556.3
CBZ 50 mg/L	0.777 ± 0.280	6132.1 ± 674.5
CBZ 100 mg/L	0.446 ± 0.101	4019.9 ± 522.6
solvent control 0.5 mL/L MeOH	1.708 ± 0.325	13305.0 ± 1729.7
solvent control 1 mL/L MeOH	1.577 ± 0.162	14239.2 ± 1423.9
solvent control 2 mL/L MeOH	1.392 ± 0.126	15771.8 ± 1892.6

Growth inhibition was calculated based on the following formula:

$$\text{Growth Inhibition (\%)} = \left(\frac{\text{Control Growth} - \text{Treatment Growth}}{\text{Control Growth}}\right) \times 100$$

As the organic solvent content is higher in this study (2, 1 and 0.5 mL/L) than recommended in the guideline (max. 100 µL/L), solvent control solutions were applied for calculation of growth inhibition.

Table 11. Growth inhibition by carbamazepine (CBZ) on *Chlorella vulgaris*.
(*source: own work*)

treated group	growth inhibition (%) by OD	growth inhibition (%) by chlorophyll-a content
CBZ 25 mg/L	35.0 ± 3.5	22.0 ± 3.1
CBZ 50 mg/L	50.7 ± 6.6	56.9 ± 6.3
CBZ 100 mg/L	67.9 ± 10.2	74.5 ± 14.9

According to the growth inhibition data it can be concluded, that EC_{50} values is around 50 mg/L CBZ for OD and chlorophyll-a content, as well. Chang *et al.* determined EC_{50} value of 27.2 mg/L on growth of *C. vulgaris* (Chang et al, 2024), while Jos *et al.* reported 36.6 mg/L EC_{50} data for population abundance (Jos et al., 2003).

5. Conclusion and proposal

Environmental pollutants enter the environment primarily as a result of human activities, posing a threat to living organisms. Drug residues are a category of organic micropollutants. In general, only a portion of medicines are absorbed by the body, with the remaining portion excreted in urine or feces. These organic pollutants often travel from households to the environment via the sewage system. For example, medications, cosmetics, oils, and other chemicals that are used or discarded can end up in the wastewater system. Pharmaceutical active compounds (PhACs) are emerging environmental concerns due to their presence in aquatic environments and their potential impact on both wildlife and humans. Carbamazepine (CBZ) is classified as a high-risk substance for aquatic systems, negatively affecting aquatic life, including algae, invertebrates, and fish. Microalgae, as a key component of the aquatic food chain, are recognized as effective indicators for assessing water quality due to their small size, short reproductive cycles, and high sensitivity to pollutants. *Chlorella vulgaris*, a common phytoplankton in freshwater ecosystems, is frequently used in toxicity testing and environmental assessments.

The target compound of this study is the pharmaceutically active substance carbamazepine (CBZ), a pollutant in surface waters. The specific objectives are: (i) to monitor CBZ levels in freshwater samples from Lake Balaton and the Danube River using enzyme-linked fluorescent immunoassay (ELFIA); (ii) to investigate the ecotoxicological effects of CBZ on green algae (*Chlorella vulgaris*) through an algal growth inhibition test.

For the monitoring study, water samples were collected from the Danube River and Lake Balaton in Hungary. A total of 8 samples from Lake Balaton and 20 samples from the Danube River were collected to determine the concentration of carbamazepine (CBZ) in surface water. The determination of the active compound, CBZ, in the collected surface water samples was performed using enzyme-linked immunofluorescent immunoassay (ELFIA), developed as part of the Aquafluosense project (NVKP_16-1-2016-0049). The limit of detection (LOD) and limit of quantification (LOQ) for the ELFIA method are 0.02 ng/mL and 0.03 ng/mL, respectively. Growth inhibition tests were carried out following the OECD 201 guideline. The most commonly used parameters for quantifying algal biomass are cell count and optical density.

For Lake Balaton, the concentration of CBZ was above both the LOD (0.02 ng/mL) and the LOQ (0.03 ng/mL) in all samples (100% occurrence). The highest concentration was recorded at the Révfülöp sampling site (4.77 ± 0.20 ng/mL), while the lowest concentration was measured at Zamárdi (0.03 ± 0.00 ng/mL). For the Danube River, the average CBZ concentrations on the first sampling date (2nd October, 2024) during the flood period were

mostly around the LOQ (0.03 ng/mL). Only the sample from Dunaújváros had a CBZ concentration below the LOD (0.02 ng/mL). On the second sampling date (9th October, 2024), which was after the flood period, CBZ concentrations were below the LOD at three sampling sites (Nagybajcs, Komárom, Baja), while concentrations at the remaining sites were 1.59 to 29.37 times higher compared to the first sampling date. These results suggest that the flood had a diluting effect on CBZ concentrations in the Danube River. Based on the growth inhibition data, it can be concluded that the EC_{50} values for CBZ, based on optical OD and chlorophyll-a content, are approximately 50 mg/L.

Based on the results, it can be concluded that CBZ is a commonly occurring pollutant in surface waters in Hungary. The toxic concentration observed in the algal acute assay was significantly higher than the concentrations found in the environment, indicating the need to investigate lower concentrations over longer exposure periods for a more comprehensive risk assessment of the active compound. The primary source of CBZ in surface water bodies is wastewater treatment plants, so the environmental load could be reduced by implementing more efficient water treatment technologies.

6. Summary

Carbamazepine (CBZ) is marked as posing prominent risks to aquatic life and is toxic to algae and invertebrates and lethal to fish. CBZ was expected to impose little or no toxic impact on the different types of aquatic species, and hence it is not very toxic in aquatic habitats. Several studies have established that the percentage of CBZ in effluent from WWTPs is usually present more often than in their corresponding surface water sources, primarily because of dilution and natural biodegradation in the environmental conditions. Furthermore, the primary contributor to CBZ contamination in terrestrial ecosystems is sewage effluent, which is often reused as treated water for agricultural and irrigation. To detecting and quantifying the concentration of CBZ in surface water, utilising enzyme-linked fluorescent immunoassay is efficient for this investigation by the numerous numbers of sample and short period of time. As from the results of the Lake Balaton, the highest CBZ concentration was measured at the Révfülöp (4.77 ± 0.20 ng/mL). For the Danube River, the average CBZ concentrations on the first sampling date (2nd October, 2024), during the flood period, were mostly around the LOQ (0.03 ng/mL). The only exception was the sample from Dunaújváros, which had a CBZ concentration below the LOD (0.02 ng/mL). On the second sampling date (9th October, 2024), which followed the flood period, CBZ concentrations were below the LOD at three sampling sites (Nagybajcs, Komárom, Baja), while at the other sites, concentrations were 1.59 to 29.37 times higher compared to the first sampling date. These findings indicate that the flood had a diluting effect on CBZ concentrations in the Danube River. Based on the growth inhibition data, it can be concluded that the EC_{50} values for CBZ, determined from optical OD and chlorophyll-a content, are approximately 50 mg/L.

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List of figures and tables

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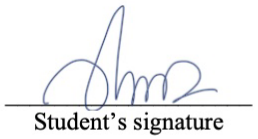

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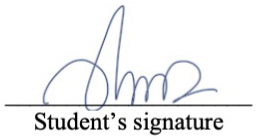

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