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Occurrence and distribution of azole antifungal agents in eight urban Romanian waste water treatment plants

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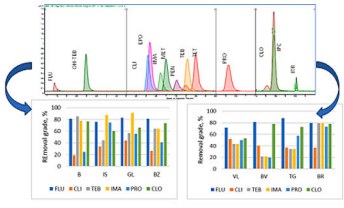
HIGHLIGHTS

- Azole antifungal agents are present in wastewater of Romania.
- Climbazole, clotrimazole, tebuconazole, prochloraz, fluconazole are partially eliminated in the sewage stations
- Climbazole can present a high risk for the plants *Lemna minor* and *Navicula pelliculosa.*
- Clotrimazole may present a high risk to the plant *Desmodesmus subspicatus* and to the invertebrate *Daphnia magna.*
- Procloraz may present high risk to the invertebrate *Mysidopsis Bahia.*

GRAPHICAL ABSTRACT



Compound	Type of living organism	Species	AF	PNEC	MEC	RQ	Risk level
CLI	Plant	Lemna minor	1000	0,019	0,247	13	High
	Plant	Navicula pelliculosa	1000	0.154	0.247	1.6	High
CLO	Desmodesmus subspicatus	Plant	1000	0,017	0,022	1,29	High
	Daphnia	Invertebrate	1000	0,02	0,022	1,10	High
	Daphnia magna	Invertebrate	1000	0,042	0,022	0,52	Medium
TEB	Danio rerio	Fish	1000	0,25	0,116	0,46	Medium
PRO	Cyprinus carpio	Fish	1000	1,355	1,031	0,761	Medium
	Mysidopsis bahia	Invertebrate	1000	0,77	2,031	2,638	High
	Scenedesmus subspicatus	Algae	1000	45,051	3,031	0,067	Medium

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ABSTRACT

Azole compounds are utilized to combat fungal infections in plants to protect them and also used for treating mycosis in humans. The LC-MS/MS method is a technique that combines liquid chromatography with tandem mass spectrometry for analysis of twelve azole compounds from wastewater (influent, effluent) and sewage sludge. The compounds were isolated from waste water using automatic extraction in the solid phase. Sludge samples were dried by lyophilization, after which they were subjected to ultrasound extraction with methanol. The quantification limits ranged from 0.3 ng/L (clotrimazole-CLO and prochloraz-PRO) to 1.5 ng/L (tetraconazole-TEB and penconazole-PEN), for wastewater samples and for sewage sludge, the LOQs ranged from 0.1 ng/g to 0.6 ng/g. High concentrations of climbazole-CLI (207–391 ng/L), tebuconazole (92–424 ng/L), and clotrimazole (6.9–93-ng/L) were observed in influent samples of the 8 urban wastewater treatment plants, followed by fluconazole (49.3–76.8 ng/L), and prochloraz (7.3–72 ng/L). The ∑Azoles had a maximum of 676 ng/L in the Galati effluent, followed by the Bucharest station 357 ng/L, and 345 ng/L in the Braila effluent. The highest value of the daily mass loading (input) level was observed for climbazole, 265 mg/day/1000 in Iasi station, followed by tebuconazole, 238 mg/day/1000 people in the Bucharest station, and 203 mg/day/1000 people for

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climbazole in the Targoviste station. The daily mass emission presented values between 0.7 and 247 mg/day/1000 people. The highest emissions were observed for climbazole, 247 mg/day/1000 people in Braila station; 174 mg/day/1000 people in the Iasi station and 129 mg/day/1000 people in the Bucharest station. The concentrations of climbazole detected in the effluent can present a high risk for the plants *Lemna minor* and *Navicula pelliculosa*. Clotrimazole may present a high risk to the plant *Desmodesmus subspicatus* and to the invertebrate *Daphnia magna*. PRO may present high risk to the invertebrate *Mysidopsis Bahia*.

1. Introduction

Azoles have various applications in different industries. They are commonly used as antifungal agents in plant protection, to treat fungal infections in humans (mycosis), and in veterinary medicine. This is because azoles have a broad-spectrum antifungal activity and are highly stable. Because the first azole compound used, imidazole, was highly toxic and produced side effects, it was replaced by the first generation of triazoles. In the 1990s, fluconazole (FCZ) and itraconazole (ITZ) were approved by the US Food and Drug Administration (FDA) to be used as drugs for the treatment of human mycosis, AIDS and in the case of cancer patients receiving chemotherapy to prevent fungal infections (Barone et al., 1998). These azole chemicals have at least one five-membered nitrogen heterocyclic ring containing two or three nitrogen atoms in their structures.

In addition to pharmaceutical products, azole compounds are also used in aircraft as anti-corrosive agents (Zarn et al., 2003), in personal care products such as shampoo, cream, setting foam, toothpaste (Allen et al., 2015). Ketoconazole (KCZ) is used in personal care products/shampoos (anti-dandruff agent), while climbazole (CBZ) is used in anti-aging cream. Azoles account for a large proportion of the annual worldwide use of fungicides amounting to approximately 300,000 t. Benzotriazole (BTZ) production is reported to be 850 t in 2012 in the USA (Giraudo et al., 2017) and 100 t per year in Australia (Loi et al., 2013). The use of climbazole in the European Union is reported in the range of 100–1000 tons per year, which represents the second largest use category (Chen and Ying, 2015; ECHA, 2013). The European Union has recently added azole compounds to its "Watch List" for monitoring purposes. *Implementing Decision (EU) no.* 2022/1307 of the Commission establishing a watch list of substances for monitoring throughout the Union under water policy includes the following azole compounds: clotrimazole (CLO), fluconazole (FLU), imazalil (IMA), ipconazole (IPC), metconazole (MET), miconazole (MIC), penconazole (PEN), prochloraz (PRO), tebuconazole (TEB), tetraconazole (TET), CE 2022/1307). The recommended method for detection is SPE-LC-MS-MS and the recommended quantification limits for each compound (20–1900 ng/L) are also specified. Chemical properties of azole antifungal compounds are presented in Table S1 (Chen 2015).

The excessive use of azole compounds in agriculture, in pharmaceuticals and in personal care products has led to the contamination of water, soil and aquatic organisms with these compounds. Once applied, azole fungicides are subsequently transported to wastewater treatment plants (WWTP) where they are processed before being released into surface waters via effluent discharge. Thus, the main source of river pollution with azole fungicides is house-hold sewage (Chen et al., 2014), with a partial contribution from hospital wastewater (Lindberg et al., 2010). Some azole fungicides were detected at concentrations of several micrograms per liter in the influent. Clotrimazole, ketoconazole and miconazole were detected both in the liquid phase and adsorbed on suspended matter particles (Peng et al., 2012). In the effluent, the concentrations of azole fungicides are significantly reduced compared to the influent, particularly within the range of tens to hundreds of nanograms per liter. The maximum reported concentrations of climbazole, clotrimazole, ketoconazole, miconazole and fluconazole were 443, 8650, 34.8, 35.7 and 448 ng/L, respectively (Assress et al., 2019, Assress et al., 2020, Casado et al., 2014, Lacey et al., 2012, Van De Steene et al., 2010, Wick et al., 2010).

Antifungal pharmaceutical compounds are partially metabolized in the human body and are therefore eliminated and end up in wastewater treatment plants (Assress et al., 2020, Giraudo et al., 2017, Peng et al., 2012,). Many personal care products, such as gels, shampoos, and perfumes, contain antifungal agents like climbazole. These agents are commonly administered onto the human body, leading to the subsequent discharge of substantial quantities of active compounds into wastewater treatment facilities. In the dewatered sludge, azole fungicides, with the exception of fluconazole, were detected at high levels of up to several micrograms per gram. Azole fungicides were also observed in raw sludge, recycled sludge and thickened sludge (Huang et al., 2012). During the wastewater treatment processes, high amounts of dehydrated sludge result. The application of sludge on the surface of the agricultural soil is a practice by which nutrients are managed to increase crop and vegetable yields (Langdon et al., 2011). This type of use of sludge in agriculture is the way through which azole fungicides reach the soil or groundwater, which are used as a source of drinking water for human consumption (Wu et al., 2010). Huang et al. (2022) discovered that azoles have negative effects on zebrafish (*Danio rerio*) by disrupting their metabolism, highlighting the potential for mitochondrial dysfunction and lipid dysregulation by triazoles. Algae and fish can experience toxic effects due to the presence of residues from azole fungicides. Azole fungicides negatively affect the mammalian endocrine system and several azoles have been identified as endocrine disruptors (Draskau et al., 2021). The acute toxicity of CLI to duckweed (*Lemna minor*), diatoms (*Navicula pelliculosa*) and green algae (*Pseudokirchneriella subcapitata*) is assessed at mean effective concentrations (EC50) of 19.0–33.9, 153.6–290.6 and 214.4–1191 μg/L, respectively (Richter et al., 2013). Regarding seaweed, clotrimazole is more toxic to periphyton, altering chlorophyll *a* content and the photoprotective cycle of xanthophyll pigments at 0.17 μg/L (Porsbring et al., 2009). At levels up to 0.14 μg/L, clotrimazole could significantly reduce the growth of marine shrimp (*Palaemon serratus*) at 18 °C (González-Ortegón et al., 2013). In addition, survival, growth and development of frog larvae (*Xenopus tropicalis*) were statistically affected when exposed to clotrimazole at the concentration of only 0.1 μg/L (Shi et al., 2012).

Considering the complex issue of the presence and potentially toxic effects of azole antifungals in the environment, within the project we proposed a sensitive and precise chromatographic method for the detection of azole compounds in wastewater and sewage sludge matrices. In the framework of the topic, the *removal yields* of azole compounds from the eight urban sewage treatment plants were estimated and the *daily mass load* level of the influent and the *daily mass emission* through the effluent were evaluated. At the same time, the ecological risk that the selected analytes may have on aquatic microorganisms was estimated. To date, there has been a lack of research conducted in Romania investigating the presence and levels of azole compounds in wastewater treatment plants (WWTPs), including influent, effluent, and sewage sludge. Understanding the concentrations of these compounds in the Romanian environment is crucial due to their detrimental effects on the aquatic organisms. Through this paper, the behavior of *antifungal azoles* in wastewater treatment plants was studied for the first time in Romania. Thus, the method was used to assess the degrees of removal of these compounds in treatment plants and the daily mass loading and daily mass emission of these stations. Finally, the ecological risk that azole compounds have on aquatic microorganisms has been determined.

2. Materials and methods

2.1. Chemicals and materials

The pure pharmaceutical standards for clotrimazole (CLO), imazalil (IMA), ipconazole (IPC), metconazole (MET), penconazole (PEN), procloraz (PRO), tebuconazole (TEB), tetraconazole (TET), climbazole (CLI), epoxiconazole (EPO), itraconazole (ITR), fluconazole (FLU), and hydroxy-tebuconazole (OH-TEB) were obtained from Sigma-Aldrich (Germany). The organic solvents acetonitrile and methanol, which were used for LC mobile phase and solid phase extraction (SPE) extraction, were acquired from Merck (Germany). Ammonium acetate (99.8 %) was obtained from Sigma-Aldrich (Steinheim, Germany). Strata-C18 and Strata-X cartridges (0.5 g, 6 mL) for solid phase extraction were purchased from Phenomenex (USA). Water for sample preparation was of HPLC grade and prepared in-house using an Milli-Q IQ 7005 system (Millipore, USA). Samples were filtered using a glass fiber filter (0.45 μm × 7 cm) from Sigma-Aldrich (Steinheim, Germany). Nylon syringe micro-filters (0.45 μm × 4 mm) were used to filter the extract samples and were obtained from VWR International (Leuven, Belgium). Standard stocks with concentrations of 500 μg/mL were obtained by weighing and dissolving the substances in a volume of 10 mL of methanol in a volumetric flask. Intermediate dilutions at concentrations of 5 μg/mL and 0.5 μg/mL were prepared in acetonitrile. Stock standard solutions were stored at −20 °C and their dilutions at 4 °C. The mixed standard calibration solutions with concentrations ranging from 1 to 100 ng/mL were prepared in the mobile phase consisting of ammonium acetate (5 mM) and acetonitrile (50/50, v/v). The mixed standard calibration solutions with concentrations in the range of 1–100 ng/mL were prepared in the mobile phase (ammonium acetate 5 mM / acetonitrile: 50/50, v/v). The internal standard (SI), *hidroxy-tebuconazol*, was added to each calibration solution (1; 5; 10; 25; 50; 100 ng/mL) in equal concentration (1 mL of SI with a concentration of 500 ng/mL was added to each standard mix 10 mL, 50 ng/mL).

2.2. Sample collections

During three consecutive days in May 2023, we collected 24-hour flow proportional composite samples (influent and effluent) from eight urban Wastewater Treatment Plants (WWTPs) located in Bucharest (B), Iasi (IS), Galati (GL), Buzau (BZ), Ramnicu-Valcea (VL), Brasov (BV), Targoviste (TG), and Braila (BR, Table S2), Romania. These samples were collected in 1 L bottles and transported to the laboratory under refrigerated conditions. The samples were extracted within 24 h of sampling. Upon arrival, they were filtered using GF filters (0.45 μm) and extracted using solid phase extraction. Thus, Station 1 serves the city of Bucharest, with a population of approximately 1,830,000; station 2 serves the city of Iasi with a population of 739,500 1000 peoples; station 3 is represented by the Glalati city station, which has an average daily effluent flow of 224,640 m/day; station 4 belongs to the city of Buzau with a population of 235,000 peoples; station 5 is the one related to the city of Ramnicu-Valcea with a population of 706,900 peoples and an average effluent flow of 110,000 m^3/day; station 6 belongs to the city of Brasov with a population of 440,701 peoples, the station of the city of Tragoviste (7) serves a population of 79,600 peoples and the last station Braila (8) serves 180,302 peoples. The wastewater treatment process is similar for all treatment plants. Thus, the biological mechanical purification is followed by a tertiary stage, which removes nutrients from the wastewater. The resulting sludge is mainly used as biosolid in agriculture.

In taking the effluent samples, it was assumed that the time required for the influent to reach the end of the purification process (residence time) was 24 h (approximately), taking composite samples. The residence time of the influent in the treatment plants was approximated to 24 h. This assumption is a common practice in the analysis of organic compounds in conventional sewage treatment plants.

2.3. Sample preparation

The pretreatment of water samples containing analytes involved using the solid phase extraction (SPE) procedure. The recovery of target analytes from the water samples was conducted using an automatic solid phase extraction system called SPE AutoTrace 280 (Thermo- Scientific-Dionex). A Strata X cartridge with a styrene-divinylbenzene polymeric adsorbent phase (0.5 g / 6 mL, Phenomenex) was employed for the extraction. To enhance analyte recovery, the aqueous samples (250 mL) underwent pH adjustment to 3, 7, and 10. Acetic acid and ammonia 2.4 % were used to adjust the pH to 3 and 10, respectively. The sorbent was conditioned with 10 mL of methanol and 10 mL of ultrapure water. Subsequently, the samples were percolated through the SPE cartridges, where the analytes were retained in the adsorbent phase while the aqueous residues were removed. To eliminate interferences from the matrix, the cartridges were washed with 10 mL of ultrapure water containing 5 % methanol. After that, the cartridges were air-dried for 20 min. The analytes present in the SPE material were then eluted with 6 mL of methanol and collected in a concentration tube. The extract was evaporated to dryness using a Biotage II evaporation system under a weak stream of nitrogen at a temperature of 45 ± 2 °C, until close to dryness. After obtaining the residue, 0.5 mL of a 50/50 mixture of acetonitrile (ACN) and ultrapure water was added.

To improve the homogeneity of the sewage sludge sample, the solid samples were dried using the Hypercool H3110 lyophilizer from Lab-Tech for 24 h. A 1 g portion of the lyophilized sludge was weighed, and then 6 mL of methanol and 0.1 mL of ammonia (2.4 %) were added. The mixture was then subjected to ultrasound in an ultrasonic bath from Bandelin, Sonorex, for 15 min, enabling liquid-solid extraction. To separate the solid phase from the liquid phase, the resulting extract was centrifuged at 3000 rpm for 10 min using the Rotofix 32 centrifuge from Hettich. The resulting supernatant was transferred to an extraction flacon. To enhance the recovery of the contaminants from the sludge, the extraction process was repeated for the remaining sludge after centrifugation. Another 6 mL of methanol were added to the sludge sample, and the established extraction procedure was followed, including ultrasonication, centrifugation, and supernatant transfer. The final organic extract was then purified using solid phase extraction (SPE) on Strata X phase cartridges (0.5 g, 6 mL) from Phenomenex, with the assistance of the automatic SPE Auto Trace 280 system from Thermo-Scientific. The methanol content was reduced to <10 % by diluting the resulting extract, approximately 12 mL, with 88 mL of ultrapure water. This eliminated the contribution of methanol to the SPE elution of the desired analytes.

2.4. LC-MS instrumentation and conditions

The analytical determinations were performed using an Agilent Technologies UHPLC-MS/MS system (model 1260-6410B) from the Waldbronn, Germany location. In positive ion mode, the liquid chromatograph was connected to a triple quadrupole mass spectrometer using an electro-spray ionization (ESI) source. Separation of the pollutants was achieved using a Zorbax Eclipse XDB C18 column (100 × 2. mm, 3.4 μm) maintained at a temperature of 30 °C. A mobile phase gradient composed of 5 mM ammonium acetate (A) and acetonitrile (B) was used for separation, with the following conditions: 0–7 min at 5 % A and a flow rate of 0.2 mL/min, 7–8 min with a gradient from 50 % to 5 % A and a flow rate of 0.2 mL/min, and 8–13 min at 5 % A isocratic. After elution of the analytes from the column, a mobile phase consisting of 50 % A at a flow rate of 0.4 mL/min was used from 13.7 min to 17.5 min for column washing and equilibration. The flow rate was then returned to 0.2 mL/min within 30 s to prepare the column for the next injection.

All determinations were performed in the positive ionization mode, and the optimized conditions were as follows: a gas temperature of 300 °C, a nebulizer gas flow rate of 10 L/min, a nebulizer pressure of 40 psi, and a cell acceleration voltage (CAV) ranging from 1 to 5 V. The

voltage applied to the capillary was set at 4000 V.

2.5. Validation study

The developed method was assessed for its linearity, sensitivity, method accuracy, repeatability, and internal reproducibility. The limit of quantitation (LOQ) was determined experimentally as the concentration that give a signal-to-noise ratio equal to 10 for a solution obtained through a specific extraction process. The LOQ calculations took into account the concentration factor resulting from the extraction of the wastewater samples (0.25 L effluent sample or 1 g sewage sludge to 0.5 mL extract). Calibration was performed using the internal standard calibration method, with an internal standard added to each standard solution. Calibration based on peak area was obtained using internal standard calibration method (OH-TEB internal standard added to each standard solution, 50 ng/mL). The B.04.00 Mass Hunter LC- MS/MS software plotted the linear regressions representing the values of the chromatographic peak areas according to the concentrations of the calibration standards.

A calibration curve with six data points was created by using standard solutions containing compounds at concentrations ranging from 1 to 100 ng/mL. Calibration graphs were generated using linear regression analysis and they were accepted if correlation coefficient was >0.99. For the assessment of the *accuracy* of the all SPE-LC-MS/MS method, effluent and sludge samples and were fortified with known concentrations of the standard (*50 ng/L or 50 ng/g*). In parallel, samples of wastewater were extracted and the determined compounds were subtracted from the spiked samples. The *recovery rate* ranging in the interval 70–120 % was accepted as good. *Matrix effect* of each selected analyte was measured by comparing the extracts from influent and effluent samples un-spiked and spiked and with calibration standard (*25 ng/mL*) in 0.5 mL of mobile phase. The evaluation of the matrix effect involved the post-extraction addition method. The extracts that were obtained had contamination from a known concentration of analyte mixture. To assess the matrix effect, we compared the variations in responses between non-spiked and spiked extracts to those obtained from a standard solution with the same concentration. The method's *precision* was assessed by calculating the relative standard deviation (RSD) for four effluent samples (250 mL) and four sludge samples, which were spiked with analytes before extraction at a concentration level of *50 ng/L and 50 ng/g*, respectively. The precision was evaluated within a single day and over a period of four consecutive days. Repeatability and reproducibility were considered acceptable if the RSD was below 15 %.

2.6. Data analysis

The degree of removal of organic compounds from the sewage treatment plant was evaluated considering the concentrations of analytes in composite water samples. Then the environmental emissions of these contaminants were calculated based on the equations

$$R\% = \left(C_{inf} - C_{efl}\right)/C_{inf} \times 100 \quad (1)$$

where C_{inf} is the analyte concentration in the influent samples, expressed in ng/L and C_{efl} is the analyte concentration in the effluent samples.

The daily mass loading (DML) and daily mass emission (DME) of the analyzed compounds were then calculated in both the influent and effluent samples using Eqs. (2) and (3) provided by Chiriac et al. (2023):

$$DML = (Q^{*}\ C\_inf)/P \quad (2)$$

$$DME = \left(Q^{*}\ C\_efl\right)/P \quad (3)$$

where, Q is the flow rate of the treatment plant in m3/day, C_{inf} is the concentration of compound in the influent and C_{efl} is the pollutant concentration in the effluent expressed in ng/L, P is the population served by the respective station.

2.7. Estimation of risk factors of azole compounds in sewage treatment effluent

To assess the potential risks associated with azole compounds on aquatic organisms, the highest concentration of each compound found in WWTP effluent discharged into surface water was utilized. As these compounds are known to be toxic to aquatic organisms and have endocrine-disrupting effects, it is crucial to estimate the risk factors (RQ) to determine the actual level of exposure for organisms in the aquatic environment. The evaluation of the environmental risk of azoles was conducted using toxicological studies documented in specialized literature. These parameters were calculated similarly to other studies (Chiriac et al., 2023) using Eqs. (4) and (5):

$$PNEC = NOEC\ (LC50 \text{ or } EC50)/AF \quad (4)$$

$$RQ = MEC/PNEC \quad (5)$$

where

MEC represents the maximum concentration determined for each compound in surface waters, PNEC the approximate concentration without effect on aquatic species

NOEC – no observed effect concentration

EC50 - the compound concentration to which the body gives a half maximum response

LC50 - the concentration of the compound at which 50 % of the organisms die

According to the technical guidance document (TGD) on risk assessment of the European Commission, the AF value was determined as 1000 for LC50 / EC50 and 100 for a long-term NOEC (European Commission, 2003; Shi et al., 2016).

An RQ value of <0.1 indicates a low risk or a potential adverse effect, while an RQ value between 0.1 and 1 indicates a moderate risk or an adverse effect. An RQ value equal to or >1 indicates a high-risk level, requiring careful attention.

The risk level was evaluated in the following manner:

- If RQ is <0.01, it is considered very low risk.
- If RQ falls between 0.01 and 0.1, it is categorized as low risk.
- If RQ is between 0.1 and 1, it is considered medium risk.
- If RQ is >1, it is classified as high risk.

Relationships between determined azole compounds in wastewater samples were evaluated by Spearman Rank correlation analysis.

3. Results and discussions

3.1. Occurrence and fate of azoles in WWTPs

3.1.1. Concentration of azole compounds in influent and effluent

Characteristics of the development of the chromatographic method for the determination of azole compounds from environmental samples can be seen in Tables S3, S4, S5, S6, S7 and in Figs. FS1, FS2, FS3, FS4, FS5.

Maximum frequencies of 100 were observed for FLU, CLI, TEB, PRO, CLO. High concentration values were observed for TEB (92–427 ng/L, mean 191 ng/L), CLI (207–391 ng/L mean 289 ng/L), CLO (6.9–93-ng/L, mean 28.2 ng /L), followed by FLU (49.3–76.8 ng/L, mean 67.3 ng/L), and PRO (7.3–72 ng/L, mean 25.5 ng/L) in the influent samples (Table 1). Three compounds were detected only in one station (Galati) as follows: EPO in Galati influent 17.8 ng/L, TET in influent 16.5 ng/L and PEN15.8 ng/L in influent and 3.2 ng/L in effluent. IMA was detected in 37.5 % of samples (Bucharest, Iasi and Galati stations), with a minimum concentration of 16.5 ng/L and a maximum of 41 ng/L, average 25.8 ng/L. MET and ITR are two compounds that were not identified in any of the water samples analyzed.

The descending order of azole compounds according to the maximum concentration determined in the influent samples was: TEB >

Table 1
The concentrations (ng/L) of azole compounds determined in the influent and effluent samples.

Compound	Influent, ng/L				Effluent, ng/L			
	Cmin	Cmax	Cmedie	Frequency, %	Cmin	Cmax	Cmedie	Frequency,%
FLU	49.3	76.8	67.3	100	8.3	17.6	13.4	100
CLI	207	391	288.6	100	123	247	185	100
EPO	17.8	–	–	12.5	–	–	–	12.5
TET	16.8	16.5	10.3	25	–	–	–	25
TEB	92	424	191	100	39	116	76.7	100
IMA	16.5	41	25.8	37.5	2	6.5	4	37.5
PEN	15.8	–	–	25	3.2	–	–	25
PRO	7.3	72	25.5	100	4.3	31	11.8	100
CLO	6.9	93	28.2	100	1.8	22	7.9	100

CLI > CLO > FLU>PRO>IMA > EPO > TET > PEN. The maximum levels of concentrations in the influent were observed in the case of Bucharest stations (TEB 424 ng/L), followed by Braila (CLI 391 ng/L) and Galati (382 ng/L, Fig. FS6).

Compounds with medium hydrophobicity (CLI log Kow 3.76, TEB log Kow 3.49) predominate the influent of WWTP's. IPC was detected in a single sample in the influent of the Galati station at a value of 17.3 ng/L. The different patterns in the influent can be attributed to the differences in the use of azole compounds in the investigated cities.

For the compounds analyzed, higher values were observed in Germany for CLI in influent 475–1350 ng/L (Wick et al., 2010). In China, similar values were detected in the sewage treatment stations, 282 ng/L (Chen et al., 2014). In Spain, similar CLO concentrations of 11–80 ng/L were determined in the influent of some sewage treatment stations (Casado et al., 2014), while in Ireland, Lacey et al. (2012) obtained a wide range of concentrations in the case of CLO from <LOQ to 900 ng/L. Also in Spain, Casado et al. (2014) obtained similar values of FLU in the influent in the range of 20–93 ng/L and in Sweden specialists determined similar values of fluconazole <LOQ-120 ng/L (Lindberg et al., 2010).

Medicines containing antifungal agents are extensively utilized, in care products (biocides), and in agriculture, so they end up having a negative impact on the environment. Along with the population growth, the level of use of these substances also increased. Antifungal azole agents are used in shampoos, and to preserve materials in paint, plastics, adhesives, paper, polymer materials such as rubber, leather. Fungicides are used in the treatment of fruits and vegetables against fungi that can affect food products. As a result, azole antifungals have become a new emerging group of pollutants in the environment that worries the academic world due to the negative effects of the non-intentional exposure of living beings to these chemicals.

Regarding the *effluents*, lower values of azole compounds were determined compared to those determined in the influents (Fig. FS7). The dominant compounds in the effluent were CLI with values between 247 and 123 ng/L, average 185 ng/L, TEB with values within the specified range of 39–116 ng/L, average 76.7 ng/L. The concentrations of PRO ranged from 4.3 to 31 ng/L (mean 11.8 ng/L) followed by CLO which ranged between 1.8 and 22 ng/L, mean 7.9 ng/L, and FLU in the range of 8.9–17.6 ng/L, mean 13.4 ng/L. The IMA presented values <10 ng/L (range 2–6.5 ng/L), and PEN was detected only in Galati (3.2 ng/L). The decreasing order of the concentration of azole compounds determined in the effluent according to the maximum mass value is the following: CLI > TEB > PRO>CLO > FLU>IMA > PEN. In the effluent samples, the azole compounds follow the same trend as that observed in the influent samples, with the maximum values being determined in the samples collected from the Braila (CLI 247 ng/L) and Bucharest (CLI 230 ng/L) Treatment Stations. Instead, TEB showed maximums of 116 ng/L and 92 ng/L in the stations in Galati and Brasov. CLI and TEB remain large in the effluent (12–247 ng/L and 39–116 ng/L), demonstrating a persistent character in the purification process.

The decrease in the concentration of azole compounds in the effluent compared to the influent is due to the processes of bio-transformation and adsorption on the sludge. CLO concentrations decreased slightly after the application of UV treatments in the British stations (Roberts and Thomas, 2006). However, the concentrations of azole compounds did not decrease significantly after UV or chlorine disinfection (Peng et al., 2012).

The sum of the compounds (∑Azoles) reached its peak level of 676 ng/L in the effluent from the Galati station, followed by the Bucharest station 357 ng/L, and 345 ng/L in the Braila effluent. The concentrations of summed azole agents varied in the range of 209–676 ng/L. These concentrations of azole compounds are discharged into the Bahlui, Dambovita, Danube rivers, from which potable water for the resident population is obtained through chemical treatment. In this way, azole contaminants can negatively impact the aquatic organisms in the receiving rivers (algae, fish, plant) and thus, the food chain has the potential to pose a risk to human health and the consumption of potentially contaminated water.

The values of the concentrations of azole antifungal agents obtained in this study were compared with those reported in literature and it was observed that in the sewage treatment stations in Germany they were similar in the case of CLI for which a range of 312–443 ng/L was reported (Wick et al., 2010). On the other hand, in China the reported values were 66.4 ng/L (Chen et al., 2014) was lower than those obtained in the present study (247–123 ng/L). CLO was determined in comparable concentrations with values of 5–11 ng/L in sewage treatment plants in Spain by Casado et al. (2014). For FLU, higher concentrations of <LOQ-140 ng/L were obtained in Sweden (Lindberg et al., 2010), 85–448 ng/L in China (Chen et al., 2014) and 37–95 ng/L in Spain (Casado et al., 2014).

The physico-chemical properties of azoles are responsible for the chemical behavior of these contaminants in WWTP's. Water solubility, hydrophobicity (log Kow), dissociation constants (pKa) influence the presence of azoles in the environment. Usually, a compound with log Kow >4 (lipophilic) should be found as adsorbed in sludge (in the solid matrix like sludge, soil, sediment), suggesting removal from the treatment plant by adsorption. A compound with log Kow <2 tends to remain in the station's liquid phase of the effluent or in the surface water. For a compound with 2 < log Kow <4 (hydrophilic-lipophilic) it can be suggested that it will exist both in the liquid phase and in the solid phase (Loi et al., 2013) suggested that FLU is hydrophilic and will be present in significant concentrations in water, while CLO, IMA, IPC, PRO, ITR should be present in major concentrations in sediment or sewage sludge. The compounds MET, PEN, TEB, TET, CLI, EPO will exist both in the form of a liquid or a solid phase. The presence of these compounds in the sewage treatment plant effluent is due to the intensive use of azole compounds for their antifungal effect in human and veterinary medicines, personal care products (shampoo, gels), application in urban gardens but also on fruits and vegetables against various harmful fungi.

3.1.2. The removal capacity of azole compounds through sewage treatment plants

The removal efficiency was evaluated for each analyte and for each treatment plant (Fig. 1). Removal capacities below 50 % (unsatisfactory)

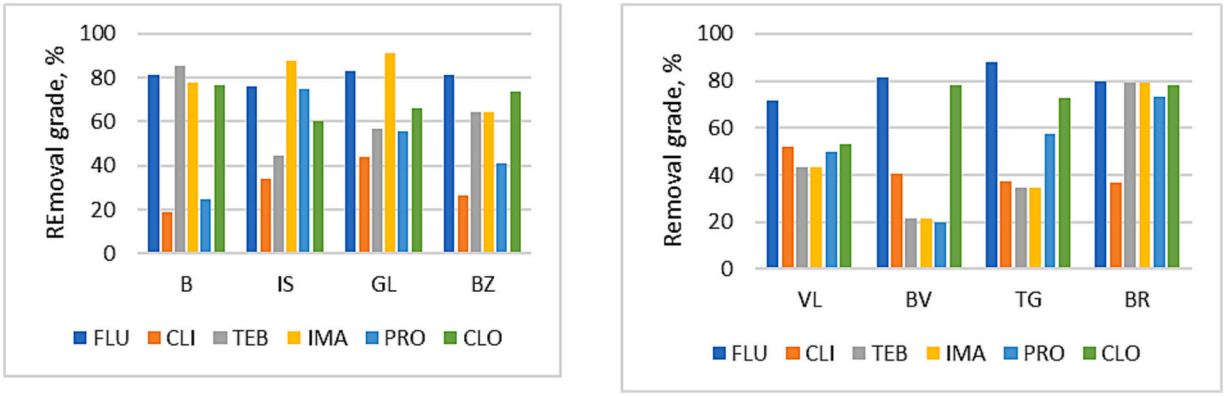

Fig. 1. Removal efficiencies of azole compounds in the studied WWTP's.

were observed for the next WWTP's: Bucharest (for CLI, PRO), Iasi (for CLI, TEB, IMA), Galati (for CLI), Buzau (for CLI, PRO), Ramnicu-Valcea (for TEB, IMA, PRO), Brasov (for CLI, TEB, IMA, PRO, CLO), Targoviste (for CLI, TEB, IMA), Braila (for CLI). The decrease in the concentration of azole compounds dissolved in the influent occurred during the biological purification processes.

Removal efficiencies of over 50 % were observed in the case of the following stations and compounds: Bucharest (FLU, TEB, IMA, CLO), Iasi (FLU, IMA, PRO, CLO), Galati (FLU, TEB, IMA, PRO, CLO), Buzau, (FLU). Spearman's correlation analysis showed strong correlations ($p < 0.05$) between the values of azoles determined in influents and those determined in effluents (B: $R^2 = 0.828$; IS: $R^2 = 0.850$; GL: $R^2 = 0.971$; BZ: $R^2 = 0.97$; VL: $R^2 = 0.992$, BR: $R^2 = 0.962$, BV $R^2 = 0.92$, TG $R^2 = 0.967$, BR $R^2 = 0.871$), indicating that all analytes determined from the effluent samples originate from the influent and were not obtained following physical or biological treatment processes from other base compounds.

Unsatisfactory removal yields (low degradability) and the environmental persistence are the main cause of the presence of azole agents in the river waters that receive the effluent loaded with this type of pollutants.

WWTPs partially remove this type of compounds, and azole concentrations as low as μg/L have been observed in effluent (Richter et al., 2013). Thus, Kahle et al. (2008) determined nine antifungals in wastewater treatment plants and rivers in Switzerland. In this way, it was found that PRO, FLU, and TEB were eliminated incompletely, but also that CLO was eliminated satisfactorily (80 %). In the case of FLU, it was observed that it is not eliminated by sewage treatment plants "due to its hydrophilic properties (log Kow=0.5) and low biodegradation rate", which causes a high presence in aquatic systems (Van De Steene et al., 2010, Wick et al., 2010, Lindberg 2010).

In Germany, Wick et al. (2010) observed that CLI was eliminated in sewage treatment plants between 34 and 67 %, values that are comparable to those obtained in the present study (19.01–52.14 %). In China, Chen et al. (2012), reported a higher removal efficiency of 76 %. For CLO, Casado et al. (2014) reported in Spain an elimination of 45–90 %, comparable to the values of the present study (53.16–78.21). In England, a 41 % removal efficiency of CLI from the treatment plant was reported (Roberts and Thomas, 2006). In China, Huang et al. (2010) and Chen et al. (2012) reported removal of 76 % and 60 % respectively for CLI. In the case of FLU, the removal efficiencies presented wide values in the literature. Thus, in Spain, a wide field of elimination for FLU of 0–60 % was observed (Casado et al., 2014) while in China, Chen et al. (2012) reported a reduced elimination of 6.1 %, and Lindberg et al. (2010) reported a low elimination of 0–16.7 % in Sweden.

3.1.3. Mass loading and emission

The daily mass loading levels for each Station are shown in Table S8. The highest value of the daily mass loading (input) level was observed for CLI 265 mg/day/1000 people in the station in Iasi, followed by TEB 238 mg/day/1000 people in the station in Bucharest, and 203 mg/day/1000 people CLI in the Targoviste station. Other high values were observed for CLI 170 mg/day/1000 people in the Galati station, CLI 112 mg/day/1000 people in the Braila station. FLU presented a daily consumption in the range of 7.7 mg/day/1000 people (Station VL) and 72.7 mg/day/1000 people in the Iasi station. PRO and CLO showed intermediate consumptions in the range of 1.4–70.5 mg/day/1000 people and 1.2–52.3 mg/day/1000 people, respectively. For IMA, reduced daily consumption values were obtained in the range of 13.6–18.3 mg/day/1000 people.

Daily mass emission -DME values for the eight sewage treatment plants were calculated using analyte concentrations in the effluent samples (Table S9). The daily mass emission presented values between 0.7 and 247 mg/day/1000 people. The highest emissions were observed for CLI: 247 mg/day/1000 people in Braila station; 174 mg/day/1000 people in the Iasi station and 129 mg/day/1000 people in the Bucharest station. CLI showed a wide range of emissions from 19 mg/day/1000 people in the Ramnicu Valcea station to 247 mg/day/1000 people. The second level of daily mass emission was obtained for TEB which presented maximums of: 79.4 mg/day/1000 people in the station in Iasi, followed by 70 mg/day/1000 people in the station in Braila and 51 mg/day/1000 people in Targoviste station. FLU, PRO and CLO showed reduced values in the ranges of 2.2–17.2 mg/day/1000 people, 0.8–17.6 mg/day/1000 people and 0.4–12.4 mg/day/1000 people.

3.1.4. Sewage sludge azole concentrations

In sewage sludge, the dominant contaminants were TEB, CLO, PRO and CLI. The calculated values varied between 128 and 258 ng/g dw for TEB, between 114 and 262 ng/g dw for CLO, between 86 and 134 ng/g dw for PRO (Table 2).

High concentrations of azole compounds were observed CLO 262 ng/g in the Iasi station and 238 ng/g in the Bucharest station followed by TEB 258 ng/g in the Bucharest station and 198 ng/g in the Iasi station (Fig. S8). TEB, PRO and CLO compounds presented similar values of the order of hundreds of ng/g. CLI presented values of the order of tens of

Table 2
Concentration (in ng/g d.w.) and RSD (%) values determined for azole compounds in sewage sludge samples ($n = 3$).

Compound (ng/g d. w.)	Minim	Maxim	Average	RSD %	Detection frequency
CLI	15	44	32,6	5,2	100
EPO	12	48	33	6,3	37.5
TEB	128	258	177	4,7	100
IMA	6	15	10,5	7,5	25
MET	2,4	4,4	3,4	5,7	100
PRO	86	134	111	4,9	100
CLO	114	262	175	5,3	100

ng/g (15–44 ng/g, average 32.6 ng/g). A maximum load in azole compounds (∑Azole) was observed for the Bucharest station 708.8 ng/g, followed by the Brasov station 625 ng/g and the Iasi station 610 ng/g.

The primary presence of these pollutants in sewage sludge can be attributed to their hydrophobic (lipophilic) characteristics. Thus, the predominant azole compounds in sludge, CLO, TEB, PRO, have high partition coefficients log Kow 5.84, 3.49, 4.12. In the case of these compounds, the elimination from the sewage treatment plants can take place through the *adsorption* of the analytes on the sewage sludge (Peng et al., 2012). But if this contaminated sludge is used in agriculture, it is necessary to evaluate the impact on the micro-organisms in the soil and the possible mobility of the azoles towards to the ground water or surface water through rain events (leaching).

In the literature, azoles have been reported in sewage treatment plant sludge. Thus, similar CLO concentrations in the range of 30–120 ng/g were observed in Spain, while CLI was reported in Germany at higher concentrations of 1160 ng/g (Casado et al., 2014; Wick et al., 2010). In China, CLI was detected in higher concentrations up to a value of 152 ng/g (Chen et al., 2012). This is the first report on the presence of azole compounds in sewage treatment plants in Romania and their potential transfer through the effluent to the receiving river. In China, Peng et al. (2012) observed that FLU goes through the purification process being found in effluent and CLO, econazole, miconazole and ketoconazole are found in the sewage sludge being subjected to the processes of biotransformation (ketoconazole) and sorption on the sludge (CLO, econazole, miconazole).

3.1.5. Ecological risk

The acute (LC50 and EC50) and chronic (NOEC/LOEC) toxicities used to calculate the risk factors are presented in table S10.

The ecological risk assessment (Table 3) highlighted that some compounds may present high risk (>1). Thus, CLI detected in the effluent can present a high risk for the plants *Lemna minor* and *Navicula pelliculosa*. CLO may present a high risk to the plant Desmodesmus subspicatus and to the invertebrate *Daphnia magna*. PRO may present high risk to the invertebrate *Mysidopsis Bahia*. On the other hand, CLO can present a medium risk for *Daphnia magna* and TEB can generate a medium risk for *Danio rerio* fish. PRO may have a medium ecological risk for the fish *Cyprinus Carpio* and the alga *Scenedesmus Subspicatus*.

4. Conclusions

A validated procedure, which is both sensitive and selective, has been established for the analysis of 12 emerging pollutants in wastewater and sludge samples obtained from eight urban wastewater treatment plants (WWTPs). Contaminants were chromatographical separated on a Zorbax Eclipse XDB C18 column. The mobile phase consisted of acetonitrile and ammonium acetate (5 mM), and a gradient was used at a flow rate of 0.2 mL/min. Detection in the mass spectrometer involved the multiple reaction monitoring mode (MRM), which utilized two transitions: precursor-quantifier and precursor-qualifier.

CLI, TEB, CLO were the most commonly occurring compounds in both influent and effluent water samples, with FLU and PRO having intermediate concentrations in the tens of ng/L range. The sum of the concentrations of azole compounds in the effluent was 676 ng/L and was recorded for the station in Galati, followed by 377 ng/L in the station in Bucharest. Assessing the mass loading of selected azoles show that the dominants compound was CLI, with a mass loading up to 265 mg/day/1000 people, followed by TEB with a mass loading up to 238 mg/day/1000 people in the Bucharest station. The compound with the dominant mass emission in the effluent was CLI (247 mg/day/1000 people in Braila, 174 mg/day/1000 people in the Iasi station and 129 mg/day/1000 people in the Bucharest station).

Removal capacities below 50 % were observed for the next WWTP's: Bucharest (for CLI, PRO), Iasi (for CLI, TEB, IMA), Galati (CLI), Buzau (CLI, PRO), Ramnicu-Valcea (TEB, IMA, PRO), Brasov (CLI, TEB, IMA, PRO, CLO), Targoviste (CLI, TEB, IMA), Braila (CLI). Environmental risk assessment proves that CLI detected in the effluent presented a high risk for the plants *Lemna minor* and *Navicula pelliculosa*. Also, CLO presented a high risk to the plant *Desmodesmus subspicatus* and to the invertebrate *Daphnia magna*. PRO presented high risk to the invertebrate *Mysidopsis Bahia.* This research enhances our understanding of the presence, elimination, and potential environmental impact of antifungal azole substances in urban wastewater and sludge.

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Table 3
PNEC, MEC values for the sewage effluent and calculated RQ indices.

Compound	Type of living organism	Species	AF	PNEC	MEC	RQ	Risk level
CLI	Plant	*Lemna minor*	1000	0.019	0.247	13	High
	Plant	Green algae	1000	6.154	0.247	0.04	Low
	Plant	Navicula peliculosa	1000	0.154	0.247	1.6	High
	Invertebrate	Daphnia	1000	4.219	0.247	0.06	Low
	Fish	Fish	1000	6.316	0.247	0.04	Low
FLU	Green algae	Plant	1000	1,162,301	0.0176	$1.5*10^{-6}$	Low
	Fish	Fish	1000	5,595,884	0.0176	$3.15–10^{-7}$	Low
CLO	Green algae	Plant	1000	0,268	0,022	0,08	Low
	Desmodesmus subspicatus	Plant	1000	0,017	0,022	1,29	High
	Daphnia	Invertebrate	1000	0,02	0,022	1,10	High
	Daphnia magna	Invertebrate	1000	0,042	0,022	0,52	Medium
	Fish	Fish	1000	0,268	0,022	0,08	Low
TEB	*Danio rerio*	Fish	1000	0,25	0,116	0,46	Medium
	Zebrra fish	Fish	1000	7,81	0,116	0,015	Low
	Daphnia magna	Invertebrate	1000	2,8	0,116	0,041	Low
	Scenedesmus subspicatus	Plant	1000	5,3	0,116	0,022	Low
IMA	Fish	Fish	1000	1,48	0,0065	$4.39 \times 10\text{-}^{3}$	Low
	Algae- *Selenastrum capricornutum*	Plant	1000	0,87	0,0065	7.47×10^{-3}	Low
PRO	*Oncorhynchus mykiss*	Fish	1000	1,5	0,031	0,021	Low
	Cyprinus carpio	Fish	1000	1355	1031	0,761	Medium
	Mysidopsis bahia	Invertebrate	1000	0,77	2031	2638	High
	Scenedesmus subspicatus	Algae	1000	45,051	3031	0,067	Medium

Ethical approval

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Consent to participate

Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Vasile-Ion Iancu: Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura-Florentina Chiriac:** Visualization, Validation, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Iuliana Paun:** Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Florinela Pirvu:** Software, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Cristina Dinu:** Software, Methodology, Investigation, Formal analysis, Conceptualization. **Lidia Kim:** Formal analysis, Data curation, Conceptualization. **Luoana Florentina Pascu:** Data curation, Conceptualization. **Marcela Niculescu:** Data curation, Conceptualization.

Declaration of competing interest

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Data availability

The authors are unable or have chosen not to specify which data has been used.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170898.

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