



Ecotoxicological risk and seasonal removal of licit and illicit drugs in wastewater treatment plants: A comparison of secondary and tertiary processes

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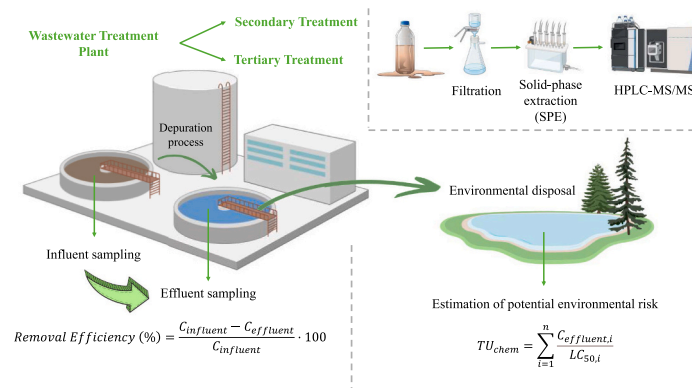
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HIGHLIGHTS

- Hydrophilic, biodegradable drugs were efficiently removed by secondary treatment.
- Ozone and ozone–UV processes enhanced removal of persistent emerging pollutants.
- Tertiary treatments reduced toxicity even with partial compound removal.
- Algae were the most sensitive organisms to residual toxicity in effluents.
- Ozone–UV treatment showed the lowest TU_{chem} values among WWTPs studied.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Emerging contaminants
Wastewater treatment
Advanced oxidation processes
Illicit drugs
Risk assessment

ABSTRACT

This study evaluates the effectiveness of different wastewater treatment methods in removing emerging contaminants, including licit and illicit drugs. 168 samples were collected over four seasons from three wastewater treatment plants (WWTPs), comparing conventional secondary biological treatments with advanced oxidation processes (ozone and ozone-UV). Hydrophilic compounds like caffeine and ethyl sulfate showed high removal efficiencies (>85%), while compounds such as MDMA and methamphetamine had lower and more variable removal rates. No consistent seasonal trend was found for most substances, although some exhibited improved removal in warmer months, likely due to enhanced microbial and photodegradation. Ecotoxicological risk was assessed using toxic units (TU_{chem}), calculated from effluent concentrations and EC₅₀/LC₅₀ values for algae, daphnia, and fish. WWTPs with tertiary treatments generally showed lower TU_{chem} values, indicating a potential reduction in environmental risk. Algae were the most sensitive organisms. The combined ozone and UV treatment achieved the lowest TU_{chem} values, reflecting lower residual concentrations of the measured compounds.

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<https://doi.org/10.1016/j.jhazmat.2025.140290>

Received 28 July 2025; Received in revised form 19 October 2025; Accepted 26 October 2025

Available online 27 October 2025

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These results reveal limitations of conventional secondary treatments and emphasize the need to optimize tertiary processes. The observed compound-specific variability highlights the importance of regular monitoring and prioritization to improve the ecological safety of treated wastewater discharged into aquatic environments.

1. Introduction

The Manzanares River has recently been ranked as the most polluted river in Europe, exhibiting alarming contamination levels that pose serious risks to both aquatic ecosystem and human health [53]. In densely populated urban areas such as Madrid, wastewater treatment plants (WWTPs) serve as critical barriers against pollution. However, population growth and the emergence of new contaminants have increased the pollutant load in aquatic ecosystems [43,8].

WWTPs discharge a wide variety of organic and inorganic micropollutants into surface waters, which negatively affect aquatic organisms such as cyanobacteria, algae, crustaceans and fish [20,22]. Among them, emerging contaminants (ECs) pose a threat even at trace concentrations, making their removal essential [52].

Treatment usually begins with a primary stage focused on the sedimentation of large solids and suspended particles, followed by a secondary stage which uses biological processes to degrade organic matter and reduce pathogens and nutrients. Therefore, conventional treatment typically includes pretreatment, primary sedimentation, a secondary biological process (such as activated sludge), and a final disinfection step, usually by chlorination. However, these processes do not completely remove ECs, resulting in their continuous release into the aquatic environment [52]. These ECs include pharmaceuticals, pesticides, and industrial compounds, characterized by high environmental persistence and adverse effects on ecosystems and human health, such as endocrine disruption, anatomical disorders, and cancer in humans, as well as bioaccumulation in fish, crustaceans, and agricultural crops [49].

To address this issue, in recent years, tertiary treatments have been implemented applying advanced techniques such as membrane filtration, activated carbon adsorption, and oxidative processes including ozonation, or ultraviolet (UV) irradiation [21,45,56]. These processes enhance the removal capacity of micropollutants, although they carry the drawback of potentially generating byproducts with higher ecotoxicity [52]. In this regard, advanced oxidation processes (AOPs) stand out as they are able to generate hydroxyl radicals ($\text{HO}\bullet$), which are capable of oxidizing ECs into CO_2 , H_2O , salts, and mineral acids [6].

The effectiveness of removing these contaminants depends to a high extent on both the type of treatment applied and factors such as the chemical properties of the compounds, plant configuration, hydraulic retention time (HRT), operational conditions (pH, temperature), and the presence of industrial wastewater [23].

In response to growing concern over micropollutants, new technologies are increasingly being implemented to improve their removal [48]. Reflecting this concern, in 2022 the European Commission proposed the implementation of a quaternary treatment that combines chemical and physical processes to eliminate these worrying compounds that are continually being discharged into the environment [13].

When pharmaceuticals (morphine, codeine, ketamine, etc.), legal substances (nicotine, caffeine or alcohol) or illegal drugs (amphetamines, cocaine, THC, etc.) are consumed, part of them are excreted unchanged, while another part is transformed into metabolites. Both the parent compounds and their metabolites are excreted from the human body through urine, saliva, sweat, and other biological fluids. These compounds reach WWTPs where they are eliminated in different percentages depending on their chemical structure and the treatment applied in the WWTP. However, a part of them remains and is discharged into the environment causing pollution of rivers, lakes or seas [51]. As a result, licit and illicit drugs along with their metabolites, are considered recent emerging pollutants that have caused environmental

concern in recent years. The term emerging pollutants highlights the current lack of information related to the potential effects caused by the incorporation of these compounds into the environment [38].

Due to their pharmacological or biological activity, these compounds can persist, bioaccumulate and transfer along the aquatic food chain, generating significant ecosystem impacts and potential risks to human health [38]. Although the concentrations of these emerging contaminants detected in the environment are typically in the ng/L range, their continuously released from WWTPs results in the continuous exposure of non-target organisms to these chemicals. This may lead to chronic effects on the fauna inhabiting the affected sites [51]. In addition, PhACs can be transported into drinking water consumed by humans, or into the food chain as a result of exposure of animals to them in nature, or because of their entry into areas where food crops or vegetables are grown through contaminated sludge used as fertilizer or through contaminated irrigation water [35]. Given this complexity and the wide variety of compounds discharged from WWTPs, further related research is needed due to the large number of organisms exposed to them [51]. Furthermore, it should be considered that transformation compounds formed after the purification process may cause greater damage to the environment than native drugs or their metabolites because of the generation of products with a higher ecotoxic capacity [35].

Despite recent advances in ECs research, significant knowledge gaps remain. In particular, the influence of seasonal variations on the removal efficiency of these compounds under real operational conditions in urban WWTPs is not fully understood yet. Moreover, there is a lack of comparative studies systematically evaluating the performance of secondary treatments versus advanced tertiary processes in the removal of pharmaceuticals, legal and illicit drugs, and their metabolites. These limitations hinder the design of effective strategies to reduce the release of these contaminants into the aquatic environment. To address these gaps, the present study conducts a comparative analysis of three WWTPs in Madrid employing different treatment configurations: one secondary and the other two tertiary advanced plants (oxidation alone or combined with UV). The study integrates the assessment of seasonal removal of licit and illicit drugs with an ecotoxicological risk analysis, providing novel evidence on the combined influence of treatment type and seasonal conditions on contaminant release. Accordingly, the main aim of this study is to evaluate the removal efficiency of ECs (particularly, licit and illicit drug residues) across the three WWTPs. Seasonal variability is assessed by analysing samples collected during winter, spring, summer, and autumn, to assess whether meteorological conditions influence the effectiveness of contaminant removal. In addition, the study conducts an ecotoxicological risk assessment based on toxic units (TU_{chem}), calculated from chemical concentrations and published $\text{EC}_{50}/\text{LC}_{50}$ values for aquatic organisms. TU_{chem} is a widely used metric that estimates the potential acute ecotoxicological risk of complex contaminant mixtures, integrating both concentration and toxicity information across different aquatic trophic levels [12,27,10]. The findings aim to identify which drug-related substances reach the aquatic environment under different treatment and seasonal scenarios, and to evaluate their potential ecological impact, thereby contributing to the design of more effective purification strategies.

2. Materials and methods

2.1. Chemicals and reagents

The target compounds were selected based on their environmental relevance, representativeness of human consumption, and potential

ecological impact. Both frequently used illicit and licit drugs in the urban population, as documented in wastewater monitoring studies, were included. Compounds with diverse chemical properties were selected to validate the analytical methods comprehensively and to evaluate their behaviour in treatment processes and aquatic ecosystems.

The reference standards of illicit drugs (ecgonine methyl ester, morphine, 6-acetylmorphine, mephedrone, heroine, fentanyl, cocaine, amphetamine, methamphetamine, 3,4-ethylenedioxy-methamphetamine (MDMA), benzoylcegonine, ketamine, 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THC-COOH) and codeine) and of licit drugs (nicotine, cotinine, trans-3'-hydroxycotinine, caffeine, 1,7-dimethyluric acid and ethyl sulfate) were purchased from Merck (Steinheim, Germany) in liquid form or as powder form of analytical grade. Individual stock solutions were prepared in methanol (MeOH) or acetonitrile (AcN) following the manufacturer's instructions except for 1,7-dimethyluric acid, which stock solution was prepared in MeOH/water (50: 50) at pH around 10 (adjusted with 25 % ammonium hydroxide solution). A mixture of illicit drugs standards at 1 mg/L was prepared in MeOH. Also, a mixture of licit drugs at 1 mg/L was prepared in MeOH.

The isotopically labelled compounds (ISs) were also purchased from Merck, and these were ecgonine methyl ester- d_3 , morphine- d_6 , mephedrone- d_3 , heroine- d_9 , fentanyl- d_5 , benzoylcegonine- d_3 , amphetamine- d_5 , methamphetamine- d_9 , MDMA- d_5 , THC-COOH- d_9 , cocaine- d_3 , caffeine- $^{13}C_3$, 1,7-dimethyluric acid- $^{13}C_4$ - $^{15}N_3$, ethyl sulfate- d_5 , nicotine- d_4 , cotinine- d_3 , trans-3'-hydroxycotinine- d_3 and. Labelled internal solutions were prepared separately in MeOH or AcN, except for 1,7-dimethyluric acid- $^{13}C_4$ - $^{15}N_3$ which was prepared in MeOH/water (50: 50) at pH around 10. A mixture of all isotopically labelled licit drugs was prepared, with a final concentration of 2 mg/L for 1,7-dimethyluric acid- $^{13}C_4$ - $^{15}N_3$ and for ethyl sulfate- d_5 , while the final concentration of the rest of the compounds was 1 mg/L. A mixture of isotopically labelled illicit drugs at 1 mg/L was also prepared.

Ultrapure water was obtained using a Milli-Q Millipore system (Bedford, MA, USA). Solvents were of reagent grade or higher. HPLC-grade MeOH, HPLC-grade AcN and ammonium hydroxide solution (25 %) were purchased from Scharlab (Barcelona, Spain). SPE cartridges used were Oasis HLB (3 mL, 60 mg) from Waters Corp., Milford, MA, USA. Two different reversed-phase HPLC columns were used: Synergi Fusion-RP; length 150 mm, internal diameter 3 mm, particle size 4 μ m and pore size 80 Å from Phenomenex (Madrid, Spain) for licit substances and HPLC capillary Avantor ACE Excel C18-PFP column (100 mm \times 2.1 mm, 2 μ m), acquired from Symta S.L.L. (Madrid, Spain) for illicit compounds.

2.2. Wastewater sampling

Composite 24-hour volume or time-proportional samples were collected at three WWTPs in Madrid: WWTP 1 (Viveros de la Villa), WWTP 2 (La Gavia), and WWTP 3 (Sur Oriental). Samples were taken from the influent (raw wastewater entering each plant before any treatment) and the effluent (final treated wastewater after all applied treatment stages). WWTP 3 continuously applies secondary treatment with biological processes in three stages (anaerobic, anoxic, and aerobic). In contrast, WWTP 1 and WWTP 2 combine secondary treatment with advanced tertiary oxidation processes during the first four days of sampling in our study (Friday to Monday), reverting to secondary treatment only for the remaining days of the week.

To obtain representative information on removal efficiencies under these different treatment configurations, samples were collected during seven consecutive days at each plant. Four sampling campaigns (December 2023, April 2024, June 2024 and October 2024) were carried out in all the WWTP analysed. The main characteristics of the WWTPs and the sampling periods are listed in Table S1. All the samples were collected in polyethylene high-density bottles (500 mL), immediately frozen after collection and transported to the laboratory, where they were stored at $-20^\circ C$ until extraction, typically within one month

after arrival at the laboratory.

2.3. Sample preparation and analysis

To analyze all the above-mentioned compounds, two different SPE methods were used due to the considerable differences in concentration levels between illicit drugs and legal substances in the wastewater samples. Firstly, drugs were analyzed by applying the method described by [18], which had been previously optimized and validated for wastewater samples. Briefly, the wastewater samples were thawed at room temperature and a 60 mL aliquot was spiked with the illicit drugs ISs mixture to achieve a final concentration of 0.5 μ g/L. The samples were then filtered under vacuum using a 0.45 μ m pore size cellulose filter purchased from Whatman (Kent, UK) and 50 mL of the filtrate were passed through a SPE Oasis HLB cartridge (3 mL, 60 mg) previously conditioned with 6 mL MeOH and 6 mL ultrapure water. The cartridges were dried under vacuum for 15 min and the analytes were eluted with 6 mL MeOH. The solvent was evaporated with a nitrogen stream at $35^\circ C$ and the extract was reconstituted in 0.5 mL MeOH-water (5–95, v/v), reaching a 100-times pre-concentration factor. Instrumental analysis was carried out by applying liquid chromatography coupled to mass spectrometry (HPLC-MS/MS) using an EVOQ Qube triple quadrupole (Bruker, Darmstadt, Germany) after separation of the compounds using an ACE Excel C18-PFP (100 mm \times 2.1 mm, 2 μ m) column with the Elute HPG 1300 pumps and Elute Autosampler (Bruker). The column was maintained at $40^\circ C$ and the mobile phase consisted of ultrapure water (eluent A) and MeOH (eluent B), both 0.1 % HCOOH. The percentage of eluent B changed as follows: 0 min, 5 %; 0.5 min, 5 %; 10 min, 100 %; 13 min, 100 %; 13.1 min, 5 %; 16 min, 5 %. The flow rate was maintained at 0.4 mL/min and the injection volume was 15 μ L. LC-MS/MS Bruker Compass EDM 1.2 and TQ control software were used to process quantitative data. Experimental MS conditions are given in Table S2 and in more detail in [18].

For the analysis of legal substances, a method previously optimized and validated for wastewater samples was applied. Briefly, a 10 mL aliquot was spiked with the IS mixture of legal compounds to reach a final concentration of 40 μ g/L for 1,7-dimethyluric acid- $^{13}C_4$ - $^{15}N_3$ and for ethyl sulfate- d_5 , while the final concentration was 20 μ g/L for the rest of the ISs. Each aliquot was vacuum filtered using a 0.45 μ m pore size cellulose filter. Subsequently, 5 mL of the filtrate was loaded on an Oasis HLB cartridge (3 mL, 60 mg), previously conditioned with 6 mL of MeOH and 6 mL of ultrapure water. The cartridges were dried under vacuum for 15 min and the analytes were eluted with 4 mL of MeOH. The solvent was evaporated to dryness with a stream of nitrogen at $35^\circ C$. Dry residues were reconstituted in 500 μ L of ultrapure water; thus, the samples were pre-concentrated 10 times. Subsequently, analyses were done by high-performance liquid chromatography (1200 Series system, Agilent Technologies, Madrid, Spain) coupled to a triple quadrupole mass spectrometer (Agilent G6410B). The compounds were separated using a Synergi Fusion-RP C18 column (150 mm \times 3 mm, ID 4 μ m, pore size 80 Å) using water (eluent A) and AcN (eluent B), both with 0.1 % HCOOH, with the following gradient program: 95 % of eluent A for the first 2 min; linear gradient to 40 % of eluent B until minute 10 followed by 2 min linear gradient to 100 % of eluent B, 3 min isocratic elution and 3 min linear gradient back to 95 % of eluent A, which was held for 2 min to equilibrate the column. The flow rate was maintained at 0.5 mL/min and the injection volume was 5 μ L. Masshunter Data Acquisition B.04.01, Masshunter Optimizer Triple Quad B.04.01 and Masshunter Qualitative Analysis B.07.00 (Agilent Technologies, Waldbronn, Germany) were used for data acquisition and processing. Experimental MS conditions are given in Table S3.

2.4. Calculation of the removal efficiency of wastewater treatment plants

The removal efficiency (RE) of each compound in the different WWTPs was calculated as described by [14]. For this purpose, the

concentration of each analyte measured in the influent (C_{influent}), i.e., before the depuration process, was compared to the concentration in the (C_{effluent}), i.e., after the depuration process. A total hydraulic retention time (HRT) of approximately 24 h was assumed for the calculation of removal efficiencies, representing a typical treatment duration from influent entry to effluent collection. This value aligns with operational data provided by two of the WWTPs included in the study, where HRTs in the biological treatment stages ranged between 18 and 22 h. For the third WWTP, specific operational HRT data were not available. Therefore, adopting a standardized HRT of 24 h provides a consistent basis for comparing removal efficiencies across the different plants and sampling campaigns. The RE was determined using the following equation:

$$\%RE = \frac{C_{\text{influent}} - C_{\text{effluent}}}{C_{\text{influent}}} \cdot 100 \quad (1)$$

2.5. Calculation of risk assessment

To estimate the potential environmental risk that could be associated with the release of these compounds, the theoretical toxic units (TU) of each analyzed substances were calculated taking into account the mean effluent concentrations and LC_{50} values obtained from the ECOSAR database applied to fish, daphnid and algae [10]. The specific LC_{50} values used can be found in Table S6. In addition, in order to estimate what the overall toxic effect of the whole mixture discharged into the environment, the theoretical toxic unit (TU_{chem}) was also calculated [27]:

$$TU = \frac{C_{\text{effluent}}}{LC_{50}} \quad (2)$$

$$TU_{\text{chem}} = \sum_{i=1}^n \frac{C_{\text{effluent},i}}{LC_{50,i}} \quad (3)$$

3. Results and discussion

3.1. Removal efficiency

This study evaluated the efficiency of removing licit and illicit drugs in three wastewater treatment plants (WWTPs) located in Madrid over four seasonal campaigns: winter (December 2023), spring (April 2024), summer (June 2024), and autumn (October 2024). The plants studied were WWTP 1 (Viveros de la Villa), WWTP 2 (La Gavia) and WWTP 3 (Sur Oriental). A total of 168 wastewater samples (84 influent and 84 effluent) were analyzed through a sample collection during one full week at each WWTP. The WWTPs differ in their treatment configurations: WWTPs 1 and 2 implement advanced oxidation processes (ozone+UV and ozone, respectively) during the first four days of the sampling (Friday to Monday), reverting to secondary treatment on the remaining days, while WWTP 3 operates continuous secondary treatment with a three-stage biological process (anaerobic, anoxic, and aerobic). Removal efficiencies were calculated as the reduction between influent and effluent concentrations (Eq. 1), as a percentage of influent concentration.

In order to assess the removal efficiency of the tested compounds, they were divided into three main categories: (i) licit drugs and their metabolites (nicotine, cotinine, trans-3'-hydroxycotinine, caffeine, 1,7-dimethyluric acid and ethyl sulfate); (ii) illicit drugs and their metabolites (cocaine, benzoylecgonine, ecgonine methyl ester, amphetamine, methamphetamine, MDMA, THC-COOH, mephedrone, heroine and 6-acetylmorphine); and (iii) medications of abuse (ketamine, codeine, morphine and fentanyl). Table S4 shows the percentage of positive findings of the selected compounds during the four seasonal campaigns.

3.2. Licit drugs and their metabolites

As shown in Fig. 1 and summarized in Table S5, none of the licit drugs and their metabolites were completely removed (i.e. 100 % value). However, the results allowed for the identification of two distinct groups. The first group, including caffeine, trans-3'-hydroxycotinine, 1,7-dimethyluric acid, and ethyl sulfate, exhibited removal efficiencies above 85 %, indicating high but incomplete removal, as these compounds were still detected in the effluent. The second group, consisting of nicotine and cotinine, showed efficiencies below 85 %, indicating intermediate removal efficiencies. Notably, cotinine's removal efficiencies fluctuated around the 85 % threshold, suggesting moderate and variable elimination depending on the treatment plant and sampling period.

These results are consistent with previous studies that have evaluated the removal efficiency of some of these compounds. For example, in a WWTP located in Jerez de la Frontera (Spain), removal efficiencies above 85 % were reported for caffeine [5]. Similarly, [50] observed removal ranges from 57 % to 99 % for nicotine, 46–99 % for cotinine, 66–99 % for trans-3'-hydroxycotinine, and 80–99 % for ethyl sulfate in treatment plants employing activated sludge processes. This variability is attributed to both the specific operational conditions prevailing in different plants and the physicochemical properties of each compound.

The lower removal efficiencies of nicotine and cotinine could be explained by several factors. Metabolically, nicotine is rapidly transformed into cotinine in the liver, primarily via the enzyme CYP2A6, [40]. Both compounds are largely excreted in conjugated forms: approximately 3–5 % as nicotine-glucuronide (8–10 % as free nicotine) and 12–17 % as cotinine-glucuronide (10–15 % as free cotinine). During transport through the sewer network, these conjugates may undergo deconjugation, releasing the parent compound from glucuronides before reaching the WWTP, thereby increasing concentrations in the effluent [15,16]. Additionally, the limits of quantification (LOQs) are higher in the influent than in the effluent (Table S4), introducing uncertainty in the efficiency calculation. Consequently, the observed low removal rates may underestimate the actual extent of transformation or elimination processes.

From a physicochemical perspective, the difficulty in removing these compounds could also be attributed to their log Kow values. Studies indicate that removal efficiency is inversely proportional to octanol-water partition coefficients (log Kow); that is, more hydrophilic compounds may be removed more efficiently than hydrophobic ones [25]. Nicotine has a log Kow of 1.2 [30], indicating moderately hydrophobic behavior, while compounds such as cotinine (−0.3) [33], caffeine (−0.1) [29], trans-3'-hydroxycotinine (−0.9) [32], 1,7-dimethyluric acid (−0.2) [31], and ethyl sulfate (−0.6) [34] show more hydrophilic characteristics. These values suggest that the lower removal of nicotine may be related to its hydrophobic nature, which may hinder its effective elimination in WWTPs, compared to more hydrophilic compounds like caffeine. Regarding cotinine, the removal efficiencies observed fluctuated around the 85 % threshold, reflecting an intermediate or variable behaviour depending on the treatment plant and the sampling period. This variability may be influenced by differences in treatment technologies or operational conditions among the WWTPs, highlighting the complex relationship between intrinsic compound properties and process performance. However, few studies have found a consistent relationship between the physicochemical properties of emerging contaminants and their removal efficiency [17].

Regarding seasonal variation, data from Fig. 1 indicate no significant differences in removal efficiencies for any of the investigated compounds across different sampling periods. Although some variations can be observed at specific sampling points, no consistent seasonal trend is apparent. This suggests that, for the selected substances and the studied WWTPs, treatment performance remained relatively stable across seasons, in line with previous studies that also reported mixed or inconsistent evidence regarding seasonal influences on contaminant removal

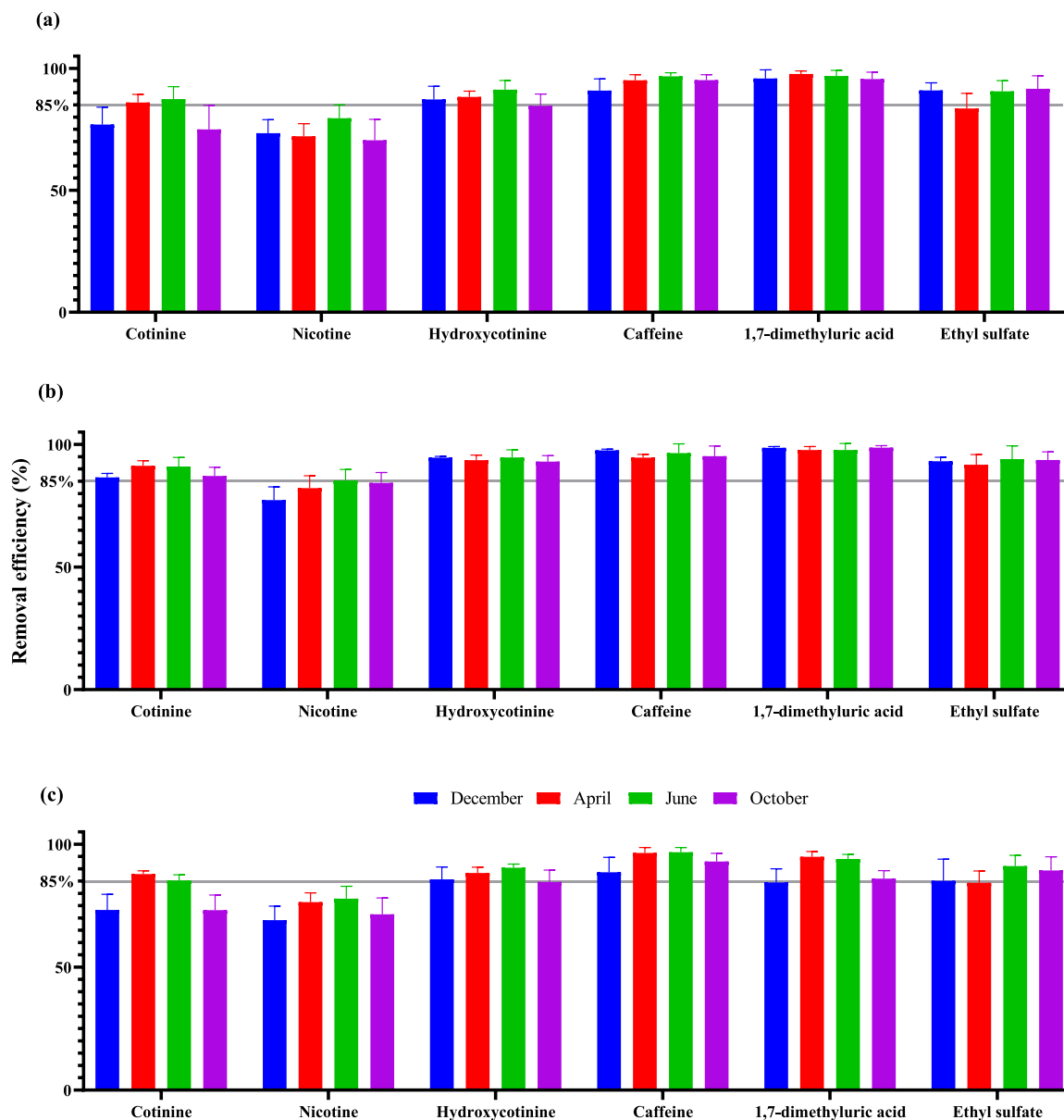


Fig. 1. Seasonal variation in the removal efficiency of licit drugs and/or their metabolites across three WWTPs: (a) WWTP 1 (tertiary treatment during four days, secondary treatment the rest); (b) WWTP 2 (tertiary treatment during four days, secondary the rest); (c) WWTP 3 (continuous secondary treatment).

in WWTPs [9].

3.3. Illicit drugs and their metabolites

Illicit drugs and their metabolites (Fig. 2 and Table S5) showed a more heterogeneous removal efficiencies pattern compared to licit substances. In this case, three general groups can be distinguished: one with high removal efficiencies (above 85%), including cocaine, benzoylecgonine, ecgonine methyl ester, amphetamine and THC-COOH; other group characterised by poor elimination, particularly methamphetamine and MDMA, with average removal efficiencies below 60% and higher variability between sampling campaigns, and the third one composed by compounds which have not been detected in either influent or effluent wastewater, including mephedrone, heroine and its metabolite 6-acetylmorphine.

The poor removal efficiency for amphetamine and MDMA could be due to their similar chemical structure, both containing branched amines, which can hinder biodegradation and adsorption processes [54]. In addition, the variability in removal efficiency observed in the

present study has been reported in other studies and may be related to differences in hydraulic retention times, the microbial composition of the samples analysed and the physicochemical properties of the compounds, such as hydrophobicity and persistence in terms of chemical stability [44]. Recent research also suggests that synthetically stimulants have been found to show persistence in removal during wastewater treatment processes [11]. Furthermore, other studies have reported similar results for the removal of amphetamine, cocaine and its metabolites using different treatments in different WWTPs, showing efficiencies exceeding 90%. These results are consistent with global assessments and confirm that these substances are susceptible to biodegradation under aerobic conditions [2,44]. In addition, THC-COOH, the main urinary metabolite of cannabis, despite its lipophilic nature also shows high elimination, probably due to adsorption and partial biodegradation mechanisms [51].

Regarding seasonal variation, no substantial differences were observed for most illicit drugs and metabolites. However, methamphetamine and MDMA showed more pronounced seasonal fluctuations, with removal efficiencies tending to be higher during warmer months

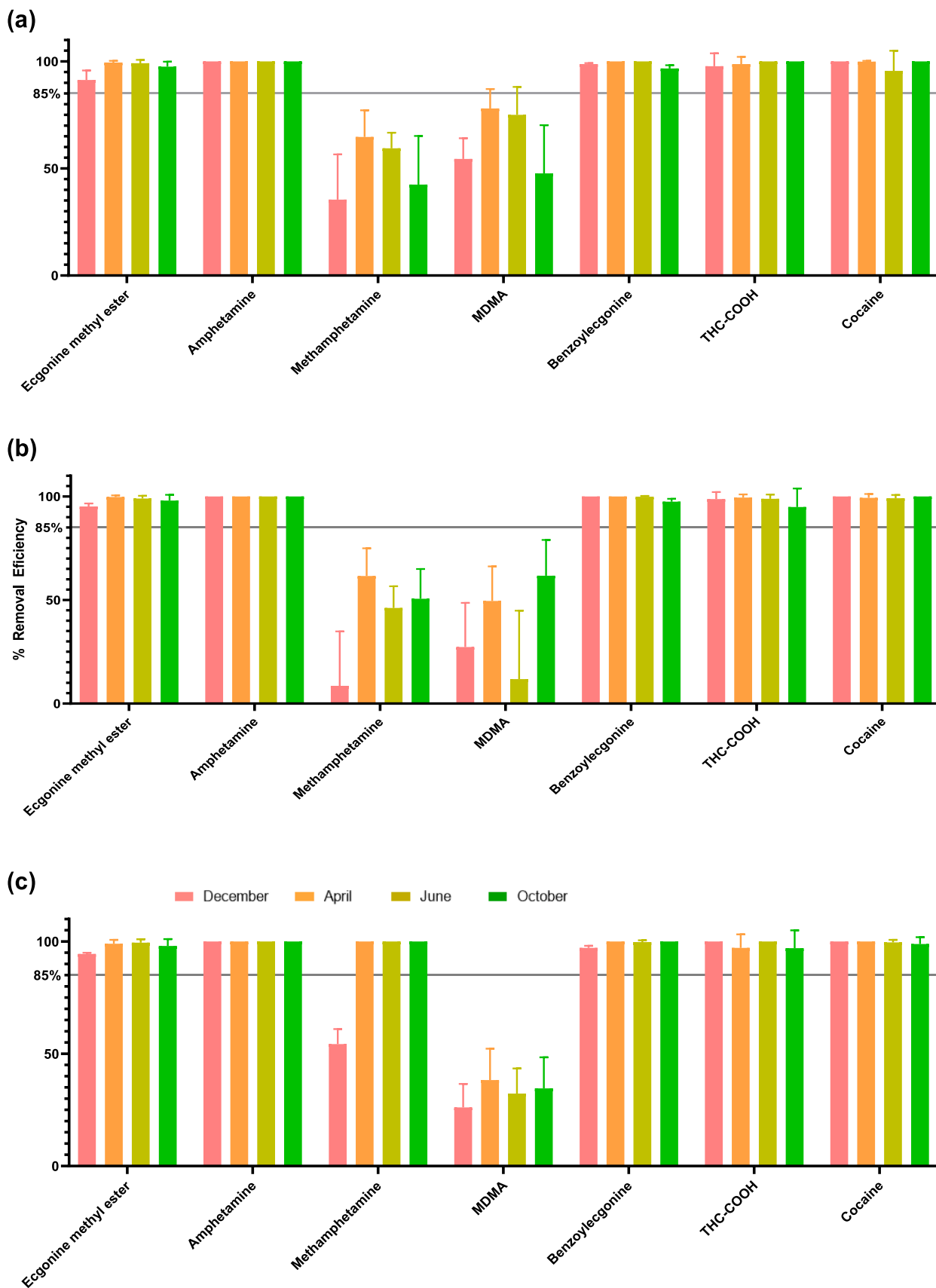


Fig. 2. Seasonal variation in the removal efficiency of illicit drugs and/or their metabolites across three WWTPs: (a) WWTP 1 (tertiary treatment during four days, secondary treatment the rest); (b) WWTP 2 (tertiary treatment during four days, secondary the rest); (c) WWTP 3 (continuous secondary treatment).

(April and June). Specifically, for methamphetamine removal efficiencies in WWTP 1 ranged from approximately 35 % in December to 65 % in April. WWTP 2 showed variable values across seasons, with a minimum of around 10 % in December and a maximum of 60 % in April. In WWTP 3, removal efficiencies were complete (100 %) in all the sampling campaigns except in December (54 %). In the case of MDMA, removal efficiencies in WWTP 1 varied between 54 % and 78 % across all seasons, while in WWTP 2 stronger fluctuations were observed, ranging from approximately 12 % in June to 62 % in October. In WWTP 3 they were notably lower and regular, below 40 %. The general increased of the removal efficiency during warmer months could be related to increased levels of microbial activity at higher temperatures, which may contribute to the elimination of these types of substances [51], as well as to promote photodegradation and biodegradation processes [10].

3.4. Medications of abuse

Fentanyl was not detected in any influent or effluent sample across the four seasonal campaigns. Among the medications of abuse detected, codeine, morphine and ketamine showed a more variable and, in some cases, limited removal efficiencies across the four seasonal campaigns and the three WWTPs, as shown in Fig. 3 and summarized in Table S5. Among them, morphine consistently showed the highest removal efficiency, with values generally above the 85 % threshold in all WWTPs and campaigns, indicating effective removal by the wastewater treatment processes employed. This effective removal is consistent with its known biodegradability under aerobic conditions, particularly in advanced biological treatments [51]. In contrast, codeine showed moderate and variable removal efficiencies depending on the season and wastewater treatment plant analysed. Although its removal exceeded 50 % in most campaigns, in both WWTP 1 and 2, removal efficiencies were consistently below in WWTP 3, where only conventional treatment was applied. This pattern has been also observed in other studies for various compounds treated under similar conditions [17,46,51]. In this WWTP, removal was negative in December, as influent concentrations were lower than those detected in effluent samples. The partial transformation of codeine and its low adsorption potential may contribute to these results, as observed in previous studies reporting intermediate removal efficiencies for opioids in conventional activated sludge systems [55,57]. Ketamine showed the most heterogeneous removal elimination among the three medications of abuse, with low or even negative removal efficiencies across the WWTPs and campaigns. These negative values may reflect a combination of factors such as poor biodegradability, retroformation from mainly glucuronide-conjugated metabolites and fluctuations in influent and effluent concentrations due to sampling variability or deconjugation processes during sewer transit [55,57]. Furthermore, ketamine is widely recognised as a compound which is resistant to conventional biological degradation [47], often persisting through activated sludge systems due to its chemical stability and moderate hydrophobicity [24]. Other studies have shown that the effective removal of a wide variety of compounds, which may include ketamine, often requires advanced oxidation processes such as ozonation or combinations of ozone and UV [36]. However, in this study, these processes were applied only during the first four days of each sampling week in WWTPs 1 and 2, with conventional secondary treatment used on the remaining days. Since the data presented in this section represent average values across all sampling days, without separating by treatment configuration, this may contribute to the low and variable removal efficiencies observed.

Overall, when comparing the three groups of compounds (licit drugs, illicit drugs and medications of abuse), clear differences in removal efficiency were observed. Licit drugs, in particular caffeine, trans-3'-hydroxycotinine and ethyl sulfate, showed the most consistent and efficient elimination, often exceeding 85 %, largely due to their hydrophilic nature and compatibility with biological degradation processes.

Illicit drugs showed a more heterogeneous behaviour: substances such as cocaine and its metabolite benzoylecgonine were generally well eliminated (>90 %), while compounds such as MDMA and methamphetamine showed lower and more variable elimination rates. In contrast, medications of abuse showed different removal efficiencies depending on the compound and the treatment applied: morphine showed removal efficiencies that generally exceeded 85 % in WWTPs and seasons, codeine presented lower and more seasonally variable efficiencies, while ketamine showed variable and negative removal efficiencies in most cases. These general differences highlight the need for treatment strategies tailored according to the physicochemical and metabolic characteristics of each group of substances.

3.5. Comparison between secondary and tertiary wastewater treatment removal efficiency

Since no clear seasonal differences in the removal efficiency of the analyzed compounds were observed, the data were averaged across all sampling periods. For WWTPs 1 and 2, average removal efficiencies were calculated separately for the days when tertiary treatments (ozone+UV or ozone) were applied and the days when only secondary treatment was used. This allowed us to compare the impact of secondary versus tertiary treatment on the removal of licit drugs, illicit drugs, and medications of abuse.

Focusing first on the group of licit drugs and their metabolites, the comparison showed that WWTP 2 achieved slightly higher removal efficiencies for nicotine, cotinine, and trans-3'-hydroxycotinine. In contrast, the removal of caffeine, 1,7-dimethyluric acid, and ethyl sulfate was broadly constant across all WWTPs, regardless of the treatment applied (Fig. 4a). These results suggest that tertiary treatments do not always guarantee an improvement.

In the case of illicit drugs and their metabolites (Fig. 4b), tertiary treatment processes appeared to have a greater impact, especially for compounds with limited biodegradability such as MDMA and methamphetamine. In particular, these compounds showed higher removal efficiencies in tertiary treatments (WWTP 1 and 2), probably due to their structural stability and resistance to microbial degradation. As previously mentioned, both MDMA and methamphetamine are synthetic stimulants that often contain branched amine groups, which reduces their susceptibility to conventionally activated sludge systems [55]. In contrast, ecgonine methyl ester, amphetamine, benzoylecgonine, THC-COOH and cocaine showed uniformly high removal efficiencies in both secondary and tertiary treatments, suggesting that these compounds are either more biodegradable or are adequately removed by adsorption mechanisms that are effective in both treatment types [57]. An exception to this trend was observed for methamphetamine in WWTP 3, where secondary treatment achieved higher removal efficiency than tertiary processes. This observation suggests that under optimized conditions, secondary systems can overcome certain conditions of ozonation and UV of more advanced processes for specific substances. Several factors could explain this pattern, including that methamphetamine is susceptible to microbial degradation under aerobic conditions, especially in systems with high sludge retention times [55]. If the tertiary process involves a shorter hydraulic retention time or introduces oxidants that do not react directly with methamphetamine, the benefit may be limited, or even detrimental if the transformation results in persistent products. Similar anomalies have been reported in previous studies, where tertiary systems did not consistently improve the removal of some amphetamine-type stimulants [42,51], probably due to insufficient ozone and UV inputs [28,58], differences in the microbial composition of the wastewater samples or the presence of interfering substances in the tertiary treatment zones [1]. Furthermore, these results confirm observations made in studies in Mediterranean regions such as Valencia, where high removal rates were consistently observed for benzoylecgonine and amphetamine, regardless of treatment sophistication, while MDMA showed lower and more variable clearance efficiencies, often

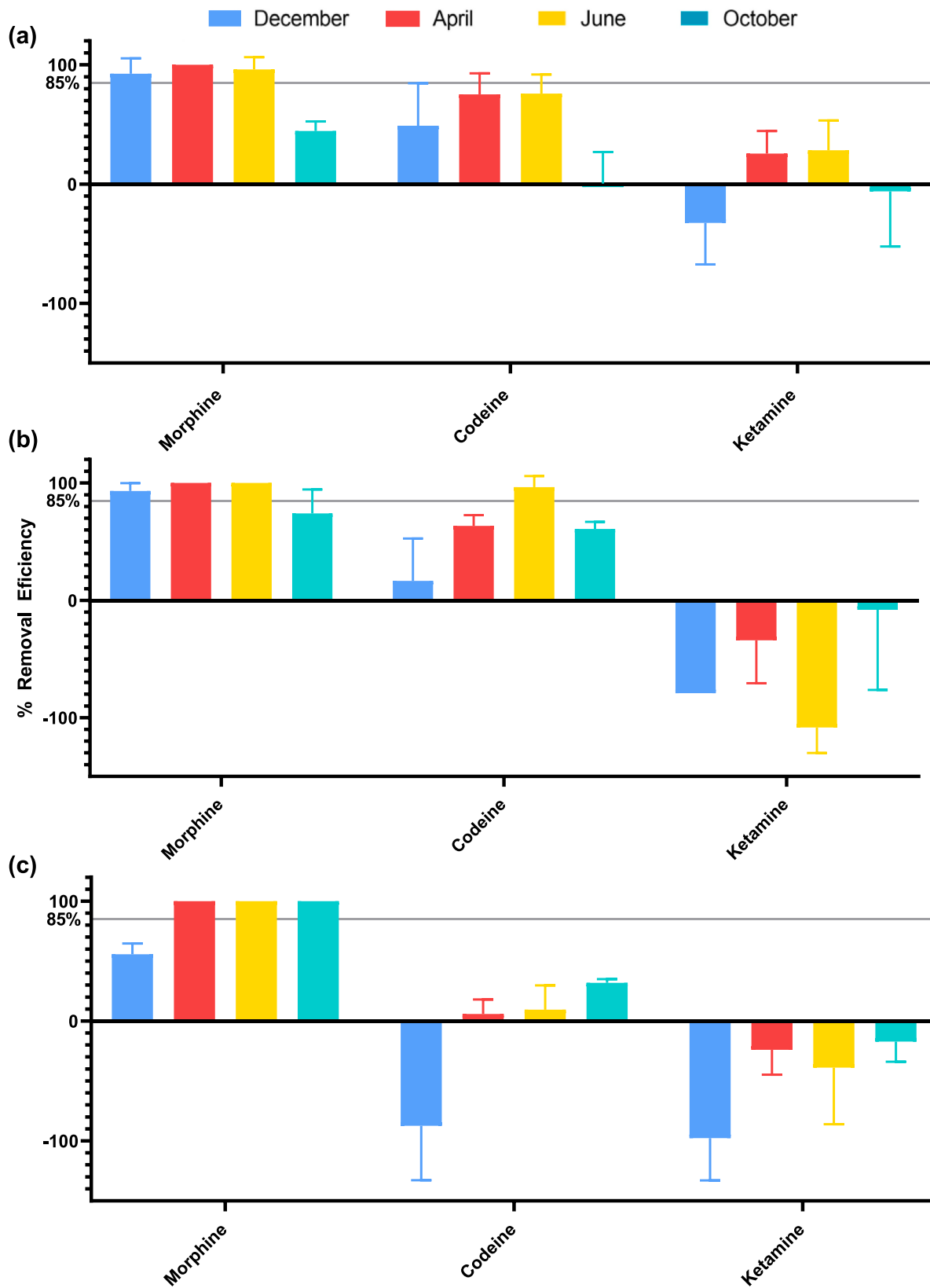


Fig. 3. Seasonal variation in the removal efficiency of medications of abuse across three WWTPs: (a) WWTP 1 (tertiary treatment during four days, secondary treatment the rest); (b) WWTP 2 (tertiary treatment during four days, secondary the rest); (c) WWTP 3 (continuous secondary treatment).

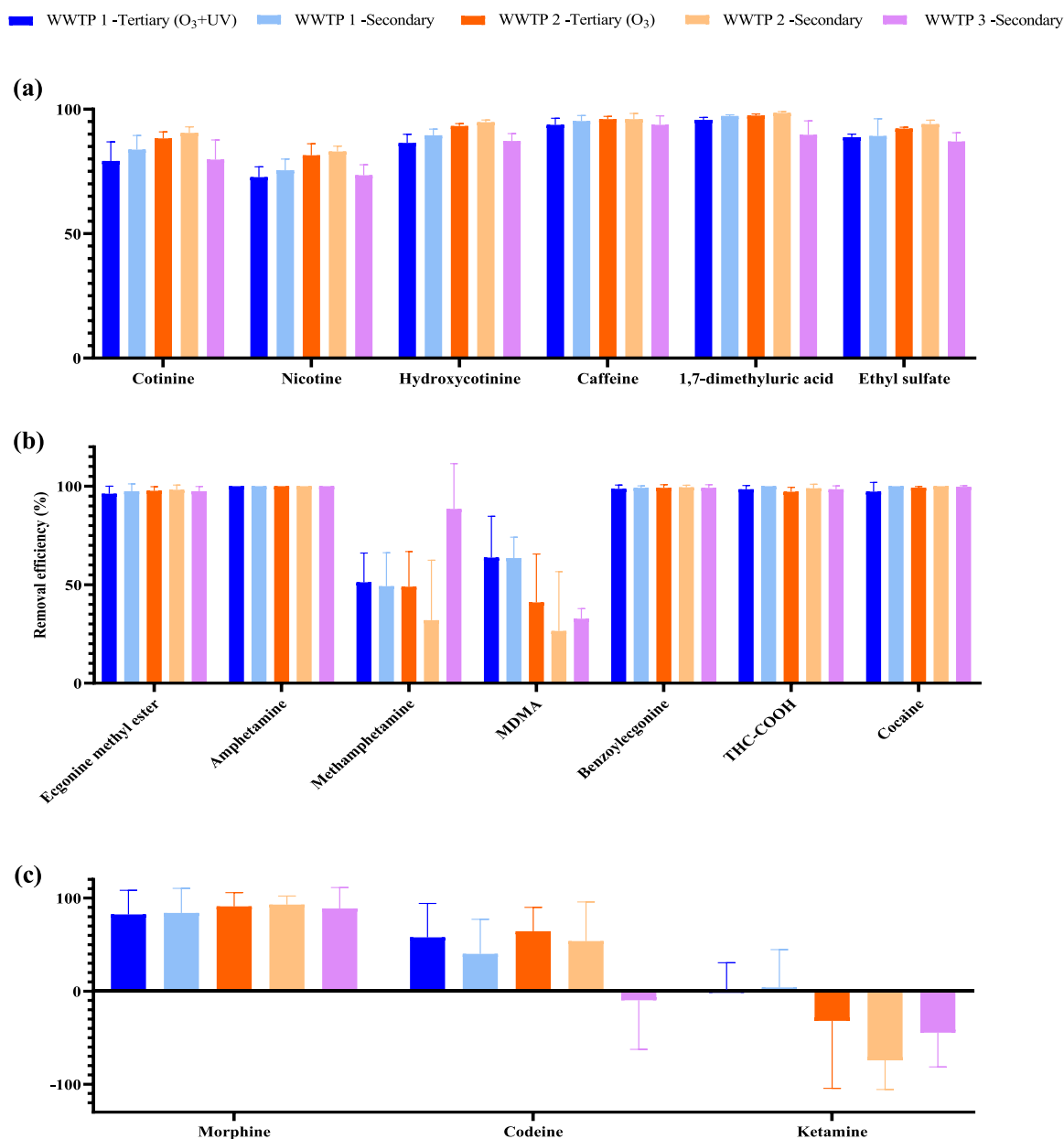


Fig. 4. Removal efficiencies (a) for licit drugs and/or their metabolites, (b) for illicit drugs and/or their metabolites, and (c) for medications of abuse, at the three wastewater treatment plants. WWTPs 1 and 2 operated under both treatment configurations (tertiary and secondary) during the four seasonal sampling campaigns, whereas WWTP 3 applied only secondary treatment.

below 60 % [47].

For medications of abuse (Fig. 4c), different elimination patterns were observed. Morphine consistently showed high removal (>85 %) in both secondary and tertiary treatments, indicating that it is effectively biodegraded even in conventional systems. Codeine showed moderate and treatment-dependent removal elimination, with higher efficacy in tertiary treatments, probably due to its partial resistance to biological processes and moderate hydrophobicity. The most persistent compound in this group was ketamine, which showed low or even negative elimination values under both secondary and tertiary treatments. These observations are in agreement with those presented in previous studies that highlight the persistence of ketamine, overcoming biological degradation, and requiring more aggressive oxidative conditions for its effective elimination [47] than those used in the present study. In addition, partial or negative removal could also be the result of deconjugation processes in the sewage system, where conjugated metabolites are cleaved, releasing the parent compound, leading to apparent higher

effluent concentrations and negative efficiencies, a phenomenon already described for other compounds [7].

Overall, these observations align with previous studies, which have indicated that the removal efficiency of emerging contaminants does not depend exclusively on the treatment applied, but on a combination of factors. These include: the physicochemical properties of the compounds, such as the presence of functional groups, heteroatoms, and their hydrophobic or hydrophilic character, expressed through the log Kow; associated processes like sorption and desorption; the formation of transformation products; uncertainties related to sampling; biodegradability and microbial dynamics; and the operational conditions of each plant, including hydraulic retention times and sludge retention times [37,46].

3.6. Evaluation of advanced oxidation treatment

In order to directly assess the effect of tertiary treatment processes

under the specific operational conditions applied, a total of 16 effluent samples (8 from WWTP 1 and 8 from WWTP 2) were collected over four consecutive days in October 2024 as case study. At each plant, two samples were taken each day: one immediately before and one immediately after the oxidation treatment stage. In WWTP 1 the tertiary treatment consisted of ozone (3.5 mg/L) combined with UV radiation (58 mJ/cm²), whereas WWTP 2 employed ozonation alone at a concentration of 3 mg/L.

Figures S1-S3 shows effluent concentrations of licit drugs, illicit drugs, medications of abuse distinguishing between samples collected before and after the oxidation stage at both WWTPs. Based on these concentration values, the corresponding reduction percentages were calculated and are presented in Fig. 5. The reduction percentages were calculated as follows:

$$\%Reduction = \frac{C_{without\ oxidation} - C_{with\ oxidation}}{C_{without\ oxidation}} \cdot 100 \quad (5)$$

This calculation enabled a specific assessment of the contribution of the oxidation (ozone or ozone-UV) step within the tertiary treatment process.

For licit compounds, overall, no significant reductions in concentration were observed after the application of tertiary treatments, except for 1,7-dimethyluric acid in WWTP 2. Other compounds, such as trans-3'-hydroxycotinine and cotinine, showed nearly zero reduction percentages, indicating similar concentrations before and after treatment. In the case of compounds such as nicotine, caffeine, and ethyl sulfate, slight increases in concentration were observed on certain days after treatment, resulting in negative reduction percentages. These increases might be explained by the possible release of conjugated forms during tertiary treatment. Altogether, these results suggest that the applied oxidation conditions may not have been sufficient to ensure effective degradation of certain licit drug residues under real operating conditions.

For illicit drugs, advanced oxidation treatments showed variable results depending on the compound analyzed (Figure S2). Benzoyllecgonine did not show a reduction in concentration after the application of ozone or ozone-UV treatments. In contrast, MDMA showed a decrease of approximately 20 % in WWTP 1 and 50 % in WWTP 2, indicating moderate effectiveness. Methamphetamine concentration was reduced by approximately 40 % in WWTP 2 after ozonation while the concentration remained stable or even slightly increased in WWTP 1, potentially due to the transformation of conjugated metabolites. These results suggest that the oxidation conditions applied were not sufficient

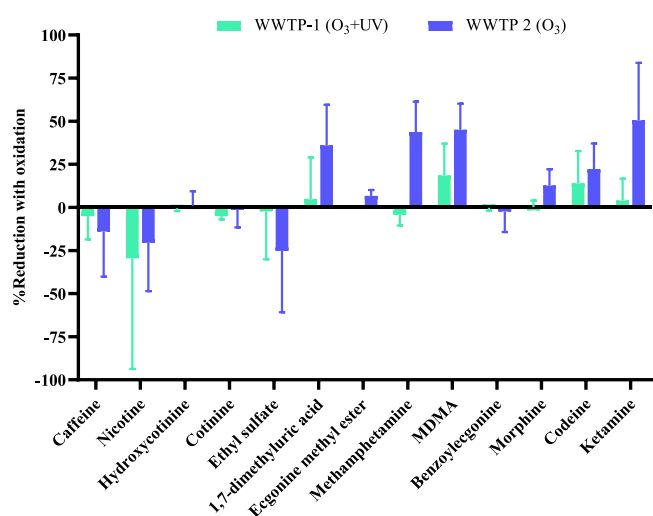


Fig. 5. Percentage reduction of target compounds in WWTP 1 and WWTP 2 after tertiary oxidation (ozone-UV or ozone), based on effluent samples collected over four days.

to achieve a complete degradation of synthetic stimulants. Other studies have shown that higher ozone doses are necessary to achieve significant removal [3,58], especially for compounds with aromatic rings or amine groups, since they provide oxidative resistance [42]. Furthermore, the incomplete oxidation of certain compounds can generate persistent oxidation transformation products (OTPs), such as OTP-213 from MDMA, which can be as harmful to the environment as the parent compound [42]. Similarly, methamphetamine is relatively resistant to UV disinfection and only responds effectively to advanced oxidation processes based on strong hydroxyl radicals, such as UV/H₂O₂ or TiO₂ photocatalysis [19]. For amphetamine, no reduction could be calculated because it was not detected in any of the samples. THC-COOH and cocaine were only detected in one out of the 16 samples, so no reduction was estimated for these compounds.

In the case of medications of abuse (Figure S3), the three compounds showed reductions after oxidation in both WWTPs, with ketamine showing the most consistent decrease. These results suggest that the ozone and UV doses applied in the WWTPs analyzed were insufficient for the complete removal of these substances. Furthermore, other studies have shown that compounds such as ketamine and codeine are only partially removed by low intensity ozonation conditions, and that more advanced oxidation processes (for example UV/H₂O₂) may be necessary to achieve higher levels of degradation [19,42]. In addition, the presence of natural organic matter wastewater may compete for oxidants, reducing the overall treatment efficiency [26].

Overall, these results can be explained by a combination of factors. On the one hand, the ozone doses applied might have been insufficient. Previous studies show that the removal of ECs depends on the ozone dose. For instance, at 6 mg/L for 10 min, many pharmaceuticals and personal care products were degraded up to, or below detection limits, while others were not completely removed, highlighting the resistance of certain drugs [39]. Similarly, other studies report that some illicit stimulants, antidepressants and their metabolites are inherently resistant to ozonation due to the absence of reactive functional groups, further limiting removal efficiency [58]. This indicates that the process efficiency mainly depends on the contaminant, as some compounds exhibit high reactivity with ozone or generated radicals, whereas others are more resistant and require higher concentrations [26]. On the other hand, the presence of endogenous dissolved organic matter (EfOM) may act as a sink for ozone and hydroxyl radicals (•OH), decreasing their availability to react with contaminants [26]. Regarding the UV radiation applied in WWTP 1, no additional improvement was observed. This result aligns with the literature, which finds that typical UV doses used for disinfection are insufficient to degrade certain emerging contaminants. In some cases, even increases in concentration have been documented, attributable to the phototransformation of conjugated forms that release the parent compound upon photolytic decomposition [4].

These results reflect the limitations of advanced oxidation treatments, the efficiency of which depends on parameters such as the ozone dose applied, the composition of the aqueous matrix and the chemical nature of the target compounds. In general, the data indicates that the oxidation conditions applied in this study were not enough to significantly reduce the concentrations of these substances. Therefore, it is necessary to optimize the dose and to combine ozone treatments with more reactive processes to improve the elimination of resistant compounds.

3.7. Risk assessment of sewage effluent elimination into the aquatic environment

The assessment of the chemical toxicity of effluents using Toxic Units for Chemicals (TU_{chem}) is a key tool for analyzing the potential toxicity of complex mixtures of pollutants. This measure is based on the ratio between the mean concentration of each compound detected in the effluent and its corresponding toxic reference value (EC₅₀ for algae and LC₅₀ for daphnia and fish). TU_{chem} allows the quantification of the acute

ecotoxicological risk at different trophic levels of the aquatic ecosystem [12]. However, it should be noted that TU_{chem} is a simplified indicator, as it does not account for possible interactions (synergistic, additive, or antagonistic) among contaminants in the mixture. Despite this limitation, it is widely used in the literature as a standardised and comparable first approximation of ecotoxicological risk.

Following this approach, the theoretical toxic units were calculated (Eq. 3) in order to determine the potential ecotoxicological risk posed by the discharge of the analyzed compounds to the aquatic environment [27]. These values were calculated taking into account the mean values of the concentrations obtained in the four samplings carried out in each of the three WWTPs, considering only the days of tertiary treatment application in WWTP 1 and 2, and the same days for the secondary treatment in WWTP 3 (Table 1) and LC_{50} and EC_{50} values that were estimated using the ECOSAR database (Table S6).

The TU_{chem} values obtained for the representative organisms of three trophic levels (algae, daphnia and fish) revealed significant differences between the three WWTPs studied. Particularly, WWTP 1, with a tertiary treatment based on ozone and UV radiation, presented the lowest TU_{chem} values for the three bioindicator groups. This observation is consistent with the expected higher efficiency of advanced treatment processes in the removal of ecotoxic compounds, although differences in influent concentrations may also contribute to the observed TU_{chem} values. In contrast, WWTP 3, without advanced tertiary treatment, exhibited the highest levels of algal toxicity, indicating an increased sensitivity of photosynthetic organisms to the mixture of pollutants that are not effectively removed by conventional treatment processes [41].

By analyzing the ratios between the concentration and LC_{50} value performed to calculate the TU_{chem} , it is possible to identify the compounds that contribute with a higher relative weight in the overall toxicity of the wastewater mixture [12]. For example, ketamine (EC_{50} for algae of 0.722 mg/L) showed a high contribution to TU_{chem} in all the WWTP due to its low effective concentration (EC_{50}) and persistent presence at relatively high concentrations in the effluent. Likewise, compounds such as MDMA and THC-COOH, despite their relatively high removal efficiency, also contributed significantly to TU_{chem} . Conversely, compounds such as benzoylecgonine or cotinine, despite being present at higher concentrations, contributed less to TU_{chem} . This is due to their high lethal effective concentrations (LC_{50}/EC_{50} values) [10], reflecting a low toxicity in the models used to estimate the overall toxicity of the wastewater.

Furthermore, interpretation of the TU_{chem} values in relation to ecotoxicological thresholds suggests that three WWTPs may pose

Table 1

Mean concentrations of compounds detected in effluent wastewater samples from the three wastewater treatment plants (WWTP) and corresponding toxic units (TU_{chem}) for algae, daphnids and fish.

Compound	Mean concentration ($\mu\text{g/L}$)		
	WWTP-1	WWTP-2	WWTP-3
Ketamine	0.16	0.52	0.43
Morphine	0.01	0.01	0.01
Codeine	0.06	0.09	0.17
Ecgonine methyl ester	0.03	0.02	0.02
Methamphetamine	0.06	0.10	0.00
MDMA	0.07	0.15	0.11
Benzoylecgonine	0.02	0.02	0.01
THC-COOH	0.01	0.02	0.01
Cocaine	0.06	0.02	0.01
Cotinine	0.45	0.43	0.46
Nicotine	1.34	1.30	1.12
Trans-3'-Hydroxycotinine	0.60	0.49	0.62
Caffeine	1.65	1.32	1.83
1,7-dimethyluric acid	1.40	1.15	2.71
Ethyl sulfate	2.13	1.83	1.92
TU_{chem} Algae	0.50	1.17	1.42
TU_{chem} Daphnid	0.27	0.64	0.52
TU_{chem} Fish	0.05	0.12	0.09

ecotoxicological risks to aquatic organisms; however, WWTP 2 and WWTP 3 represent the highest risk, especially for microalgae, since values above 0.1 have been proposed to indicate a relevant ecotoxicological risk potential [10]. These results are especially concerning when taking into account the chronic exposure of aquatic organisms to these and other environmental pollutants [12].

In this context, it is noteworthy that although both WWTP 1 and WWTP 2 apply ozonation as a tertiary treatment, WWTP 1 combines ozone with UV radiation. The consistently lower TU_{chem} values obtained for WWTP 1 likely reflect a combination of influent characteristics, treatment performance, and a potential additional contribution of UV to the degradation of persistent compounds, although direct measurements of individual compound removal before and after the UV stage indicated limited effect under the operational conditions applied. This highlights the relevance of assessing advanced oxidation processes not only by their removal efficiency for individual contaminants, but also by their ability to reduce the overall acute toxicity of effluents. These findings support the need to design and optimize integrated advanced oxidation processes configurations to achieve a greater reduction of ecological risk, particularly for sensitive organisms like algae.

4. Conclusions

Comparative analysis of three WWTPs in Madrid revealed that removal efficiencies vary depending on the physicochemical properties of the compounds and the treatment technologies applied. While conventional biological secondary treatments were generally effective for hydrophilic and biodegradable compounds such as caffeine, advanced oxidation processes (ozone and ozone-UV) achieved higher removal efficiencies for more persistent compounds. However, these advanced treatments did not guarantee significant removal of all target analytes, highlighting their current limitations. The ecotoxicological assessment using chemical toxic units (TU_{chem}) showed that tertiary treatments substantially reduce the potential acute toxicity of the effluents, especially for algae, which are the most sensitive trophic group studied. The WWTP applying ozone and UV treatment showed the lowest TU_{chem} values (algae: 0.50; daphnia: 0.27; fish: 0.05), reflecting the combined influence of influent characteristics and tertiary treatment performance. These results underline the importance of optimizing integrated advanced oxidation processes to minimize the ecological impact associated with emerging contaminants. Although removal efficiencies were generally stable across seasons, certain compounds such as MDMA and methamphetamine exhibited improved removal during warmer months in some WWTPs, likely due to enhanced microbial activity and photodegradation processes. These findings suggest that seasonal factors may influence treatment performance for specific substances and should be considered in monitoring strategies.

As a limitation, this study included only three WWTPs and assessed removal efficiency based solely on treatment type and seasonal variation, without considering other operational or environmental factors. Future research should expand to more plants, evaluate long-term stability through multi-year studies, and conduct pilot-scale experiments to optimize treatment processes.

Overall, this study highlights the need to optimize tertiary treatment conditions, including ozone and UV dosage, to improve the removal of persistent and toxic pollutants. In addition, incorporating toxicity-based metrics such as TU_{chem} into the routine monitoring and regulatory frameworks would allow a more comprehensive evaluation of environmental risks associated with wastewater discharges and better inform improvements in treatment strategies.

Environmental implication

This study highlights the limitations of conventional wastewater treatments in removing certain emerging contaminants, including licit and illicit drugs, and demonstrates that tertiary treatments based on

ozone and UV generally result in lower ecotoxicological risk. By using toxicity-based metrics (TU_{chem}), it emphasizes the importance of assessing both removal efficiencies and ecological impacts. The calculated TU_{chem} values indicate that algae are the most sensitive trophic level to the studied compounds. These findings support the optimization of advanced oxidation processes and the inclusion of compound-specific monitoring strategies to better protect the environment from the discharge of hazardous emerging contaminants.

CRedit authorship contribution statement

Natalia Melones-Peña: Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing - original draft. Writing - review & editing. **Elsa C. Maroto-Melle:** Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing - original draft. Writing - review & editing. **Susana Torrado-Del Rey:** Investigation, Data curation. **Azara Valenciano-Pérez:** Investigation, Data curation. **M Justina Martín-Gutiérrez:** Resources, Supervision. **Emma Gracia-Lor:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Government Delegation for the National Drugs Plan (Ministry of Health, Spain), with funds from the Recovery, Transformation, and Resilience Mechanism of the European Union - NextGenerationEU, project number EXP2022/008817. It was also supported by Madrid Salud, and by the Spanish Ministry of Science, Innovation and Universities through PID2023-148425NB-I00 project funded by MCIU/AEI/10.13039/501100011033 and FEDER, UE. The authors sincerely thank the Madrid City Council and the personnel from the sewage treatment plants who were involved in sample collection, as well as the Analysis Service Unit facilities of ICTAN.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2025.140290](https://doi.org/10.1016/j.jhazmat.2025.140290).

Data availability

Data will be made available on request.

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