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Removal of residues of psychoactive substances during wastewater treatment, their occurrence in receiving river waters and environmental risk assessment

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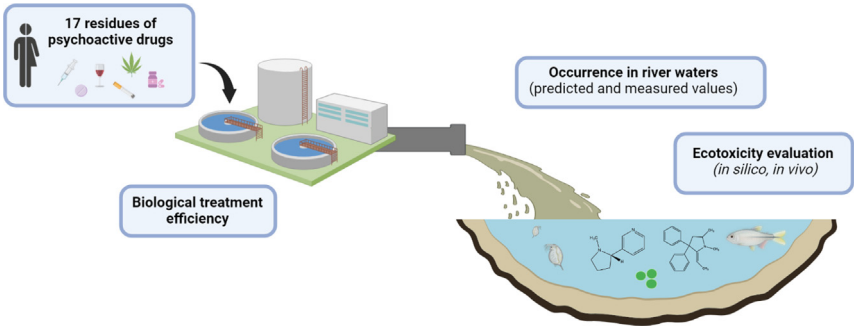
HIGHLIGHTS

- Removal of psychoactive drug residues with activated sludge and MBR were comparable.
- MBBR was less efficient at removing nicotine and cocaine residues.
- Predicted and measured levels in river water were in good agreement.
- *Chlamydomonas reinhardtii* growth was uninhibited by drug residues at 1 mg/L.
- Five drug residues exceeded in silico predicted PNEC.

GRAPHICAL ABSTRACT



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ABSTRACT

Continuous consumption combined with incomplete removal during wastewater treatment means residues of psychoactive substances (licit drugs, medications of abuse and illicit drugs) are constantly introduced into the aquatic environment, where they have the potential to affect non-target organisms. In this study, 17 drug residues of psychoactive substances were determined in wastewater influent, effluent and in receiving rivers of six Slovene municipal wastewater treatment plants employing different treatment technologies. Variations in removal efficiencies (REs) during spring, summer and winter were explored, and ecotoxic effects were evaluated using in silico (Ecological Structure-Activity Relationships software–ECOSAR) and in vivo (algal growth inhibition test) methods. Drug residues were detected in influent and effluent in the ng/L to μg/L range. In receiving rivers, biomarkers were in the ng/L range, and there was good agreement between measured and predicted concentrations. On average, REs were highest for

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nicotine, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH), cocaine residues, and amphetamine (>90 %) and lowest for methadone residues (<30 %). REs were comparable between treatments involving activated sludge and membrane bioreactors, while the moving biofilm bed reactor (MBBR) removed cotinine, cocaine, and benzoylecgonine to a lesser extent. Accordingly, higher levels of nicotine and cocaine residues were detected in river water receiving MBBR discharge. Although there were seasonal variations in REs and levels of drug residues in receiving rivers, no general pattern could be observed. No significant inhibition of algal growth (*Chlamydomonas reinhardtii*) was observed for the tested compounds (1 mg/L) during 72 h and 240 h of exposure, although effects on aquatic plants were predicted in silico. In addition, environmental risk assessment revealed that levels of nicotine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), morphine, and 3,4-methylenedioxymethamphetamine (MDMA) pose a risk to aquatic organisms. Since nicotine and EDDP can have acute and chronic effects, the authors support regular monitoring of receiving surface waters, followed up by regulatory actions.

1. Introduction

Once administered, psychoactive drugs cross the blood-brain barrier and act upon the central nervous system affecting mental processes such as perception, consciousness, cognition, mood and emotions (Viana et al., 2012; WHO, 2022). They are consumed licitly for recreational purposes (e.g., nicotine and alcohol), medicinally (e.g., morphine, ketamine, codeine, and methadone), or illicitly (e.g., cocaine, amphetamines, and heroin) (Viana et al., 2012). Like most drugs, they are excreted from the body in urine, faeces, sweat and saliva as either the parent compound or as metabolites (drug residues) and enter the sewer system where, in most cases, they are delivered to a local wastewater treatment plant, WWTP (Jin et al., 2022; Mohan et al., 2021; Zuccato et al., 2005). Once at the WWTP, they are removed to differing degrees (from negative removal efficiencies, RE, to >99 %) depending on influent concentration and their physicochemical properties, treatment technology and environmental parameters (Deng et al., 2020; Di Marcantonio et al., 2020; Evgenidou et al., 2015; Hedgespeth et al., 2012; Jin et al., 2022; Verovšek et al., 2022; Yadav et al., 2017). Consequently, drug residues, e.g. parent compounds and their metabolites, are found in receiving surface waters (e.g., rivers, lakes, and seawater) in the ng/L–μg/L range, making wastewater effluent a major source of drug residues in the environment (Evgenidou et al., 2015; Jin et al., 2022; Verovšek et al., 2022).

The presence of psychoactive drug residues in receiving surface waters raises ecotoxicological concerns due to their psychoactive properties, especially since their presence in the environment through continuous release (pseudo-persistence) poses a risk to non-target organisms (Ebele et al., 2017; Mohan et al., 2021; Rosi-Marshall et al., 2015). Medium to long-term exposure may result in chronic effects (ecological and evolutionary) even at low environmental concentrations (ng/L range). Moreover, their simultaneous presence may lead to additive or synergistic effects (Evgenidou et al., 2015; Jin et al., 2022). Unfortunately, research on their effects on aquatic organisms is limited regarding the number of drug residues tested and the variety of aquatic organisms exposed (Mohan et al., 2021). However, available data indicate that drug residues could harm aquatic organisms. For example, plant-derived substances, such as cocaine, cannabinoids, opioids, nicotine, and amphetamines, have antimicrobial properties (Baran et al., 2020; Radulović et al., 2013; Rosi-Marshall et al., 2015). Nicotine, cocaine and tetrahydrocannabinol (THC) were shown to affect invertebrates, such as water fleas (*Daphnia magna* and *Daphnia pulex*) and mussels (Zebra mussel, *Dreissena polymorpha*), while cocaine was proven to affect vertebrates (European eel, *Anguilla anguilla*) (Binelli et al., 2012; De Felice et al., 2019; Gay et al., 2013; Oropesa et al., 2017; Parolini and Binelli, 2014). So far, no published studies have addressed the ecotoxicological effect of psychoactive drug residues on algae, although it is widely known that the disturbance of algae as primary producers in the aquatic food chain affects higher trophic levels (Geis et al., 2000).

This study aimed to fill knowledge gaps by determining (i) the efficiency of six Slovenian WWTPs differing in size and configuration (including MBBR) for removing psychoactive drug residues; (ii) their presence in receiving waters determined using liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) and predicted based on effluent concentrations and river flows; (iii) their potential aquatic toxicity using an algal growth inhibition test (*Chlamydomonas reinhardtii*) for the first time; and (iv) a risk assessment based on measured concentrations of drug residues in receiving rivers and effect concentrations estimated using Ecological Structure-Activity Relationships software (ECOSAR).

2. Methods

2.1. Compounds of interest

Seventeen residues of licit drugs, medications of abuse and illicit drugs were targeted in wastewater (influents and effluents) and receiving river waters (Table 1). All details regarding reagents are provided in the Supplementary material (see SM: 1.1. Chemicals and Materials).

2.2. Sampling and sample preparation

Six WWTPs varying in catchment size (25,414–270,305 inhabitants) and type of treatment technology (activated sludge – AS, sequential batch reactor – SBR, SBR with UV disinfection, membrane bioreactor – MBR and moving biofilm bed reactor – MBBR) were included in the study (Table S2). Wastewater influent and effluent samples (24-h composites) were collected using time- or volume-proportional sampling taking hydraulic-retention times (HRTs) into account. Receiving waters (Table S2) differing in hydrological conditions, e.g., dilution factor (4.22–887), expressed as the ratio between the receiving river water and wastewater effluent flow, were collected as grab samples from the riverbank, approximately 100 m downstream of the WWTPs outflows. In the case of one WWTP, collection of receiving river water was not possible (Table S2). All water samples were collected in spring and summer (2019) and winter (2020) and stored at −20 °C until analysis. Full details about the WWTPs and receiving waters are given in the SM (1.2. Sampling).

Spring sample analysis is based on previously published methods (Verovšek et al., 2021a, 2021b). Pre-concentration of compounds was achieved using solid-phase extraction (SPE) with Oasis MCX cartridges, followed by analysis using UPLC-MS/MS. In the case of nicotine and alcohol residues, which were present in much higher concentrations, the direct injection method was used. Due to contamination of the MS when using direct injection and ion-pairing reagent (Verovšek et al., 2022), the method was later optimised and used to analyse the summer and winter samples. Both methods are described in the SM (1.3. Sample preparation: waste- and surface waters). In this case, waste- (influent: 125 mL, effluent: 250 mL) and river (0.5 L) water samples were spiked with isotopically-labelled internal standards (ISs) of each drug residue (Table S1). For the determination of alcohol residue (ethyl sulphate), liquid-liquid extraction (LLE) with acetonitrile was used. Nicotine residues were extracted from wastewater influent using supported liquid extraction – SLE (ISOLUTE SLE+, 400 μL, Biotage, Sweden) and from effluent and river water using SPE (Oasis PRIME MCX, 150 mg/6 mL, Waters, Milford, MA, USA). Residues of illicit drugs and medications of abuse were extracted from waste- and river water by SPE (Oasis PRIME MCX). In order to determine THC-

Table 1
Compounds of interest (human metabolic residues).

Psychoactive drug	Compound of interest	Abbreviation
Licit drugs		
Nicotine (tobacco)	Nicotine[a]	NIC
	Cotinine[a]	COT
	Trans-3′-hydroxycotinine[a]	HCOT
Ethanol (alcohol)	Ethyl sulphate	EtS
Medications of abuse		
Morphine	Morphine[a]	MOR
Codeine	Codeine	COD
Methadone	Methadone	MTHD
	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine[a]	EDDP
Ketamine	Ketamine	KET
Illicit drugs		
THC	11-Nor-9-carboxy-Δ9-tetrahydrocannabinol[a]	THC-COOH
Cocaine	Cocaine[a]	COC
	Benzoylecgonine[a]	BE
	Cocaethylene	COE
Amphetamine	Amphetamine[a]	AMP
Methamphetamine	Methamphetamine	MAMP
Ecstasy	3,4-Methylenedioxymethamphetamine[a]	MDMA
Heroin	6-Acetylmorphine	6-AM
Algal growth inhibition test (spike of mixture):		
MIX_1: Nicotine and EDDP		
MIX_2: Nicotine, cotinine, HCOT, EDDP and benzoylecgonine		
MIX_3: Nicotine, cotinine, HCOT, morphine, EDDP, cocaine, benzoylecgonine, amphetamine and MDMA		

[a] Compounds used in algal growth inhibition test (individual spike).

COOH, an additional clean-up was introduced (Strata NH_2, 200 mg/3 mL, Phenomenex, Torrance, California, USA).

2.3. Chemical analysis and method validation

All samples were analysed using reverse-phase UPLC-MS/MS. The method is described in full in the SM (1.5. Sample analysis), and the methods' performance was evaluated by determining the following parameters: linearity, limits of detection (LOD), limits of quantification (LOQ), filtration recovery (FR), extraction recovery (ER), matrix effect (ME), accuracy and repeatability on at least two concentration levels. Artificial wastewater influent and effluent, potable water and blank TRIS acetate phosphate (TAP) medium were used as blank matrices for validation (see SM: 1.6. Method validation). Methods used for analysis were validated in the frame of the Sewage analysis CORe group – Europe (SCORE) interlaboratory comparison study (SCORE, 2022).

2.4. Removal efficiency calculation

Removal efficiency (RE) was calculated according to Eq. (1).

$$RE\ (\%) = 100 - \left(\frac{c_{eff}}{c_{inf}} \times 100\right) \quad (1)$$

where c_{eff} is the concentration of the target compound (ng/L) in wastewater effluent and c_{inf} is the concentration of the target compound in wastewater influent. In the case where the c_{inf} was <LOQ in wastewater influent, the RE was not calculated, whereas when the c_{eff} was <LOQ in wastewater effluent, LOQ was used.

2.5. Predicting environmental concentration of drug residues in receiving rivers

An approach based on generating dilution factors for predicting the environmental concentration of down-the-drain chemicals in surface waters was adapted from Keller et al., 2014. The concentration of drug residues in receiving surface waters was predicted using calculated dilution factors (Table S2) and measured concentrations in wastewater effluents (Tables S11–S13). Estimated concentrations were compared with those measured in the receiving rivers (Tables S11–S13).

2.6. Algal growth inhibition test

Ten drug residues (Table 1) were selected based on their occurrence in wastewater and predicted ecotoxicity (ECOSAR) and spiked (individually and as mixtures) in TAP medium (Table S3). An algal growth inhibition test was then conducted following OECD Test No. 201 guideline (OECD, 2022) with minor modification of prolonged exposure with individual drug residues. The guideline consists of the following three validation criteria: (i) growth rate of at least 0.92 per day, (ii) coefficients of variation between each test day <35 % and (iii) coefficients of variation for individual control cultures during whole test <10 %. Each week of the experiment, green algae (*Chlamydomonas reinhardtii*) culture was prepared in agar (2 g of agar mixed with 250 mL sterile TAP medium). The stock culture was prepared by mixing a portion of the agar culture into a liquid TAP medium and incubated under controlled conditions for three days. The stock culture was inoculated into the test chambers (100 mL glass Erlenmeyer® flasks, sterilised) filled with 10 mL TAP medium to achieve an initial algae concentration of 2×10^4 cells/mL. For cell counting, an aliquot of each liquid culture (50 μL) was transferred to a 96-well plate (Brand GMBH + CO KG, Germany) and injected into a flow cytometer (MACSQuant Analyser 10: Miltenyi Biotec, Germany). Inoculated TAP medium was spiked with ten drug residues (individually or in a mixture) at a nominal value of 1 mg/L. Non-spiked inoculated TAP medium was used for the negative control, and TAP medium with added methanol and acetonitrile (separately and in a mixture) as a control for the effect of solvents of drugs' stock standard solutions (Table S1) on algal growth. All tests were performed in an algae growth chamber (LTH, Slovenia) at room temperature (22 ± 1 °C) under constant light (80–120 μE/m^2s, Sylvania GRO-Lux F 18 W/GRO-T8) and shaking (80 rpm, GFL 3017, Germany). Total exposure time was 240 h for individual drug residues and 72 h for mixtures of drug residues. Algal growth was measured by flow cytometer after 24, 48, 72, and 240 h (each time in triplicate). Specific growth rate (Eq. (2)) and inhibition of growth rate (Eq. (3)) were calculated for each time interval. The levels of drug residues spiked in the TAP medium were determined (at 0, 72, and 240 h) as follows: a portion of the TAP medium was sequentially centrifuged (14,000 RCF, 3 min), spiked with ISs of each targeted analyte and filter-centrifuged using modified nylon centrifugal filters (0.2 μm, 14,000 RCF, 3 min, VWR, Vienna, Italy). The drug residues were then extracted either by SLE or LLE into acetonitrile (see SM: 1.4. Sample preparation: TAP medium).

The specific growth rate (μ) for an individual period was calculated as the logarithmic increase in the cell density (Eq. (2)), where μ_{i-j} is the specific growth rate (cell/mL) at times i to j (hours), X_i is the cell density at the time i and X_j is the cell density at time j. The specific growth rate was calculated for individual measurement within the individual replicate.

$$\mu_{i-j}\left(h^{-1}\right) = \frac{\ln X_j - \ln X_i}{t_j - t_i} \quad (2)$$

The inhibition of growth rate (I_r) for individual measurement within an individual replicate was calculated in percentages as presented in Eq. (3), where μ_C is the average specific growth rate (h^{-1}) in the control group, and μ_T is the growth for individual measurement.

$$I_r(\%) = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad (3)$$

2.7. Environmental risk assessment

An environmental risk assessment (ERA) addresses the ecological threat associated with drug residues in receiving waters. In this study, the ERA is based on Commission Directive 93/67/EEC on Risk assessment for new notified substances, Commission Regulation (EC) no. 1488/94 on Risk assessment for existing substances, directive 98/8/EC (European Directive

93/67/EC, 1488/94 and 98/8/EC, Part III) and US Environmental Protection Agency (EPA) guidelines (US EPA, 2022) by calculating risk quotients (RQ) according to Eq. (4):

$$RQ = \frac{MEC}{PNEC} \quad (4)$$

The measured environmental concentration (MEC) is the average and maximal amount of each drug residue in the receiving water body. When the measured concentrations were < LOQ, the LOQ was used in the calculation. The predicted no-effect concentration (PNEC) was calculated by dividing the lowest concentration for a single species short-term (median lethal concentration – LC_{50} or median effect concentration – EC_{50}) or the long-term (no-observed-effect concentration – NOEC) effect concentration with the assessment factor – AF (Table S5). As no experimental data is available for the majority of drug residues except for nicotine (HSDB, 2022), the EC_{50}/LC_{50} were predicted (Table S5) using ECOSAR software (v2.2), which predicts the toxicity of new/untested compounds based on their structural similarities with compounds with known experimental effect levels and physicochemical properties. The lowest experimental EC_{50}/LC_{50} offered in ECOSAR was used for nicotine. For all residues, the NOEC was calculated by dividing the chronic value (ChV) derived using ECOSAR with √2 (European Directive 93/67/EC, 1488/94 and 98/8/EC, Part III). An AF of 1000 was applied to address acute effects and an AF of 50 to assess chronic risks (European Directive 93/67/EC, 1488/94 and 98/8/EC, Part III). The RQ expressing risk to aquatic organisms were compared to levels of concern (LOC) determined by the US EPA (Table S6) (US EPA, 2022), where RQs >1 indicate an acute risk for aquatic plants and RQ >0.05 represents an acute risk to aquatic animals. An RQ >1 represents a chronic effect on aquatic animals. Only an acute risk assessment was made for aquatic plants.

2.8. Statistical analysis

Univariate and multivariate analysis was used to explore differences in removal efficiencies (variation between WWTPs and seasonal variations) and in the occurrence of drug residues in receiving waters (variation between rivers and seasonal variations). A Student's *t*-test (when data normality and equality of variance were assumed), Welch's t-test (when data normality but equality of variance is not assumed) or Mann-Whitney Rank Sum Test (when the normality of the data was not assumed) at the 95 % confidence level ($\alpha = 0.05$) were used to evaluate the differences between two groups. The normality of the data was tested using the Shapiro-Wilk test and the equality of variance using the Brown-Forsythe test (95 % confidence level, $\alpha = 0.05$). The variation within the dataset was explored using principal component analysis – PCA (unsupervised), while orthogonal projection to latent squares-discriminant analysis – OPLS-DA (supervised) was used to determine the importance of the variables (drug residue) in the projection (VIP) score (95 % confidence interval). Overfitting of the OPLS-DA model was excluded using a permutation test ($n = 100$). For PCA and OPLS-DA, the data were either UV or Par scaled, and logarithmic transformation was applied where necessary. In the algal growth inhibition test, the differences in the inhibition of growth rate between the negative control (inhibition set on 0) and the samples (spiked with solvents and drug resides) were evaluated using repeated measures (RM) one-way ANOVA with Dunnett's multiple comparisons post-hoc test (95 % confidence interval, $\alpha = 0.05$). Statistical evaluations were performed using SigmaPlot 14.0, Origin 2020, GraphPad Prism 9 and Simca 15.0.

3. Results and discussion

3.1. Method validation parameters

A linear response ($R^2 > 0.99$) was observed between LOQ–1000 ng/mL for all drug residues except methadone (LOQ–500 ng/L) in artificial wastewater influent (Table S7), between LOQ and at least 300 ng/L in artificial wastewater effluent (Table S8), between LOQ and at least 250 ng/L in potable water (Table S9) and between LOQ and at least 500 ng/L in TAP medium (Table S10). The LOD and LOQ were in the ng/L range (Tables S7–S10), except for LOD/LOQ for licit drug residues in artificial wastewater influent (LOD: 109.5–1166 ng/L and LOQ: 155–3341 ng/L), ethyl sulphate in artificial wastewater effluent (LOD: 739 ng/L and LOQ: 2419 ng/L) and potable water (LOD: 884.9 ng/L and LOQ: 2947 ng/L). Signal suppression or enhancement (Tables S7–S10) was observed in artificial wastewater influent (ME: −7–46.2 %) wastewater effluent (ME: −13.7–15.7 %), potable water (ME: −84.7–32.6 %) and TAP medium (ME: −18–8.5 %). The relative extraction recoveries (Tables S7–S10) were 19–112 % in artificial wastewater influent (the lowest for ethyl sulphate), 29–110 % in artificial wastewater effluent (the lowest for morphine), 23–108 % in potable water (the lowest for ethyl sulphate) and 65–107 % in TAP medium (the lowest for THC-COOH). Despite lower recoveries and higher matrix effects in exceptional cases, good accuracies were obtained for all compounds of interest in tested matrices (86–110 %; Tables S7–S10). Only ethyl sulphate showed an accuracy of 76 % at the lowest spike in potable water. Instrumental and inter-day repeatability was ≤20 %RSD.

3.2. Occurrence in wastewaters

Fourteen out of the 17 targeted drug residues had a high (≥80 %) detection frequency – DF (percentage of the samples containing drug residue >LOD) in the wastewater influents (Fig. S1), while DF of ketamine (8.3 %) and 6-acetylmorhine (20 %) was low (≤20 %). Quantities of drug residues in wastewater influents are, among others (e.g., excretion rate), closely related to the level of drug use (Yadav et al., 2017). The levels of drug residues in aqueous samples sampled in spring, summer and winter are given in Tables S11–S13. As tobacco and alcohol (ethanol) are the two most commonly used drugs in Slovenia, i.e., approximately 24 % and 68 % of the population between 15 and 64 years of age, respectively (NIJZ, 2019a), high detection (DF = 100 %) and high measured concentrations (756–60,900 ng/L) of their residues were expected and in comparable concentrations (μg/L) to that reported in the literature (Verovšek et al., 2022). The medication of abuse with the highest concentration was morphine (9.40–1634 ng/L), agreeing with its higher prescription rate than other studied medications, i.e., in 2019, morphine was prescribed at least 6500 times, codeine 2400 and methadone 30 times (NIJZ, 2019b). In wastewater, morphine may also originate from other sources, such as the metabolism of codeine and heroin (Gracia-Lor et al., 2017). Among illicit drug residues, benzoylecgonine had the highest concentration (180–2900 ng/L), followed by cocaine (54–1096 ng/L) and THC-COOH (up to 736 ng/L).

Nine out of 17 targeted drug residues in wastewater effluent had a high DF (>80 %, Fig. S1), while ethyl sulphate (1.6 %) and amphetamine (2.4 %) had low DFs. Ketamine showed higher DF in wastewater effluents (27 %) than influents (8.3 %), which may be explained by the 3–4 times lower LOD/LOQ obtained for wastewater effluent (Tables S7–S8). Similarly, Bijlsma et al. (2012) found ketamine mainly in wastewater effluents of five Dutch WWTPs ($DF_{influents}$ = 22 %, $DF_{effluents}$ = 88 %). In our study, 6-acetylmorphine was not detected. The levels of drug residues in wastewater effluent depend on their removal efficiency during wastewater treatment (Yadav et al., 2017). As expected, most drug residues had lower concentrations in wastewater effluents than influents (Tables S11–S13), agreeing with the literature data (Yadav et al., 2017; Verovšek et al., 2022). Licit drug residues were detected in wastewater effluents up to 1075 ng/L (nicotine), residues of medications of abuse up to 494 ng/L (morphine) and illicit drug residues up to 218 ng/L (MDMA).

3.3. Removal efficiencies

The highest average REs (>90 %) were observed for nicotine residues, THC-COOH, cocaine residues, and amphetamine, with methadone residues

Table 2
Individual drug residue removal efficiencies (average and range).

Drug residue	Average RE (range) [%]	Drug residue	Average RE (range) [%]
Ethyl sulphate	75 (30–96)	THC-COOH	98 (88–99)
Nicotine	99 (87–99.999)	Cocaine	97 (62–99.9)
Cotinine	97 (83–99.8)	Benzoylecgonine	94 (53–99.9)
HCOT	99 (85–99.99)	Cocaethylene	95 (66–99.7)
Morphine	88 (−6–99)	Amphetamine	97 (78–99.7)
Methadone	27 (−59–74)	Methamphetamine	51 (35–95)
EDDP	20 (−87–86)	MDMA	47 (−155–90)
COD	53 (−64–98)	6-acetylmorphine	42 (29–56)

EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, RE – removal efficiency, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

having the lowest RE (<30 %; Table 2). The result agrees with already published studies (Baker and Kasprzyk-Hordern, 2013; Bijlsma et al., 2012; Ekpeghere et al., 2018; Nefau et al., 2013; Nguyen et al., 2018; Postigo et al., 2008; Postigo et al., 2010; Terzic et al., 2010). Negative REs for morphine, methadone residues, codeine and MDMA (Table 2) indicate that these drug residues were present in higher amounts in wastewater effluent than the influent. This difference can be explained by the formation/transformation of the compounds during wastewater treatment, e.g., the transformation of parent compound/precursor, deconjugation of glucuronide conjugates or transformation of conjugated metabolites into parent compound and by an inadequate pairing of wastewater influent and effluent samples (sampling not in range of HRT) (Bijlsma et al., 2012; Subedi and Kannan, 2014; Terzic et al., 2010; Yadav et al., 2019).

The differences in the RE of the six WWTPs for the studied compounds were explored using PCA (Fig. 1). Only WWTP_6 formed a distinct group. For the projection, the difference in removal of cotinine, cocaine and benzoylecgonine was important (OPLS-DA), with significantly lower removals obtained for WWTP_6 (cotinine: $U_{statistic} = 14$, $p \leq 0.001$, $\alpha = 0.05$; cocaine; $U_{statistic} = 0$, $p \leq 0.001$, $\alpha = 0.05$; benzoylecgonine: $U_{statistic} = 0$, $p \leq 0.001$, $\alpha = 0.05$). The dependence of RE on commonly applied wastewater treatment technologies are already well known (Yadav et al., 2017) and in terms of efficiency is as follows: MBR > AS > trickling filters (Baker and Kasprzyk-Hordern, 2013; Kasprzyk-Hordern et al., 2009; Petrovic et al., 2009; Verovšek et al., 2022). The present study observed no difference (grouping) in REs between MBR and AS (Fig. 1). To the best of our knowledge, the removal of drug residues by MBBR was studied for the first time and indicated poorer removal of cotinine, cocaine, and benzoylecgonine.

Seasonal variation in RE is expected since environmental factors, namely wastewater temperature and dissolved oxygen, besides treatment processes and operational parameters, play an important role in removing drug residues by (micro)biological processes (Yadav et al., 2017). Indeed, differences in RE regarding season were observed (PCA: small level of grouping) for five out of the six studied WWTPs (Fig. S2), with important differences in the RE of nicotine residues (WWTP_4 and WWTP_5), codeine (WWTP_1 and WWTP_6), cocaine (WWTP_3), and benzoylecgonine (WWTP_4). In the case of codeine, cocaine, and benzoylecgonine, significantly higher REs were obtained in summer compared to winter and, in some cases (codeine – WWTP_6, benzoylecgonine – WWTP_4), in comparison to spring (Table S14). Higher REs during summer are expected because higher wastewater temperatures (14.2–25.3 °C compared to 9.90–18.3 °C in spring and 9.4–15.3 °C in winter) generally enhance microbial activity (Castiglioni et al., 2006). In contrast, nicotine residues (nicotine: WWTP_4 and WWTP_5, cotinine and HCOT: WWTP_4) showed significantly higher REs in spring than in summer (Table S14). However, although seasonal variations in REs were observed, they were only significant for specific drug residues at specific WWTPs (Tables S11–S13), suggesting that seasonal RE variation is compound and WWTP specific.

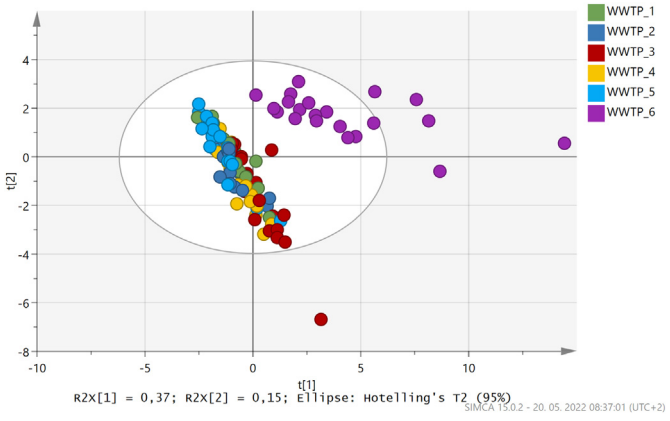

Fig. 1. PCA scatter plot showing variation in REs' dataset.

3.4. Occurrence in receiving river waters

The presence of drug residues in receiving waters (Tables S11–S13) is expected due to their occurrence in the effluent (see Occurrence in wastewaters). Nine out of 17 drug residues had high DF (>80 %, Fig. S1), with DF ≤ 20 % obtained for ethyl sulphate (1 %), ketamine (14 %), THC-COOH (8 %) and amphetamine (12 %). 6-acetylmorphine was not detected. Nicotine, HCOT, cocaine residues, amphetamine and methamphetamine, had a higher DF in receiving river waters than wastewater effluents (Fig. S1), which can be explained by either the much lower LOD/LOQ (2–17-times) obtained for river water (Tables S8–S9) or by the input of drug residues from sources other than WWTPs, namely the clandestine release of illicit drugs/precursors into the soil, nicotine wash-out from cigarette butts and ashes and leaking sewer system (Barbosa et al., 2016; Castiglioni et al., 2015). Otherwise, as expected, due to dilution (Table S2), lower levels of drug residues were detected in receiving waters compared to wastewater effluents (Tables S11–S13). In general, licit drug residues were detected in receiving river waters in concentrations up to 312 ng/L (nicotine), residues of medications of abuse in concentrations up to155 ng/L (EDDP) and illicit drug residues in concentrations up to 190 ng/L (BE).

The variations in the occurrence (level) of psychoactive drug residues were explored in terms of location (different rivers with different dilution factors) and season (spring, summer and winter) using multi-variant analysis. The scatter plot (Fig. 2) shows how R_6 (receiving effluent from WWTP_6) is grouped separately. For the projection, the difference in the occurrence of nicotine residues and benzoylecgonine was important (OPLS-DA), with significantly higher concentrations measured in R_6 (nicotine: $U_{statistic} = 229, p \leq 0.001$, $\alpha = 0.05$; cotinine: $U_{statistic} = 9$, $p \leq 0.001$, $\alpha = 0.05$; HCOT: $U_{statistic} = 100$, $p \leq 0.001$, $\alpha = 0.05$; benzoylecgonine: $U_{statistic} = 0$, $p \leq 0.001$, $\alpha = 0.05$). The result can be explained by the lower removal of cotinine and cocaine residues and its lower dilution factor (R_6: 4.22–14.4, all studied rivers: 4.22–887), making the influence of wastewater effluent on the quality of receiving river water more significant.

Seasonal variation in levels of drug residues in the receiving waters is shown to be connected to variations in WWTP discharge, REs, dilution (i.e., river flow) and environmental conditions affecting the degradation of compounds (water temperature and exposure to sunlight) (Baker and Kasprzyk-Hordern, 2013; Mendoza et al., 2014). We observed seasonal variations (PCA: grouping of the samples) for R_3, R_5 and R_6 (Fig. S3). For the projections, nicotine residues, morphine, codeine, methadone, EDDP, methamphetamine and cocaethylene were important variables (OPLS-DA), with significantly higher concentrations obtained in winter and spring (Table S15). Exceptions were nicotine residues in R_6, where significantly higher concentrations were observed in the summer and cocaethylene concentrations in R_3, which were significantly higher in spring. Surprisingly, no seasonal variation (e.g., no grouping) was observed for R_1 and R_2 when data were analysed using PCA (Fig. S3). Similar to REs, seasonal variations in drug residues in receiving waters are also compound and receiving water-specific (significant variation observed for a limited number of compounds in different river waters).

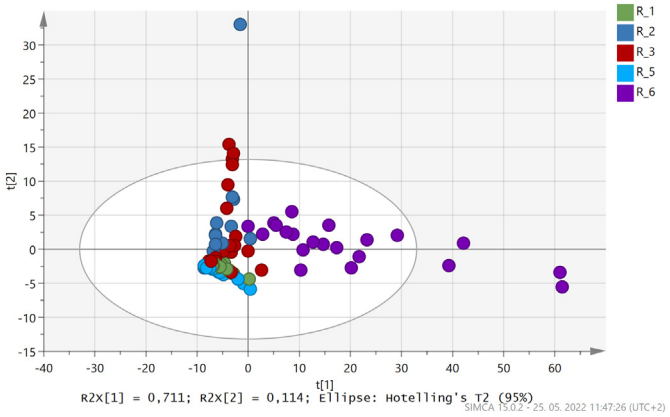

Fig. 2. Scatter plot (PCA) of samples with data on the occurrence of drug residues in the studied rivers (R_1 to R_6).

3.5. Drug residues in river water: measured vs predicted values

A comparison between measured and estimated levels of drug residues in river waters revealed that differences are within an order of magnitude (the average ratio between measured and estimated values is 10.06: 1, without WWTP_5). With only a few exceptions, measured values are higher than predicted (Fig. 3) and are likely sampling related. Sampling water from the river bank means that the complex hydrodynamics of the river section is not captured. Also, it should be noted that the river water samples were taken as grab samples. Grab sampling makes obtaining a representative river water sample more challenging (Verovšek et al., 2022). The assumption that the hydrodynamics of the river is not captured is further supported by the fact that the differences between measured and estimated concentrations are more pronounced in rivers with higher flow rates. The differences may also be related to uncertainties in the river discharge data and the representativeness of the selected stations. Such discrepancies are most noticeable for WWPT_5, where the dilution is most pronounced (WWTP_5: 108–887, other WWTPs: 4.22–121), and the nearest available hydrological station is located approximately 10 km downstream. At this site, differences between measured and estimated values are also the greatest (max ratio measured *vs* estimated values: 5691). All three nicotine residues stand out in particular, which may also be related to untreated wastewater or sources other than WWTP discharge (see Occurrence in receiving river waters). Despite some deviation, the results demonstrate that predicting environmental concentrations when reliable data on river flows are available can be an alternative to analysing river water, especially considering the cost of analysis and at locations where sampling is difficult.

3.6. Algal growth inhibition test results

Cell growth was measured in spiked samples (individually or as a mixture of drug residues) and the negative controls (Table S16). The results show that cell growth in the spiked samples was similar to that in the negative controls (Fig. 4), with negative inhibition (growth enhancement) during 0–24 h (Table S17), while no pronounced enhancing or inhibiting effect was observed with prolonged exposure (72 h and 240 h). Although based on predicted EC_{50} (Table S19), EDDP (EC_{50} = 0.04 mg/L) and THC-COOH (EC_{50} = 0.05 mg/L) were expected to affect algal growth (spiked concentrations were above predicted EC_{50}; Table S5), no significant difference in inhibition of growth rate was observed when spiked individually ($p > 0.05$, $\alpha = 0.05$). The result may be explained by (i) a reduction in the concentration of drug residues (Table S18), which may have occurred due to their transformation, adsorption (test vessel or algal cells) and uptake into algal cells (Elersek et al., 2021; Castiglioni et al., 2013) and (ii) insensitivity of the tested species (*Chlamydomonas reinhardtii*) to a particular drug residue (Rojíčková and Maršálek, 1999). Given that this is the first study looking at the effects of drug residues on green algae, no comparison with the literature is possible.

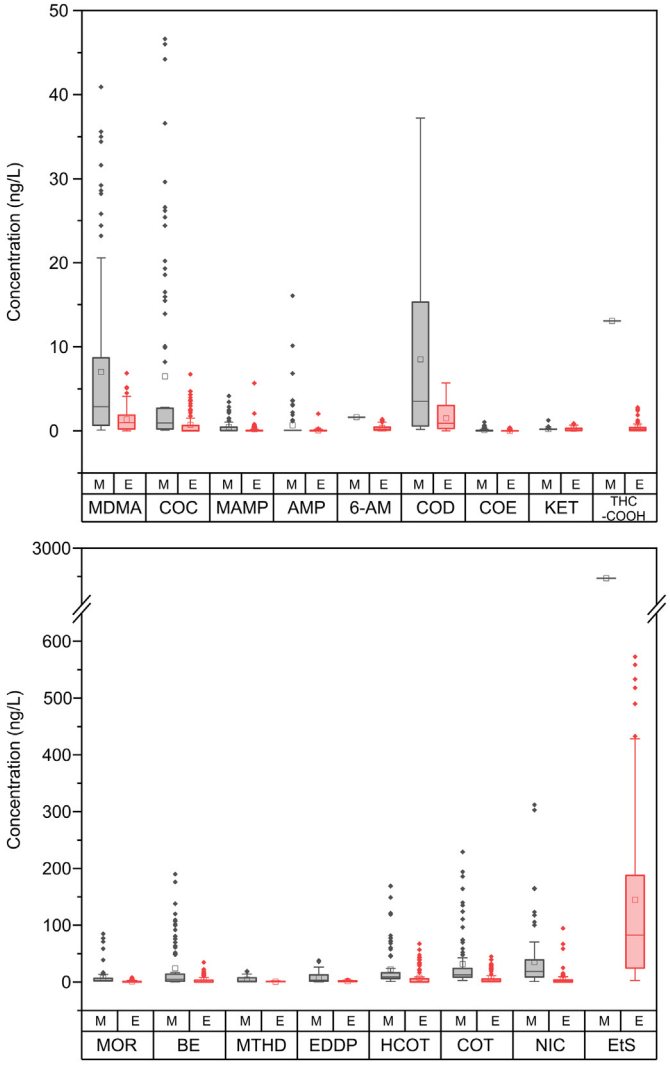

Fig. 3. Comparison and distribution (median, mean, P25–P75, min-max values and outliers) of measured (M) and estimated (E) concentrations of individual compounds in river water.

3.7. Environmental risk assessment

The ECOSAR results are given in Tables S19–S24. Although interactions between different psychoactive compounds may induce synergistic and additive effects on aquatic organisms (la Farré et al., 2008), only the effect of individual drug residues was predicted in this study. The results show that only EDDP at average (47 ng/L, RQaverage = 1.18) and maximal (155 ng/L, RQmax = 3.87) measured concentrations pose an acute risk for aquatic plants in R_2 during winter sampling.

Nicotine was the only measured drug residue in river water (except for R_5) with predicted acute effects on aquatic animals at average (3.87–185 ng/L, RQaverage = 0.06–0.92) and maximal (8.92–312 ng/L, RQmax = 0.09–1.56) levels during all seasons. The results agree with the toxic potential of nicotine reported in studies conducted in Spanish (RQ > 1, maximal concentration: 2500 ng/L (Oropesa et al., 2017)) and Italian receiving waters (RQ > 1, all sampling sites: 670–6430 ng/L [Riva et al., 2019]).

Among the medications of abuse, acute risks to aquatic animals of methadone were predicted in R_2 in winter (average concentration: 20.8 ng/L, RQaverage = 0.06; maximal concentration: 65.8 ng/L, RQmax = 0.19). Also, acute effects were predicted for EDDP at average (6.42–47.0 ng/L,

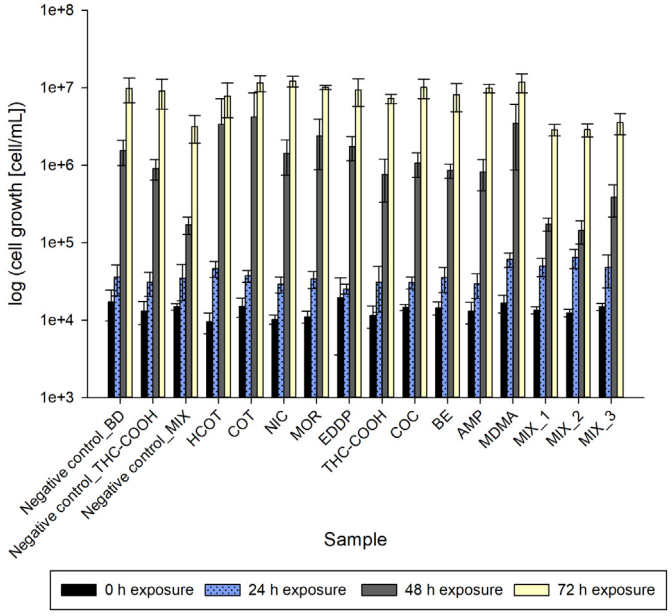

Fig. 4. Cell growth in the negative controls and individual samples after an exposure time of 0, 24, 48 and 72 h. Variability is shown as the standard deviation between all replicates.

RQaverage = 0.06–0.43) and maximal (9.38–155 ng/L, RQmax = 0.09–1.41) measured concentrations in three receiving rivers R_2, R_3 and R_6 during all seasons (except for R_3 in summer). Mastroianni et al. (2016) also reported the effects of EDDP (concentration: <50 ng/L) on aquatic organisms (at two out of 77 studied sites: RQEDDP >1; at others: RQEDDP accounted for >82 % of cumulative RQ). Although Riva et al. (2019) found that morphine had no potential toxic effect on the aquatic environment at concentrations measured in Italian surface waters (<8.2 ng/L), our data show that in R_3 (spring), morphine has the potential to affect aquatic animals (RQmax = 0.06) at the highest measured concentration (85.0 ng/L).

Among illicit drug residues, only MDMA posed acute risks to aquatic animals at the highest measured concentration (13.5 ng/L, RQmax = 0.06) in R_2 during winter and in R_6 during winter (average concentration: 23.5 ng/L, RQaverage = 0.11; maximal concentration: 35.0 ng/L, RQmax = 0.16), summer (average concentration: 30.1 ng/L, RQaverage = 0.14; maximal concentration: 40.9 ng/L, RQmax = 0.19) and spring (maximal concentration: 24.4 ng/L, RQmax = 0.11). Chronic risks for aquatic animals were predicted only for worst-case scenarios, i.e., the highest measured concentrations for nicotine (R_6, winter: 312 ng/L, RQmax = 1.10) and EDDP (R_2, winter: 155 ng/L, RQmax = 1.09).

Although the results suggest that methadone, morphine and MDMA could pose an acute risk to aquatic organisms, monitoring and regulatory actions may be warranted for nicotine and EDDP since acute and chronic effects were predicted.

4. Conclusion

While it is known that residues of psychoactive drugs are present in wastewater, there is still a lack of comprehensive data on their removal in connection with occurrence in environmental waters and especially ecotoxicity. This study aimed to fill the knowledge gaps by testing the efficiency of biological treatments (AS, MBR and MBBR) for removing known drug residues from wastewater and determining their occurrence in receiving rivers over three seasons using water analysis (LC-MS/MS) and prediction. Ecotoxicity of drug residues was predicted using in silico (ECOSAR prediction) and in vivo (algal growth inhibition test) methods. For the first time, MBBR efficiency in the removal of drug residues and ecotoxicity of drug residues for primary producers in the aquatic food chain (green algae, *Chlamydomonas reinhardtii*) were investigated. The study showed that the treatment technology employed at the WWTP affects the presence of drug residues in the receiving water, while the influence of seasons was not visible. Similar results were obtained using water analysis and prediction of environmental concentrations. Although no biologically pronounced enhancement or inhibition of green algae (*Chlamydomonas reinhardtii*) was observed, drug residues may still affect other algae species and aquatic organisms. Indeed, environmental risk assessment using ECOSAR predicted EC_{50} values indicating that nicotine, methadone, EDDP, morphine and MDMA could affect aquatic organisms at levels detected in the studied rivers. The data suggest monitoring and regulatory actions may be warranted for individual drug residues. Also, future studies may consider extending the target compound list and including a suspect and non-target analysis to obtain better insight into pollution and the effects of psychoactive substances in the environment.

CRediT authorship contribution statement

Taja Verovšek: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Ariana Šuštarič:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Maria Laimou-Geraniou:** Conceptualization, Methodology, Validation, Writing – review & editing. **Ivona Krizman-Matasic:** Conceptualization, Methodology, Writing – review & editing. **Helena Prosen:** Writing – review & editing, Supervision, Project administration. **Tina Eleršek:** Writing – review & editing, Supervision, Project administration. **Vlasta Kramarič Zidar:** Methodology. **Vesna Mislej:** Methodology. **Boštjan Mišmaš:** Methodology. **Marjeta Stražar:** Methodology. **Marjetka Levstek:** Methodology. **Bernardka Cimrmančič:** Methodology. **Simon Lukšič:** Methodology. **Nataša Uranjek:** Methodology. **Tjaša Kozlovič-Bobič:** Methodology. **Tina Kosjek:** Methodology. **David Kocman:** Methodology, Writing – original draft, Visualization. **David Heath:** Conceptualization, Writing – review & editing, Visualization. **Ester Heath:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Data availability

Data will be made available on request.

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.

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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.2022.161257.

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