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Investigation of drugs of abuse in educational institutions using wastewater analysis

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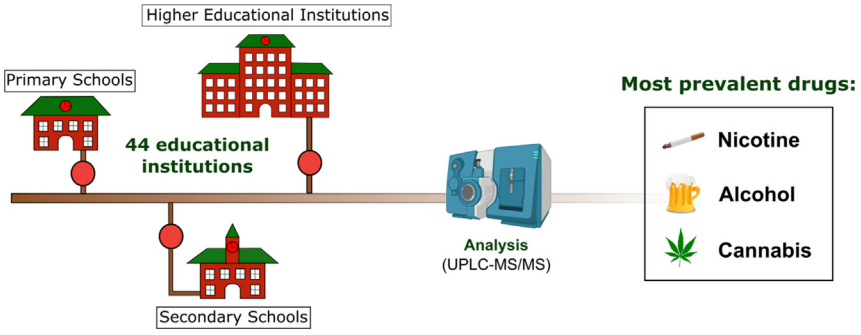
HIGHLIGHTS

- Drug prevalence in educational institutions was studied using wastewater analysis.
- Residues of drugs of abuse were present in all samples.
- Residues of nicotine, alcohol and cannabis were the most frequently detected.
- Educational level of the institute influenced consumption patterns the most.
- Drugs are present in young people's life in early stages of their education.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater analysis was used to investigate drug prevalence in primary and secondary schools and institutes of higher education located in urban and non-urban areas of six municipalities in Slovenia. Seven-hour composite raw wastewater samples from 44 educational institutions, including 19 primary schools (6–15 yrs.), ten secondary schools (15–19 yrs.), nine higher education institutions (19+ yrs.) and six mixed secondary and higher education institutions (15+ yrs.), were collected at the end of the 2018/2019 academic year. Metabolic residues of licit drugs (nicotine and alcohol), medications of abuse (morphine, codeine and methadone) and illicit drugs (cannabis, cocaine, amphetamine, methamphetamine, ecstasy and heroin) were targeted in the study. The analysis was carried out using solid-phase extraction and direct injection combined ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Biomarkers of nicotine, alcohol and cannabis intake were the most frequently detected, indicating a high prevalence of these drugs. Morphine and codeine were also detected, while among the stimulants, benzoylecgonine had the highest detection frequency. Drug differences were found between different levels of educational institution, geographic location (inter-municipality comparison) and degree of urbanization. However, t-distributed stochastic neighbour embedding (t-SNE) revealed that the level of educational institution was the main factor influencing the differences in drug prevalence. Although a good agreement between data from this study and other studies implementing wastewater analysis was observed, there was a discrepancy with Slovenian epidemiological survey data. Finally, despite certain drawbacks of the method, its application to detect drug residues in educational institutions provides a non-invasive insight into drug use trends.

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1. Introduction

Licit and illicit drug use can negatively impact a young person's health, such as cognitive functioning, psychological well-being, and future socio-economical position and success (Koprivnikar et al., 2018). The earlier a person begins using psychoactive substances, the greater the possibility that he or she will develop harmful patterns of drug use, which can lead to addiction and behavioural problems later in life; hence in Slovenia, many prevention programmes are targeted towards adolescents and young adults (Koprivnikar et al., 2018). For example, in 2017 and 2018, Slovenian non-governmental organizations organised various drug prevention and harm reduction programmes, workshops, and lectures aimed at young people, parents and school workers at the community level to aid young people in making healthy life choices (NIJZ, 2019b). However, proper implementation and evaluation of prevention programmes such as these require accurate knowledge about the extent of substance abuse (EMCDDA, 2019; López-García et al., 2019).

In Slovenia, data on drug use among 15-16-year-olds have been gathered through the European School Survey Project On Alcohol And Other Drugs (ESPAD Group, 2020) and from the Cross-National Survey On Health Behaviour In School-Aged Children (HBSC) study (Inchley et al., 2020), which surveyed 11-, 13-, 15- and 17-year-olds. Unfortunately, recall bias, the time lag in reported data and the respondents' subjectivity prevent surveys from gathering objective and timely information (Zuccato et al., 2008b). A more objective alternative is to determine selected metabolic excretion products (biomarkers) of consumed drugs in wastewater from which drug use in a community can be estimated (Gracia-Lor et al., 2017). This approach, called wastewater-based epidemiology (WBE), can provide evidence-based, objective, non-invasive, and near real-time estimates of community drug use at the regional, national and international level (Castiglioni et al., 2011; González-Mariño et al., 2019; Krizman-Matasic et al., 2019; Mastroianni et al., 2017; Zheng et al., 2017).

To date, few researchers have performed WBE studies of educational institutions (Burgard et al., 2013; Gatidou et al., 2016; Gushgari et al., 2018; Heuett et al., 2015; Panawennage et al., 2011; Verovšek et al., 2020; Zuccato et al., 2017). There have also been no studies exploring the prevalence of licit drugs, including medicines of abuse and illicit drugs, in primary and secondary schools and institutes of higher education, and knowledge of drug consumption patterns among educational institutions in areas with varying degrees of urbanization is lacking. With this in mind, paper seeks to address this knowledge gap by using wastewater analysis to investigate drug prevalence in Slovenian educational institutions in different locations classed as urban and non-urban by the Statistical Office of the Republic of Slovenia and compare the findings with available epidemiological data. In order to achieve our aim, wastewater samples from primary, secondary schools, higher education institutions (HEIs) and mixed secondary and higher education institutions (SHEIs) were analysed for metabolic residues of licit drugs (nicotine and alcohol), medications of abuse (morphine, codeine, methadone) and illicit drugs (cannabis, cocaine, amphetamine, methamphetamine, ecstasy and heroin).

2. Methods

2.1. Materials

Standard solutions (1 mg/mL) of 16 metabolic residues of licit drugs: nicotine (nicotine, cotinine, trans-3′-hydroxycotinine – HCOT) and alcohol (ethyl sulphate), medications of abuse: morphine (morphine), codeine (codeine) and methadone (methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine – EDDP), and illicit drugs: cannabis (11-nor-9-carboxy-Δ9-tetrahydrocannabinol – THC-COOH), cocaine (cocaine, benzoylecgonine and cocaethylene – a cocaine and alcohol co-consumption biomarker), amphetamine (amphetamine), methamphetamine (methamphetamine), ecstasy (3,4-methylenedioxymethamphetamine, MDMA) and heroin (6-acetylmorphine) and their labelled analogues (1 or 0.1 mg/mL) were purchased from Ceriliant Corp. (Round Rock, Texas, USA). Labelled analogues for each compound were used except for nicotine metabolites, where (±)-cotinine-d3 was used for all of them (Table S1). Working standards were prepared with final concentrations of 10 mg/L (analytes), 2 mg/L (labelled analogues of residues of medications of abuse and illicit drugs), and 0.5 mg/L (alcohol and nicotine residues labelled analogues). Methanol was purchased from JT Baker (Philipsburg, USA), LC-MS grade formic acid (HCOOH) and phosphoric acid (H_3PO_4) from Fluka (Switzerland), aqueous ammonia solution (NH3, 25%) from Merck (Darmstadt, Germany) and ammonium formate and tetrabutylammonium bromide (ion-pair reagent) from Sigma Aldrich (Missouri, USA). Millipore Direct-Q purifying system was used to obtain Milli-Q water.

2.2. Wastewater sampling

Forty-four educational institutions participated in the study. These consisted of 19 primary schools (6–15 yrs.), ten secondary schools (15–19 yrs.) comprising four gymnasiums (general education), three vocational, technical and other professional schools and three multi-programme schools, nine HEIs (19+ yrs.) comprising two institutions of social and seven of natural sciences, and six SHEIs (15+ yrs.). The location of each institution was defined as either urban or non-urban according to the Statistical Office of the Republic of Slovenia's definition. This definition considers the number of inhabitants, a surplus of jobs over the number of working people, the built-up area with urban character and number of workplaces and share of farms (Pavlin et al., 2004; SURS website). In our case, institutes were located in 37 urban and seven non-urban areas within seven municipalities (M1–7) from six different statistical regions (Fig. 1).

Sampling times, sampling location and methodology were based on a one-week preliminary study conducted at a secondary school in April 2019. Briefly, the samples obtained were analysed for 15 drug residues (ethyl sulphate was not included in the preliminary study) and results (see 3.2. Preliminary study results) were used to set appropriate days for sampling. For the full study, 40 composite raw wastewater samples (one sample per sampling site: 100 mL every 5 min over 7 h) were obtained mid-week, i.e., on either Tuesday, Wednesday or Thursday. Sampling took place at the end of the academic year 2018/2019 (May and June) except for two samples taken in March (Table S2). In specific cases, the sewer layout meant that some samples contained wastewater from more than one educational institution.

2.3. Sample preparation

For the determination of nicotine, cotinine, HCOT and ethyl sulphate, the samples were filtered through GF/D (2.7 μm, Whatman, USA), GF/C (1.2 μm, Whatman, USA) and cellulose membrane filters (0.45 μm, Sartorius, Gottingen, Germany) and spiked with labelled internal standards (final concentration: 10 ng/mL). For the determination of ethyl sulphate, the ion-pair agent (tetrabutylammonium bromide, TBA) was added to the samples (final concentration: 50 mM) to improve compound retention, peak shape and signal response (Rodríguez-Álvarez et al., 2014).

For basic drug residues, i.e., morphine, codeine, methadone, EDDP, cocaine, benzoylecgonine, cocaethylene, amphetamine, methamphetamine, MDMA and 6-acetylmorphine, and cannabinoid (THC-COOH) we used a modified method based on that of Senta et al. (2013). Briefly, each sample (125 mL) was spiked with labelled internal standards (final concentration: 60 ng/mL) and filtered through GF/D and GF/C filters (Whatman, USA). Sample pH was adjusted to pH 2 using concentrated H_3PO_4. Drug residues were extracted using solid phase extraction

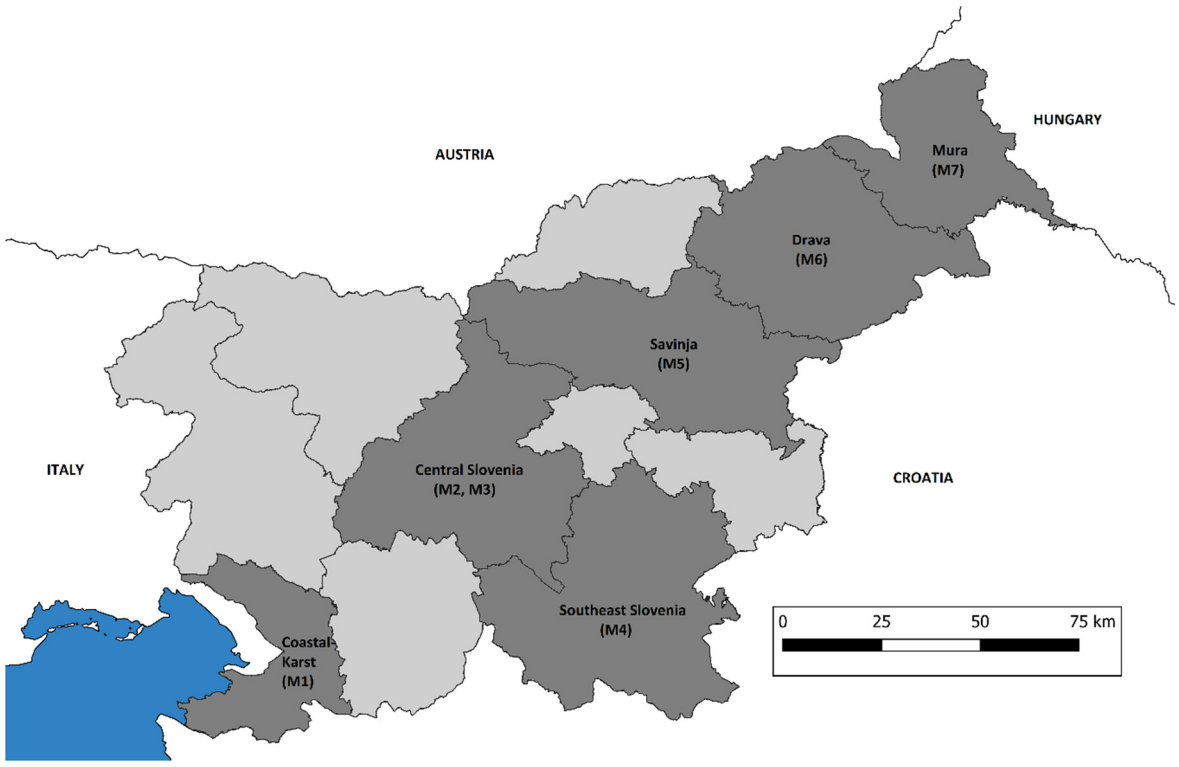

Fig. 1. Map of Slovenia showing sampling regions (dark grey) (Eurostat, GISCO; MOP, Geodesy Office, e-data; QGIS Geographic Information System).

(Oasis MCX, 150 mg/6 mL cartridges, Waters, Milford, MA, USA). After conditioning with methanol (5 mL), Milli-Q water (5 mL) and 25 mM H_3PO_4 (5 mL) and sample loading, a two-step elution was performed. Cannabinoids were eluted in the first fraction (methanol, 6 mL) and basic drug residues in the second (0.5% ammonium solution in methanol, 6 mL). Further purification of the acidified cannabinoid fraction (concentrated HCOOH, 60 μL) was performed using Strata NH_2 cartridges (200 mg/3 mL, Phenomenex, Torrance, California, USA). The analytes were eluted using 1% HCOOH in methanol (4 mL). All extracts were dried (40 °C, N_2) and reconstituted in 500 μL (250-times concentration) of either Milli-Q and methanol, 80:20, v/v with 0.1% formic acid (basic drug residues extract) or Milli-Q water and methanol, 30:70, v/v (cannabinoids-containing extract).

2.4. Analysis

Analysis was performed using a Shimadzu ultra-performance liquid chromatograph hyphenated to an AB Sciex 4500 QTRAP detector mass spectrometer (UPLC-MS/MS) with electrospray ionization (positive and negative) and operated in the multiple reaction monitoring (MRM) mode. Retention times, both transitions and the ratio between the transition peak areas were used to identify the targeted drug residues (European Union Commission Decision 2002/657/EC).

For alcohol residue analysis, 10 μL of the sample was injected on an Ascentis® Express C18 column (2 μm, 50 mm × 2.1 mm, Supelco, Pennsylvania, USA) at 40 °C. The mobile phase (flow rate: 0.3 mL/min) consisted of Milli-Q water (A) and methanol (B), containing 0.1% formic acid (elution gradient: 2% B, increase to 15% B at 10 min, 95% B at 11 min [hold: 1 min], then 2% B at 13 min). The ESI was in negative ionization mode (ESI−). For nicotine and basic drug residues, the method of Senta et al. (2013) was used. Briefly, 10 μL of the extract was injected onto a Synergi Polar-RP column (2.5 μm, 30 mm × 2 mm, Phenomenex, Torrance, California, USA) at 40 °C. In this case, the mobile phase (flow rate: 0.3 mL/min) was Milli-Q water (A) and Methanol (B) containing 5 mM ammonium formate and 0.1% formic acid (elution gradient: 2% B to 50% B at 6.9 min, 55% B at 7.3 min, 85% B at 8.7 min, 88% B at 10.7 min, and 100% B at 11 min [hold: 1.4 min]). The ESI was in the positive ion mode (ESI+). The MRM™ algorithm (MRM detection window: 120 s) was applied during acquisition. Separation of cannabinoids was achieved by injecting 10 μL of the extract on a Supelco Ascentis® Express C18 column (2 μm, 50 mm × 2.1 mm, Supelco, Pennsylvania, USA) at 40 °C using Milli-Q water (A) and methanol (B) as the mobile phase at a flow rate: 0.3 mL/min (elution gradient: 10% B to 50% B at 1.5 min, 60% B at 3.0 min [hold 4 min], increase to 85% B at 12.5 min, 10% B at 13 min [hold 2 min]). Here the ESI was operated in the negative ion mode (ESI-).

2.5. Method validation

The method was validated in terms of linearity, limits of detection (LOD), limits of quantification (LOQ), relative extraction recovery (for residues of medications of abuse and illicit drugs), matrix effect (ME), accuracy and repeatability. Linearity was determined from the calibration curve (peak area ratio of the analyte vs its labelled analogue as a function of analyte concentration) and described using the coefficient of determination (R^2). The LOD/LOQ were determined by spiking the wastewater with labelled analyte analogues at low concentrations and calculated by averaging the signal-to-noise ratios (S/N = 3 or S/N = 10) of five replicates. Relative extraction recoveries, ME, accuracy and repeatability were also calculated using spiked samples.

2.6. Statistical analysis

A statistical evaluation was performed using Excel (Microsoft, USA), SigmaPlot (version: 14.0) and R (version: 4.0.3) (Eftimov et al., 2017). The data were analysed using the non-parametric Chi-Squared test for two or more samples (95% confidence level, $\alpha = 0.05$). When differences among multiple groups were observed, a Chi-Squared post-hoc test using adjusted residuals and Bonferroni adjustment of the significance level (adjusted α) was applied. Additionally, singular value

decomposition (SVD) was used to obtain a new representation of data instances, visualized using the dimensional reduction technique t-distributed stochastic neighbour embedding (t-SNE, R library: Rtsne). Perplexity was set to ten.

2.7. Ethics and consent

Although the benefit of WBE is that it avoids many of the ethical issues associated with surveys and drug testing, privacy concerns may arise when applied to smaller specific sites, such as educational institutions (Prichard et al., 2015). Guidelines for the researchers using wastewater analysis were developed by Prichard et al. (2015) and are hosted on the SCORE (Sewage Analysis CORe group Europe) website (SCORE website, Ethical guidelines for WBE). According to the guidelines, consent must be obtained when sampling from a specific site. In the study, informed consent was obtained from the Head of each institution. In return, they received an outline of the study aims and sampling procedure. Notably, an anonymity agreement was also signed to avoid identifying an individual institution and prevent possible stigmatization, i.e., by sensationalised media reporting.

3. Results and discussion

3.1. Method validation

In general, a linear response ($R2 > 0.99$) was observed between LOQ–1000 ng/mL for the drug residues. The exceptions were ethyl sulphate, EDDP, methamphetamine (LOQ–500 ng/mL) and methadone (LOQ–200 ng/mL). The LOD and LOQ for medications of abuse and illicit drugs were in the ng/L range (LOD: 0.31–3 ng/L, LOQ: 1–9.6 ng/L), while for licit drugs, they were 19–305 ng/L and 64–1020 ng/L. The matrix effect ranged from −2% to −115% for most drug residues. Nicotine was the only compound whose signal was enhanced, i.e., by 77%, at the lower spiking concentration. Relative extraction recoveries for medications of abuse and illicit drugs were between 71 and 110%, except for EDDP (23%). Accuracy was in the 84–136% range, while repeatability was below 10% (RSD). The only exception was ethyl sulphate at low concentrations (14% RSD).

3.2. Preliminary study results

The number of detected residues did not differ statistically between sampled days ($\chi^2 = 0.220$, $p = 0.974$, $\alpha = 0.05$), but differences in the detection frequency of particular metabolites, namely MDMA and benzoylecgonine, were observed. Stimulants are known to have a distinctive weekly consumption pattern, i.e., their higher consumption at weekends (Krizman et al., 2016; Thomas et al., 2012; Zuccato et al., 2008b) may explain the presence of MDMA and benzoylecgonine only on Mondays and Fridays. Therefore, to avoid the influence of weekend use, we chose Tuesday, Wednesday and Thursday (mid-week) as the most appropriate sampling days.

3.3. General findings

Despite certain limitations (e.g., the influence of the dose, excretion rate, sample preparation and LOD on the detection of individual analytes), drug prevalence was evaluated based on detection frequency (DF = percentage of samples containing drug residues above LOD) rather than consumption estimates (Verovšek et al., 2020; Zuccato et al., 2008b). The reason being that sampling in time-proportional mode meant that, although the sampling frequency was high (100 mL/5 min), small and inconsistent wastewater flows, i.e., episodes without wastewater in the sewer, prevented the collection of some composite subdivisions. Moreover, dilution with kitchen wastewater occurred at some sampling sites, making it difficult to compare all institutions quantitatively. The latter could be overcome by normalising the data (concentrations) to mass loads using flow data. However, the data on flow was impossible to obtain for all sampled sites and accordingly, mass loads could not be calculated. Aside from accurate flow measurements, flow-proportional sampling and the use of passive samplers (e.g., Polar Organic Chemical Integrative Sampler – POCIS and passive-active samplers) are also possible solutions (Amato et al., 2021; Verovšek et al., 2020). However, the application of passive samplers is only feasible under optimum conditions (i.e., passive samplers should not dry out during sampling), and further studies on the efficacy of passive samplers are needed (Verovšek et al., 2020).

The results show that between four to ten residues were detected in the samples (see Supplementary material: Fig. S1), with six being the most frequent (25% of the samples). High DFs of nicotine biomarkers (cotinine and HCOT; Table 1) suggest a high prevalence of nicotine use in Slovenian educational institutions. Since nicotine and alcohol metabolic residues were the most commonly detected in the study, we compared their detection frequencies. Compared to nicotine biomarkers, fewer samples contain ethyl sulphate ($\chi^2 = 13.514$, $p = 0.00024$, $\alpha = 0.05$), reflecting possible differences in consumption patterns, i.e., daily use of nicotine versus recreational use of alcohol, with peak consumption occurring over the weekend (Lai et al., 2018; Reid et al., 2011; Ryu et al., 2016).

Among the opioids, morphine had the highest DF (Table 1). Morphine is the second most commonly prescribed opioid (6500 prescriptions in 2019) in Slovenia after oxycodone (NIJZ, 2019a). Besides medical morphine, morphine may also originate from the metabolism of codeine and heroin (Baselt, 2000; Zuccato et al., 2008a). It is also produced in-sewer from the degradation of 6-acetylmorphine and glucuronide conjugates (Gracia-Lor et al., 2017; Senta et al., 2014; Zuccato et al., 2008b). Accordingly, we would expect to be able to detect its presence. Also present was codeine, which is a drug prescribed to treat mild to moderate pain or as a codeine-based cough syrup, and in some instances, people can purchase it as a codeine-based over-the-counter medication (CBZ website). It is less regulated than some opiates, but users risk developing tolerance and eventually dependence (Thai et al., 2016; van Dyken et al., 2014). Heroin use is unlikely to be the primary source of morphine in the samples since 6-acetylmorphine was <LOD. Also, because only 1.3% of the heroin dose is excreted as 6-acetylmorphine (Gracia-Lor et al., 2016; Postigo et al., 2008), the amounts of 6-acetylmorphine in wastewater is expected to be small (dilution) and in-sample transformation (degradation) could result in it being below the LOD.

Table 1
Detection frequencies of targeted drug residues in all of the obtained samples (n = 40).

Drug	Drug residue	DF [%]
Tobacco (nicotine)	HCOT[a]	98
	Cotinine[a]	100
	Nicotine	100
Alcohol	Ethyl sulphate[a]	80
Morphine	Morphine[a]	40
Codeine	Codeine[a]	23
Methadone	Methadone	n.d.
	EDDP[a]	n.d.
Cannabis	THC-COOH[a]	93
Cocaine	Cocaine	75
	Benzoylecgonine[a]	50
	Cocaethylene	8
Amphetamine	Amphetamine[a]	5
Methamphetamine	Methamphetamine[a]	13
Ecstasy	MDMA[a]	15
Heroin	6-Acetylmorphine[a]	n.d.

n.d. – not detected.
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.
[a] Drug biomarker used for consumption estimation.

Aside from licit drugs (nicotine and alcohol), cannabis was the most prevalent substance in Slovenian schools (Table 1). In fact, the DF of THC-COOH was higher than ethyl sulphate. A statistical evaluation of differences between their DFs reveals that cannabis is as prevalent as alcohol, i.e., the difference in the DFs of THC-COOH and ethyl sulphate was not statistically significant ($\chi^2 = 2.635$, $p = 0.1045$, $\alpha = 0.05$). Although cannabis use is widespread among young people in Slovenia (NIJZ, 2019b), it can only be obtained legally for medical purposes (Group II: illegal drugs regulated only for medical application), whereas alcohol can be legally purchased at 18 years of age (NIJZ, 2019b).

Compared to its metabolite benzoylecgonine, cocaine was detected in a significantly higher percentage of samples ($\chi^2 = 5.333$, $p = 0.0209$, $\alpha = 0.05$; Table 1). A possible explanation is the disposal of cocaine into the sewer, either from direct disposal or handwashing or wiping residual cocaine from the surfaces into the toilet/sink after its' use. This finding may be explained by the shorter time to peak concentration (Tmax) of cocaine (2–5 h) in urine in comparison to benzoylecgonine (4–8 h) (Cone et al., 1998; Huestis et al., 2007; Jufer et al., 2000), suggesting that cocaine use is taking place in the institutions. Other stimulants such as amphetamine, methamphetamine and MDMA were only detected sporadically, which agrees with their typical consumption patterns, i.e., peak consumption occurring over the weekends (Krizman et al., 2016; Thomas et al., 2012; Zuccato et al., 2008b), or it may indicate their overall low prevalence in educational institutions in general. Notably, although drug use in Slovenian educational institutions is discussed, except for the cocaine results, the presence of drug residues in samples may not necessarily indicate drug use directly in the institutions since the majority of drug residues under investigation have long excretion times (Table S3) and may be consumed elsewhere and only excreted in the institutions, e.g., at home.

3.4. Drug prevalence vs the level of educational institution

On average, six drug residues (min = 4, max = 9) were detected in primary school samples (Table S4). A significant difference ($\chi^2 = 14.843$, $p = 0.0006$, $\alpha = 0.05$) was observed in the number of samples containing nicotine, alcohol and cannabis biomarkers (Table 2). The results also reveal a significantly higher number of primary school samples containing nicotine biomarkers (Chi-squared post hoc test: $\chi2 = 9.896$, $p = 0.0035$, adjusted $\alpha = 0.0083$) and a significantly lower number of primary school samples containing ethyl sulphate (Chi-squared post hoc test: $\chi^2 = 13.194$, $p = 0.0007$, adjusted $\alpha = 0.0083$). The order of prevalence of drugs in primary schools was nicotine>cannabis>alcohol. Among medications of abuse, the use of morphine and codeine was observed (Table 2). The results also reveal the use of cocaine (DF of benzoylecgonine = 37%) and methamphetamine and that cocaine and alcohol were co-consumed (detection of cocaethylene). Unfortunately, since, to our knowledge, this is the first application of WBE to investigate residues of licit drugs, medications of abuse and illicit drugs in primary schools, it is not possible to compare the results with other primary schools in other countries.

Secondary school samples contained an average of eight drug residues (min = 6, max = 10, Table S4). Also, nicotine, alcohol, and cannabis were equally prevalent, and medications of abuse (e.g., morphine and codeine) were detected (Table 2). Amphetamine was the only stimulant not detected in secondary schools (Table 2). The findings agree with Zuccato et al. (2017), who studied drug consumption in eight Italian secondary schools (age 15–19). Contrary to our study, methamphetamine and MDMA were not detected. On average, a different number of drug residues was observed in samples from secondary schools implementing different educational programmes (Table S5), although the difference was not significant ($\chi^2 = 0.711$, $p = 0.7008$, $\alpha = 0.05$). There were, however, differences in the type of drugs detected (Table 3). For example, only in vocational and technical schools were morphine, codeine, and MDMA found together, while methamphetamine was only identified in multi-programme schools. Although ethyl sulphate, cocaine, and benzoylecgonine were found in all samples from vocational and technical schools (Table 3), cocaethylene indicating alcohol and cocaine co-consumption was not detected. The lack of cocaethylene suggest that these substances were consumed separately (by different persons); however, their low concentration in wastewater, possibly due to low excretion in urine (0.7% of cocaine dose excreted in 24 h (Gracia-Lor et al., 2017)) and sampling difficulties may explain why they were not detected. Further studies, including a higher number of participating secondary schools, are needed to obtain more accurate data.

On average, eight drug residues (min = 5, max = 10) were detected in HEI (Table S4). Nicotine, alcohol and cannabis were also the most common suggesting their equal prevalence (Table 2). Among the medications of abuse, morphine showed a high prevalence (DF = 83%). Overall, the results agree with WBE studies previously conducted on different university campuses. For example, alcohol consumption was observed in a university campus in Greece with ethyl sulphate detected in all samples (Gatidou et al., 2016), a high DF of morphine (79%) was obtained on a US university campus (Heuett et al., 2015), and cannabis was reported to be one of the most frequently used drugs in two studies conducted in the US (Heuett et al., 2015; Panawennage et al., 2011). In these studies, cocaine and amphetamine were frequently reported. In the present study, the use of stimulants, such as cocaine (DF of benzoylecgonine = 67%), amphetamine (DF = 33%) and MDMA (DF = 50%) was observed, and although amphetamine was not as prevalent as cocaine, it was only specific to HEIs (Table 2). Also, a difference in the number of drug residues detected in HEIs offering different higher educational programmes (Table S5) is observed but is not statistically significant ($\chi^2 = 2.522$, $p = 0.1123$, $\alpha = 0.05$). However, there is an observable difference in the DF of drug residues, namely all residues had DF = 100% in HEIs offering social sciences, while different DFs were obtained in HEIs offering natural sciences (Table 3). Despite this, only amphetamine was observed in a statistically higher number of HEIs offering social sciences ($\chi^2 = 6.0$, $p = 0.0143$, $\alpha = 0.05$), suggesting its higher prevalence in those HEIs, although a higher number of samples from each type of HEIs would be needed to confirm this finding. Similar to vocational and technical secondary schools, alcohol and cocaine can be assumed to be consumed separately (ethyl sulphate, cocaine and benzoylecgonine were detected in all samples, while cocaethylene was <LOD; Table 3). However, non-detection due to the low concentration of cocaethylene in wastewater cannot be excluded. The SHEI samples contained an average of seven drug residues (min: 5, max: 10, Table S3). This finding is similar to that for secondary schools

Table 2
Detection frequencies (%) of targeted drug residues in samples from educational institutions of different level.

Drug residue	Primary schools (n = 19)	Secondary schools (n = 8)	HEIs (n = 6)	SHEIs (n = 7)
HCOT	95	100	100	100
Cotinine	100	100	100	100
Nicotine	100	100	100	100
Ethyl sulphate	58	100	100	100
Morphine	37	25	83	29
Codeine	21	38	17	14
THC-COOH	84	100	100	100
Cocaine	68	100	67	71
Benzoylecgonine	37	63	67	57
Cocaethylene	11	n.d.	n.d.	14
Amphetamine	n.d.	n.d.	33	n.d.
Methamphetamine	16	13	n.d.	14
MDMA	n.d.	13	50	29

Methadone, EDDP, 6-acetylmorphine were not detected in any of the samples.
n.d. – not detected (<LOD).
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, HEI – higher education institution, MDMA – 3,4-methylenedioxymethamphetamine, SHEI – mixed secondary and higher education institution, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

Table 3
Detection frequencies (%) of targeted drug residues in samples of educational institutions regarding educational programme classification.

Drug residue	Gymnasiums (n = 3)	Vocational and technical schools (n = 2)	Multi-programme schools (n = 3)	HEIs for natural sciences (n = 4)	HEIs for social sciences (n = 2)
HCOT	100	100	100	100	100
Cotinine	100	100	100	100	100
Nicotine	100	100	100	100	100
Ethyl sulphate	100	100	100	100	100
Morphine	n.d.	50	33	75	100
Codeine	67	50	n.d.	25	n.d.
THC-COOH	100	100	100	100	100
Cocaine	100	100	100	50	100
Benzoylecgonine	67	100	33	50	100
Cocaethylene	n.d.	n.d.	n.d.	n.d.	n.d.
Amphetamine	n.d.	n.d.	n.d.	n.d.	100
Methamphetamine	n.d.	n.d.	33	n.d.	n.d.
MDMA	n.d.	50	n.d.	25	100

Methadone, EDDP, 6-acetylmorphine were not detected in any of the samples.
n.d. – not detected (<LOD).
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, HEI – higher education institution, MDMA – 3,4-methylenedioxymethamphetamine, SHEI – mixed secondary and higher education institution, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

regarding the type of drugs present (Table 2), although cocaine and alcohol co-consumption was detected in SHEIs (Table 2).

Overall, no statistical difference in the number of detected residues was observed between primary, secondary schools, HEIs and SHEIs ($\chi^2 = 5.345$, $p = 0.1482$, $\alpha = 0.05$) although different types of drugs prevailed. Additionally, the data was visualized, and similarities between samples were explored using a dimensional reduction technique (t-SNE). In this case, two groups are observed when the data are arranged (in 2D space) based on the level of educational institution (Fig. 2). Group1 (lower left) mainly consisted of primary school samples and Group2 (upper right) of institutions offering secondary and higher education, suggesting a difference in drug prevalence observed is influenced by the level of the educational institution. Mostly, there is a difference between primary schools and the others.

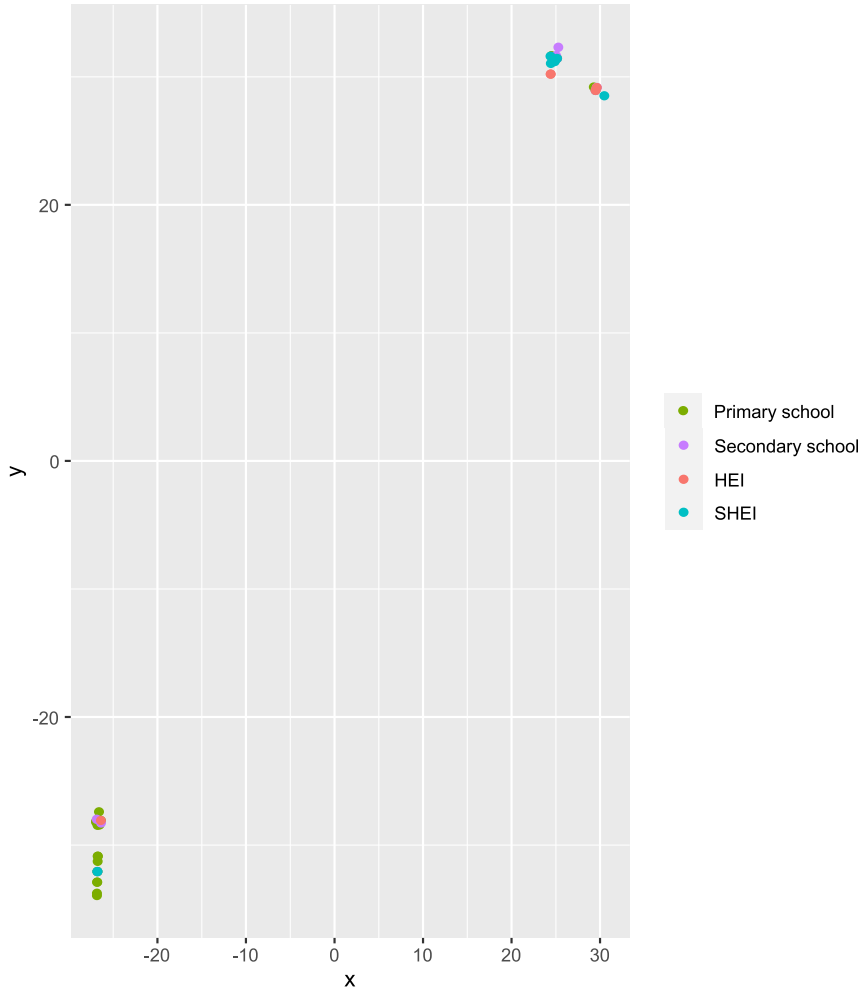

Fig. 2. SVD followed by t-SNE visualization of the data in 2D space (HEI – higher educational institution, SHEI – secondary and higher educational institution).

3.5. Geographic location vs drug prevalence

On average, a different number of drug residues were detected in samples from primary, secondary schools, HEIs and SHEIs from different municipalities. However, the differences were not statistically significant (Table S6). In addition, regardless of educational institute level, similar numbers of drugs were present in each municipality ($\chi^2 = 1.981$, $p = 0.9214$, $\alpha = 0.05$). Except for tobacco, alcohol, cannabis and cocaine, all other drug types varied. All investigated drugs were detected (biomarkers >LOD) in M2, i.e., Ljubljana, Slovenia's capital (Table 4). At least one prescription drug (morphine or codeine) was used in educational institutions in each municipality, except for M4 (primary schools and SHEIs), where neither were detected. Cocaine and alcohol co-consumption was observed in three municipalities: M2 (SHEIs), M5 (primary schools) and M6 (primary schools). Although ethyl sulphate and cocaine metabolites (cocaine and benzoylecgonine) were detected in all secondary school samples from M2 and M5 and SHEI samples from M4, cocaethylene was not detected. As already discussed, its absence suggests that alcohol was not co-consumed with cocaine suggesting consumption by different persons, but there is the possibility that cocaethylene is below the LOD. Amphetamine was detected only in M2, which is expected since it is specific to HEIs, seven of which are located in this municipality. Methamphetamine was detected in a higher number of municipalities than MDMA from

Table 4
Detection frequencies (%) of drug residues in educational institutions.

Statistical region		Costal-Karst	Central Slovenia		Southeast Slovenia	Savinja	Drava	Mura
Municipality (number of obtained samples)		M1 (n = 2)	M2 – Ljubljana (n = 16)	M3 (n = 6)	M4 (n = 4)	M5 (n = 6)	M6 (n = 3)	M7 (n = 3)
Drug residue	Samples	Detection frequency (%)						
HCOT	Primary school samples	100	100	100	100	100	67	100
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Cotinine	Primary school samples	100	100	100	100	100	100	100
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Nicotine	Primary school samples	100	100	100	100	100	100	100
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Ethyl sulphate	Primary school samples	100	60	33	100	67	33	50
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Morphine	Primary school samples	n.d.	20	67	n.d.	n.d.	100	50
	Secondary school samples	100	25	n.d.	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	80	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	n.d.	n.d.	n.a.	100
Codeine	Primary school samples	n.d.	n.d.	33	n.d.	33	67	n.d.
	Secondary school samples	n.d.	75	n.d.	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	20	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	n.d.	n.d.	n.a.	n.d.
THC-COOH	Primary school samples	100	100	100	50	100	67	50
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Cocaine	Primary school samples	100	80	67	100	33	100	n.d.
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	80	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	100	50	n.a.	100
Benzoylecgonine	Primary school samples	n.d.	40	33	50	33	67	n.d.
	Secondary school samples	100	75	n.d.	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	80	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	100	50	n.a.	n.d.
Cocaethylene	Primary school samples	n.d.	n.d.	n.d.	n.d.	33	33	n.d.
	Secondary school samples	n.d.	n.d.	n.d.	n.a.	n.d.	n.a.	n.d.
	HEI samples	n.a.	n.d.	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	n.d.	n.d.	n.a.	n.d.
Amphetamine	Primary school samples	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Secondary school samples	n.d.	n.d.	n.d.	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	40	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.	n.d.
Methamphetamine	Primary school samples	n.d.	20	33	n.d.	n.d.	n.d.	50
	Secondary school samples	n.d.	n.d.	50	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	n.d.	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	n.d.	n.a.	50	n.d.	n.a.	n.d.
MDMA	Primary school samples	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Secondary school samples	n.d.	25	n.d.	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	60	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	n.d.	n.a.	100	n.d.	n.a.	n.d.

Methadone, EDDP, 6-acetylmorphine were not detected in any of the samples.
n.a. – not applicable (no samples were obtained), n.d. – no detected (<LOD).
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, HEI – higher education institution, MDMA – 3,4-methylenedioxymethamphetamine, SHEI – mixed secondary and higher education institution, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

different statistical regions of Slovenia (four vs two). Both stimulants were detected in M2 (primary, secondary schools and HEIs) and M4 (SHEIs), while none were detected in M1, M5 and M6. No grouping of the results based on geographic location was observed in the t-SNE visualization (Fig. S2), suggesting other variables than municipality play a role in drug use trends.

3.6. Urban vs non-urban areas

An insufficient number of samples meant that only primary schools in urban (n = 13) and non-urban (n = 6) areas were used to explore differences due to urbanization. On average, six drug residues were detected in both urban (min = 5, max = 9) and non-urban (min = 4 max = 8) locations (Table S7), with no significant difference in the number of drugs residues detected ($\chi^2 = 0.425$, $p = 0.5144$, $\alpha = 0.05$). However, there was a difference in DFs of drug residues (Table 5). Although results obtained in other studies conducted in different sized cities (Krizman et al., 2016) and population type, i.e., capital and villages (Gatidou et al., 2016) suggest a higher drug consumption in urban areas. No such conclusion can be drawn in our study since individual drug consumption estimates were not calculated. When DFs of THC-COOH and ethyl sulphate are compared, the prevalence of cannabis was high and similar to that of alcohol (urban samples: $\chi^2 = 3.467$, $p = 0.0626$, $\alpha = 0.05$, non-urban samples: $\chi^2 = 0.343$, $p = 0.5582$, $\alpha = 0.05$). Urban samples also contained significantly more cocaine than its metabolite, benzoylecgonine ($\chi^2 = 3.939$, $p = 0.0472$, $\alpha = 0.05$), suggesting a connection between cocaine availability or its use in institutions and urbanization (see 3.3. General findings). According to the "Report on the drug situation in 2019 of the Republic of Slovenia" ("NIJZ, 2019b"), illicit drugs are more readily obtained in larger urban areas, which explain their availability in educational institutions. However, additional information is needed to support this claim. Interestingly, cocaine co-consumption with alcohol was only observed in urban areas (cocaethylene >LOD; Table 5). Also, there was no statistical difference ($\chi^2 = 2.030$, $p = 0.1542$, $\alpha = 0.05$) between the DF of methamphetamine in urban and non-urban samples. Despite this, no urban–non-urban grouping was observed in the t-SNE visualization (Fig. S3). However, there are only seven non-urban samples, so a higher number of samples from educational institutions located in non-urban areas are needed to confirm this finding.

3.7. Comparison with available epidemiological data

The results were compared with survey data from ESPAD 2015 (NIJZ, 2017) and HBSC 2018 (NIJZ, 2019b) and WBE data from SCORE 2019 covering six Slovenian municipalities (EMCDDA, Wastewater-based epidemiology and drugs topic page; SCORE-ES1307 COST Action). Despite our results, caution should be taken when extrapolating the results to schoolchildren and students since residues may also originate from members of staff and visitors since wastewater analysis only provides drug consumption of the whole population. There is also no socio-epidemiological data on drug consumption by specific groups (e.g., teachers, staff and visitors) associated with education in Slovenia, making it impossible to estimate the contribution from individual groups in educational institutions.

Differences in the DF of nicotine and alcohol biomarkers suggest a higher prevalence of nicotine than alcohol in Slovenian educational institutions ($\chi^2 = 13.514$, $p = 0.00024$, $\alpha = 0.05$). This finding contradicts the survey data, which suggests that alcohol use is more significant than tobacco among adolescents (Table S8). Differences in nicotine and alcohol use may explain this discrepancy, i.e., daily vs recreational use (Lai et al., 2018; Reid et al., 2011; Ryu et al., 2016).

The presence of codeine and morphine could indicate their therapeutic use, although codeine misuse cannot be ruled out. Codeine, for example, can be easily purchased over-the-counter in Slovenia (CBZ website), and it is reported in the ESPAD 2015 (NIJZ, 2017) that painkillers were used to get high by 2% of 15–16-year olds (Table S8). However, the low percentage of students using painkillers to get high is likely to mean that higher DFs (Tables 2 and 3) originate from (medical) usage by other groups of people present, although additional data is needed to support such a claim. In contrast, neither methadone nor EDDP was detected. Their absence could be explained by the fact that 65% of problem opioid users are between the ages 31 and 40 according to the Opioid Substitution Treatment program data (OST) (NIJZ, 2019b), whereas 41.8% of people ending higher education were <25 of years of age (SURS website).

Cannabis was the most common illicit drug in Slovenian educational institutions. This finding agrees with the survey data (Table S8), although the observed equal prevalence of cannabis and alcohol ($\chi^2 = 2.635$, $p = 0.1045$, $\alpha = 0.05$) does not, i.e., the survey data report alcohol as the most common drug (Table S8). Another discrepancy is in the use of stimulants. In our study, cocaine was the most prevalent, while amphetamine, methamphetamine and MDMA were detected only occasionally, whereas the survey data (ESPAD 2015; 15-16-years-olds and HBSC 2018; 17-year-olds) show the lifetime use of stimulants to be much more prevalent (Table S8). However, the results agree with the SCORE 2019 data (the year the study was performed), which show a higher prevalence of cocaine over other stimulants in the general population (EMCDDA, Wastewater-based epidemiology and drugs topic page; SCORE-ES1307 COST Action). Heroin was not detected, even though 1% of 15–16-year olds and 0.8% of 17-year olds reported using the drug (Table S8).

Overall, the results are inconsistent with those obtained by epidemiological surveys conducted in Slovenia. However, the discrepancy may result from differences in the methodology used (wastewater analysis vs questionnaires), time and mode of sampling/surveying, and reporting of results. Moreover, the number of samples containing individual drug biomarker(s) was used to predict drug prevalence, which means that no information can be obtained on the actual number of drug users at individual sites, while survey results offer direct insight into the number of users.

Table 5
Detection frequencies (%) of drug residues in primary schools located in urban and non-urban areas.

Drug residue	Urban	Non-urban
HCOT	92	100
Cotinine	100	100
Nicotine	100	100
Ethyl sulphate	62	50
Morphine	38	33
Codeine	23	17
THC-COOH	92	67
Cocaine	77	50
Benzoylecgonine	38	33
Cocaethylene	15	n.d.
Amphetamine	n.d.	n.d.
Methamphetamine	8	33
MDMA	n.d.	n.d.

Methadone, EDDP, 6-acetylmorphine were not detected in any of the samples.
n.d. – not detected.
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

4. Conclusions

The prevalence of licit drugs, medicines of abuse and illicit drugs was investigated in Slovenian educational institutions. In general, nicotine, alcohol and cannabis had the highest DFs. The most common medications of abuse were morphine and codeine, while cocaine was the most commonly detected stimulant. The number of detected residues did not vary between educational institutions regarding the level of education offered, geographic location and urbanization, but there were

differences in DFs and the type of drugs present. Amphetamine, for example, was detected only in HEIs. Ljubljana (M2) produced the greatest variety of drug residues, and urban areas were related to higher cocaine availability (evidenced by unused cocaine entering the sewer) and alcohol co-consumption. The level of educational institutions mainly influenced drug use patterns, i.e., differences were observed mainly between primary schools and other institutions. The observed DFs also agreed with other WBE studies conducted in educational institutions and the SCORE 2019 WBE study. Other variables that influence drug consumption patterns, however, require further exploration.

Overall, wastewater analysis is useful for investigating drug use in site-specific settings such as educational institutions since it is non-invasive and produces objective data in near-real-time. Importantly, the result obtained in this study may deepen our understanding of when drugs enter young peoples' lives and which drugs are the most common in different stages of education. Despite its many advantages, applying wastewater analysis to educational institutions is not without limitations. Inconsistent wastewater flows (e.g. periods with no flow) and different dilution factors (e.g. institutions with kitchens and those without), and, in this case, a lack of accurate flow data meant that it was impossible to quantify drug use, and such issue will need to be addressed in future site-specific studies. However, it did allow a comparison of drug types used. Also, maintaining an institution's anonymity in small catchments can be problematic, and disclosure could result in negative attention. Finally, caution is needed when interpreting the results since it is difficult to distinguish between students, staff and visitors.

CRediT authorship contribution statement

Taja Verovšek: Conceptualisation, Methodology, Validation, Investigation, Formal analysis, Data Curation, Writing – Original Draft, **Ivona Krizman-Matasic:** Conceptualisation, Methodology, Writing – Review & Editing, **David Heath:** Conceptualisation, Writing – Review & Editing, **Ester Heath:** Conceptualisation, Writing – Review & Editing, Supervision, Project administration.

All authors reviewed the manuscript and contributed to improving the quality of this paper.

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.2021.150013.

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