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Screening for new psychoactive substances in wastewater from educational institutions

Taja Verovšek [a,b], Alberto Celma [c,d], David Heath [a], Ester Heath [a,b,**], Félix Hernández [d], Lubertus Bijlsma [d,*]

[a] Jožef Stefan Institute, Jamova 39, 1000, Ljubljana, Slovenia
[b] Jožef Stefan International Postgraduate School, Jamova 39, 1000, Ljubljana, Slovenia
[c] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences (SLU), SE-750 07, Uppsala, Sweden
[d] Research Institute for Pesticides and Water, University Jaume I, Avda Sos Baynat s/n, 12006, Castellón, Spain

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ABSTRACT

Drug (ab)use among young people is a serious issue, negatively impacting their well-being and prospects. The emergence of new psychoactive substances (NPS) further complicates the situation as they are easily accessible (e.g., online), but users are at high risk of intoxication as their chemical identity is often unknown and toxicity poorly understood. While surveys and drug testing are traditionally used in educational institutions to comprehend drug use trends and establish effective prevention programs, they are not without their limitations. Accordingly, we investigated the occurrence of NPS in educational institutions through wastewater analysis and critically evaluated the viability of the approach. The study included eight wastewater samples from primary schools (ages 6–15 years), six from secondary schools (ages 15–19 years), three from institutions for both secondary and higher education (ages 15+), and six from higher educational institutions (ages 19+). Samples were obtained mid-week and evaluated in two Slovenian municipalities; the capital Ljubljana and a smaller one (M1). Samples were screened using liquid chromatography-ion mobility-high-resolution mass spectrometry (LC-IMS-HRMS), and NPS identified at three levels of confidence (Level 1: unequivocal, Level 2: probable, Level 3: tentative) from a suspect list containing over 5600 entries. NPS were identified in all types of educational institutions. Most were synthetic stimulants, with 3-MMC, ephedrine, 4-chloro-α-PPP, and ethcathinone being unequivocally identified. Also, NPS were present in wastewater from all educational institution types revealing potential spatial but no inter-institutional trends. Although specific groups cannot be targeted, the study, as a proof-of-concept, demonstrates that a suspect screening of wastewater employing LC-IMS-HRMS can be used as a radar for NPS in educational institutions and potentially replace invasive drug testing.

1. Introduction

According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the term New Psychoactive Substances (NPS) refers to "narcotic or psychotropic drugs, in pure form or in preparation, that are not controlled by the United Nations drug conventions, but which may pose a public health threat comparable to that posed by substances listed in these conventions" (EMCDDA, 2022a). NPS are not necessarily newly synthesized substances, as the term suggests, but rather substances that have recently emerged on the drug market (UNODC, 2023). They are a broad and diverse category of drugs that are usually designed to mimic the effects of illegal drugs such as cocaine, cannabis and ecstasy and can be classified as synthetic cannabinoids, synthetic hallucinogens, dissociative drugs, synthetic stimulants, synthetic depressants and natural psychoactive compounds. (EMCDDA, 2022b). The NPS market is large and rapidly evolving due to the relative ease of synthesising NPS through minor modifications in their chemical structure. For example, the EU's Early Warning System reported the emergence of 52 NPS in 2021 alone (EMCDDA, 2022a; EMCDDA, 2022b). Furthermore, a wide range of NPS can be easily obtained online with little to no information about their identity and toxicity, increasing the risk of intoxication and drug-related death, as evidenced by

* Corresponding author. University Jaume I, Avda Sos Baynat s/n, 12006, Castellón, Spain.
** Corresponding author. Jožef Stefan Institute, Jamova 39, 1000, Ljubljana, Slovenia.
E-mail addresses: Ester.Heath@ijs.si (E. Heath), bijlsma@uji.es (L. Bijlsma).

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drug-induced fatalities associated with the emergence of new benzodiazepines (EMCDDA, 2021; EMCDDA, 2022b).

Experimenting with NPS use among young people is of particular concern since, according to European School Survey Project on Alcohol and Other Drugs (ESPAD), 3.4% of 15-16-year-olds have reported using any NPS at least once (ESPAD, 2019). Studying NPS among young people is also essential to understand the motivations and risks associated with their use and to successfully implement prevention, education, and harm reduction programs, especially since the onset of risky drug consumption patterns has been linked to early drug use (NIJZ, 2019). Additionally, it can provide valuable insights into the changing drug landscape and inform public health strategies and law enforcement authorities (EMCDDA, 2021).

Educational institutions have been identified as an important setting for studying trends in drug use among young people (Sznitman et al., 2012), where surveys are commonly used to address substance and NPS use among schoolchildren and students, such as ESPAD. However, in addition to biased reporting and time lag in reported data, surveys raise ethical considerations and may require parental or respondent consent to be conducted (ESPAD, 2019). In another approach, known as "drug testing", pupils can be tested for drug use by either targeted or random urine analysis. Drug testing can be intrusive and seen as an invasion of privacy. Accordingly, it may create a hostile atmosphere, discouraging pupils from engaging in healthy behaviour (Sznitman et al., 2012). Moreover, due to the targeted nature of drug testing, substances can go undetected, especially for NPS, which are constantly changing (Sznitman et al., 2012; Vaccaro et al., 2022). Equally, there is no evidence to suggest that drug testing effectively deters drug use among young people (Sznitman et al., 2012).

Wastewater-based epidemiology (WBE) has become an increasingly popular approach for estimating drug consumption by analysing wastewater for metabolic drug residues (biomarkers) and has proven effective in providing complementary and near real-time objective data (Bade et al., 2023; Bijlsma et al., 2019; Salgueiro-González et al., 2022). Its non-invasive nature also makes it suitable for investigating drug prevalence in vulnerable populations, given that the data obtained cannot be traced to individuals, thereby ensuring anonymity (Verovšek et al., 2020). However, studies looking into NPS in educational institutions have focused on the targeted analysis of a few selected biomarkers, thus limiting the number of NPS that can be covered (Heuett et al., 2015; Panawennage et al., 2011; Zuccato et al., 2017). A potential solution is to use high-resolution mass spectrometry (HRMS), which provides full-spectrum accurate-mass data and allows non-targeted analysis and suspect screening for thousands of compounds (Klingberg et al., 2022). In addition, coupling HRMS with advanced separation techniques, such as ion mobility (D'Atri et al., 2017), can provide additional dimensions to the screening process, including collision cross-section values (CCS), thereby increasing confidence in compound identification (Bade et al., 2020; Celma et al., 2020). This extra information is especially important when monitoring NPS, as it is typically impossible to have all reference standards available in the laboratory for unequivocal identification (Bijlsma et al., 2021). Moreover, when acquiring in data independent acquisition mode, ion mobility allows to obtain cleaner mass spectra which facilitates data interpretation (Bijlsma et al., 2021).

In this study, we conducted a comprehensive screening strategy to assess the presence of NPS in wastewater samples from primary schools, secondary schools, institutions for secondary and higher education (SHEIs), and higher educational institutions (HEIs) within two different-sized municipalities in Slovenia. We included an extensive dataset of over 5600 entries in the screening workflow and compared the obtained data with available socio-epidemiological information. The goal of this proof-of-concept study was twofold: first, to evaluate the viability of using a complementary target and suspect screening for the detection and identification of NPS in wastewater, and second to get more insight on the presence of NPS in educational institutions by employing the WBE approach.

2. Methods

2.1. Wastewater samples and participating educational institutions

Raw wastewater samples (n = 23), consisting of 7-h composites, collected mid-week at the end of the 2018/2019 academic year in March, May and June, were taken directly from the main sewer outlet of an individual institution using an autosampler working in the time-proportional mode, *i.e.,* every 5 min, 100 mL of wastewater was sampled (Verovšek et al., 2021a, 2021b). Although the sewer layout was examined and sampling conducted by trained personnel from municipal WTTPs, the construction of sewers in two cases made it impossible to obtain samples from the two institutions individually. Nevertheless, the results were included since, in all cases, the samples cover educational institutions of the same type.

Samples collected included eight samples from primary schools (6–15 yrs), six from secondary schools (15–19 yrs), three from SHEIs (15+ yrs) and six from HEIs (19+ yrs) located within two municipalities; Ljubljana and smaller municipality (M1). Both municipalities offer all levels of education but differ in character and size, with Ljubljana being the capital city and M1 being more provincial and a third the size. Seventeen of the 23 samples were from Ljubljana, and six were from M1. After collection, samples were stored at −20 °C. Samples were shortly defrosted once for the analysis of illicit drugs, prior to the analysis of NPS.

2.2. Chemicals and materials

Methanol (HPLC and LC-MS grade) was purchased from J.T. Baker (Philipsburg, USA), orthophosphoric (H_3PO_4, ≥85%, LC-MS grade) and formic (HCOOH) acid from Fluka (Switzerland) and aqueous ammonium (25%) from Merck (Darmstadt, Germany). Filters, namely glass microfiber filters (GF/D and GF/C) and nylon centrifugal filters, were obtained from Whatman (USA) and VWR (USA), respectively. Oasis (HLB and prime MCX) extraction cartridges were purchased from Waters (Milford, MA, USA) and Strata NH_2 extraction cartridges from Phenomenex (Torrance, California, USA). CORTECS chromatographic column was from Waters (Milford, MA, USA).

2.3. Sample preparation

All samples were processed using previously developed protocols (A and B). In order to ensure the extraction of the broadest possible range of psychoactive substances, these methods involved solid-phase extraction (SPE) using two stationary phases: Oasis HLB in protocol A (Celma et al., 2019) and MCX in protocol B (Verovšek et al., 2023).

2.3.1. Protocol A

Briefly, 25 mL of centrifuged wastewater (6000 rpm, 5 min; Domel, Centric CF 48, Slovenia) was loaded on an Oasis HLB (60 mg) SPE cartridge preconditioned with 6 mL of methanol and 6 mL of Milli-Q (Millipore Direct-Q purifying system). After sample loading, the cartridge was washed with 50 mL of Milli-Q and left under vacuum to dry. The retained compounds were eluted using 1 mL of methanol, and the resulting eluent was then dried using N_2 gas at 40 °C. Samples were reconstituted in 250 μL of methanol: Milli-Q (10:90, v/v) and filter-centrifuged (0.2 μm modified nylon centrifugal filter) at 14,000 RCF for 3 min before the analysis. All samples were stored at −20 °C prior to analysis.

2.3.2. Protocol B

The sample (125 mL) of filtered wastewater (glass microfiber filters: GF/D – 2.7 μm and GF/C – 1.2 μm) was first acidified (pH 2, ≥85% H_3PO_4) before being loaded on Oasis prime MCX (150 mg) SPE cartridge and dried using a vacuum manifold. Retained compounds were first eluted with 6 mL of methanol (fraction B1) and then with 6 mL of 0.5% ammonia in methanol (fraction B2). After elution, fraction B2 was dried (40 °C, N_2), reconstituted in 500 μL of 0.1% HCOOH and filter-centrifuged (14,000 RCF, 3 min, modified nylon, 0.2 μm), while fraction B1 was acidified (60 μL of HCOOH) and purified using a Strata NH_2 (200 mg) SPE cartridge. After preconditioning with 4 mL of 1% HCOOH in methanol, the eluate (acidified B1) was passed through the cartridge and recollected, allowing interfering compounds to be retained. After drying (40 °C, N_2), the recollected fraction B1 was reconstituted in 500 μL of methanol: Milli-Q (70:30, v/v) and filter-centrifuged (14,000 RCF, 3 min, modified nylon, 0.2 μm). Both fractions B1 and B2 of all samples were stored at −20 °C prior to analysis.

2.4. Sample analysis

Sample analysis is based on Celma et al. (2020). The compounds were separated on a CORTECS C18 fused core column (2.1 × 100 mm, 2.7 μm) maintained at a temperature of 40 °C. Mobile phases consisting of Milli-Q (A) and methanol (B), both containing 0.01% HCOOH, were used, and the flow rate was set to 0.3 mL/min. After sample injection (5 μL), gradient elution was as follows: 10% B at 0 min, linear increase to 90% B over 14 min, hold the condition for 2 min, decrease to 10% B at 16.1 min and hold the condition for 2 min for the column to equilibrate.

Analysis was performed on an Acquity I-Class UPLC system (Waters, Milford, MA, USA) coupled to a VION IMS-QTOF, with an electrospray ionization interface operating in the positive mode. Mass spectral data were acquired in HDMSe mode over the range *m/z* 50–1000, with leucine encephalin used for mass correction. Low energy (LE, collision energy of 6 eV) and high energy (HE, collision energy ram of 28–56 eV) scans were done independently, with 0.3 s scan time. Data were examined using in-house built accurate mass screening workflow using the UNIFI platform (version 1.9.4). Additional data on VION parameters can be found in Table S1.

2.5. Suspect screening workflow

Empirical data for each chromatographic peak (feature) with an intensity >1000 cps was compared with library data. Comprehensive screening of NPS from various classes was conducted using two databases simultaneously: (1) the HighResNPS database (April 2021 version) – a extensive (5600 entries) centralised collection of NPS mass spectra provided by laboratories around the world (HighResNPS database, 2021), and (2) an in-house database including empirical data (LC, IMS and HRMS data) for 128 psychoactive substances (Celma et al., 2020), which were obtained by analysing reference standards under the same LC, IMS and HRMS conditions to the ones applied to wastewater samples within this study. Since no guidelines on compound identification in wastewater exist, threshold values, i.e., the acceptable deviation between empirical and library data was selected (Table 1) based on the SANTE/11312/2021 (2021) guidelines, and our previous experience in wastewater sample analysis, i.e., the higher deviation in retention times as proposed in SANTE was taken into account due to the influence of the wastewater matrix composition (Celma et al., 2020).

Table 1
Threshold values used for compound identification.

Analytical technique	Identification feature	Threshold value/criteria
HRMS	Mass accuracy	<5 ppm
	Number of fragment ions	Protonated molecule ion and the presence of (at least) one fragment ion
	Peak intensity	>1000 counts
LC	t_R	±0.2 min
IMS	CCS	±2%

CCS – collision cross-section values, HRMS – high-resolution mass spectrometry, IMS – ion mobility separation, LC – liquid chromatography, t_R – retention time.

2.6. Levels of confidence

Compounds were identified according to different confidence levels (Celma et al., 2020) depending on the information available: the availability of reference standards and the accuracy of the acquired chromatographic and mass spectral data. Briefly, **Level 3** – tentative identification: this is the lowest level of confidence reported in this study. It is used when identified compounds meet the minimum requirements for tentative identification, *i.e.,* an accurate mass and conformational mass fragment, but more than one possible candidate can be assigned to a single feature. **Level 2** – probable identification: represents compounds that meet the requirements for tentative identification, with the difference that an exact structure of a substance could be proposed based on empirical data, leading to a single candidate. Within Level 2, Level 2a represents a probable identification by LC-HRMS library match, and Level 2b represents probable identification by *in silico* or GC-HRMS library data. **Level 1** – unequivocal identification: this is the highest level of confidence and is used when data from reference standards support compound identity in all dimensions: chromatographic, IMS and mass spectral data. However, numerous spectral interferences in wastewater can affect mass accuracy and retention times, and accordingly, deviations beyond the established threshold (Table 1) for the HRMS or LC metrics (but not both simultaneously) were deemed acceptable and labelled as Level 1*.

2.7. Ethics

Although WBE studies raise limited ethical issues, as individuals cannot be identified, caution must be taken in specific sites, such as educational institutions, where there is a risk of stigmatisation and sensationalisation of the results. Therefore, we followed the ethical research guidelines developed by Prichard et al. (2014), where consent was obtained from the Heads of each institution, and an anonymity agreement was signed to protect the participants' identities. Additionally, the smaller municipality was anonymised to prevent tracking. All institutions willingly agreed to participate in the study and to the publication of the anonymised data. It should be noted that with the current study design, only data on the presence of psychoactive substances at the institution could be obtained, and the data cannot be inferred to a particular group, e.g., students, staff or visitors (see 4.1. Study strength, limitations and future perspectives).

3. Results

NPS were present in wastewater from all institutional types (Table 2). Four compounds, namely 3-MMC, ephedrine, 4-chloro-α-PPP and ethcathinone, were identified unequivocally (Level 1), with 4-chloro-α-PPP present in at least one sample from each institution type. Three compounds met Level 2 criteria, namely levorphanol, embutramide, and kavain and two features were related to 15 tentative (Level 3) candidates. More details to support the obtained results, i.e., relevant data on observed mass (m/z), mass error (ppm), retention time (min), CCS, and fragment ions, can be found in Table S2, while more data on (tentatively) identified compounds can be found in Table S3.

Out of nine features, six belonged to synthetic stimulants, two synthetic opioids and one to the natural plant-based psychoactive substance kavain, which was identified in 18 of the 23 wastewater samples. Overall, a similar number of features relating to NPS candidates were identified in samples from each type of institution (primary: n = 6, secondary: n = 6, SHEIs: = 6 and HEIs: n = 7), with Ljubljana having all of the features present and two being detected in M1.

Table 2
Summary of NPS identified for each relevant feature regardless of extraction method, organised according to NPS classification and level of confidence. Their legal status in Slovenia is also provided (Decree RS 157/20, 2020).

Feature	Exact mass[a] [g/mol]	NPS *(legality status in Slovenia)*	Confidence Level[c]	NPS class	Number of samples (n = 23)					
					Educational institution				Municipalities	
					Primary school (n = 8)	Secondary school (n = 6)	SHEI (n = 3)	HEI (n = 6)	Ljubljana (n = 17)	M1 (n = 6)
1	177.1154	3-MMC *(illicit)*	Level 1	Synthetic stimulants	1	0	0	0	1	0
2	165.1154	1R-2S-(−)-Ephedrine[b] *(licit)*	Level 1		0	1	0	3	4	0
3	237.0920	4-Chloro-α-PPP *(licit)*	Level 1[a]		1	1	1	2	5	0
4	177.1154	Ethcathinone *(licit)*	Level 1[a]		1	0	1	0	2	0
5	174.1157	5-IT, AMT *(illicit)* N-methyltryptamine, 6-IT *(licit)*	Level 3		6	1	3	1	9	2
6	191.1310	3-MEC, Pentedrone, 2-MEC *(illicit)* 2,3-DMMC, 2,4-DMMC, 4-MDMC, Isopentedrone, N- Ethylbuphedrone, 4-MPH, 2-NMC, N-Acetylmethamphetamine *(licit)*	Level 3		1	2	2	2	7	0
7	257.1780	Levorphanol *(illicit)*	Level 2a	Synthetic depressants (opioids)	0	1	1	1	3	0
8	293.1991	Embutramide *(licit)*	Level 2a		0	0	0	1	1	0
9	230.0943	Kavain *(licit)*	Level 2a	Natural (plant-based) psychoactive compounds	5	5	3	5	15	3

[a] (PubChem, 2023).
[b] Ephedrine can also be of natural origin (herb Ma-huang: *Ephedra sinica*).
[c] Level 1 = unequivocal, Level 1* = identification features align with reference standards, with a slight deviation in mass accuracy or retention times (but not both simultaneously; see 2.6. Levels of confidence), Level 2a = probable, Level 3 = tentative (Celma et al., 2020).

4. Discussion

4.1. NPS findings

In this study, four NPS were unequivocally identified, three met Level 2 criteria with high confidence, and 15 were tentatively identified as possible candidates (Level 3). The latter does not necessarily indicate the presence of all 15 different NPS in the wastewater samples. At Level 3 identification, multiple NPS could be assigned to an individual feature based on structural similarities but insufficient evidence to differentiate between candidates. Moreover, reference standards are required for final confirmation of the identity of NPS. Despite this, most (tentatively) identified NPS were synthetic stimulants, which agrees with their higher relative prevalence in Slovenia as reported by other socio-epidemiological data, namely from drug analysis, toxicological reports and questionnaires (NLZOH, 2021; NIJZ, 2019). Interestingly, synthetic cannabinoids, also common in Slovenia, were not detected. A possible reason could be their rapid metabolism in the human body (leading to lower concentrations) and hydrophobic properties, removing them from the aqueous phase of the wastewater (Bijlsma et al., 2019, 2021).

Among the unequivocally identified stimulants, i.e., 3-MMC, ephedrine, 4-chloro-α-PPP and ethcathinone, ephedrine is the only one that can be of synthetic or natural origin. It has many known uses, i.e., as medication in traditional and Eastern medicine, as a drug of abuse (by athletes and drug users) and as a precursor in the clandestine synthesis of methamphetamine (Gad et al., 2021). As a common ingredient in preparations to treat colds, asthma and narcolepsy (Gad et al., 2021), ephedrine is expected to be found in wastewater. However, in Slovenia, medical ephedrine is only available as an injection of ephedrine hydrochloride, used to treat hypotension during general and local anaesthesia (CBZ, 2023). Therefore, given the sample set (educational institutions), the presence of ephedrine is most likely related to its recreational use. This assertion gains further support from the reported instances of recreational ephedrine use in Slovenia (NIJZ, 2019).

Notably, 4-chloro-α-PPP was the only NPS unambiguously identified in at least one sample from all institution types. While this drug has been previously detected in street drug samples (NFL, 2022), it is not as commonly used or as well-known as 3-MMC, a controlled substance in Slovenia (NIJZ, 2019; Decree RS 157/20, 2020). 3-MMC, widely referred to as “ice cream” on the streets, gained popularity during the period of ecstasy and cocaine shortages, which coincided with economic and migrant crises. However, its popularity has since waned over time. Regardless, it is still among the most used and well-known NPS among drug users, including young users, and has been associated with drug intoxication (NIJZ, 2019). Although it has been reported as one of the most commonly used NPS among students at the University of Ljubljana (NIJZ, 2019, 2021), our study only detected its presence in a sample obtained from a primary school in Ljubljana.

Among probable candidates for synthetic stimulants, AMT and 6-IT (feature five), and MEC-type NPS, pentedrone, 2,3-DMMC, 4-MDMC and 4-MPH (feature six) are known to have been present on the Slovenian drug market (NIJZ, 2019, 2021). However, according to reports, only illicit MEC-type NPS were used by students of the University of Ljubljana in 2017/2018 and 2019/2020 (NIJZ, 2019, 2021), providing greater confidence in their identification of the samples analysed. Among all probable candidates, the most unusual and least expected is N-acetylmethamphetamine, as there are no indications (reports) that this substance is consumed as the parent compound (Barnes et al., 2019). N-acetylmethamphetamine is a by-product of methamphetamine synthesis and thus can be found in methamphetamine as an impurity. However, no methamphetamine was found in samples where N-acetylmethamphetamine was identified (Verovšek et al., 2021a, 2021b). Interestingly, except for 3-MMC, no other synthetic cathinones detected in Slovenian municipal wastewaters in 2016 (mephedrone, methylone and α-PVP), 2017 (mephedrone), and during the Christmas-New Year period in 2021/2022 (eutylone) (Castiglioni et al., 2021; Bade et al., 2023) were identified in wastewater from educational institutions analysed in this study. Their absence could be attributed to the dynamic nature of the NPS market or, alternatively, may be influenced by catchment specificities or their low concentration levels, which may fall below the detection limit of our method (Bade et al., 2020; NIJZ, 2021).

Although opioids were the least expected psychoactive substances, since unlike the stimulants, they do not increase alertness, morphine and codeine were found in wastewater from educational institutions in

Slovenia (Verovšek et al., 2021a). In this study, opioids levorphanol and embutramide met Level 2 identification criteria. Although opioid-type psychoactive substances are often used as a readily accessible substitute for prescription medications subject to medical supervision (Andersson and Kjellgren, 2016), we are unaware of any metadata regarding their occurrence in Slovenia to confirm their street use.

Kavain was the only naturally occurring (plant-based) psychoactive substance identified (Level 2) in 18 samples. Its high prevalence can be supported by easy accessibility, i.e., it can be readily obtained online in Slovenia (Google search by authors). It is one of the most abundant kavalactones in kava (*Piper methysticum*) and is responsible for the plant's psychoactive effect. Beyond its use in religious rituals in Melanesian societies, kava is known for its calming effect and is used in Western countries as a prescription-free alternative to benzodiazepines and recreationally as an alcoholic substitute (Chua et al., 2016). Despite being marketed as an alternative medicine in Slovenia, its safety was called into question in Europe between 1999 and 2000 due to a surge in reports highlighting its potential liver toxicity and lack of clinical evidence supporting its health benefits. (Kuchta et al., 2015). Accordingly, kavain (and the kava plant) was banned in several European countries, but its legal use was restored due to a lack of proper clinical research (Kuchta et al., 2015).

Regarding inter-institutional trends, we expected a higher number and diversity of NPS in HEIs, as NPS can be easily purchased online (Bijlsma et al., 2019), and the first instance of illicit drug use in Slovenia typically occurs between the ages of 19 and 23 (NIJZ, 2022). Moreover, NPS are often used as legal substitutes for illicit drugs, making their presence more likely among this age group and older. However, our study revealed a similar number of NPS features (six and seven) across all educational institutions, suggesting that the occurrence of NPS is comparable regardless of the institution type. Also, it remains unclear whether this reflects their prevalence in the general population, as no comparable data on wastewater-based epidemiology (WBE) exist for Slovenia.

Regarding potential spatial trends, all NPS corresponding features ($n = 9$) were found in Ljubljana, the capital of Slovenia, while in M1, there were only two features, none of which correspond to unequivocally identified NPS. Also, in M1, none of the likely candidates corresponds to synthetic opioids. Given the difference in the number of detected features, the results suggest that NPS were more prevalent in Ljubljana, which is a larger, more diverse municipality between the two addressed in the study, despite NPS being easily obtained online. The difference in the number of samples obtained from each municipality (17 *vs* 6) could also be a contributing factor to the results, as it highlights a substantially larger target population in Ljubljana. Nevertheless, a city-characteristical use of NPS was also confirmed by Brandeburová et al. (2020).

4.2. Study strengths, limitations and future perspectives

The non-invasiveness of WBE makes it an attractive technique for studying vulnerable populations, and it has already been applied successfully to prisons, festivals and educational institutions (Verovšek et al., 2020). Also, the integration of non-target data acquisition further enhances its effectiveness as a surveillance tool (radar) for NPS detection, which makes it particularly valuable (Bijlsma et al., 2019). However, the method is not without its limitations. For example, sampling wastewater in specific sub-catchments, such as single facilities, is challenging since it requires good knowledge of the sewer system, power availability when using an autosampler, and optimised sampling frequency to adjust to inconsistent wastewater flow (Verovšek et al., 2020). While extending the sampling period in this study was not feasible, it is recommended that future research considers sampling over multiple days; otherwise, it provides only a snapshot of drug use at a given time (Ort et al., 2010; Verovšek et al., 2020).

Another limitation specific to NPS is the need for reference standards for unequivocal identification. For this reason, having pre-knowledge of NPS used in countries from other socio-epidemiological sources is essential since it adds confidence to identification when no reference standards are available. Likewise, despite having an extensive database available, it can take time for NPS to be identified and included and may not appear in the study. One advantage of non-target data acquisition is that it allows for retrospective analysis, which can be performed at any future time to uncover additional information from the acquired data (Bijlsma et al., 2021). Moreover, due to limitations in method sensitivity and extraction specificity, certain compounds may remain undetected, despite utilising two different SPE cartridges to broaden the range of extracted NPS.

The main limitation of using wastewater analysis to investigate NPS use among younger individuals by sampling from educational institutions is that, at the institution level, it is challenging to distinguish between the target population (pupils and students) and other groups such as staff members or visitors (Verovšek et al., 2021a). Therefore, caution should be taken when extrapolating results to a particular group. Also, this makes comparison with socio-epidemiological study data problematic given that such data always relate to a particular group, e.g., the general population (15-64-year olds), students, or drug users. However, as stated above, such a comparison is essential in screening for NPS to gain confidence in the identification. A possible solution would be to leverage existing sewer infrastructure to target areas where pupils are known to congregate, such as bathrooms and locker rooms similar to that used in prison settings (van Dyken et al., 2014).

Despite the limitations identified, the combination of WBE and suspect analysis conducted in educational institutions holds significant promise. This approach provides valuable insight into the presence of psychoactive substances and serves as an effective tool (radar) for detecting NPS in environments where they should not be present, regardless of whether they are being consumed by pupils or staff. In that way, valuable data on when and with which NPS children and students come in contact are gathered. In addition, wastewater analysis can serve as a practical and non-invasive substitute for traditional drug testing methods to detect the presence of drugs, particularly in educational institutions, where drug testing may carry a social stigma and create a sense of shame for those who test positive. It is known that the association with drug use can also cause long-term psychological and social harm and encourage punitive actions rather than early prevention and education (Dupont et al., 2013). However, further research on replacing drug testing with wastewater analysis and evaluating its impact on drug use among young people is needed to support such a claim.

5. Conclusions

This study is the first to identify the presence of NPS in wastewater samples obtained from all levels of educational institutions, including primary schools, where data on the prevalence of psychoactive substances, including NPS, is scarce even at a global level. Also, no trends in NPS prevalence were observed when different types of institutions were compared, while more diverse substances were present in the larger of the two studied municipalities. Importantly, our proof of concept confirms that wastewater analysis has the potential to provide insight into the presence of NPS in educational institutions. It is worth noting that with specific adaptation (e.g., leveraging parts of existing sewer infrastructure covering parts of the institutions where pupils are congregating), this analysis may be able to attribute NPS consumption to a particular group of individuals within the institution (e.g., students). However, even in its current form, this analysis can still provide data on the presence of drugs in educational institutions, which is needed since these institutions are expected to maintain a drug-free environment. Accordingly, wastewater analysis could serve as a valuable non-invasive substitute for drug testing, which is currently carried out in various countries worldwide.

Credit authors statement

Taja Verovšek: Writing – original draft, Methodology, Formal analysis, Data curation, Visualization; **Alberto Celma:** Writing – review & editing; Investigation, Formal analysis; **David Heath**: Conceptualization, Data Curation, Writing – review & editing; **Ester Heath**: Project administration, Funding acquisition, Conceptualization, Supervision, Writing – review & editing; **Félix Hernández**: Resources, Writing – review & editing; **Lubertus Bijlsma:** Funding acquisition, Conceptualization, Supervision, Data curation, Writing – review & editing.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.117061.

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