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DOI
10.1016/j.scitotenv.2021.152139

Publication date
2022

Document Version
Final published version

Published in
Science of the Total Environment

Science of the Total Environment 811 (2022) 152139

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

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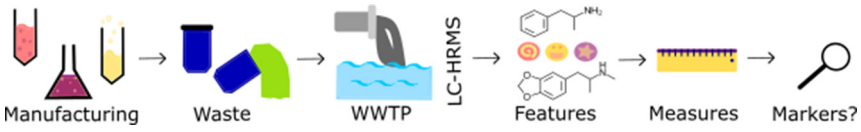
HIGHLIGHTS

- Suspect and non-target screening of drug manufacturing waste in wastewater
- A novel prioritization approach was implemented based on distance measures.
- Selection of 28 presumed markers of production of MDMA and amphetamine
- Detect disposal of illegal waste and monitor synthesis route

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 October 2021
Received in revised form 27 November 2021
Accepted 28 November 2021
Available online 3 December 2021

Editor: Kevin V. Thomas

Keywords:
MDMA
Amphetamine
Manufacturing
(pre-)precursors
Wastewater
LC-HRMS

ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA) and amphetamine are commonly used psychoactive stimulants. Illegal manufacture of these substances, mainly located in the Netherlands and Belgium, generates large amounts of chemical waste which is disposed in the environment or released in sewer systems. Retrospective analysis of high-resolution mass spectrometry (HRMS) data was implemented to detect synthesis markers of MDMA and amphetamine production in wastewater samples. Specifically, suspect and non-target screening, combined with a prioritization approach based on similarity measures between detected features and mass loads of MDMA and amphetamine was implemented. Two hundred and thirty-five 24 h-composite wastewater samples collected from a treatment plant in the Netherlands between 2016 and 2018 were analyzed by liquid chromatography coupled to high-resolution mass spectrometry. Samples were initially separated into two groups (i.e., baseline consumption versus dumping) based on daily loads of MDMA and amphetamine. Significance testing and fold-changes were used to find differences between features in the two groups. Then, associations between peak areas of all features and MDMA or amphetamine loads were investigated across the whole time series using various measures (Euclidian distance, Pearson's correlation coefficient, Spearman's rank correlation coefficient, distance correlation and maximum information coefficient). This unsupervised and unbiased approach was used for prioritization of features and allowed the selection of 28 presumed markers of production of MDMA and amphetamine. These markers could potentially be used to detect dumps in sewer systems, help in determining the synthesis route and track down the waste in the environment.

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA) is an entactogen drug, often associated with nightlife settings. It is consumed in the form of crystals, powder or tablets – in which case it is referred to as ecstasy, XTC or Molly (European Monitoring Centre for Drugs and Drug Addiction, 2019; Saleemi et al., 2017). The prevalence of use is higher in the age range 15–24, with 1.3 million (2.3% of EU population) estimated to have

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http://dx.doi.org/10.1016/j.scitotenv.2021.152139

used MDMA in 2018 (0.8% for age group 15–64), and mass loads of the drug in the wastewater increased between 2011 and 2018 in most European cities that were monitored (European Monitoring Centre for Drugs and Drug Addiction, 2019; European Monitoring Centre for Drugs and Drug Addiction, 2016a). With amphetamine, MDMA dominates the market of synthetic stimulants in Europe (European Monitoring Centre for Drugs and Drug Addiction, 2016a). Though different molecules, some criminal groups are involved in the manufacturing of both drugs (European Monitoring Centre for Drugs and Drug Addiction, 2016a).

Production of these drugs for the global market mainly takes place in Europe, even though most precursors and other chemicals needed for the production are imported from elsewhere, especially China (European Monitoring Centre for Drugs and Drug Addiction, 2016a). In The Netherlands, the production of synthetic drugs started more than 40 years ago and the country has become a European hub of manufacturing, along with Belgium. The annual revenues of the Dutch production of synthetic drugs were estimated at over 18.9 billion euros in 2017 (Tops et al., 2018). The manufacturing is dependent on the availability of starting materials, with precursors being controlled substances and/or suffering from natural shortages (European Monitoring Centre for Drugs and Drug Addiction, 2019; Tops et al., 2018). Nowadays, producers have found ways to overcome regulations and increasingly resort to using "(pre)pre-precursors" or masked precursors that can relatively easily be transformed into the desired precursor. However, the use of these chemicals adds further steps to the synthesis, generating increasing amounts of waste (Tops et al., 2018; Schoenmakers et al., 2016). As a representative example, 6 to 10 kg of waste are produced by the manufacture of 1 kg of MDMA by reductive amination, and the synthesis of 1 kg of amphetamine from its precursor BMK (benzyl methyl ketone, also called phenylacetone, phenyl-2-propanone, or P2P) can produce 20 to 30 kg of waste (European Monitoring Centre for Drugs and Drug Addiction, 2016a; Emke et al., 2018), that can be highly acidic (pH < 1) or highly basic (pH > 10) (Ort et al., 2018). This complex mixture contains all kinds of products, such as precursors, intermediates, by-products, impurities and end-products. All this waste has to be disposed of and methods include burying, incinerating, abandonment in nature (e.g., field, forest, river) or in a confined environment (e.g., apartment, stolen car), or direct disposal in the sewers (e.g., through floor drain or in car wash) have been reported, all of which create health risks and can have significant environmental impacts (European Monitoring Centre for Drugs and Drug Addiction, 2016a; Emke et al., 2018; Ort et al., 2018). It was estimated that each year, millions of tons of toxic and hazardous waste from drug production are released into the environment worldwide (European Monitoring Centre for Drugs and Drug Addiction, 2016a). In the Netherlands, the number of recognized dumping sites is on the rise (+550% between 2010 and 2014, +130% between 2014 and 2017) (Schoenmakers et al., 2016; Politie et al., 2018). Considering that 307 tons of amphetamine and 153 tons of MDMA are synthesized each year in the country, roughly 7′000 tons of toxic waste is also produced (6140 and 918 tons for each drug, respectively) (European Monitoring Centre for Drugs and Drug Addiction, 2016a; Tops et al., 2018). Moreover, synthesis routes change constantly (depending on the availability and/or regulation of starting materials), and so does the composition and amount of chemical waste which is being generated (European Monitoring Centre for Drugs and Drug Addiction, 2016a).

Here, an innovative approach is presented, using a combination of suspect and non-target screening (NTS) through retrospective analysis, combined with various similarity metrics to find relations between detected features and levels of MDMA and amphetamine measured in wastewater samples using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS). The proposed approach is used to prioritize potentially relevant features detected in wastewater samples, which might be associated with the synthesis of these drugs and might provide clues about the synthesis routes being used. In fact, given that synthesis waste contains substantial concentrations of the final substance (Emke et al., 2018), the hypothesis is that if such waste is disposed of in sewers, precursors, intermediates, and by-products will follow the same trend as the target drug. To our knowledge, this is the first time that this kind of approach is used to prioritize features detected in this context. The acquired information can help in determining the synthesis route used to produce illicit drugs, detect illegal dumping of synthesis waste and help to tackle the challenges faced when trying to estimate illicit drug use in countries where production is widespread.

2. Materials and methods

2.1. Sampling

With 450,000 inhabitants living in the catchment area, the wastewater treatment plant (WWTP) of Eindhoven is one of the largest in the Netherlands, and the catchment consists of both rural and urban areas (Emke et al., 2018). Composite samples were collected at the influent of the WWTP after the fine screen. Samples were collected each day for 90 consecutive days in 2016 (from April 26th to July 24th), for 50 consecutive days in 2017 (from July 3rd to August 21st) and for 100 consecutive days in 2018 (from June 4th to September 14th). No data was collected on May 22nd and June 2nd, 2016 due to a malfunction of the autosampler, and on a three-day break from August 13th to August 15th, 2018. Volume proportional samples were collected every 800 m^3 and for 24 h cycles. The composite sample is the resulting mixture of approximately 200 individual subsamples, and thus, representative of the preceding 24 h period. During collection, samples were stored at 4 °C. Composite samples were stored in HDPE bottles and frozen at −20 °C until analysis. Before analysis, samples were thawed, adjusted to pH 7, spiked with a mix of deuterated internal standards and then filtered (more details about sample preparation are provided below). The mixture of isotopically labelled internal standards used for samples collected in 2016 consists of morphine-D3, amphetamine-D11, MDMA-D5, cocaine-D3 and atrazine-D5. In 2017 and 2018, cocaethylene-D3 and diazepam-D5 are used instead of cocaine-D3 and atrazine-D5 respectively.

2.2. Chemical analysis

Two different methodologies were used; 2016 samples were first extracted using Solid Phase Extraction (SPE) while samples from 2017 and 2018 were directly injected into the chromatographic system. As this is a retrospective study, each yearly campaign was analyzed separately, hence the different methodologies.

In the SPE process, samples were first filtered through 1 μm pores of a type A/E glass fiber filter and then through a 0.20 μm pore polyethersulfone filter. Extraction was made with Oasis HLB 6 cc cartridges (Waters, Milford, USA) and analytes were eluted with methanol. 2016 samples were then analyzed by high performance liquid chromatography (HPLC) (ThermoFisher Scientific, Bremen, Germany) with an XBridge BEH C18 column (3.5 μm, 2.1 × 150 mm) (Waters, Etten-Leur, The Netherlands). The detection system was a hybrid linear ion trap quadrupole Fourier transform (LTQ-FT) Orbitrap mass spectrometer (Thermo Electron, Waltham, USA). All details about the chromatographic separation and mass spectrometry settings can be found in Voogt et al. (2011).

For 2017–8 samples, the setup consisted of an HPLC (ThermoFisher Scientific), with an XBridge BEH C18 XP column (2.5 μm, 2.1 × 150 mm) (Waters, Etten-Leur, the Netherlands) coupled to a Tribrid Orbitrap Fusion mass spectrometer (ThermoFisher Scientific). All details about the chromatographic separation and mass spectrometry settings can be found in Emke et al. (2018).

All analyses were carried out in data-dependent acquisition (DDA) in positive electrospray ionization (ESI) mode. Quantitative analysis of MDMA and amphetamine in wastewater was performed using a fully validated LC-MS/MS method as reported in Bijlsma et al. (2012, 2013).

2.3. Suspect list

A suspect list was created through literature mining (Supporting Information (S.I.), Excel file). All the references used are presented in the S.I.

The suspect list was made in order to develop an inventory of all substances linked to the production of MDMA and amphetamine. Precursors, intermediates or by-products, and impurities reported (in the final product or at a previous stage) were added to the list, as well as adulterants. A total of 186 and 65 compounds were added to the list for MDMA and amphetamine, respectively.

2.4. Group-based prioritization

Prioritization of features in the group-based approach was performed by separating samples into two distinct groups according to whether measured MDMA or amphetamine levels (determined using the quantitative method described previously) were considered as originating from consumption or from dumping of synthesis waste. This was done by assuming that samples showing particularly high loads compared to a defined baseline level corresponded to days during which synthesis waste had been dumped illegally in the sewers. Baseline levels were determined arbitrarily and corresponded to the average daily loads during days where no sudden increase in MDMA or amphetamine levels was observed (see Section 3.2). Weekends and other special events were not taken into account when deciding on the baseline.

LC-HRMS data processing for suspect and non-target screening was a retrospective analysis carried out using *Compound Discoverer 3.0* (Thermo Fisher Scientific Inc., USA), with the workflow illustrated in the S.I. (Fig. S1). As wastewater is considered a dirty sample, a minimum peak intensity was set to 500′000 counts. Features found in procedural blanks (i.e., ultra-pure water spiked with internal standards and processed as wastewater samples) whose peak area was not at least 10-times that of the blank were flagged as background. Searches were performed with a 2 ppm mass tolerance and allowed a maximum shift of 0.1 min in retention times for features alignment. Library searches were conducted against *mzCloud* (HighChem Ltd., Slovakia), *mzVault* (Thermo Fisher Scientific Inc., USA) (with the *mzVault May 2018* library), and *Chemspider* (Royal Society of Chemistry, USA) (with *EAWAG biocatalysis/biodegradation*, *EPA DSSTox*, *EPA toxcast*, *Drugbank*, *ACToR*, and *FDA UNII – NLM* databases). Suspect screening was performed against the compiled suspect list via the mass list node in Compound Discoverer. Samples from each yearly campaign were analyzed in a single run and the data for each year was processed individually. The aim of this group-based prioritization approach consisted in determining if compounds compiled in the suspect list could be (tentatively) detected in wastewater samples. A system of confidence levels was implemented based on the scale developed by Schymanski et al. (2014). Features (referred to as the accurate mass, retention time and peak area of detected compounds) that had a “hit” with the suspect list were further selected if there was a positive log 2-fold change of the peak area between the group *dump* and the group *consumption*. A principal component analysis (PCA) was computed to determine if samples could be grouped based on the peak area of all detected features. Prior to calculating the PCA, the data was scaled to unit variance. Statistical significance was tested using the Tukey HSD test after an analysis of variance (ANOVA). The Benjamini-Hochberg correction was used on the p-value in order to control the false discovery rate (Benjamini and Hochberg, 1995; Thermo, 2018). If p-value < 0.05, then the peak area of the feature was considered significantly different between the two groups (*dump* vs *consumption*). Volcano plots, illustrating the log 2–fold change in peak area between features in the two groups as a function of the significance (Benjamini-Hochberg corrected p-value), were computed to visualize relevant features. The latter were selected as they were assumed to indicate production waste dumping in wastewater.

2.5. Similarity-based prioritization

In this work, an alternative approach was used to prioritize features related to the synthesis of MDMA or amphetamine, namely one which relies on the assumption that features related to synthesis waste (e.g., impurities and synthesis by-products) will follow a similar pattern compared to MDMA or amphetamine loads. This assumption was made because MDMA and amphetamine synthesis waste is known to contain high residual concentrations of the final product and will hence cause sudden important increases in concentrations measured in wastewater, should this waste be disposed of in sewers. For this purpose, various metrics were used to determine if a relationship could be established between features intensities (i.e., peak area) and trends in MDMA and amphetamine loads determined using the quantitative method described previously.

All features detected through the described workflow were taken into account, this includes both those that gave a hit with the suspect list and those that did not (non-targeted). Peak areas of features were extracted and then normalized by the peak area of the closest internal standard in terms of retention time. Similarity metrics were computed between the peak areas of the normalized features detected in samples and the corresponding loads of MDMA or amphetamine.

Five different metrics were used: Euclidean distance, Pearson's correlation coefficient (R), Spearman's rank correlation coefficient (rho ρ), the distance correlation (dCor) and the maximum information coefficient (MIC). Metrics obtained between MDMA and amphetamine peak areas and their corresponding concentrations were used as a control to assess whether the selected measure was capable of picking up true relationships.

For each substance, year and considered metric, the top three features (i.e., having the strongest relationship with either amphetamine or MDMA) were selected. In the final approach, results from Euclidean distance were excluded due to unsatisfactory results between MDMA/amphetamine feature peak areas and corresponding concentration. For the other metrics, the following criteria was established: values with a distance measure below 0.5 were not considered (weak relationship), except for amphetamine in 2016 given that all distance measures were low, while if the distance value was above 0.7 (strong relationship), all features were selected. Euclidian distance, Pearson correlation and Spearman Rho were calculated with the built-in functions of R, while the distance correlation was measured using the *Energy* package (Szekely et al., 2007) and the maximum information coefficient was calculated using the *Minerva* package (Albanese et al., 2013).

Relevant candidates were further inspected. Improved identification was attempted by reprocessing the data on *Compound Discoverer 3.0*. MS2 data was used against *mzCloud* and *mzVault* spectral libraries, used for spectral tree searches for structure identification (on *mzCloud*), Fragment Ion Search (FISh scoring – which compares predicted fragments to the experimental scans) in *Compound Discoverer 3.0*, and *MetFrag* queries with the ChEBI (*Chemical Entities of Biological Interest*), KEGG (*Kyoto Encyclopedia of Genes and Genomes*) and PubChem databases (Thermo, 2018; Brunner et al., 2019; Ruttkies et al., 2016). All analyses and visualizations were made with *RStudio 1.2.1335* (RStudio Team, Boston, USA).

3. Results and discussion

3.1. Amphetamine and MDMA loads

Daily mass loads of MDMA and amphetamine during the sampling periods are presented in Fig. 1. For amphetamine, loads were much higher in 2016, with a maximum of 6625 g/day on May 27th. On average, the load was 326 g/day over the whole sampling period, while in 2016, the average was 566 g/day. With 450,300 people contributing to the wastewater, this corresponds to a population normalized load of 1256 mg/day/1000 inhabitants, whereas values range between 50 and 120 mg/day/1000 inhabitants in other cities, including known drug use hubs (e.g. Amsterdam, London, Barcelona, Zurich) (European Monitoring Centre for Drugs and Drug Addiction, 2019; González-Mariño et al., 2020). In the following years (2017–8), the average daily load decreased. These findings seem to suggest that during 2016, waste was being disposed of frequently in the sewers while in 2017–2018 it occurred only intermittently. These extremely high population normalized loads and previous chiral analysis are the reason why it has been assumed that disposal of amphetamine synthesis waste takes place in the city of Eindhoven (Emke et al., 2014).

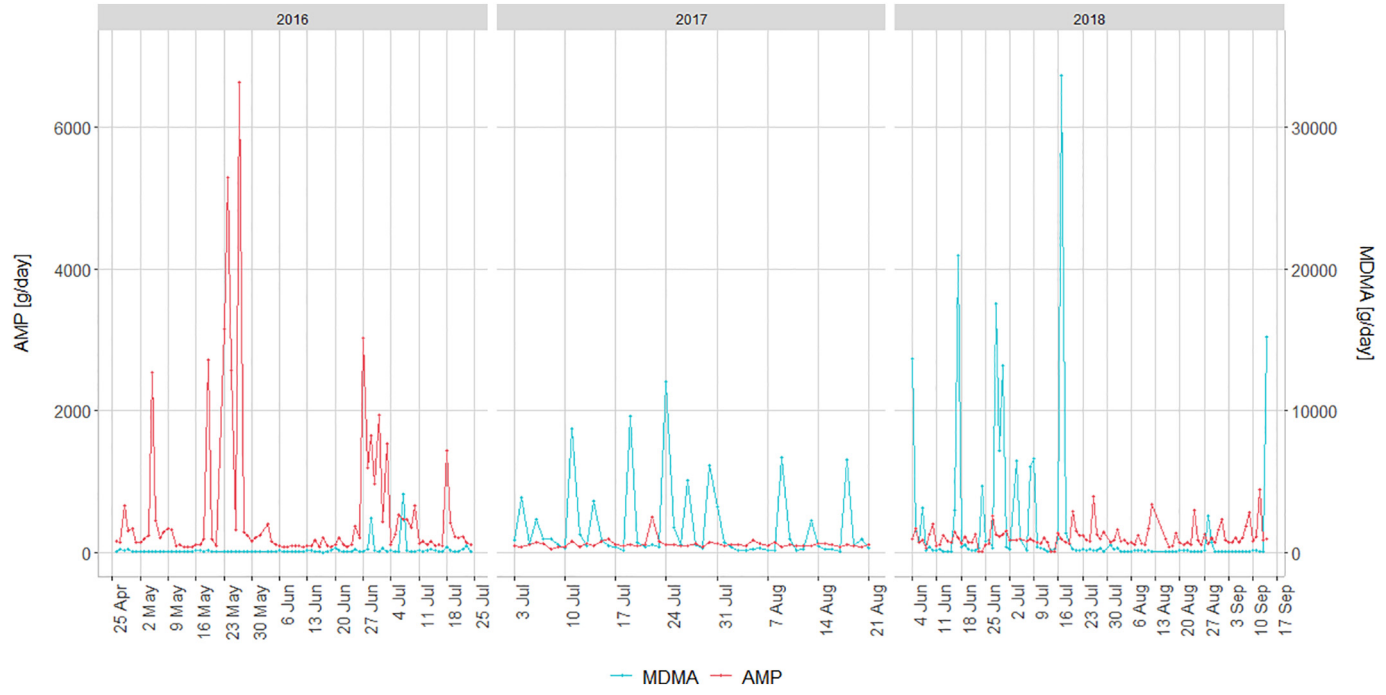

Fig. 1. Daily mass loads of amphetamine (AMP) (left axis) and MDMA (right axis) during sampling periods. AMP loads are highest in 2016, while MDMA loads show a yearly increase.

For MDMA, loads increased year by year, reaching a peak of 33,656 g/day on July 17th, 2018. The average MDMA load during the sampling period was 1179 g/day, corresponding to a population normalized load of MDMA of 2618 mg/day/1000 inhabitants (25–50 mg/day/1000 inhabitants for other cities) (González-Mariño et al., 2020). With a correction factor of 4.4, which has been suggested by Gracia-Lor et al. to back-calculate MDMA consumption from loads measured in wastewater (Gracia-Lor et al., 2016), this would correspond to 5000 g of pure MDMA consumed each day if measured loads would only be due to consumption. Assuming a daily dose of 100 mg of pure MDMA per person and per day, this would correspond to a prevalence (daily use) of almost 19% for people aged 20–64 years (compared to an estimate of 0.8% of MDMA use in 2018 in the European Union for adults of 15 to 64 years old (European Monitoring Centre for Drugs and Drug Addiction, 2019)). Similar to findings for amphetamine, these figures are unrealistic and it can therefore be assumed that MDMA waste is being dumped very frequently, with waste chemicals slowly reaching the WWTP from various locations across its catchment. When put in perspective with other data (e.g., police information), some of the observed values can be explained. For example, between the 15th and the 18th of July 2018, four dumping locations were found in Eindhoven, Geldrop, Heeze, and Mierlo and it is possible that waste containing still high levels of MDMA leaked into the sewers, therefore explaining the aberrant load of 33,656 g/day on the 17th of July 2018. However, given the size of the catchment and widespread manufacturing, it is not possible to exclude that the observed peak was due to another (or multiple) laboratory disposing of its waste in the sewer system.

3.2. Group-based prioritization

In the group-based prioritization approach, loads of MDMA and amphetamine were used to classify samples into two categories: if daily mass loads were above a given threshold, then it was assumed that a dump took place, otherwise the mass loads were considered to be due to consumption only. Baseline levels corresponded to the average daily loads during days where no sudden increase in MDMA or amphetamine levels was observed. The threshold for 2016 was established at 200 g/day for both drugs and at 280 g/day for the following years. It should be noted that this approach suffers from one limitation, namely that it does not take into account that dumping of waste might still occur although no sudden peak in MDMA or amphetamine mass loads is visible (e.g., due to lower amounts of residual MDMA or amphetamine in the disposed waste). Similarly, this approach does not allow to take into account dispersion and residence time in sewers, which might cause features related to a specific dumping event to be detected hours or days after the actual event took place. Furthermore, it is known that some special events (e.g., music festivals, national holidays), as well as weekends, are concomitant with an increase in drug consumption (Been et al., 2014; Foppe et al., 2018; González-Mariño et al., 2017). Benaglia et al. (2020) showed that a music festival could increase 3–5 fold the amount of drugs found in wastewater (Benaglia et al., 2020). In this context, however, it can be safely assumed that observed peaks are due to dumping events because their magnitude is substantially higher compared to what is known about festivals. A preliminary visualization of grouping between samples was explored using PCA. As shown in Fig. 2, no clear separation between *Consumption* and *Dump* could be observed when considering all features jointly. Higher principal components were also investigated yet no further separation could be obtained. Only in the case of data from 2016, two clusters can be separated along the first principal component, however these do not seem to correspond to the established groups.

Volcano plots were subsequently generated to select features that showed significant differences between the two groups (see Fig. 3). Among features with significantly higher peak areas (shown in red in Fig. 3), those which showed matches with the suspect list (confidence level 3 according to Schymanski et al. (2014)) or those for which a structure could either be tentatively attributed (confidence level 2, match with MS2 spectra from used databases) or even confirmed (confidence level 1, match with reference standard) were selected. In particular, these were amphetamine, N-cyclohexylacetamide, BEK (benzyl ethyl ketone, or 1-phenyl-2-butanone), PMK (piperonyl methyl ketone, also called MD-P2P, or 3,4-methylenedioxyphenylpropan-2-one), methylephedrine, MDMA, MDEA (3,4-methylenedioxy-N-ethylamphetamine), MBDB (1,3-benzodioxolyl-*N*-methylbutanamine), MMOM-MDPEA (*N*-methyl-2-methoxy-1-methyl-2-(3,4-methylenedioxyphenyl)-ethanamine), and cocaine. Additional information (retention time (t_R), $[M + H]^+$, molecular formula and relation

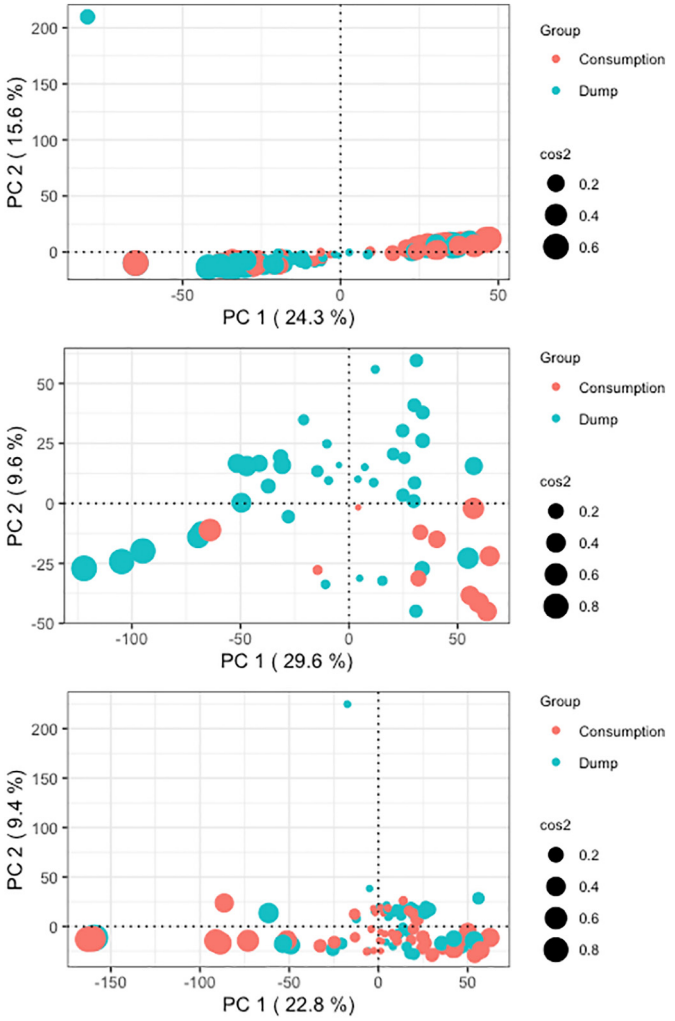

Fig. 2. Results of the principal component analysis (PCA) for the years 2016 (top), 2017 (middle), and 2018 (bottom). Component 1 and 2 are illustrated here, as well as the explained variance for each component (between brackets).

to the target drug), as well as examples of measured MS2 spectra and matches from available databases or *in-silico* predicted spectra can be found in the S.I. (Table S1, Figs. S2–S6).

N-cyclohexylacetamide was reported to be a marker of the MD-P2NP (3,4-methylenedioxyphenyl-2-nitropropene) reduction to PMK (Swist et al., 2005). The fact that this was a significant feature potentially present in the samples can be an indication that producers used this route to manufacture MDMA. PMK is the main precursor used to produce MDMA, but it is also regulated in Europe. It can, therefore, be synthesized from a pre-precursor like safrole or MD-P2NP for example (European Monitoring Centre for Drugs and Drug Addiction, 2016a). The feature attributed to BEK originally gave a hit with estragole, a common impurity found in safrole (an important precursor for MDMA). Manual comparison with spectral libraries showed that BEK was a more suitable candidate. Though BEK does not seem to have been reported in the literature to be linked with MDMA or amphetamine, it can be hypothesized that its presence is related to impurities in BMK, one of the main precursors of amphetamine.

An additional feature originally gave a hit with PMMA (paramethoxymethamphetamine), but manual comparison with spectral libraries as well as *in-silico* fragmentation suggested that methylephedrine was a more suitable candidate (additional information can be found in the S.I.). Methylephedrine is an amphetamine derivative but is known to be associated with methamphetamine manufacture. Its link to amphetamine production cannot be established, but it can be hypothesized that the clandestine labs that dispose of the waste also produced other synthetic drugs, such as methamphetamine. However, methamphetamine itself could not be detected in the analyzed samples. This could be due to the fact that up until 2019, methamphetamine was detected only sporadically in wastewater samples from Eindhoven (European Monitoring Centre for Drugs and Drug Addiction, 2021).

The peak area of MDEA was roughly two orders of magnitude lower in 2017 compared to those of MDMA, indicating it is a minor component of what was disposed of in the sewers. MDEA has already been reported multiple times as an impurity in MDMA tablets (Palhol et al., 2002; van Deursen et al., 2006) and is probably caused by contamination of ethylamine in the methylamine reagent. As MDEA and MDMA share a very similar structure, it is likely that they will follow the same excretion path in the human body. Therefore, MDEA in wastewater could also be linked to consumption. Furthermore, the ratios between MDMA and MDEA peak areas do not change between days labelled as *dump* and *consumption*.

MBDB has been reported as an MDMA adulterant (Cheng et al., 2003). Adulterants are pharmacologically active substances that are added to a controlled drug, in order to reproduce or enhance the effects or to increase benefits from the sales. Inactive substances can also be added as "binders" or "fillers" in order to press the drug into tablets (European Monitoring Centre for Drugs and Drug Addiction, 2016b; Giné et al., 2014). Though recent studies showed that the purity of MDMA products is on the rise after the market was previously disrupted with new legislation around controlled substances (European Monitoring Centre for Drugs and Drug Addiction, 2019; United Nations Office on Drugs and Crime, 2014), the source of MBDB can be associated with an adulteration step rather than a synthesis step.

MMOM-MDPEA is considered as a specific impurity for the reductive amination route, i.e. it is only found when manufacturing is done via this path (Stojanovska et al., 2013). The fact that this feature was potentially present in wastewater samples can be a good indicator that production waste is being dumped in the sewers and that the route used is reductive amination.

Cocaine has also already been found in MDMA tablets. In France, it was reported as a contact impurity (i.e., not linked to the manufacturing), whereas it was reported to be sometimes used as an adulterant in the USA (Saleemi et al., 2017; Palhol et al., 2002). However, in the present case, the origin of cocaine is suspected to be from consumption. Indeed, benzoylecgonine, the main cocaine metabolite, was also found in the samples and they happened to follow the same pattern (i.e., if there is a peak of cocaine there is also one of benzoylecgonine). Furthermore, it is expected that the cocaine/benzoylecgonine ratio should not exceed 0.75 or be below 0.27 if it is due to human consumption only (Nuijs et al., 2009; Postigo et al., 2010). In the samples, the ratio was always below the cut-off value of 0.75 apart for one day (13 July 2017) (see Table S2, S.I.). The fact that the feature corresponding to cocaine had an adjusted *p*-value below 0.05 between the two groups is due to the fact that when there was an increase in MDMA loads, there was sometimes also an important peak of cocaine (e.g., 11.07.17, 19.07.17, 09.08.17). As discussed previously, this increase is not necessarily always due to disposal but could also be due to weekends or specific events, where increased use of both MDMA and cocaine is likely to occur.

From these results, it seems that N-cyclohexylacetamide, BEK, PMK, and MMOM-MDPEA can be used as indicators to trace disposal of synthesis waste in wastewater and even give some information on the used synthesis route. On the other hand, the simple presence of MDEA cannot be used to differentiate a dump from a normal consumption peak.

It was noticed that there were numerous features with an adjusted *p*-value below 0.05, but these did not match with any compound present in the suspect list. Possible explanations include a non-exhaustive suspect list, but also the limitations of the methodology used to select relevant features. As mentioned earlier, the threshold between a dumping event and "baseline" consumption was defined arbitrarily. Second, still with regards to the threshold, only MDMA and amphetamine were taken into account. However, the waste composition (thus the residual amount of MDMA or

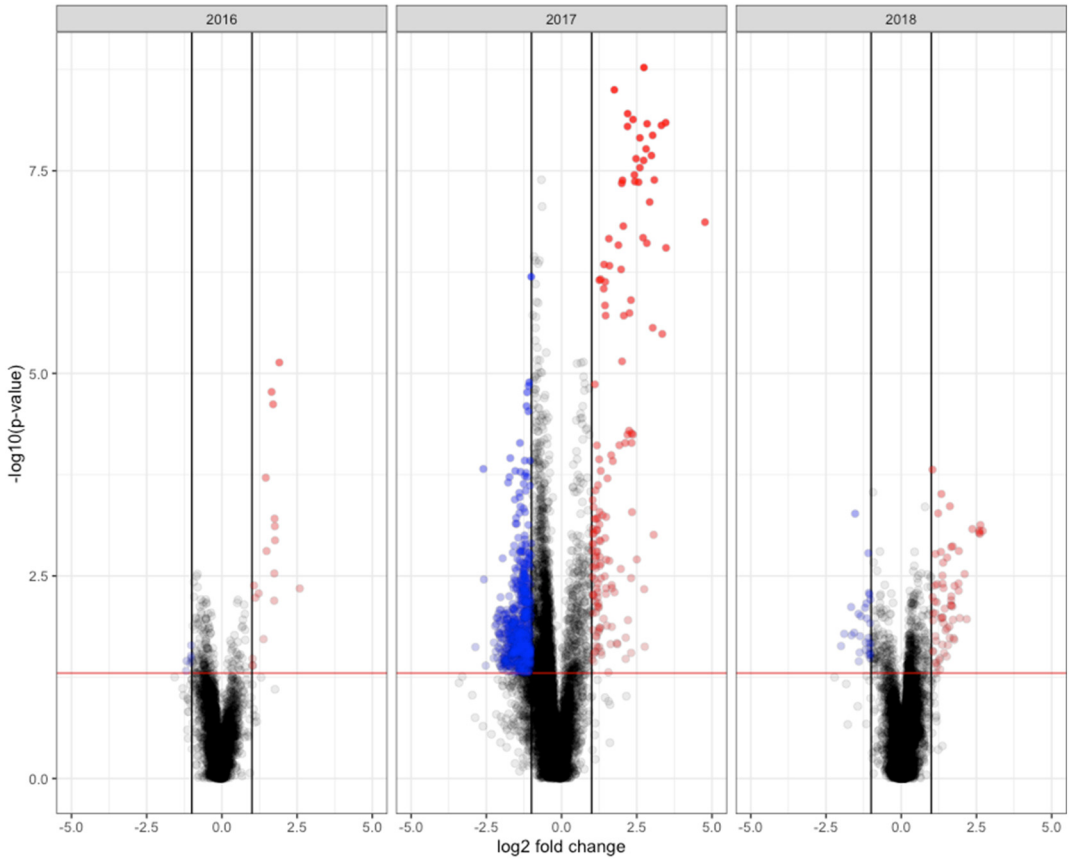

Fig. 3. Volcano plots of features detected in the two groups. Features showing a positive log 2-fold change (red) exhibited a higher peak area in samples considered to be a "dump" while features marked in blue had higher intensities in the "consumption" group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

amphetamine present) can change, depending on the synthesis route used or even on the cook. Lastly, groups (*dump* versus "baseline" *consumption*) were defined using loads whereas the *p*-value is calculated only on feature peak areas. In other words, samples were separated taking into account flows, yet this was not the case when adjusted *p*-values were calculated as these were based solely on feature peak areas. As the WWTP of Eindhoven has a combined sewer system, rainwater runoff impacts the flow. Therefore, features might have very different intensities due to dilution effects. If flows would be considered, this would not necessarily be the case as dilution would be accounted for. Similarly, defining a threshold based on concentrations of MDMA or amphetamine could also be potentially biased, given that dumping during a rainy day might be masked by dilution.

3.3. Similarity-based prioritization

Based on the control step, it was decided to exclude the Euclidian distance, as it did not show a strong relationship with MDMA and amphetamine peak areas and their respective concentrations.

A total of 28 features that appeared to be related to the target drugs have been selected and are presented in Table 1. They represent the features with the highest values (correlation or association) to MDMA or amphetamine. Fig. 4 illustrates the fluctuations of the loads (MDMA or amphetamine) and two related features during the sampling periods.

It was observed that if R's value was high, dCor's value was likely to be also high. In most cases, features with high MIC values (good association) also showed at least one good correlation value (R, ρ, dCor). As a high level of identification was not achieved for all the features, it is not known whether the MIC or a correlation measure can be used alone to find compounds related to the synthesis. It would be recommended to keep various similarity measures, for example, MIC and dCor.

Measures for amphetamine were generally significantly lower than the ones for MDMA. For 2017 samples, it was already suggested that amphetamine was being dumped occasionally, for example, when several trucks discharged pooled urine in the catchment area (July 23rd, 2017), causing a sudden increase in concentrations/loads. On the other hand, there are suspicions that MDMA was being dumped continuously. When applying this logic to all samples, the low correlation and association values can be explained. As not many amphetamine dumps took place, not many peaks were detected. Because the synthesis waste is never the same and because there were not many dumps, it is less likely to find the same compound multiple times. With MDMA, the almost daily dumping ensured that it was more likely to detect the same compound in different peaks, therefore explaining why the relations are stronger.

Further identification using multiple tools (e.g., MS2 spectral libraries and in-silico fragmentation) allowed improving the level of identification. From the seven selected compounds in 2016, none of them generated a MS2 spectrum, and thus only a tentative formula could be assigned. Out of the remaining 21 compounds for 2017–8, all had sufficient intensities to generate a MS2 spectrum. Only four (MDEA (present twice), PMK and safrole) were identified with a level 2 confidence. 9 other features were assigned a speculative structure (level 3) based on a high FISh score (>70). For the other features, they did not appear in *mzCloud* nor *mzVault* and showed a poor score with the spectral tree search, FISh scoring, and *MetFrag* queries. (2Z)-2-acetamido-3-(4-methoxyphenyl)acrylic acid was assigned (level 3) to two different features in samples from 2017.

Only 8 of the 28 features matched with a compound of similar $[M + H]^+$ from the suspect list, reinforcing the idea that the list is not exhaustive. This is most likely due to constant changes and deviations from synthesis routes, which cause the formation of numerous and previously unknown intermediary products and impurities. Similarly, besides MDEA

Table 1
Features supposedly related to MDMA/amphetamine (AMP) detected with the similarity-based prioritization approach. Features with an asterisk (*) were also detected using the group-based prioritization approach. **Confidence level according to Schymanski et al. (2014).

Time series	**Level	Match in suspect list	Name	[min]	[M+H]+	Tentative Formula	R	ρ	dCor	MIC
2016 MDMA	4			4.929	196.13274	$C_{11}H_{17}NO_2$		0.656		0.609
	4	√		12.575	222.11235	$C_{12}H_{15}NO_3$	0.960		0.844	
	4			15.975	228.1959	$C_{13}H_{25}NO_2$	0.638		0.529	
	4			17.124	314.30511	$C_{19}H_{39}NO_2$	0.570		0.549	
2017 MDMA	3		4'-(imidazole-1-yl)acetophenone	2.044	187.08636	$C_{11}H_{10}N_2O$	0.787		0.831	
	3		(2Z)-2-acetamido-3-(4-methoxyphenyl)acrylic acid	5.901	236.09145	$C_{12}H_{13}NO_4$		0.717		0.781
	3		Monoisopropylphosphorylserine	6.103	228.06294	$C_6H_{14}NO_6P$	0.929	0.705	0.920	0.666
	2	√	MDEA*	6.39	208.13297	$C_{12}H_{17}NO_2$		0.766		
	3		(2Z)-2-acetamido-3-(4-methoxyphenyl)acrylic acid	6.521	236.09151	$C_{12}H_{13}NO_4$		0.744		0.626
	2	√	PMK	9.609	179.07005	$C_{10}H_{10}O_3$			0.804	
	2	√	safrole	10.444	163.07515	$C_{10}H_{10}O_2$	0.831		0.836	
	4			13.641	688.14043	$C_{36}H_{30}N_6OP_2S_2$	0.791		0.817	
2018 MDMA	2	√	MDEA	8.704	208.13306	$C_{12}H_{17}NO_2$			0.962	0.676
	5	√		9.118	276.08377		0.959		0.960	0.624
	3		1-ethyl-2,3,4,9-tetrahydro-1H-beta-carboline-3-carboxylic acid	9.17	245.12823	$C_{14}H_{16}N_2O_2$		0.802		0.634
	3		(2Z)-2-acetamido-3-(4-methoxyphenyl)acrylic acid	11.234	236.09117	$C_{12}H_{13}NO_4$	0.968		0.955	
2016 AMP	4			5.967	186.10291	$C_{11}H_{11}N_3$	0.355	0.642	0.569	
	4			11.284	368.24104	$C_{17}H_{36}O_8$				0.640
	4	√		12.257	164.10716	$C_{10}H_{13}NO$	0.361	0.703	0.572	
2017 AMP	4			5.098	269.16040	$C_{11}H_{24}O_7$		0.765		
	3	√	N-cyclohexylacetamide*	5.967	142.12250	$C_8H_{15}NO$			0.785	0.928
	5			6.458	319.17212		0.656		0.794	
	5			9.473	378.11142				0.805	
	4			12.062	235.12991	$C_{10}H_{14}N_6O$	0.710			
2018 AMP	3		Hopantenic acid	6.686	234.13335	$C_{10}H_{19}NO_5$		0.619	0.538	0.509
	3		2-butylnorleucine	7.827	188.16464	$C_{10}H_{21}NO_2$		0.577	0.532	
	4			9.428	462.23288	$C_{14}H_{40}N_9O_6P$				0.539
	5			12.526	171.09166		0.569	0.596	0.572	

and (2Z)-2-acetamido-3-(4-methoxyphenyl)acrylic acid that were selected both in 2017 and 2018, no other compound was recurrent over the three year period. This could again be an indication that production techniques are constantly changing, probably due to modifications in the legislation affecting the availability of (pre-)precursors (Emke et al., 2018). Furthermore, the disposal method can also have an impact – whether waste is being mixed and disposed of at once or step-by-step.

From the selected features, only a few were already considered as significant using the group-based prioritization approach, namely N-cyclohexylacetamide, MDEA, and PMK. However, it is worth mentioning that PMK was detected in 2018 using the group-based prioritization method and in 2017 with the similarity-based prioritization. Therefore, it can be considered that only two compounds were detected in both analyses (N-cyclohexylacetamide and MDEA). As previously mentioned, one of the main problems with the way the group-based prioritization was carried out is that it did not account for changes in flows, which on the other hand was the case here. Furthermore, this approach does not require splitting samples between two groups based on an arbitrary threshold.

3.4. Markers to detect disposal of synthesis waste

The Netherlands is a hub of drug production, which, at least for the considered catchment, can be confirmed by the results of wastewater analysis. This situation complexifies the estimation of community-wide drug use via WBE because of the disposal of synthesis waste or even unused drugs (e.g., fly-tipping) (Emke et al., 2014). If the selected features are included in routine wastewater monitoring campaigns, they could help to detect illegal disposal of synthesis waste of MDMA and amphetamine, or even methamphetamine, in the sewers and differentiating them from peaks due to consumption only.

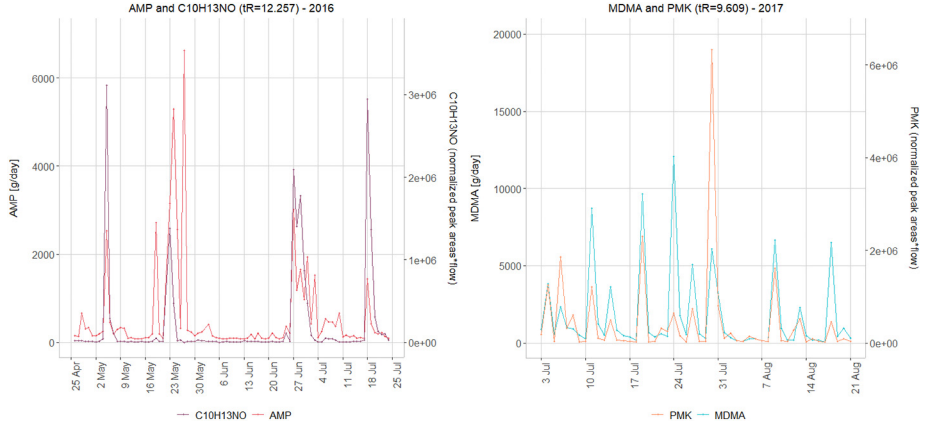

Fig. 4. On the left: fluctuation of the daily load of AMP and a related feature (C10H13NO) in 2016. Not all AMP peaks have a matching C10H13NO peak but a similar pattern was picked up by the methodology. On the right: fluctuation of the daily load of MDMA and a related feature (PMK) in 2017. The feature shows a similar pattern and the strong relationship was picked up by the methodology. The left y-axis in each graph shows loads of AMP/MDMA in [g/day]. The right y-axis is the normalized peak area of the feature multiplied by the flow.

MDEA presented a strong relation with MDMA in samples from both 2017 and 2018. However, it has already been reported multiple times as an impurity in the synthesis process (Palhol et al., 2002; van Deursen et al., 2006) and, it is present in the finished product, it will most likely be ingested and follow the same excretion route as MDMA. Thus, it cannot be determined whether the presence of MDEA is linked to consumption or production waste.

Safrole showed a high correlation with MDMA in 2017. It is a well-known precursor of MDMA and has little licit use (global licit trade of approx. 4500 L/year, incl. safrole-rich oils) (European Monitoring Centre for Drugs and Drug Addiction, 2016a). Similarly, PMK also showed a good correlation with MDMA and is almost exclusively used for the production of MDMA as there is practically no licit trade (European Monitoring Centre for Drugs and Drug Addiction, 2016a).

N-cyclohexylacetamide is a marker of reduction of MD-P2NP to PMK and thus can be linked to MDMA synthesis (Swist et al., 2005). The way the other features with level 3 identification (see Table 1) connect with MDMA/amphetamine could not be established (i.e., they have not been reported to be part of the manufacturing process, not even as an impurity or an adulterant). However, from the obtained results, it can be assumed that they are related. Analogous reasoning can be used for other features with lower confidence levels.

Preliminary results indicate that the selected features can be used as markers of dumps of waste from MDMA/amphetamine synthesis in wastewater (except for MDEA, as previously explained). In the context of this study, the focus was set on the development of the analytical and statistical procedure to select relevant features. The proposed methodology can be used for feature prioritization. It works as an unsupervised and unbiased approach to prioritize features which have a strong relationship with amphetamine or MDMA. The obtained results show that the method was robust enough to detect related features even though these were not present systematically in all dumping events (see Fig. 4). However, it is not known whether this method is able to detect a feature that will appear only in one event, as all the selected features were present on multiple occasions.

Considering that ρ and dCor both derive from R, only one of the three measures could be used next time, alongside MIC. In future studies, stronger confidence could be given by formally identifying prioritized features by purchasing and analyzing reference standards, provided these are available. Further analysis including targeted MS/MS acquisition of the most promising compounds, for which no MS2 were obtained in the first analysis, could allow to (tentatively) identify more structures and thus provides additional clues about the synthesis route used. Furthermore, combining additional (external) sources of information would allow strengthening the hypothesis that the features detected are useful markers. For instance, monitoring the pH to detect sudden acidification or basification in sewers or using data from the police to see if a raid happened in a production lab (Ort et al., 2018; Emke et al., 2014) would be useful for future studies. Assessing the impact of public events might also be desirable, however it would remain difficult to do so considering the period in which samples have been taken for this study (i.e., spring/summer which can have numerous events happening simultaneously).

The advantages of having such markers are multiple. Illegal dumping of MDMA/amphetamine production waste can be tracked down; if features listed in Table 1 are detected, then synthesis waste was likely disposed of in the sewers. These compounds can also be used to distinguish between a peak due to MDMA/amphetamine consumption and a waste dump. This gives an added value to wastewater analysis and enables to tackle the challenge of trying to estimate illicit drug consumption in countries where production is widespread. The obtained information could then be used in various ways. Firstly, for forensic intelligence purposes, such as detecting trends, guiding searches in laboratory dismantling or at customs. Secondly, the information could be used for further research purposes (i.e., detect new impurities or health risks), as well as for legislative/regulatory purposes (i.e., adapting legislation on controlled substances by including new (pre-)precursors).

3.5. Environmental aspects

Thanks to its large capacity, the high loads of drugs did not impact the treatment process in the studied WWTP (Emke et al., 2018). However, the removal efficiency of MDMA in this WWTP is close to −12%, meaning it is not removed (Bijlsma et al., 2012). The fate of the synthesis markers during treatment is yet to be determined. It can be hypothesized that compounds of similar structure (e.g., MDEA, PMK, and safrole) will also be poorly eliminated, meaning that they will be released in receiving waters. Though half-lives can be short, the chemicals released in the environment exhibit a "pseudo-persistence" in surface waters due to continuous dumping and release to the environment. Chronic exposure could bear potential risks for human health, just as aquatic biota has been shown to be negatively affected by the presence of licit and illicit drugs (Rosi-Marshall et al., 2015). Through leakages in sewer networks, synthesis waste could also contaminate groundwater. This is of particular concern when considering that clandestine laboratories often also dispose of their waste in the environment (e.g., fields, forests and rivers), potentially contaminating both surface and groundwater systems. Similarly, when waste is being buried, groundwater contamination can occur (European Monitoring Centre for Drugs and Drug Addiction, 2016a; World Health Organization, 2011). In the Netherlands, a case was reported where production waste had been mixed with manure that was subsequently applied to a cornfield (van Dam, 2016). Runoffs from manure polluted surface waters, the soil contaminated groundwater, and traces of MDMA and amphetamine were found in crops (van Dam, 2016). Because surface and groundwater are often used for drinking-water production, disposal of these chemicals in the environment could eventually endanger human health (European Monitoring Centre for Drugs and Drug Addiction, 2016a; World Health Organization, 2011).

Synthesis markers as the ones reported here could be used to shed light on the circumstances of the incident. For instance, in case of contamination, they could be used to determine the cause of pollution (e.g., synthesis route involved). This information would allow to determine which chemicals have likely been involved, which would allow to evaluate the extent of the damages, gauge the environmental impact and the associated risks. In countries that are affected by the illegal disposal of large amounts of chemical waste, there is a need for further research on the fate and the effects of these chemicals in the environment, and in particular to determine which concentrations could pose health risks. If hazards exist, then advanced water treatment processes (e.g., reverse osmosis, ozonation and advanced oxidation processes), that showed higher removal rates on targeted pharmaceuticals (Rosi-Marshall et al., 2015; World Health Organization, 2011), will be necessary before release to the environment.

4. Conclusion

Disposal of synthesis waste from illicit drug manufacturing represents a complex issue, both from law enforcement and environmental perspectives. This work aimed at developing an innovative approach to detect markers of synthesis waste in wastewater samples collected in an area known for its widespread MDMA and amphetamine manufacturing. For this purpose, a retrospective analysis of 235 wastewater samples was implemented. The samples were collected over 3 years (2016–2018) and analyzed by LC-HRMS. Building on the presence of high concentrations of MDMA and amphetamine in disposed waste, a prioritization strategy relying on the use of various similarity and distance measures was developed and compared to conventional grouping approaches used to prioritize features of interest. The latter approach allowed to select four potential indicators to trace disposal of synthesis waste in wastewater. However, this method showed several drawbacks (e.g., samples need to be grouped based on an arbitrary threshold and it does not allow to account for wastewater dilution effects). On the other hand, the similarity-based prioritization strategy using different metrics (i.e., R, ρ, dCor, MIC) showed better success, with the selection of 28 presumed markers of production of MDMA and amphetamine. This method was robust enough to detect features that were not present in all dumping events. Moreover, in future applications, only MIC and one

correlation measure could be used. The innovative approach presented here shows potential for implementation in routine wastewater monitoring and can help detect illegal waste disposal, discriminate consumption from disposal peaks, and give some information on the synthesis route. These are valuable pieces of information when trying to understand the extent and impact of illegal disposal of chemical wastewater.

CRediT authorship contribution statement

Naomi Reymond: formal analysis, investigation, writing – original draft, visualization. **Erik Emke**: resources, writing – review & editing. **Thea Boucheron**: resources. **Thomas ter Laak**: writing – review & editing. **Pim de Voogt**: writing – review & editing. **Pierre Esseiva**: supervision, writing – review & editing. **Frederic Been**: conceptualization, supervision, visualization, writing – original draft, writing – review & editing.

Declaration of competing interest

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

Acknowledgments

Naomi Reymond would like to thank the University of Lausanne for the scholarship, Dennis Vughs and Andrea Brunner for their precious advice. The authors would like to thank the European Union's Justice Programme — Drugs Policy Initiatives, EuSeME (project number 861602). The authors also thank the WWTP personnel from the water board De Dommel (Eindhoven, the Netherlands).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.152139.

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