Wastewater-based assessment of regional and temporal consumption patterns of illicit drugs and therapeutic opioids in Croatia

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HIGHLIGHTS

• Drug consumption patterns in Croatia were studied using sewage epidemiology
• The most prevalent illicit drugs were cannabis, cocaine and heroin
• Drug consumption shows pronounced regional and seasonal differences
• Seasonal differences were observed between coastal and continental cities
• The results were compared with epidemiological data on treated addicts

GRAPHICAL ABSTRACT

A comprehensive study of spatial and temporal consumption patterns of the selected illicit drugs (heroin, cocaine, amphetamine, MDMA, methamphetamine, cannabis) and therapeutic opioids (codeine, methadone) has been performed in six Croatian cities by applying wastewater-based epidemiology. The investigated cities (Bjelovar, Vinkovci, Varazdin, Karlovac, Zadar and Zagreb) varied widely in the population size (27,000–688,000 inhabitants) as well as in the number of registered drug consumers included in compulsory and voluntary medical treatment and rehabilitation programs (30–513 persons/100,000 inhabitants of age 15–64). The most consumed illicit drugs were cannabis (10–70 doses/day/1000 inhabitants), heroin (<0.2–10 doses/day/1000 inhabitants) and cocaine (0.2–8.7 doses/day/1000 inhabitants), while the consumption of amphetamine-type drugs was much lower (<0.01–4.4 doses/day/1000 inhabitants). Enhanced consumption of illegal drugs was generally associated with larger urban centers (Zagreb and Zadar) however comparatively high consumption rate of cocaine, MDMA and methadone was determined in some smaller cities as well. The overall average dose number of 3 major illegal stimulants (cocaine, MDMA, amphetamine) was rather similar to the number of corresponding heroin doses, which is in disagreement with a comparatively much higher proportion of heroin users in the total number of registered drug users in Croatia. Furthermore, the illicit drug consumption pattern in the large continental city (Zagreb) was characterized by a significant enhancement of the consumption of all stimulants during the weekend, which could not be confirmed neither for the coastal city of Zadar nor for the remaining small continental cities. On the other hand, the city of Zadar exhibited a significant increase of stimulant drug usage during summer vacation period, as a result of pronounced seasonal changes of the population.
composition and lifestyle in coastal tourist centers. The obtained results represent a valuable complementary data source for the optimisation and implementation of strategies to combat drug abuse in Croatia.

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1. Introduction

Wastewater-based epidemiology has been increasingly used as an additional source of information on the consumption of illicit drugs (e.g. Bijlsma et al., 2014; Irvine et al., 2011; Kankaanpää et al., 2014; Khan et al., 2014; Nefau et al., 2013; Ort et al., 2014; Östman et al., 2014; Postigo et al., 2010; Terzic et al., 2010; Thomas et al., 2012; Vuori et al., 2014; Zuccato et al., 2008). The main advantage of this innovative approach is its objectivity, which is based on highly accurate chemical measurements of selected drug biomarkers in wastewater, and its suitability for near-real-time tracking of the changes in drug consumption patterns within the selected communities. Wastewater analysis can provide information about spatial and temporal variations of illicit drugs use as well as on the impact of special events such as national holidays or music festivals on drug consumption patterns. Consumption patterns were investigated at different scales: as detailed studies in one selected municipality (e.g. Karolak et al., 2010; Terzic et al., 2010), as a part of national surveys (e.g. Banta-Green et al., 2009; Kankaanpää et al., 2014; Metcalfe et al., 2010; Nefau et al., 2013; van Nuijs et al., 2009; Vuori et al., 2014) or in the international context (e.g. Ort et al., 2014; Thomas et al., 2012; Zuccato et al., 2008). The recently performed international studies (Ort et al., 2014; Thomas et al., 2012) indicated rather pronounced geographical differences in illicit drug consumption patterns across Europe. The highest cocaine use was determined in western and central Europe, while the highest consumption of methamphetamine was determined in northern and eastern Europe. Furthermore, significant differences in drug consumption patterns were also demonstrated within the same country (e.g. Kankaanpää et al., 2014; Lai et al., 2013a; Nefau et al., 2013; Thomas et al., 2012; van Nuijs et al., 2009), indicating typically higher illicit drug abuse in highly urbanized metropolitan areas. Wastewater analyses can also be used to track temporal variability in illicit drug consumption. Zuccato et al. (2011) demonstrated a significant drop of cocaine use at the beginning of economic crisis in Italy. Moreover, a number of studies demonstrated a pronounced increase in consumption of stimulants during the weekend (Bijlsma et al., 2009; Huerta-Fontela et al., 2008; Karolak et al., 2010; Lai et al., 2011; Prichard et al., 2012; Reid et al., 2011; Terzic et al., 2010; Thomas et al., 2012; van Nuijs et al., 2009; Zuccato et al., 2008) as well as during music festivals (Bijlsma et al., 2014; Lai et al., 2013b; Mackulak et al., 2014) and sport events (Gerrity et al., 2011). Lai et al. (2013a) demonstrated that the consumption of several illicit drugs, including MDMA, cocaine and methamphetamine on the Australian vacation island significantly increased during the national peak holiday season, however the number of studies on seasonal variability of drug consumption is still rather low.

There are three major specific aims to study patterns of drug abuse in Croatia. As a transition country, Croatia is characterized by fast socio-economic changes in the last two decades, some of which could be linked with the changing patterns in drug abuse. Specifically, Croatia is situated on a so-called Balkan route of drug trafficking, making it vulnerable to enhanced availability of illicit drugs originating from Middle East. After the end of the war conflicts in the former Yugoslavia, this route has become increasingly actual. Finally, Croatia’s fast growing tourism during the last couple of years has become an important issue when addressing different routes of drug trafficking. Traditionally, sources of information on illicit drug consumption in Croatia have included primarily the data on the number of treated drug addicts and police drug seizures, while general population surveys as well as wastewater-based epidemiology have been used only recently. The general population survey conducted in 2011 indicated that the lifetime prevalence rates of almost all investigated illicit drugs in Croatia were below the European average (Glavak Tkalic et al., 2013), which is in a good agreement with the results of the two recent wastewater-based Europe-wide studies (Ort et al., 2014; Thomas et al., 2012). On the other hand, the same study indicated that the lifetime prevalence of heroin consumption in Croatia in 2011 (0.4%) was at the European average (0.41%). Moreover, the wastewater-based epidemiology study conducted in the city of Zagreb in 2009 indicated that the consumption prevalence of heroin was even higher than in some other European cities (Terzic et al., 2010). Currently available wastewater-based epidemiology data for Croatia are limited only to the city of Zagreb while the data on regional differences in illicit drug consumption are still missing. The aim of this study was therefore to investigate regional variability of the consumption patterns of illicit drugs (cocaaine, heroin, amphetamine, MDMA, methamphetamine and cannabis) and two therapeutic opioids (methadone, codeine) in Croatia. The specific goals of the study included: a) a preliminary comparison of drug consumption patterns in six selected Croatian cities with regard to the city population size and geographic position; b) study of weekday-related consumption patterns; c) study of changes in drug consumption patterns associated with summer tourist season in two selected cities and d) comparison of wastewater-based consumption estimates with the available epidemiological data.

2. Material and methods

2.1. Selection of target compounds

The study included analyses of 6-acetylmorphine (6-AM), morphine (MOR) and 3-β-D-morphine glucuronide (MG) as principal heroin-derived substances, cocaine (COC) and its main metabolite benzoylecgonine (BE), amphetamine (AMP), 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), methamphetamine (MAMP) as well as two urinary metabolite of cannabis, 11-nor-9-carboxy-AΔ2-tetrahydrocannabinol (THC-COOH) and 11-hydroxy-AΔ2-tetrahydrocannabinol (THC-OH). Methadone (MTHD) and its metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrroline (EDDP) as well as codeine (COD) were monitored as representatives of therapeutic opioids. The selection of target compounds was made based on the data on drug seizures in Croatia and general population survey performed in Croatia in 2011 (Glavak Tkalic et al., 2013).

2.2. Chemicals and materials

Standard solutions of all target analytes (1 g L−1) and their deuterated analogs (0.1 g L−1) were purchased from Lipomed AG (Switzerland). Mixed standard solutions of the analytes and their deuterated analogs (used as surrogate standards) were prepared in methanol (MeOH) at concentrations of 10 mg L−1 and 2 mg L−1, respectively, and kept in the dark at −20 °C. Aqueous ammonia solution (NH3, 25%) and LC-MS grade MeOH were purchased from Merck AG (Darmstadt, Germany). Acetic acid (CH3COOH), also LC-MS grade, formic acid (HCOOH) and phosphoric acid (H3PO4) were purchased from Fluka (Switzerland). MQ water was obtained by purifying in Elix-Mili-Q-system (Millipore, Bedford, USA). Oasis MCX cartridges (150 mg / 6 mL) were produced by Waters (Milford, MA, SAD) while Strata NH2 (200 mg / 3 mL) cartridges as well as HPLC columns used for the chromatographic separation (Synergi Polar; 4 μm, 150 mm × 3 mm and Kinetex FFP; 2.6 μm, 100 mm × 2.1 mm) were manufactured by Phenomenex (Torrance, California, USA).
The samples collected in Zagreb during the 1-year sampling campaign were processed within a few hours after the collection, while all other collected samples were frozen immediately after the collection and kept in the freezer (−20 °C) until analysis. All frozen samples were processed within <1 month after collection.

2.4. Analytical methodology

The sample preparation and instrumental analysis were performed applying fully validated analytical method published before (Senta et al., 2013). Briefly, the wastewater samples (125 mL) were spiked with surrogate standards (120 ng/L), filtered using GF/C filters and subsequently enriched using Oasis MCX cartridges, cleaned-up using NH₂ cartridges and analyzed by triple-quadrupole liquid-chromatography-tandem mass spectrometry (Quantum AM, Thermo Electron, USA). The method accuracy was in the range from 83% to 116% and extraction recovery between 60% and 94%. The method quantification limits were between 0.1 and 5 ng/L.

2.5. Estimation of drug consumption

The assessment of drug consumption was performed by applying the methodology proposed by Zuccato et al. (2008). The consumption of individual drugs (expressed as number of average doses per 1000 inhabitants) was calculated by multiplying the population normalized representative average mass loads of selected drug biomarkers by the corresponding correction factors and by dividing with the corresponding average dose size.

In order to take into account possible weekday-related differences in drug consumption patterns (Terzic et al., 2010), representative average mass loads (X<sub>RP</sub>) and their corresponding standard deviations (S<sub>RP</sub>) were calculated as follows:

$$\text{X}_{\text{RP}} = \frac{5}{7} \times \text{(workday)} + \frac{2}{7} \times \text{(weekend)}$$

$$\text{S}_{\text{RP}} = \sqrt{\left(\frac{5}{7} \times \text{(workday)}\right)^2 + \left(\frac{2}{7} \times \text{(weekend)}\right)^2}$$

where X (workday), S (workday), X (weekend) and S (weekend) represent the average values and standard deviations of workday and weekend daily mass loads. The daily mass loads were calculated by multiplying the concentrations of urinary biomarkers by the corresponding daily wastewater flow. In the case when the concentrations of the individual urinary biomarkers were below the detection limit, the corresponding daily mass loads were estimated using the concentration equal to the half of the detection limit. The population normalized daily mass loads were obtained by dividing the representative average mass loads with the number of inhabitants (in thousands) served by the investigated WWTP.

Apart from some exceptions, the correction factors used in the calculation of drug consumption were taken from the paper published by Zuccato et al. (2008). The estimation of cocaine consumption was

Table 1

<table>
<thead>
<tr>
<th>Characteristics of the cities and wastewater treatment plants (WWTPs) included in the study.</th>
<th>Zagreb</th>
<th>Zadar</th>
<th>Karlovac</th>
<th>Varazdin</th>
<th>Vinkovci</th>
<th>Bjelovar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of inhabitants</td>
<td>688,163</td>
<td>71,474</td>
<td>46,833</td>
<td>38,839</td>
<td>32,029</td>
<td>27,024</td>
</tr>
<tr>
<td>Number of treated drug consumers/1000 inhabitants</td>
<td>4.19</td>
<td>5.13</td>
<td>0.81</td>
<td>2.18</td>
<td>1.09</td>
<td>0.30</td>
</tr>
<tr>
<td>No. of inhabitants served by WWTP</td>
<td>688,163</td>
<td>64,324</td>
<td>29,500</td>
<td>60000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28,000</td>
<td>23,574</td>
</tr>
<tr>
<td>WW flow&lt;sup&gt;c&lt;/sup&gt; (m&lt;sup&gt;3&lt;/sup&gt;/day)</td>
<td>252,336</td>
<td>12,750</td>
<td>11,900</td>
<td>21,514</td>
<td>9000</td>
<td>10,000</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of treated drug consumers/1000 inhabitants in the corresponding Croatian counties — data for 2013 obtained from the Croatian Institute for Public Health (Katalinic and Huslic, 2014);

<sup>b</sup> WWTP Varazdin is treating the wastewater of the city of Varazdin and other surrounding villages;

<sup>c</sup> Average dry wastewater flow; Number of inhabitants based on the 2011 Census data; The number of inhabitants served by the WWTP was calculated by multiplying the percentage of the population served by WWTP by population number derived from the 2011 Census. The number of inhabitants served by WWTP Varazdin was obtained from the WWTP manager.
made by using later proposed correction factor of 3.6 (Castiglioni et al., 2013), while the estimation of methadone consumption was performed by using recently proposed correction factor of 2.0 (Thai et al., 2015). The consumption data were transformed into the number of average drug doses by applying the data on the size of a dose presented in the Table 2.

2.6. Statistical evaluation

Statistical analysis of the results was performed with Sigma Plot 12.0 (Systat software Inc., SAD). Mostly non-parametric tests were applied because the majority of the data deviated from the normal Gaussian distribution. Statistical evaluation of temporal variability was done with Mann–Whitney test. A significance level of < 0.05 was set.

3. Results and discussion

3.1. Occurrence of drug biomarkers in municipal wastewaters of Croatian cities

The study was performed in 6 Croatian cities (Zagreb, Zadar, Karlovac, Vinkovci, Bjelovar and Varazdin) whose population size (27,024–688,163 inhabitants) as well as the number of registered drug consumers (0.3–5.15 treated drug consumers/1000 inhabitants of age 15–64) varied in a rather wide range (Table 1). The analyses of municipal wastewaters showed a ubiquitous presence of the investigated drug biomarkers in the selected Croatian cities (Table S1) with concentrations of individual drug biomarkers varying widely, depending on the sampling location and type of the compound. Almost all analyzed samples (99%–100%) contained measurable concentrations of MOR, BE, COC, COD, EDDP and THC-COOH, while most of them (83%–98%) were above the detection limit.

![Fig. 2. Representative average mass loads of 7 selected urinary drug biomarkers in investigated Croatian cities. The error bars represent the variability of the individual data sets.](image-url)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug biomarker</th>
<th>Percentage of drug dose excreted as drug biomarker (%)</th>
<th>Molar ratio</th>
<th>C_f</th>
<th>Dose (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heroin</td>
<td>6-AM</td>
<td>1.3</td>
<td>1.13</td>
<td>86.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cocaine</td>
<td>BE</td>
<td>29</td>
<td>1.05</td>
<td>3.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>AMP</td>
<td>30</td>
<td>1.00</td>
<td>3.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ecstasy</td>
<td>MDMA</td>
<td>65</td>
<td>1.00</td>
<td>1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>97&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>THC (Cannabis)</td>
<td>THC-COOH</td>
<td>0.6</td>
<td>0.91</td>
<td>152</td>
<td>125&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methadone</td>
<td>EDDP</td>
<td>55</td>
<td>1.12</td>
<td>2.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>80&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> van Nuijs et al., 2011; <sup>b</sup> Castiglioni 2013; <sup>c</sup> Zuccato et al. 2008; <sup>d</sup> Thai et al., 2015; <sup>e</sup> Office for Combating Narcotic Drug Abuse of the Government of the Republic of Croatia, data for 2013; <sup>f</sup> Croatian Institute of Public Health, data for Zagreb for 2010.
contained detectable concentrations of MG, MDMA, MTHD and THC-OH. By contrast, some of the analytes, including 6-AM and AMP were rather frequently (94–100%) detected in the wastewater of larger cities (Zagreb, Zadar), while their concentrations in the wastewater of the remaining 4 cities were mainly below the method detection limit. MAMP was the least frequently found compound in all samples and its concentration never exceeded 5 ng/L, which indicated rather low prevalence of its consumption in Croatia. The concentration of all analyzed drug biomarkers in all investigated cities were, generally, relatively low and rarely exceeded 1 ng/L. The highest average concentrations were determined for BE (190 ± 89 ng/L), MOR (178 ± 95 ng/L) and THC-COOH (132 ± 27 ng/L), while the lowest ones were determined for MG (from <0.3 ng/L to 62 ng/L) and 6-AM (<0.12 ng/L to 28 ng/L).

3.2. Regional differences in drug consumption patterns in Croatia

The comparability between the different sized cities was achieved by calculating the population normalized representative average mass loads of the selected urinary biomarkers of heroin (6-AM), cocaine (BE), amphetamine (AMP), ecstasy (MDMA), cannabis (THC-COOH), methadone (EDDP) and codeine (COD). The results are presented in Fig. 2. The highest daily mass loads were determined for COD (up to 120 mg/day/1000 inhabitants), EDDP (up to 60 mg/day/1000 inhabitants), THC-COOH (up to 59 mg/day/1000 inhabitants) and BE (up to 72 mg/day/1000 inhabitants), while the lowest daily mass loads were determined for 6-AM (<0.1–1.2 mg/day/1000 inhabitants). The daily mass loads of amphetamine-type drugs were also comparatively low (up to 13 mg/day/1000 inhabitants of AMP and 8.3 mg/day/1000 inhabitants of MDMA). The spatial distribution of the population normalized daily excretion of 6-AM, BE, AMP, and EDDP was generally associated with the size of the city, indicating the enhanced consumption rates of illegal drugs and methadone in the two larger urban centers (Zagreb and Zadar), which is in a good agreement with the findings published for some other countries (e.g., Irvine et al., 2011; Vuori et al., 2014; Kankaanpää et al., 2014). However, there were some exceptions to that rule. For example, the population normalized average daily consumption of MDMA in the small city of Vinkovci (8.2 ± 3.7 mg/day/1000 inhabitants) was rather similar to the consumption of that drug in Croatian capital (8.3 ± 2.9 mg/day/1000 inhabitants), despite the 20-fold difference in their population size. Besides that, the consumption prevalence of cannabis in two smaller continental cities (Karlovac, Bjelovar) was not significantly different than its consumption in Zadar, while, on the other hand, heroin, cocaine and amphetamine consumption was significantly higher in Zadar. The variability of therapeutic opioid compounds also showed distribution patterns independent of the city size. Obviously, the reasons for the observed geographical differences in drug consumption patterns in Croatia are complex and cannot be simply ascribed only to the differences in population size of different cities. Further studies are needed in order to address the issue of seasonal variability of illicit drug consumption and its possible impact on the observed regional differences since the sampling periods as well as the number of the collected samples per city in this study were different. However, a statistical comparison of the data sets obtained in the city of Zagreb for 1-week in March (n = 7) and a whole-year sampling period (n = 57) did not show any significant difference between the two data sets for any of the investigated illicit drugs (Krizman, 2015). Based on that, it was assumed that a similar week-to-week variability can be expected in smaller inland cities as well, provided that the sampling week did not include any special events (festivals, sport events or holidays, such as Christmas and New Year periods). Several studies conducted in major European cities demonstrated that the consumption of certain stimulants (e.g., cocaine and some amphetamine-type drugs) was significantly enhanced during the weekend as compared to the weekdays (e.g., Thomas et al., 2012; Zuccato et al., 2008). Among the cities included in this study, similar consumption pattern was statistically confirmed only for the capital of Croatia (Fig. 3), although the enhanced consumption of cocaine was determined in some other smaller inland cities as well. Statistical analysis of the results presented in Fig. 3 confirmed a significantly enhanced weekend consumption (Mann–Whitney test, p < 0.05) of all 3 stimulants in the
city of Zagreb \((n = 57)\), which is in a good agreement with our previous findings \((\text{Terzic et al., 2010})\). In contrast, the differences between the weekend and weekday consumption of these drugs were not significant neither in the city of Zadar \((n = 32)\), as the representative of coastal tourist cities, nor in the remaining 4 smaller inland cities \((n = 32)\). Nevertheless, these findings are based on relatively small data sets which included < 10% of both weekdays and weekend days and need to be further investigated in the future based on more representative data sets.

3.3. Changes in drug consumption patterns associated with summer tourist season

The absence of any clear weekly dynamics of stimulant loads in the city of Zadar suggested that the drug consumption patterns in coastal tourist centers might differ from those in large continental cities like Zagreb. In order to test this hypothesis, the comparison of the data sets determined in March and July/August period has been made for these two cities and the results are presented in Fig. 4.

According to the official statistics, the total population size of the city of Zadar during the main tourist season (July–August) increased by 16% as compared to the preseason period \(\text{data for 2013; Fig. S1,}\) which was taken into account in the calculation of population normalized mass loads presented in Fig. 4. However, it is very likely that the real increase of nonresidential population during the summer season might have been even larger due to the certain percentage of non-registered tourists visiting the city. By contrast, the contribution of the tourists to the total number of residents in the city Zagreb was considered negligible \((\leq 1\%)\). The population number in the city of Zagreb could be however somewhat lower during the summer vacation period but we were not able to obtain any official data to confirm this assumption. The statistical comparison of the data obtained in March 2013 indicated that consumption prevalence of almost all illicit drugs (heroin, MDMA, cocaine, cannabis) as well as of codeine was significantly higher \((\text{Mann–Whitney test, } p < 0.05)\) in Zagreb than in Zadar. This is in a good accordance with previously published results which indicated higher prevalence of illicit drug consumption in metropolitan cities \((\text{Irvine et al., 2011; Vuori et al., 2014; Kankaanpää et al., 2014})\). By contrast, the intercity differences in the consumption of AMP and MTHD were insignificant. In contrast, the observed intercity differences in the drug consumption were much less pronounced during the summer vacation period \(\text{(Fig. 4, especially for MDMA, whose consumption in Zadar even surpassed the one in Zagreb. This was interpreted as a result of pronounced changes both in the population structure as well as the lifestyle during the summer period. Interestingly, a significant summer decrease in the prevalence of cannabis consumption was determined in both investigated cities. The results obtained in this study are in a good agreement with the study performed in Australia \((\text{Lai et al., 2013a})\), which indicated a significant increase of MDMA, cocaine and methamphetamine consumption, accompanied by a significant decrease of cannabis consumption in a tourist area during the annual key holiday.}

3.4. Drug consumption assessment and comparison with epidemiological data

The estimations based on wastewater-based epidemiology indicated that cannabis was the most prevalently used drug in all investigated

![Fig. 4](image-url) Seasonal variability of representative average mass loads of selected urinary drug biomarkers in a large continental city (Zagreb) and a medium-size coastal city (Zadar). \((6-\text{AM} = 6\text{-acetylmorphine}; \text{AMP} = \text{amphetamine}; \text{MDMA} = 3.4\text{-methylendioxymethamphetamine}; \text{BE} = \text{benzoylecgonine}; \text{THC-COOH} = 11\text{-nor-9-carboxy-11\text{-nor-9-carboxy-}\Delta 9\text{-tetrahydrocannabinol}}; \text{EDDP} = 2\text{-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine}; \text{COD} = \text{codeine})\). The error bars represent the variability of the individual data sets.
cities (10–70 doses/day/1000 inhabitants; Fig. 5), which is in a good agreement with the results of the studies performed before in Croatia (Glavak Tkalic et al., 2013; Terzic et al., 2010) as well as in some other countries (e.g. Zuccato et al., 2008; Lai et al., 2013a, 2013b). It should be stressed that the correction factor of 152 applied for the back-calculation of cannabis (THC) consumption (Zuccato et al., 2008) does not take into account fecal excretion of THC-COOH, which could potentially lead to an overestimation of cannabis (THC) consumption (Khan and Nicell, 2012). Nevertheless, the estimations obtained by using the correction factor of 152 for the back-calculation of THC consumption would indicate that 1–7% of the population in the investigated 6 cities might be cannabis consumers, if a typical consumer takes one dose per day. Such figure would be in a relatively good agreement with the figure on the last-year prevalence of cannabis use in Croatia (5%) in 2011, reported by Glavak Tkalic et al. (2013). However, if the average consumption frequency is lower, the number of cannabis users estimated using wastewater-based epidemiology would be in disagreement with the official figures on cannabis use in Croatia. Apart from cannabis, the most consumed illicit drugs in Croatian cities were heroin (<0.2–10 doses/day/1000 inhabitants) and cocaine (<0.2–8.7 doses/day/1000 inhabitants), while the consumption of amphetamine (<0.03–4.4 doses/day/1000 inhabitants) and MDMA (<0.01–0.1 doses/day/1000 inhabitants) was lower. The comparison of our data with those published for some other countries indicated either similar or much lower consumption of illicit drugs in Croatian cities (e.g. Lai et al., 2013a, Kankaanpää et al., 2014; Thomas et al., 2012; Zuccato et al., 2008), which is in line with the findings published by Glavak Tkalic et al. (2013).

The only available epidemiological data for the investigated cities were those on the number of registered drug consumers, included in compulsory and voluntary medical treatment and rehabilitation programs (Fig. 5, data from Katalinic and Huskic, 2014). Official statistics on drug users in Croatia distinguishes two groups of users: heroin addicts and users of other drugs, which include primarily amphetamine-type drugs, cocaine and cannabis. Spatial distribution of registered drug users indicated, as expected, the highest drug consumption prevalence in the two largest cities, which is in agreement with the findings of our study. As can be seen in Fig. 5, heroin users still represent a large majority of all recorded illicit drug users in Croatia. Wastewater-based epidemiology data indicated that the collective consumption rate of heroin and illicit stimulants in the city of Zagreb was much higher than in the city of Zadar, which was not directly reflected in the epidemiological data (Fig. 5). Moreover, measurable per capita daily consumption of heroin, based on 6-AM excretion, were determined only in Zagreb (10 doses/day/1000 inhabitants) and Zadar (6.2 doses/day/1000 inhabitants), while its consumption in the remaining 4 cities was lower than the minimal detectable amount (<0.2–0.4 doses/day/1000 inhabitants), which indicated rather heterogeneous distribution of heroin consumption in Croatia.

It should be pointed out that the regional distribution patterns of the heroin and methadone consumption were rather similar with the exception of the city of Vinkovci, which was characterized by a
comparatively much lower heroin-to-methadone ratio. This can probably be related to the enhanced ratio between the registered heroin users, receiving methadone therapy, and non-registered heroin users in that city. If we assume that most of the methadone in wastewater originated from the treatment of heroin users, the number of doses should generally reflect the number of registered addicts. However, the comparison between the spatial distribution patterns of methadone consumption and number of treated heroin consumers (Fig. 5) showed some discrepancies. For example, in spite of 5-fold difference in the number of treated heroin users in the cities of Zadar (4.87 heroin addicts/1000 inhabitants age 15–64) and Vinkovci (0.96 heroin addicts/1000 inhabitants age 15–64), the consumption rates of methadone, which is the main therapeutic drug used in a treatment of heroin addicts in Croatia, was rather similar in these two cities (1 dose/day/1000 inhabitants). On the other hand, comparatively high number of heroin users in the city of Varazdin (1.73 treated heroin users/1000 inhabitants age 15–64) was not associated with enhanced methadone consumption in that city (≤0.3 doses/day/1000 inhabitants). The observed discrepancies can probably be assigned to the regional differences in the procedures applied in the heroin addiction treatment in Croatia. Namely, the common practice of the treatment in Croatia includes the substitution therapy either by methadone or by buprenorphine, which might be used in different proportions in different cities. Besides that, the number of methadone doses was calculated based on the average dose for the city of Zagreb (Table 2), while the average doses for other cities might be somewhat different. An interesting comparison between heroin and other drugs can be made based on the relationship between the corresponding consumption rates and the number of treated addicts. It should also be noticed that the average cumulative daily consumption of 3 major illegal stimulants (cocaine, MDMA, amphetamine) was rather similar to or even higher than the consumption of heroin in all investigated cities. However, this was not reflected in the available epidemiological data on treated users, suggesting that only a small percentage of other drug users was included in the treatment programs. The observed discrepancies between the two data sets may be caused by several factors, including a rather long time gap between the initial drug consumption and the involvement of the consumers in the treatment.

4. Uncertainties

Since the wastewater-based epidemiology approach involves a number of steps, such as sampling, chemical analyses, estimation of population size and back-calculations of consumption, the assessment of drug consumption patterns includes several sources of uncertainties (Castiglioni et al., 2013). Taking into account considerations by Castiglioni et al. (2013), it can be expected that most of the uncertainties in our study were associated with sampling procedures. The study was based on time-proportional 24-h composite sampling with sampling intervals varying from 6 min in Bjelovar to 30 min in Varazdin. The longer sampling interval (30 min) in the city of Varazdin might have affected the reliability of the results since the longer sampling intervals can affect the sample representativeness, especially for smaller cities (Ort et al., 2010). Furthermore, the comparison of the cities, selected for the preliminary study, was performed in different seasons, which possibly introduced some errors associated with temporal (week-to-week) variability. These uncertainties, which may affect the significance of the differences between the cities, were minimized by making sure that the selected sampling weeks did not include any special events (festivals, sport events or holidays). Moreover, the extensive observations in the city of Zagreb indicated that day-to-day variabilities were much smaller (from 14% for BE to 55% for MDMA) than the differences between the cities. For example, per capita mass loads of illicit drugs in the city of Zagreb were several times higher than those in the small inland cities (1.2 to 4.3 times for THC-COOH; 0.9 to 8.9 times for MDMA; 11 to 22 times for AMP; 6.2 to 35 times for BE). Therefore, the observed regional differences can be considered realistic, although some follow-up studies are warranted, especially in the city of Varazdin.

5. Conclusion

This study represents the first comprehensive investigation of illicit drug consumption patterns in different Croatian cities, including the role of city size and the role of summer tourist season. Comparison of the wastewater-based estimates of the consumption with the available epidemiological data showed that classical epidemiological data on the number of treated drug-abusing patients do not necessarily reflect current situation in drug abuse. Consequently, wastewater-based epidemiology provides an important complementary source of data which needs to be increasingly used in the process of creating new preventive measures as well as checking the efficiency of the existing ones.

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Appendix A. Supplementary data

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References
