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Abstract: A comprehensive study aimed at monitoring of temporal variability of illicit drugs (heroin, cocaine, amphetamine, MDMA, methamphetamine and cannabis) and therapeutic opiate methadone in a large-sized European city using wastewater-based epidemiology (WBE) was conducted in the city of Zagreb, Croatia, during an 8-year period (2009-2016). The study addressed the impact of different sampling schemes on the assessment of temporal drug consumption patterns, in particular multiannual consumption trends and documented the possible errors associated with the one-week sampling scheme. The highest drug consumption prevalence was determined for cannabis (from 59 ± 18 to 156 ± 37 doses/day/1000 inhabitants 15-64 years), followed by heroin (from 11 ± 10 to 71 ± 19 doses/day/1000 inhabitants 15-64 years), cocaine (from 8.3 ± 0.9 to 23 ± 4.0 doses/day/1000 inhabitants 15-64 years) and amphetamine (from 1.3 ± 0.9 to 21 ± 6.1 doses/day/1000 inhabitants 15-64 years) whereas the consumption of MDMA was comparatively lower (from 0.18 ± 0.08 to 2.7 doses ± 0.7 doses/day/1000 inhabitants 15-64 years). The drug consumption patterns were characterized by clearly enhanced weekend and Christmas season consumption of stimulating drugs (cocaine, MDMA and amphetamine) and somewhat lower summer consumption of almost all drugs. Pronounced multiannual consumption trends were determined for most of the illicit drugs. The investigated 8-year period was characterized by a marked increase of the consumption of pure cocaine (1.6-fold), THC (2.7-fold), amphetamine (16-fold) and MDMA (15-fold) and a concomitant decrease (2.3-fold) of the consumption of pure heroin. The heroin consumption decrease was associated with an increase of methadone consumption (1.4-fold), which can be linked to its use in the heroin substitution therapy. The estimated number of average methadone doses consumed in the city of Zagreb was in a good agreement with the prescription data on treated opioid addicts in Croatia.

Response to Reviewers: Reviewer #1: The authors report a WBE study in which they monitor temporal variability in biomarkers of heroin, cocaine, amphetamine, MDMA, methamphetamine and cannabis and the therapeutic opiate methadone in the waste water of Zagreb, Croatia over an 8-year period (2009-2016). The study assessed the reliability of one week vs annual sampling strategies on estimated temporal drug consumption patterns, weekday variations in the use of these drugs, and trends over time in the use of these drugs.

Their main findings were similar to those of studies in other European and high income countries in that:

* the drug with the highest consumption prevalence of use was for cannabis, followed by heroin, cocaine and amphetamine, with MDMA use much lower;
* There were enhanced weekend and holiday consumption of cocaine, MDMA, and amphetamine;
* Consumptions was marginally lower in summer for almost all drugs, reflecting population movements;
* Over the 8-year study period there was increases in the consumption of cocaine and THC and a more marked increase in use of amphetamine (16-fold) and MDMA (15-fold). There was a large decrease in the consumption of heroin over the study period and an increase in the last year of study.
* The decline in heroin use was associated with an increase in methadone consumption that was linked to its increased use as a substitution treatment for heroin.
* The estimated average daily methadone dose in the city of Zagreb agreed well with the prescription data on the number of opioid addicts in Croatia enrolled in methadone treatment.

The last two findings are major novelties that have not been previously reported so far as I am aware, namely, a decline in indicators of heroin use occurring as there was increased use of methadone; and showing that methadone consumption estimated from waste water biomarkers closely agreed with data on the amount of methadone dispensed.

Q: I had one minor issue: what was the justification for the "arbitrary" definition of a significant ratio of weekend to weekly use of a drug, i.e. 1 plus or minus -0.2?

R: The criterion was selected based on the initial insight into the day-to-day variability of various non-stimulating drugs (in particular morphine, codeine and methadone) in the city of Zagreb with moderate relative standard deviations (RSD) of average daily loads being in the range up to 11 to 17%, which indicated robustness of the collective excretion rates as an indicator of drug abuse in larger populations. Moreover, this criterion is well above the possible limitations posed by mere repeatability of the analytical method.

Reviewer #2: General Comments

The manuscript presents a 7-year monitoring of selected drug consumption patterns in the city of Zagreb. The study presents an extensive monitoring data and is within the scope of STOTEN. Although similar studies have published before, the authors have tried to give a new perspective to the study by comparing monitoring data for different sampling periods and look for specific trends. I recommend this manuscript for publication following some major corrections.

Major comments:
* It appears that the Authors have tried to make relatively generalized conclusions about any "large-sized European city" using the example of Zagreb with limited number of drugs considered. However, EMCDDA reports have shown the trends of drug use is very region-dependent and different for each drug. I suggest the authors to be more moderate and corroborate their outcomes with the studies in the same region and cities with similar population size.

R: We do not agree with this comment. As it was clearly emphasized in the title, one of the primary goals of the paper was testing different sampling strategies (sampling schemes) and estimation of the robustness of the applied sampling schemes to assess relatively small changes in consumption rates by taking into account possible sources of temporal variability (weekly dynamics, seasonal variability and impact of special events). These are important methodological issues of general character applicable to any large sized city. Our study did not intend to make any generalization regarding the drug consumption trends in other large-sized European cities based on the data from the city of Zagreb. We rather demonstrated that considering the proper sampling schemes can significantly improve the reliability of the trend monitoring making possible detection of relatively small changes.

* Using the term "sampling strategies", especially in the title is misleading. In fact, the study does not consider different sampling strategies (e.g. flow, volume, time proportional with different sampling intervals) but it rather considers different "sampling periods".

R: The term "sampling strategy" was systematically replaced with the term "sampling scheme(s)".

* The impact of in-sewer transformations was neglected in the manuscript. Number of studies (including previous author's studies) have shown that 6-AM, BE, THC-COOH are subject to transformation or formation in the sewer. How do the results would change if the authors consider such transformations? If these in-sewer processes are not included in the estimation of consumption rates (e.g. not through correction factors), at least the possible impacts should be discussed.

R: The impact of possible in-sewer transformations was not taken into account when estimating drug consumption. A model experiments which were performed at 10oC and 20oC, with the wastewater from the city of Zagreb, indicated rather higher stability of all urinary biomarkers within the wastewater in-sewer residence time (<5 h) in the city of Zagreb. Our study (Senta et al., 2016. Sci Tot Environ, 487, 659-665), showed that even the most labile biomarkers such as 6-AM, BE, THC-COOH are not expected to be transformed more than 10% (which we accepted as a margin of error). Furthermore, the study was performed within the same city (the same sewer system). Consequently, possible in-sewer transformations is not expected to have a significant effect either on the determined weekday/workday and holiday consumption patterns or on multiannual consumption trends.

A possible impact of in-sewer transformations is now briefly discussed in the revised version (Section 3.2.3.).

* Devault et al. 2017 has shown that the stability of 6-AM and THC-COOH is greatly influenced by temperature. Since this manuscript presents results related to March and August how does the temperature difference can explain the difference between the results presented in Fig. 4. Unfortunately the temperature is not reported in the manuscript and the impact is not discussed.

R: The typical in-sewer temperature in the city of Zagreb in March and Jul/Aug periods is 12oC and 20.5oC, respectively. Our model experiments which were performed at 10oC and 20oC, with the wastewater from the city of Zagreb, indicated rather higher stability of all urinary biomarkers presented in Fig. 4 at both investigated temperature conditions (Senta et al., 2016. Sci Tot Environ, 487, 659-665). Since the in-sewer wastewater residence time in the city of Zagreb is relatively short (<5 h), a significant impact of in-sewer degradation on the results presented on Figure 4 is not very likely.

A possible impact of in-sewer transformations is now briefly discussed in the revised version (Section 3.2.3.) and the reference Devault et al., 2017 is included.

* As compared to the actual outcomes, the conclusion section is rather short and incomplete. This can be supplemented with some details as outlined in the objectives (Lines 110-114) together with some recommendations for future monitoring campaigns.

R: The suggestion has been accepted. The conclusion section has been modified.

Detailed comments:
Line 142-144: When was the beginning and ending sampling in each day?

R: The samples were collected from 8 a.m. of the previous day to 8 a.m. of the sample collection day. This info was added to the manuscript (Section 2.3.)

Line 147-149: Are there any data that presented here but published before e.g. Krizman et al. 2016, Senta et al. 2015 or SCORE monitoring? This should be clarified in the manuscript.

R: The sentence: "Since the study covers a rather long time-period, some of the data, resulting from the sampling campaigns described above, were partially used in previously published studies (e.g. Krizman et al., 2016; Ort et al., 2014b; Terzic et al., 2010; Thomas et al., 2014)." has been added to the manuscript.

Line 153: "The total number of samples per year varied from 21 to 46". These numbers do not much with the "number of analyzed samples" in Table 2 (7 to 72). Is there any difference between number of samples and number of analyzed samples?

R: The total number of samples per year collected within the whole-year sampling scheme was 21-46. However, the total number of the samples presented in the Table 2 includes all samples collected and analyzed within a specified year (e.g. the number of samples collected within the one-week sample scheme plus the number of samples collected within the whole-year sample scheme, plus the number of samples collected within the Christmas-New Year period).

Line 173 - 188: This is entirely a copy-paste from Krizman et al. 2016 (STOTEN 566-567 (2016) 454-462).

R: The applied methodology for the estimation of drug abuse is the same as described in Krizman et al (2016). We did our best to change the sentences of this part of the Section 2.4. The changes are clearly marked in the revised version.

Line 195-197: What about correction factor for heroin?

R: The following text was added to the last sentence of the Section 2.4.: "whereas heroin consumption was calculated from 6-AM mass loads, using a correction factor of 86.9 (van Nuijs et al. 2011)

Line 209: 213: This seems to belong to Materials and Methods

R: This sentence was omitted from the revised version of the manuscript.

Line 210: what does the age of registered drug addicts (15-64) relate to your wastewater data? As you keep mentioning this range of age in your results, how can you make sure that people with age not included in the range did not contribute to your collected samples? I suggest you bring a strong evidence or remove it from the manuscript and Fig. 6.

R: We do not agree with this comment and suggestion. The epidemiological data (e.g. number of registered drug users are frequently normalized on the population of age 15-64 years. It does not mean that all users are in that age group. Consequently, the WBE data are frequently normalized on the population of age 15-64 years old and it does not mean that only the population of the age 15 -64 years contributed to the sample.

250-251: this is a repeated sentence (Line 156-157), suggest to remove

R: Suggestion accepted. Removed.

Line 269: "higher than" instead of "higher then"

R: Corrected

Line 325-331: Ort et al. 2014b, only assessed the back-calculation of COC using BE. So the relative error of 60% was for this specific chemicals. Whereas in this manuscript the chemicals are completely different and the error varies a lot as shown in Figure 5. So this comparison and generalization is not entirely valid.

R: We do not agree with this comment. Ort and coworkers (2014b) addressed the challenges of surveying wastewater drug loads of small populations and generalizable aspects on optimizing monitoring design by comparing the results obtained for cocaine biomarker mass loads (BE and COC) using different sampling schemes in one small city (7160 inhabitants). Fig. 5 contains the data for BE as well. The variability for BE for both sampling schemes (one-week and whole-year) was lower then 20% in all investigated years. Therefore, we think that the performed comparison is appropriate.

Line 340: please check with the formatting standard of the journal when you refer to supplementary material.

R: The expression "Electronic Supplementary Material" was replaced by "Supplementary Material".

Line 351: "… in some other WBE studies". This is very general. In which regions where those studies conducted? what population size? Which chemicals?

R: The following text was added to the revised manuscript:

„In principle, the determined drug consumption patterns and rates were rather similar to those determined in some other Mediterranean countries, like Spain and Italy (Mastroianni et al., 2017; Zuccato et al., 2016), although some differences regarding the prevalence of individual drugs as well as regarding the temporal trends were observed. For example, cannabis and cocaine were the most prevalently consumed illicit drugs in Barcelona (Spain) and investigated Italian cities, whereas a heroin consumption was reported to be much lower (Mastroianni et al., 2017; Zuccato et al., 2016)."

Line 410: "… are much smaller than those for the small communities". Based on which comparison this conclusion is made? This statement requires detailed comparison.

R: The sentence was slightly changed as follows:
"The errors associated with day-to-day and intra-annual variability of BE (<20%) determined in the city of Zagreb (>500 000 inhabitants) study were much smaller from those reported for small communities (Ort et al. 2914b), which indicated enhanced robustness of the estimates obtained for large sized cities."

Figures: Please define in the figure captions what do error bars mean.

R: Defined. Error bars represent standard deviations.

Reviewer #3: The manuscript presents the monitoring of drug consumption in Zagreb using wastewater-based epidemiology from 2009-2016.
The manuscript is fairly well written and cover a range of topics. I think this work is of relevance for readers of Science of the Total Environment and only have minor comments for the authors to remedy:
* Despite an apparently comprehensive literature search, I believe the authors are missing some references that could strengthen the introduction. A group from South Australia have been performing bimonthly (every two months) sampling and it would be pertinent to include this somewhere in the introduction to show that there are other groups who don't just do one-week sampling. References could be Bade et al Analytical and Bioanalytical Chemistry 2018, 529-542 and Tscharke et al Science of the Total Environment 2016, 384-391. Furthermore, Jiang et al (Environmental Science and Technology 2015, 792-799) also present the use of wastewater-based epidemiology for analyzing drug consumption during a festival. I encourage the authors to cite these articles within the introduction.

R: The suggested references are included in the revised manuscript.

* The authors should be consistent with nomenclature. E.g. Line 231 (Figure 1) then all subsequent references to figures are (Fig. 2 etc.) The authors should stick with one.

R: Corrected.

* Line 277-279 is not needed. It is replicated at the beginning of the next section.

Removed from the revised version.

* Line 304: Why were Sunday and Tuesday chosen as sampling says for the year-long campaign? By only sampling one weekend day, the majority of the stimulants would be underestimated as described later in the section.

R: Sunday and Tuesday were selected as representatives of weekend day and week-day, respectively, for practical reasons. However, we don't think that we underestimated stimulants by sampling only one weekend day. Namely, as clearly described in our methodology we calculated representative average mass loads using the weight factors of 2 and 5 for weekend and weekday, respectively.

* The authors should replace "bimonthly" with "fortnightly" as bimonthly can be confused with "every two months".

R: Replaced.

* Line 407: The authors state in the conclusion that whole-year sampling showed a clear advantage over the seven-consecutive-day sampling scheme. However, in line 323, the authors state that one-week sampling may provide a reliable base the estimate of the annual consumption if most classical illicit drugs. These two sentences seem contrasting. In my opinion, there is no clear opinion voiced by the authors in section 3.3 as to which sampling scheme should be used. If the authors do believe that year-long sampling is advantageous, they should state that in section 3.3.

R: We agree with the reviewer's comment. To avoid misinterpretations, the sentence in the Section 3.3. was rephrased as follows:
"Nevertheless, although some previous studies, addressing the issue of multiannual changes, demonstrated the applicability of one-week sampling scheme (Ort et al. 2014; Mastroianni et al. 2017), our data show that such a scheme is insufficiently reliable for the drugs exhibiting high day-to-day and/or intra-annual variability, even in case of larger cities like the city of Zagreb."

* Figure 6: Why was methamphetamine not included in this figure?

R: Concentration of methamphetamine in most of the samples was below MQL and quantifiable concentrations appeared only sporadically. Therefore, its consumption was not included in Fig 6 which illustrates multiannual trends in Zagreb since, under the circumstances, no reliable trends could be observed

*   Figure 7: What are "stimulants" in the epidemiological figure? Within the manuscript, stimulants are described as the cocaine, methamphetamine, amphetamine and MDMA. However, cocaine is separate in this figure. The authors should specify precisely what these stimulants cover to ensure comparability with the wastewater data.

R: Stimulants in the epidemiological figure include amphetamine-type drugs. The explanation is added to the Figure captions.

Reviewer #4: The manuscript entitled, "Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: comparison of sampling strategies for the assessment of multiannual trends" provides an interesting study for estimating drugs consumption in a European city. The paper is relevant, well written and logically constructed. The paper is of general interest. Even though the research is not novel as back-calculation methods have been use in many papers to estimate illicit drugs in untreated wastewater. I would like to recommend acceptance of this manuscript however there are important sections of data which should be explained in more detail before publication to ensure the results and methodologies applied are transparent and adequately quality assessed.

Comments
1.In my opinion the graphical abstract is not attractive. It could be improved.

R: This comment is not very informative. It is difficult to know what the reviewer means by “not attractive”. However, we hope the reviewer is going to find the graphical abstract being more attractive in its revised form.

2. Line 30 and 42: According to the data, a 7-year period was conducted. Therefore, change 8-year period to 7-year period.

R: The study was performed within an 8-year period (2009-2016) but the data set includes data from 7 years (2009, 2011, 2012, 2013, 2014, 2015, 2016).

3. Line 39-40. Please, specify that "holiday" refers to Christmas time.

R:“holiday” replaced with “the Christmas season”

4. Line 106: To be consistent with the rest of the paper, replace WBA by WBE.

R: Corrected

5. Line 134. MQ = Milli-Q water, I guess

R: Corrected

6. Line 128. Which deuterated standards did you use?

R: All analytes had their deuterated analogues. The analytical details are given in the analytical method (Senta et al, Analytical and Bioanalytical Chemistry, 405, 3255-3268).

7. Sampling data is confusing. I suggest adding the exact dates for sampling regarding lines 150 to 157.

R: The Section 2.3. is changed in the revised version.

8. Specify the exact total number of samples you analyzed.

R: A following sentence was included in the revised manuscript: “A total number of 282 samples with an average pH of 7.6 ± 0.2 was collected.”

9. Which was the pH sample?

R: A following sentence was included in the revised manuscript: “A total number of 282 samples with an average pH of 7.6 ± 0.2 was collected.”

10. Brief information about liquid-chromatography as well as MS/MS conditions should be mentioned in the text

R: Some additional information on HPLC and MS conditions was added. We think that this should suffice considering the word count limitations. According to the Journal instructions, the methods which are already published should be summarized, and indicated by a reference, which is done.

11. Lines 186-189: "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, which was based on 2011 Census data". However, data are referred to population
15-64 years along the text. Please, clarify.

R: Some of the published WBE data available in the literature are normalized to the total population (e.g. Zuccato et al. Drug Alcohol Depend 2016), whereas some of them are normalized to the population of age 15-64 years (e.g. Mastroianni et al. 2017). To facilitate the comparison with the literature, the drug consumption data in the Table S2 (Supplementary Material) are expressed in 8 different units (mg/day/1000 inh.; mg/day/1000 inh. 15-64 years; doses/day/1000 inh.; doses/day/1000 inh. 15-64 years; g/day; kg/year; kg/year - street purity). Only the Fig. 7 includes consumption data normalized to the population (in thousands) of age 15-64 years since the epidemiological data which are included in this figure are normalized to the population 15-64 years old.
Both the total population number served by the WWTP and the population number of age 15-64 years served by the WWTP are based on 2011 Census data.

12. For the back-calculation of heroin from MOR, did you take into account the contribution of therapeutic MOR? It should be subtracted when back-calculating heroin consumption

R: Heroin consumption was calculated from 6-AM. Please, check the Table 1.

13. Line 259-262. Is there any explanation about the increase of MOR?

R: No, currently we do not know the reason.

14. Line 262 and 378: Typing error: replace "concomittant" by "concomitant"

R: Corrected.

15. Line 269: Change then for than in sentence "Christmas holiday season were 2 - 3.9-fold higher then during the average weekday"

R: Corrected.

16. Line 287-289. I partially agree with the authors because in summer there is a decrease of residential population but many tourists visit the city.

R: Even in summer, the contribution of tourists to the city population is negligible (<1%; official data), whereas at the peak of summer season (25.7-15.8.) a significant percentage of residential population (unfortunately, official data are not available) leave Zagreb. This information was included in the revised section 3.2.3.

17. Lines 292-294. This statement should be explained in more detailed.

R: The discussion in the Section 3.2.3. has been amended to address possible reasons for lower summer biomarker mass loads.

18. Line 298: "was based" should be replace by "were based"

R: Corrected.

19. Line 311. There is a typing error. Replace "occasional" by "occasional"

R: Corrected

20. Line 366. Add a reference for official data on the purity of seized drugs in the same period

R: The data on the purity of seized drugs were provided by the Office for Combating Narcotic Drug Abuse of the Government of the Republic of Croatia. This info was added to the revised manuscript.

21. Please, explain how you calculated the amounts of the street-purity drugs (line 367-368).

R: Following sentence was added to the manuscript (Section 2.4.): "The amounts of street-purity drugs which circulated on the illegal market in Zagreb were calculated from the estimated annual consumption of pure drugs (expressed in kg/year), which were divided by the corresponding drug purity presented in Table S1.”

22. Conclusions: As you have not compared errors in large cities vs small communities, this sentence should be modified
.
R: This section was thoroughly modified.

23. Table 1. Put a space in "Castiglioniet al" between Castiglioni and et

R: Corrected.

24. Table 1. Refined correction factors have been recently proposed for the back-calculation of the illicit drugs considered in this work. I suggest the authors to check the most recently published works (for instance, Gracia-Lor et al. 2016)

R: The refined correction factors proposed by Gracia-Lor et al. (2016) are applied in the revised version.

25. Figure 2. A legend about the meaning of the horizontal lines should be included.

R: The following text was added to the figure caption: „Horizontal lines represent arbitrarily assumed weekend to workday mass load ratio of 1.0 ± 0.2“.

26. In Figure 2, 7 and S1 it is difficult to distinguish among data due to similar coloured bars. Kindly, use a different means for identifying each analyte.

R: Corrected.

27. References: line 479, change Horder to Hordern

R: Corrected.

28. Table S2. Typing error: Change "wastwater" to wastewater

R: Corrected

Reviewer #5: This manuscript presents a long-term monitoring study of drug consumption in Zagreb - Croatia using wastewater-based epidemiology. In addition to 1 week of samples per year which is common in other multiannual wastewater-based epidemiology studies, the authors have also looked at higher sampling frequencies for a couple of years and compared this with the results they would have otherwise got based on only 1 week of sampling. The authors have also looked at drug consumption during holiday periods. My only major concern is that the authors appear to have used a static population size when the study has been conducted over an 8 year period and thus the data may not be truly population normalised and thus I think this needs to be addressed or at the very minimum discussed. My minor comment is that there are numerous grammatical errors throughout the text which would have been addressed from proper editing prior to submission.

R: We do not expect that the population in Zagreb changed significantly over the investigated period. For example, the difference in the number of city inhabitants obtained by CENSUS 2001 and CENSUS 2011 was lower than 2%. The number of tourists visiting Zagreb never exceeds 1% of the total population (official data). The only period with a significant change in population number might be summer vacation season (25th July – 15th August.) due to the outward migrations of residential population (official data not available). Therefore, the mass loads determined during summer might be somewhat underestimated, which was discussed in Section 3.2.3.

Individual points:
Abstract
Line 30 - grammar "an 8-year"

R: Corrected

Line 66 - grammar "of the WBE approach"

R: Corrected

Line 67 - grammar "of the WBE approach"

R: Corrected

Introduction

Line 106 - grammar and spelling "an initial WBE"
R: Corrected

Line 107 - grammar "of the other"

R: Corrected

Chemicals and materials
Line 135 - grammar "purifying with an Elix-Mili-Q-system"

R: Corrected

Line 136 - grammar "were purchased from Waters"

R: Corrected

Line 138 - grammar "were purchased from Phenomenex"

R: Corrected

Line 139 - grammar "were purchased from Whatman"

R: Corrected

Wastewater sampling and analysis

Lines 144 to 149 - the way this is written is unclear

R: This part of the Section 2.3. is rewritten. We hope it is clear now.

Line 162 - grammar "where performance"

R: Corrected

Lines 186 to 189 - This is a long time to normalize to a static population size. Did the population change over this period? What about for the holiday period comparison? Were population markers assessed?

R: We do not expect that the population in Zagreb changed significantly over the investigated period. For example, the difference in the number of city inhabitants obtained by CENSUS 2001 and CENSUS 2011 was < 2%. The number of tourists visiting Zagreb never exceeds 1% of the total population (official data). The only period with a significant change in population number might be summer vacation season (25th July – 15th August.) due to the outward migrations of residential population (official data not available). Therefore, the mass loads determined during summer might be somewhat underestimated, which was discussed in Section 3.2.3.

Line 197 - grammar "using the later"

R: Corrected

Line 213 - grammar "in the treatment"

R: Corrected

The impact of holiday season on drug consumption patterns

Line 252 - grammar "load"

R: Corrected

Line 255 - grammar "seasons"

R: Corrected

Line 262 - grammar - remove "a" before "holiday-related"
R: Corrected

Line 264 - be consistent with "holiday season" and "holiday-season"

R: Corrected

Lines 264 to 267 - without using de facto population sizes it seems like these differences might not be due to higher "per capita consumption" or may only be increased to a lesser extent

R: We do not agree with this comment. It is not likely that the data presented in Fig 3 can be significantly affected by the changes in population in the city of Zagreb.

Line 277 - grammar "on a one-week"

R: The sentence was omitted from the revised manuscript.

Lines 287 to 294 - Other studies have shown numerous markers of population in wastewater which even without a thorough calibration for the investigated catchments would at least reflect relative change in population size. Why have the authors ignored this aspect?

R: As indicated in our response above, the official data on the population of the city of Zagreb do not suggest any significant changes during the period covered by this study.

Impact of sampling strategy on the estimation of drug consumption in multiannual studies

Line 298 -replace "was" with "previously conducted were"

R: Corrected.

Line 301 - replace "the" with "an"

R: Corrected.

Multiannual trends in drug consumption patterns and comparison with available epidemiological data

Line 379 - spelling "substitution therapy"

R: Corrected.

Line 395 - grammar "the outcome"

R: Corrected.

Line 396 - grammar "surveys"

R: Corrected.

Line 412 - too many uses of "moreover"

R: Corrected.

Reviewer #6: Dear Editor,
Thank you for your invitation to review manuscript STOTEN-D-18-06314 entitled "Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: Comparison of sampling strategies for the assessment of multiannual trends."

Monitoring studies are useful and the topic is of interest, so I consider this paper is interesting to be published in STOTEN after some minor changes.

General comments

- Why do you write sometimes 7-year study (highlights, page 6, line 114) and sometimes 8-year period (page 2, line 30 and 42; page 18, line 355)?

R: The study was performed within an 8-year period (2009-2016) but the data set includes data from 7 years (2009, 2011, 2012, 2013, 2014, 2015, 2016). To be more consistent, the corrections were made in the text wherever needed.

- Page 4, lines 62-65, 68-70: Some references are quite old. There are a lot of monitoring research studies on wastewater-based epidemiology in the last 5 years so I recommend to authors to update references.

R: Some additional references are included in the revised version. However, the literature on WBE of illicit drugs has become rather large and, since this is not a review paper, there has to be some selection.

- Page 6, line 106: I think you want to say 'WBE'.

R: Corrected.

- I suggest extending the discussion in sections 3.1 Occurrence of drug biomarkers in municipal wastewater of the city of Zagreb and 3.2 Drug consumption patterns. Please, compare your results with other European countries.

R: In our opinion there is no need for the extensive comparison of the results from Zagreb with the results from other European cities in these 2 sections. According to the Journal's instructions, extensive citations and discussion of published literature should be avoided.

- Table 2. Delete vertical line between Mass load and Average (first line of AMP data).

R: Corrected.

- Figure 1 and 5. Exchange decimals in commas for decimal points.

R: Corrected.

Reviewer #7: The article titled "Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: Comparison of sampling strategies for the assessment of multiannual trends" is based on the analysis of WBE data over an eight-year period. Their study analyses trends revealed by the longitudinal data and compares sampling techniques currently used in many WBE studies. Based on their results, the authors propose a different sampling strategy different from what has been currently used in many WBE studies.

With more regions implementing WBE for community drug monitoring, the results from this study could prove significant to improving regional and multi-regional sampling. I believe this study will be of interest to many STOTEN readers and even more the WBE scientists. I recommend the article for publication.

I have only few comments and suggest a thorough read-through to correct some typos.

1. Line 183-184 authors used daily flow rates for mass load calculation, as such I assume you have all the flow rate data. Line 290-292. Do the seasonal changes in population affect the WWTP flow rates?

R: Yes, the data on wastewater mass flow expressed in m3/day were obtained from the Central WWTP of the city of Zagreb. However, the sewer system of the city of Zagreb receives either municipal and industrial wastewater as well as rain water and even some stream waters. The flow rates are therefore more influenced by precipitations than by changes in population size and cannot be used as indicators of population size changes.

2. Additionally, though the proposed multiannual and seasonal sampling techniques applied in this study were useful in providing insight on drug use dynamics and better drug use estimations for Zagreb. It is difficult without a comparison site to tell if the same sampling technique would apply as well or have significant impact on a different city (smaller vs bigger; rural vs urban) even in Croatia.

R: We believe that the improvements achieved through the use the whole-year sampling scheme described in this paper strongly suggest that, in spite of possible variations in weekly and seasonal dynamics, large sized cities provide a robust systems for multiannual monitoring of illicit drugs.

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Zagreb, 30 July 2018

Dear dr. Pico,

please find enclosed the revised version of the manuscript entitled „Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: Comparison of two sampling schemes for the assessment of multiannual trends". We carefully considered all reviewers' comments and provided an itemized list of responses. The changes made to the manuscript are clearly marked in the revised version of the manuscript using the track changes option. The version of the revised manuscript with the accepted changes is also submitted. We are very grateful to all 7 reviewers whose valuable comments, suggestions and questions helped us to improve the manuscript.
We hope that the revised manuscript is now acceptable for the publication in the Science of the Total Environment.
Please send all further correspondence to me (terzic@irb.hr).

Sincerely yours,

dr. Senka Terzic

Title Page

Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: Comparison of two sampling schemes for the assessment of multiannual trends

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***Responses to Reviewers Comments**

ANSWERS TO THE REVIEWERS

Reviewer #1: The authors report a WBE study in which they monitor temporal variability in biomarkers of heroin, cocaine, amphetamine, MDMA, methamphetamine and cannabis and the therapeutic opiate methadone in the waste water of Zagreb, Croatia over an 8-year period (2009-2016). The study assessed the reliability of one week vs annual sampling strategies on estimated temporal drug consumption patterns, weekday variations in the use of these drugs, and trends over time in the use of these drugs.

Their main findings were similar to those of studies in other European and high income countries in that:
* the drug with the highest consumption prevalence of use was for cannabis, followed by heroin, cocaine and amphetamine, with MDMA use much lower;
* There were enhanced weekend and holiday consumption of cocaine, MDMA, and amphetamine;
* Consumptions was marginally lower in summer for almost all drugs, reflecting population movements;
* Over the 8-year study period there was increases in the consumption of cocaine and THC and a more marked increase in use of amphetamine (16-fold) and MDMA (15-fold). There was a large decrease in the consumption of heroin over the study period and an increase in the last year of study.
* The decline in heroin use was associated with an increase in methadone consumption that was linked to its increased use as a substitution treatment for heroin.
* The estimated average daily methadone dose in the city of Zagreb agreed well with the prescription data on the number of opioid addicts in Croatia enrolled in methadone treatment.

The last two findings are major novelties that have not been previously reported so far as I am aware, namely, a decline in indicators of heroin use occurring as there was increased use of methadone; and showing that methadone consumption estimated from waste water biomarkers closely agreed with data on the amount of methadone dispensed.

Q: I had one minor issue: what was the justification for the "arbitrary" definition of a significant ratio of weekend to weekly use of a drug, i.e. 1 plus or minus -0.2?

R: The criterion was selected based on the initial insight into the day-to-day variability of various non-stimulating drugs (in particular morphine, codeine and methadone) in the city of Zagreb with moderate relative standard deviations (RSD) of average daily loads being in the range up to 11 to 17%, which indicated robustness of the collective excretion rates as an indicator of drug abuse in larger populations. Moreover, this criterion is well above the possible limitations posed by mere repeatability of the analytical method.

Reviewer #2: General Comments

The manuscript presents a 7-year monitoring of selected drug consumption patterns in the city of Zagreb. The study presents an extensive monitoring data and is within the scope of STOTEN. Although similar studies have published before, the authors have tried to give a new perspective to the study by comparing monitoring data for different sampling periods and look for specific trends. I recommend this manuscript for publication following some major corrections.

<u>Major comments</u>:
* It appears that the Authors have tried to make relatively generalized conclusions about any "large-sized European city" using the example of Zagreb with limited number of drugs considered. However, EMCDDA reports have shown the trends of drug use is very region-dependent and different for each drug. I suggest the authors to be more moderate and corroborate their outcomes with the studies in the same region and cities with similar population size.

R: We do not agree with this comment. As it was clearly emphasized in the title, one of the primary goals of the paper was testing different sampling strategies (sampling schemes) and estimation of the robustness of the applied sampling schemes to assess relatively small changes in consumption rates by taking into account possible sources of temporal variability (weekly dynamics, seasonal variability and impact of special events). These are important methodological issues of general character applicable to any large sized city. Our study did not intend to make any generalization regarding the drug consumption trends in other large-sized European cities based on the data from the city of Zagreb. We rather demonstrated that considering the proper sampling schemes can significantly improve the reliability of the trend monitoring making possible detection of relatively small changes.

* Using the term "sampling strategies", especially in the title is misleading. In fact, the study does not consider different sampling strategies (e.g. flow, volume, time proportional with different sampling intervals) but it rather considers different "sampling periods".

R: The term "sampling strategy" was systematically replaced with the term "sampling scheme(s)".

* The impact of in-sewer transformations was neglected in the manuscript. Number of studies (including previous author's studies) have shown that 6-AM, BE, THC-COOH are subject to transformation or formation in the sewer. How do the results would change if the authors consider such transformations? If these in-sewer processes are not included in the estimation of consumption rates (e.g. not through correction factors), at least the possible impacts should be discussed.

R: The impact of possible in-sewer transformations was not taken into account when estimating drug consumption. A model experiments which were performed at 10°C and 20°C, with the wastewater from the city of Zagreb, indicated rather higher stability of all urinary biomarkers within the wastewater in-sewer residence time (<5 h) in the city of Zagreb. Our study (Senta et al., 2016. Sci Tot Environ, 487, 659-665), showed that even the most labile biomarkers such as 6-AM, BE, THC-COOH are not expected to be transformed more than 10% (which we accepted as a margin of error). Furthermore, the study was performed within the same city (the same sewer system). Consequently, possible in-sewer transformations is not expected to have a significant effect either on the determined weekday/workday and holiday consumption patterns or on multiannual consumption trends.

A possible impact of in-sewer transformations is now briefly discussed in the revised version (Section 3.2.3.).

* Devault et al. 2017 has shown that the stability of 6-AM and THC-COOH is greatly influenced by temperature. Since this manuscript presents results related to March and August how does the temperature difference can explain the difference between the results presented in Fig. 4. Unfortunately the temperature is not reported in the manuscript and the impact is not discussed.

R: The typical in-sewer temperature in the city of Zagreb in March and Jul/Aug periods is 12°C and 20.5°C, respectively. Our model experiments which were performed at 10°C and 20°C, with the wastewater from the city of Zagreb, indicated rather higher stability of all urinary biomarkers presented in Fig. 4 at both investigated temperature conditions (Senta et al., 2016. Sci Tot Environ, 487, 659-665). Since the in-sewer wastewater residence time in the city of Zagreb is relatively short (<5 h), a significant impact of in-sewer degradation on the results presented on Figure 4 is not very likely.

A possible impact of in-sewer transformations is now briefly discussed in the revised version (Section 3.2.3.) and the reference Devault et al., 2017 is included.

* As compared to the actual outcomes, the conclusion section is rather short and incomplete. This can be supplemented with some details as outlined in the objectives (Lines 110-114) together with some recommendations for future monitoring campaigns.

R: The suggestion has been accepted. The conclusion section has been modified.

Detailed comments:
Line 142-144: When was the beginning and ending sampling in each day?

R: The samples were collected from 8 a.m. of the previous day to 8 a.m. of the sample collection day. This info was added to the manuscript (Section 2.3.)

Line 147-149: Are there any data that presented here but published before e.g. Krizman et al. 2016, Senta et al. 2015 or SCORE monitoring? This should be clarified in the manuscript.

R: The sentence: "Since the study covers a rather long time-period, some of the data, resulting from the sampling campaigns described above, were partially used in previously published studies (e.g. Krizman et al., 2016; Ort et al., 2014b; Terzic et al., 2010; Thomas et al., 2014)." has been added to the manuscript.

Line 153: "The total number of samples per year varied from 21 to 46". These numbers do not much with the "number of analyzed samples" in Table 2 (7 to 72). Is there any difference between number of samples and number of analyzed samples?

R: The total number of samples per year collected within the whole-year sampling scheme was 21-46. However, the total number of the samples presented in the Table 2 includes all samples collected and analyzed within a specified year (e.g. the number of samples collected within the one-week sample scheme plus the number of samples collected within the whole-year sample scheme, plus the number of samples collected within the Christmas-New Year period).

Line 173 - 188: This is entirely a copy-paste from Krizman et al. 2016 (STOTEN 566-567 (2016) 454-462).

R: The applied methodology for the estimation of drug abuse is the same as described in Krizman et al (2016). We did our best to change the sentences of this part of the Section 2.4. The changes are clearly marked in the revised version.

Line 195-197: What about correction factor for heroin?

R: The following text was added to the last sentence of the Section 2.4.:
"whereas heroin consumption was calculated from 6-AM mass loads, using a correction factor of 86.9 (van Nuijs et al. 2011)

Line 209: 213: This seems to belong to Materials and Methods

R: This sentence was omitted from the revised version of the manuscript.

Line 210: what does the age of registered drug addicts (15-64) relate to your wastewater data? As you keep mentioning this range of age in your results, how can you make sure that people with age not included in the range did not contribute to your collected samples? I suggest you bring a strong evidence or remove it from the manuscript and Fig. 6.

R: We do not agree with this comment and suggestion. The epidemiological data (e.g. number of registered drug users are frequently normalized on the population of age 15-64 years. It does not mean that all users are in that age group. Consequently, the WBE data are frequently normalized on the population of age 15-64 years old and it does not mean that only the population of the age 15 -64 years contributed to the sample.

250-251: this is a repeated sentence (Line 156-157), suggest to remove

R: Suggestion accepted. Removed.

Line 269: "higher than" instead of "higher then"

R: Corrected

Line 325-331: Ort et al. 2014b, only assessed the back-calculation of COC using BE. So the relative error of 60% was for this specific chemicals. Whereas in this manuscript the chemicals are completely different and the error varies a lot as shown in Figure 5. So this comparison and generalization is not entirely valid.

R: We do not agree with this comment. Ort and coworkers (2014b) addressed the challenges of surveying wastewater drug loads of small populations and generalizable aspects on optimizing monitoring design by comparing the results obtained for cocaine biomarker mass loads (BE and COC) using different sampling schemes in one small city (7160 inhabitants). Fig. 5 contains the data for BE as well. The variability for BE for both sampling schemes (one-week and whole-year) was lower then 20% in all investigated years. Therefore, we think that the performed comparison is appropriate.

Line 340: please check with the formatting standard of the journal when you refer to supplementary material.

R: The expression "Electronic Supplementary Material" was replaced by "Supplementary Material".

Line 351: "… in some other WBE studies". This is very general. In which regions where those studies conducted? what population size? Which chemicals?

R: The following text was added to the revised manuscript:

„In principle, the determined drug consumption patterns and rates were rather similar to those determined in some other Mediterranean countries, like Spain and Italy (Mastroianni et al., 2017; Zuccato et al., 2016), although some differences regarding the prevalence of individual drugs as well as regarding the temporal trends were observed. For example, cannabis and cocaine were the most prevalently consumed illicit drugs in Barcelona (Spain) and investigated Italian cities, whereas a heroin consumption was reported to be much lower (Mastroianni et al., 2017; Zuccato et al., 2016)."

Line 410: "… are much smaller than those for the small communities". Based on which comparison this conclusion is made? This statement requires detailed comparison.

R: The sentence was slightly changed as follows:
"The errors associated with day-to-day and intra-annual variability of BE (<20%) determined in the city of Zagreb (>500 000 inhabitants) study were much smaller from those reported for small communities (Ort et al. 2914b), which indicated enhanced robustness of the estimates obtained for large sized cities."

Figures: Please define in the figure captions what do error bars mean.

R: Defined. Error bars represent standard deviations.

Reviewer #3: The manuscript presents the monitoring of drug consumption in Zagreb using wastewater-based epidemiology from 2009-2016.
The manuscript is fairly well written and cover a range of topics. I think this work is of relevance for readers of Science of the Total Environment and only have minor comments for the authors to remedy:

* Despite an apparently comprehensive literature search, I believe the authors are missing some references that could strengthen the introduction. A group from South Australia have been performing bimonthly (every two months) sampling and it would be pertinent to include this somewhere in the introduction to show that there are other groups who don't just do one-week sampling. References could be Bade et al Analytical and Bioanalytical Chemistry 2018, 529-542 and Tscharke et al Science of the Total Environment 2016, 384-391. Furthermore, Jiang et al (Environmental Science and Technology 2015, 792-799) also present the use of wastewater-based epidemiology for analyzing drug consumption during a festival. I encourage the authors to cite these articles within the introduction.

R: The suggested references are included in the revised manuscript.

* The authors should be consistent with nomenclature. E.g. Line 231 (Figure 1) then all subsequent references to figures are (Fig. 2 etc.) The authors should stick with one.

R: Corrected.

* Line 277-279 is not needed. It is replicated at the beginning of the next section.

Removed from the revised version.

* Line 304: Why were Sunday and Tuesday chosen as sampling says for the year-long campaign? By only sampling one weekend day, the majority of the stimulants would be underestimated as described later in the section.

R: Sunday and Tuesday were selected as representatives of weekend day and week-day, respectively, for practical reasons. However, we don't think that we underestimated stimulants by sampling only one weekend day. Namely, as clearly described in our methodology we calculated representative average mass loads using the weight factors of 2 and 5 for weekend and weekday, respectively.

* The authors should replace "bimonthly" with "fortnightly" as bimonthly can be confused with "every two months".

R: Replaced.

* Line 407: The authors state in the conclusion that whole-year sampling showed a clear advantage over the seven-consecutive-day sampling scheme. However, in line 323, the authors state that one-week sampling may provide a reliable base the estimate of the annual consumption if most classical illicit drugs. These two sentences seem contrasting. In my opinion, there is no clear opinion voiced by the authors in section 3.3 as to which sampling scheme should be used. If the authors do believe that year-long sampling is advantageous, they should state that in section 3.3.

R: We agree with the reviewer's comment. To avoid misinterpretations, the sentence in the Section 3.3. was rephrased as follows:
"Nevertheless, although some previous studies, addressing the issue of multiannual changes, demonstrated the applicability of one-week sampling scheme (Ort et al. 2014; Mastroianni et al. 2017), our data show that such a scheme is insufficiently reliable for the drugs exhibiting high day-to-day and/or intra-annual variability, even in case of larger cities like the city of Zagreb."

* Figure 6: Why was methamphetamine not included in this figure?

R: Concentration of methamphetamine in most of the samples was below MQL and quantifiable concentrations appeared only sporadically. Therefore, its consumption was not included in Fig 6 which illustrates multiannual trends in Zagreb since, under the circumstances, no reliable trends could be observed

* Figure 7: What are "stimulants" in the epidemiological figure? Within the manuscript, stimulants are described as the cocaine, methamphetamine, amphetamine and MDMA. However, cocaine is separate in this figure. The authors should specify precisely what these stimulants cover to ensure comparability with the wastewater data.

R: Stimulants in the epidemiological figure include amphetamine-type drugs. The explanation is added to the Figure captions.

Reviewer #4: The manuscript entitled, "Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: comparison of sampling strategies for the assessment of multiannual trends" provides an interesting study for estimating drugs consumption in a European city. The paper is relevant, well written and logically constructed. The paper is of general interest. Even though the research is not novel as back-calculation methods have been use in many papers to estimate illicit drugs in untreated wastewater. I would like to recommend acceptance of this manuscript however there are important sections of data which should be explained in more detail before publication to ensure the results and methodologies applied are transparent and adequately quality assessed.

Comments
1.In my opinion the graphical abstract is not attractive. It could be improved.

R: This comment is not very informative. It is difficult to know what the reviewer means by “not attractive”. However, we hope the reviewer is going to find the graphical abstract being more attractive in its revised form.

2. Line 30 and 42: According to the data, a 7-year period was conducted. Therefore, change 8-year period to 7-year period.

R: The study was performed within an 8-year period (2009-2016) but the data set includes data from 7 years (2009, 2011, 2012, 2013, 2014, 2015, 2016).

3. Line 39-40. Please, specify that "holiday" refers to Christmas time.

R:“holiday” replaced with “the Christmas season”

4. Line 106: To be consistent with the rest of the paper, replace WBA by WBE.

R: Corrected

5. Line 134. MQ = Milli-Q water, I guess

R: Corrected

6. Line 128. Which deuterated standards did you use?

R: All analytes had their deuterated analogues. The analytical details are given in the analytical method (Senta et al, Analytical and Bioanalytical Chemistry, 405, 3255-3268).

7. Sampling data is confusing. I suggest adding the exact dates for sampling regarding lines 150 to 157.

R: The Section 2.3. is changed in the revised version.

8. Specify the exact total number of samples you analyzed.

R: A following sentence was included in the revised manuscript: "A total number of 282 samples with an average pH of 7.6 ± 0.2 was collected."

9. Which was the pH sample?

R: A following sentence was included in the revised manuscript: "A total number of 282 samples with an average pH of 7.6 ± 0.2 was collected."

10. Brief information about liquid-chromatography as well as MS/MS conditions should be mentioned in the text

R: Some additional information on HPLC and MS conditions was added. We think that this should suffice considering the word count limitations. According to the Journal instructions, the methods which are already published should be summarized, and indicated by a reference, which is done.

11. Lines 186-189: "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, which was based on 2011 Census data". However, data are referred to population
15-64 years along the text. Please, clarify.

R: Some of the published WBE data available in the literature are normalized to the total population (e.g. Zuccato et al. Drug Alcohol Depend 2016), whereas some of them are normalized to the population of age 15-64 years (e.g. Mastroianni et al. 2017). To facilitate the comparison with the literature, the drug consumption data in the Table S2 (Supplementary Material) are expressed in 8 different units (mg/day/1000 inh.; mg/day/1000 inh. 15-64 years; doses/day/1000 inh.; doses/day/1000 inh. 15-64 years; g/day; kg/year; kg/year - street purity). Only the Fig. 7 includes consumption data normalized to the population (in thousands) of age 15-64 years since the epidemiological data which are included in this figure are normalized to the population 15-64 years old.
Both the total population number served by the WWTP and the population number of age 15-64 years served by the WWTP are based on 2011 Census data.

12. For the back-calculation of heroin from MOR, did you take into account the contribution of therapeutic MOR? It should be subtracted when back-calculating heroin consumption

R: Heroin consumption was calculated from 6-AM. Please, check the Table 1.

13. Line 259-262. Is there any explanation about the increase of MOR?

R: No, currently we do not know the reason.

14. Line 262 and 378: Typing error: replace "concomittant" by "concomitant"

R: Corrected.

15. Line 269: Change then for than in sentence "Christmas holiday season were 2 - 3.9-fold higher then during the average weekday"

R: Corrected.

16. Line 287-289. I partially agree with the authors because in summer there is a decrease of residential population but many tourists visit the city.

R: Even in summer, the contribution of tourists to the city population is negligible (<1%; official data), whereas at the peak of summer season (25.7-15.8.) a significant percentage of residential population (unfortunately, official data are not available) leave Zagreb. This information was included in the revised section 3.2.3.

17. Lines 292-294. This statement should be explained in more detailed.

R: The discussion in the Section 3.2.3. has been amended to address possible reasons for lower summer biomarker mass loads.

18. Line 298: "was based" should be replace by "were based"

R: Corrected.

19. Line 311. There is a typing error. Replace "occasional" by "occasional"

R: Corrected

20. Line 366. Add a reference for official data on the purity of seized drugs in the same period

R: The data on the purity of seized drugs were provided by the Office for Combating Narcotic Drug Abuse of the Government of the Republic of Croatia. This info was added to the revised manuscript.

21. Please, explain how you calculated the amounts of the street-purity drugs (line 367-368).

R: Following sentence was added to the manuscript (Section 2.4.): "The amounts of street-purity drugs which circulated on the illegal market in Zagreb were calculated from the estimated annual consumption of pure drugs (expressed in kg/year), which were divided by the corresponding drug purity presented in Table S1."

22. Conclusions: As you have not compared errors in large cities vs small communities, this sentence should be modified

.

R: This section was thoroughly modified.

23. Table 1. Put a space in "Castiglioniet al" between Castiglioni and et

R: Corrected.

24. Table 1. Refined correction factors have been recently proposed for the back-calculation of the illicit drugs considered in this work. I suggest the authors to check the most recently published works (for instance, Gracia-Lor et al. 2016)

R: The refined correction factors proposed by Gracia-Lor et al. (2016) are applied in the revised version.

25. Figure 2. A legend about the meaning of the horizontal lines should be included.

R: The following text was added to the figure caption: „Horizontal lines represent arbitrarily assumed weekend to workday mass load ratio of 1.0 ± 0.2“.

26. In Figure 2, 7 and S1 it is difficult to distinguish among data due to similar coloured bars. Kindly, use a different means for identifying each analyte.

R: Corrected.

27. References: line 479, change Horder to Hordern

R: Corrected.

28. Table S2. Typing error: Change "wastwater" to wastewater

R: Corrected

Reviewer #5: This manuscript presents a long-term monitoring study of drug consumption in Zagreb - Croatia using wastewater-based epidemiology. In addition to 1 week of samples per year which is common in other multiannual wastewater-based epidemiology studies, the authors have also looked at higher sampling frequencies for a couple of years and compared this with the results they would have otherwise got based on only 1 week of sampling. The authors have also looked at drug consumption during holiday periods. My only major concern is that the authors appear to have used a static population size when the study has been conducted over an 8 year period and thus the data may not be truly population normalised and thus I think this needs to be addressed or at the very minimum discussed. My minor comment is that there are numerous grammatical errors throughout the text which would have been addressed from proper editing prior to submission.

R: We do not expect that the population in Zagreb changed significantly over the investigated period. For example, the difference in the number of city inhabitants obtained by CENSUS 2001 and CENSUS 2011 was lower than 2%. The number of tourists visiting Zagreb never exceeds 1% of the total population (official data). The only period with a significant change in population number might be summer vacation season (25^{th} July – 15^{th} August.) due to the outward migrations of residential population (official data not available). Therefore, the mass loads determined during summer might be somewhat underestimated, which was discussed in Section 3.2.3.

Individual points:
Abstract
Line 30 - grammar "an 8-year"

R: Corrected

Line 66 - grammar "of the WBE approach"

R: Corrected

Line 67 - grammar "of the WBE approach"

R: Corrected

Introduction

Line 106 - grammar and spelling "an initial WBE"
R: Corrected

Line 107 - grammar "of the other"

R: Corrected

Chemicals and materials
Line 135 - grammar "purifying with an Elix-Mili-Q-system"

R: Corrected

Line 136 - grammar "were purchased from Waters"

R: Corrected

Line 138 - grammar "were purchased from Phenomenex"

R: Corrected

Line 139 - grammar "were purchased from Whatman"

R: Corrected

Wastewater sampling and analysis

Lines 144 to 149 - the way this is written is unclear

R: This part of the Section 2.3. is rewritten. We hope it is clear now.

Line 162 - grammar "where performance"

R: Corrected

Lines 186 to 189 - This is a long time to normalize to a static population size. Did the population change over this period? What about for the holiday period comparison? Were population markers assessed?

R: We do not expect that the population in Zagreb changed significantly over the investigated period. For example, the difference in the number of city inhabitants obtained by CENSUS 2001 and CENSUS 2011 was < 2%. The number of tourists visiting Zagreb never exceeds 1% of the total population (official data). The only period with a significant change in population number might be summer vacation season (25th July – 15th August.) due to the outward migrations of residential population (official data not available). Therefore, the mass loads determined during summer might be somewhat underestimated, which was discussed in Section 3.2.3.

Line 197 - grammar "using the later"

R: Corrected

Line 213 - grammar "in the treatment"

R: Corrected

The impact of holiday season on drug consumption patterns

Line 252 - grammar "load"

R: Corrected

Line 255 - grammar "seasons"

R: Corrected

Line 262 - grammar - remove "a" before "holiday-related"
R: Corrected

Line 264 - be consistent with "holiday season" and "holiday-season"

R: Corrected

Lines 264 to 267 - without using de facto population sizes it seems like these differences might not be due to higher "per capita consumption" or may only be increased to a lesser extent

R: We do not agree with this comment. It is not likely that the data presented in Fig 3 can be significantly affected by the changes in population in the city of Zagreb.

Line 277 - grammar "on a one-week"

R: The sentence was omitted from the revised manuscript.

Lines 287 to 294 - Other studies have shown numerous markers of population in wastewater which even without a thorough calibration for the investigated catchments would at least reflect relative change in population size. Why have the authors ignored this aspect?

R: As indicated in our response above, the official data on the population of the city of Zagreb do not suggest any significant changes during the period covered by this study.

Impact of sampling strategy on the estimation of drug consumption in multiannual studies

Line 298 -replace "was" with "previously conducted were"

R: Corrected.

Line 301 - replace "the" with "an"

R: Corrected.

Multiannual trends in drug consumption patterns and comparison with available epidemiological data

Line 379 - spelling "substitution therapy"

R: Corrected.

Line 395 - grammar "the outcome"

R: Corrected.

Line 396 - grammar "surveys"

R: Corrected.

Line 412 - too many uses of "moreover"

R: Corrected.

Reviewer #6: Dear Editor,
Thank you for your invitation to review manuscript STOTEN-D-18-06314 entitled "Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: Comparison of sampling strategies for the assessment of multiannual trends."

Monitoring studies are useful and the topic is of interest, so I consider this paper is interesting to be published in STOTEN after some minor changes.
General comments
- Why do you write sometimes 7-year study (highlights, page 6, line 114) and sometimes 8-year period (page 2, line 30 and 42; page 18, line 355)?

R: The study was performed within an 8-year period (2009-2016) but the data set includes data from 7 years (2009, 2011, 2012, 2013, 2014, 2015, 2016). To be more consistent, the corrections were made in the text wherever needed.

- Page 4, lines 62-65, 68-70: Some references are quite old. There are a lot of monitoring research studies on wastewater-based epidemiology in the last 5 years so I recommend to authors to update references.

R: Some additional references are included in the revised version. However, the literature on WBE of illicit drugs has become rather large and, since this is not a review paper, there has to be some selection.

- Page 6, line 106: I think you want to say 'WBE'.

R: Corrected.

- I suggest extending the discussion in sections 3.1 Occurrence of drug biomarkers in municipal wastewater of the city of Zagreb and 3.2 Drug consumption patterns. Please, compare your results with other European countries.

R: In our opinion there is no need for the extensive comparison of the results from Zagreb with the results from other European cities in these 2 sections. According to the Journal's instructions, extensive citations and discussion of published literature should be avoided.

- Table 2. Delete vertical line between Mass load and Average (first line of AMP data).

R: Corrected.

- Figure 1 and 5. Exchange decimals in commas for decimal points.

R: Corrected.

Reviewer #7: The article titled "Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: Comparison of sampling strategies for the assessment of multiannual trends" is based on the analysis of WBE data over an eight-year period. Their study analyses trends revealed by the longitudinal data and compares sampling techniques currently used in many WBE studies. Based on their results, the authors propose a different sampling strategy different from what has been currently used in many WBE studies.

With more regions implementing WBE for community drug monitoring, the results from this study could prove significant to improving regional and multi-regional sampling. I believe this study will be of interest to many STOTEN readers and even more the WBE scientists. I recommend the article for publication.

I have only few comments and suggest a thorough read-through to correct some typos.

1. Line 183-184 authors used daily flow rates for mass load calculation, as such I assume you have all the flow rate data. Line 290- 292. Do the seasonal changes in population affect the WWTP flow rates?

R: Yes, the data on wastewater mass flow expressed in m3/day were obtained from the Central WWTP of the city of Zagreb. However, the sewer system of the city of Zagreb receives either municipal and industrial wastewater as well as rain water and even some stream waters. The flow rates are therefore more influenced by precipitations than by changes in population size and cannot be used as indicators of population size changes.

2. Additionally, though the proposed multiannual and seasonal sampling techniques applied in this study were useful in providing insight on drug use dynamics and better drug use estimations for Zagreb. It is difficult without a comparison site to tell if the same sampling technique would apply as well or have significant impact on a different city (smaller vs bigger; rural vs urban) even in Croatia.

R: We believe that the improvements achieved through the use the whole-year sampling scheme described in this paper strongly suggest that, in spite of possible variations in weekly and seasonal dynamics, large sized cities provide a robust systems for multiannual monitoring of illicit drugs.

Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: Comparison of ~~sampling strategie~~two sampling schemes~~s~~ for the assessment of multiannual trends

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Abstract

A comprehensive study aimed at monitoring of temporal variability of illicit drugs (heroin, cocaine, amphetamine, MDMA, methamphetamine and cannabis) and therapeutic opiate methadone in a large-sized European city using wastewater-based epidemiology (WBE) was conducted in the city of Zagreb, Croatia, during an 8-year period (2009-2016). The study addressed the impact of different sampling ~~strategies~~ schemes on the assessment of temporal drug consumption patterns, in particular multiannual consumption trends and documented the possible errors associated with the one-week sampling scheme. The highest drug consumption prevalence was determined for cannabis (from ~~49~~ 59 ± ~~15~~ 18 to ~~131~~ 156 ± ~~31~~ 37 doses/day/1000 inhabitants 15-64 years), followed by heroin (from 11 ± 10 to 71 ± 19 doses/day/1000 inhabitants 15-64 years), cocaine (from 8.3 ± 0.9 to 23 ± 4.0 doses/day/1000 inhabitants 15-64 years) and amphetamine (from 1.~~6~~ 3 ± ~~1.0~~0.9 to ~~25~~ 21 ± ~~7~~6.~~2~~ 1 doses/day/1000 inhabitants 15-64 years) whereas the consumption of MDMA was comparatively lower (from 0.~~06~~ 18 ± 0.~~03~~ 08 to ~~0.9~~2.7 doses ± 0.~~2~~ 7 doses/day/1000 inhabitants 15-64 years). The drug consumption patterns were characterized by clearly enhanced weekend and Christmas ~~holiday~~ season consumption of stimulating drugs (cocaine, MDMA~~,~~ and amphetamine) and somewhat lower summer consumption of almost all drugs. Pronounced multiannual consumption trends were determined for most of the illicit drugs. The investigated 8-year period was characterized by a marked increase of the consumption of pure cocaine (1.6-fold), THC (2.7-fold), amphetamine (16-fold) and MDMA (15-fold) and a concomitant decrease (2.3-fold) of the consumption of pure heroin. The heroin consumption decrease was associated with an increase of methadone consumption (1.4-fold), which can be linked to its use in the heroin substitution therapy. The estimated number of average methadone doses consumed in the city of Zagreb was in a good agreement with the prescription data on treated opioid addicts in Croatia.

Keywords: illicit drugs, opioids, multiannual trends, wastewater-based epidemiology, Zagreb, LC-MS/MS

1. Introduction

Abuse of illicit drugs has become a major global problem with numerous negative consequences including increase in crime rate, negative impacts on public health, economic damage as well as costs of treatment of drug addicts (EMCDDA, 2009). Consequently, knowing the extent and patterns of drug abuse is very important for planning timely and effective actions to mitigate these problems. The official data about illicit drug consumption usually include the information about the amount and purity of seized drugs, number of treated drug addicts and general population survey data, whose frequency in different countries may be rather different. In recent years, wastewater-based epidemiology (WBE) has been used as a complementary approach for the estimation of drug consumption across the world (e.g. Bijlsma et al., 2016; Bones et al., 2007; Huerta-Fontela et al., 2008; Kahn et al., 2014; Kankaanpää et al., 2014; Kasprzyk-Hordern et al., 2009; Irvine et al., 2011; Lai et al., 2013a, 2016; Metcalfe et al., 2010; Postigo et al., 2010; Terzic et al., 2010; van Nuijs et al., 2009; Zuccato et al., 2008).

The main advantages of the WBE approach are objectivity and suitability for near-real-time monitoring. In order to improve and expand the WBE approach, several publications addressed the problem of uncertainties associated with sample collection (Ort et al., 2010), sample stability (McCall et al., 2016; van Nuijs, 2012; Senta et al., 2014) as well as back-calculation of drug consumption (Castiglioni et al., 2013; Gracia-Lor et al., 2016; Lai et al., 2011). A number of studies have already demonstrated the potential of WBE to provide information about the spatial (Been et al., 2016; Bijlsma et al., 2016; Kankaanpää et al., 2016; Nefau et al., 2013) and temporal (Bade et al., 2018; Been et al., 2016; Lai et al., 2016; Mastroianni et al., 2017; Tscharke at al., 2016) drug consumption patterns, including large international comparative studies (Ort et al., 2014a; Thomas et al., 2012), which showed a pronounced regional and temporal varibility of drug abuse accross the Europe. In several studies, the potential of this approach as a complementary tool to support epidemiological and seizure data (Baz-Lomba et al., 2016; Been et al., 2016; Zuccato et al., 2016) was demonstrated. The WBE approach was also successfully applied to study the differences in drug consumption patterns between the large and small cities (Krizman et al., 2016; van Nuijs et al., 2009), with a clear indication that large cities represent communities with significantly enhanced drug consumption and, consequently, are very suitable for the investigation of the drug consumption patterns.

Regarding temporal variability, a significant emphasis of existing studies was on short-term consumption variability, especially regarding so-called recreational stimulating drugs. A number of WBE studies performed in different countries confirmed an enhanced consumption of stimulating illicit drugs during the weekend (e.g. Krizman et al., 2016; Terzic et al., 2010; Thomas et al., 2012), large sport events (Gerritry et al., 2011), music festivals (Bijlsma et al., 2014; Jiang et al., 2015; Lai et al., 2013b; Mackuľak et al., 2014) and the peak of tourist season in the vaccation areas (Krizman et al., 2016; Lai et al., 2013c). In contrast, only few reports addressed the issue of multiannual changes in drug consumption patterns within the selected population (e.g. Kankaanpää et al., 2016; Mastroianni et al., 2017; Ort et al., 2014a; Tscharke at al., 2016; Zuccato et al. 2016). Most of the~~The~~ published multiannual studies were based on the comparison of one-week wastewater sampling campaigns in a given time-period (Kankaanpää et al., 2016; Mastroianni et al., 2017; Ort et al., 2014a; Zuccato et al. 2016.). In such cases, possible week-to-week variability during the particular year was not taken into account, which might increase the uncertainties related to the annual consumption estimates. In order to get a more accurate estimate, representative of average annual drug consumption, a recent study by Ort et al. (2014b) recommended the use of stratified annual sampling to minimize the errors associated with day-to-day varibility. The importance of sampling scheme for the assessment of consumption was also discussed in Humphries et al. (2016).

In this study we investigated the multiannual trends in the consumption of 6 illicit drugs (cannabis, cocaine, heroin, MDMA, amphetamine, methamphetamine) and one therapeutic opioid (methadone) in the city of Zagreb in the period 2009-2016, by applying two different sampling schemes (one-week sampling scheme and a whole-year sampling scheme). The city of Zagreb is the capital and the largest Croatian city, representing almost 20% of Croatia's population. ~~Moreover~~Furthermore, an initial ~~WBA~~ WBE study conducted in Zagreb (Terzic et al., 2010) indicated specific drug consumption patterns which were different from those reported for most of the other European cities, in particular regarding comparatively higher prevalence of heroin consumption and lower prevalence of cocaine and amphetamine drug consumption.

The specific goals of the present study included: a) long-term study of the weekday-related drug consumption patterns; b) impact of the holiday season on drug consumption patterns; c) seasonal changes in drug consumption patterns; d) testing different sampling ~~strategies~~ schemes for the assessment of multiannual trends; e) tracking the multiannual changes of the drug consumption over a period of ~~7~~ 8 years and comparison with the available epidemiological data.

2. Materials and methods

2.1. Selection of target compounds

The selection of target compounds was ~~made~~ based on the available data on drug consumption patterns in Croatia (Glavak Tkalic et al., 2013) and in the city of Zagreb (Krizman et al., 2016; Terzic et al., 2010). Selected analytes included morphine (MOR), morphine-3-glucuronide (M3G) and 6-acetylmorphine (6-AM) as principal heroin-derived substances as well as benzoylecgonine (BE), amphetamine (AMP), methamphetamine (MAMP), 3,4-methylendioximethamphetamine (MDMA), 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH) and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) as principal biomarkers of cocaine, amphetamine, methamphetamine, MDMA, cannabis and methadone consumption, respectively.

2.2. Chemicals and materials

Standard solutions of all target analytes (1 g/L) and their deuterated analogues (0.1 g/L) were purchased from Lipomed AG (Switzerland). Mixed standard solutions of the analytes and their deuterated analogues, used as surrogate standards, were prepared in methanol (MeOH) at concentrations of 10 mg/L and 2 mg/L, respectively, and kept in the dark at −20 °C. Aqueous ammonia solution (NH_3, 25%) and LC-MS grade MeOH were purchased from Merck AG (Darmstadt, Germany). Acetic acid (CH_3COOH), also LC-MS grade, formic acid (HCOOH) and phosphoric acid (H_3PO_4) were purchased from Fluka (Switzerland). Milli-Q~~MQ~~ water was obtained by purifying ~~in~~ with an Elix-Mili-Q-system (Millipore, Bedford, USA). Oasis MCX cartridges (150 mg / 6 mL) were ~~produced~~ purchased ~~by~~ from Waters (Milford, MA, SAD) whereas Strata NH_2 (200 mg / 3 mL) cartridges as well as HPLC columns used for the chromatographic separation (Synergi Polar; 4 µm, 150 mm × 3 mm, Kinetex PFP; 2.6 µm, 100 mm × 2.1 mm) were ~~manufactured~~ purchased ~~by~~ from Phenomenex (Torrance, California, USA). Glass-fiber filters (GF/C) were ~~delivered~~ purchased ~~by~~ from Whatman (USA).

2.3. Wastewater sampling and analysis

The 24-h composite samples (from 8 a.m. of the previous day to 8 a.m. of the sample collection day) of untreated wastewater were collected at the inlet of the central WWTP of the city of Zagreb in the period 2009-2016, except in 2010. All collected samples were time-proportional, with the sampling time interval of 15 min. A total number of 282 samples, having an average pH of 7.6 ± 0.2, was collected. Depending on the specific research goals, different sampling schemes were applied to cover both short-time and long-term variability: one-week sample scheme, a whole-year sampling-scheme and Christmas season sampling scheme.

All investigated years included at least one one-week sampling period (25 March - 2 April 2009; 26 August - 3 September 2009, 9-15 March 2011, 17-24 March 2012, 6-12 March 2013, 24 July - 31 August 2013, 11-18 March 2014, 17-23 March 2015, 9-15 March 2016).

In addition, in 2009 and further throughout the period 2012-2016, samples were also collected over the whole year, two to four times per month, and uniformly covered all seasons (whole-year sampling scheme). In principle, a whole-year sampling scheme included a collection of equal number of weekend (Sunday) and weekday (Tuesday) samples. The total number of samples collected within one whole-year sampling scheme varied from 21 to 46. Special time-periods such as Christmas holiday season and major festivals were avoided within the one-week and whole-year sampling schemes. Christmas season sampling scheme included two Christmas holiday seasons in the period: 21 December 2012 – 4 January 2013 (*n*=15) and 20 December

2013 – 3 January 2014 (n=14). ~~Depending on the specific research goals, different sampling strategies were applied to cover both short-time and long-term variability. All investigated years included at least one period of seven to nine consecutive days (25 March - 2 April 2009; 26 August - 3 September 2009, 9-15 March 2011, 17-24 March 2012, 6-12 March 2013, 24-31 July 2013, 11-18 March 2014, 17-23 March 2015, 9-15 March 2016).~~

~~In addition, in 2009 and throughout the period 2012-2016, samples were collected over the whole year, two to four times per month, and uniformly covered all seasons. The sampling scheme included collection of a weekend sample followed by collection of a subsequent weekday (Tuesday) sample. The total number of samples per year varied from 21 to 46 samples. Special time-periods such as Christmas holiday season and major festivals were avoided during the regular sampling. To investigate the impact of special events on drug consumption, samples were collected during two holiday seasons in the period: 21 December 2012 – 4 January 2013 (n = 15) and 20 December 2013 – 3 January 2014 (n = 14).~~

The samples collected ~~during~~ within the one-week sampling ~~periods~~ scheme ~~as well as the samples collected during the holiday periods were~~and the Christmas holiday sampling scheme were frozen immediately after collection and kept frozen until analyses, whereas all other samples were processed within a few hours after collection. Since the study covers a rather long time-period, some of the data, resulting from the sampling campaigns described above, were partially used in previously published studies (e.g. Krizman et al., 2016; Ort et al., 2014b; Terzic et al., 2010; Thomas et al., 2014).

The sample preparation and LC-MS/MS analysis were performed by applying already published and validated analytical method (Senta et al., 2013). The~~,~~ ~~which~~ performance of the method was repetitively confirmed in ~~7~~ 6 international intercalibration studies performed during the period 2011-2016 (van Nuijs et al., 2018). Briefly, samples of wastewater (125 mL) were spiked with surrogate standards (120 ng/L) and after equilibration filtered using GF/C filters. After filtration, samples were enriched on Oasis MCX cartridges. The basic drugs were eluted with 6 mL of 0.5% NH_3 in MeOH whereas THC-COOH was eluted with methanol and additionally cleaned-up using Strata NH_2 cartridges. These two fractions were analyzed separately by triple-quadrupole liquid chromatography tandem mass spectrometry (Quantum AM, Thermo Electron, USA). Chromatographic separation of basic drug biomarkers was performed using a gradient elution on Synergy 4µ POLAR-RP 80 Å column (Phenomenex, 150 x 3 mm), whereas for the analyses of THC-COOH, Kinetex 2.6 µm PFP 100 Å (Phenomenex, 100 x 2.1 mm) column was used. Eluents used for the separation of basic analytes included 0.1% acetic acid in H_2O (*v*/*v*) and 0.1% acetic acid in MeOH (*v*/*v*), whereas THC-COOH analyses were performed using H_2O and MeOH as eluents. THC-COOH was analyzed in negative ionization mode (NI) whereas the analyses of all other analytes were performed in positive ionization mode (PI). Identification and quantification was performed using two characteristic transitions for each analyzed compound (MRM mode). Quantitation of all analytes was performed using corresponding deuterated internal standards for all analytes.

2.4. Estimation of drug consumption

~~The assessment of drug~~Estimation of drug consumption was performed as described earlier by Krizman et al. (2016), applying the methodology originally proposed by Zuccato et al. (2008). ~~To minimize possible weekday-related differences in drug consumption patterns (e.g. Terzic et al., 2010), r~~The representative average mass loads (X_{rp}) and their corresponding standard deviations (S_{RP}) used for the assessment of drug consumption were calculated using the ~~as~~ following equations~~s~~:

$$XRP = \frac{5}{7}x\,(workday) + \frac{2}{7}\,x\,(weekend)$$

$$SRP = \sqrt{\left(\frac{5}{7}S\,(workday)\right)^2 + \left(\frac{2}{7}S\,(weekend)\right)^2}$$

in which ~~where~~ ~~X~~X (workday), S (workday), X (weekend) and S (weekend) represent the average values and standard deviations of workday and weekend daily mass loads. The ~~The~~ concentration equal to the half of the detection limit was applied in all cases when the analyzed urinary biomarkers were not detectable. ~~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 drug 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, which~~

The number of inhabitants as well as the number of inhabitants of age 15-64 years, served by the WWTP, was based on 2011 Census data. The ~~consumption of individual drugs expressed as the number of average doses per 1000 inhabitants was calculated by dividing the population~~ normalized ~~drug~~ consumption of individual drugs, expressed as the number of doses per 1000 inhabitants, was calculated using the corresponding ~~with the corresponding~~ average dose size listed in ~~(~~Table 1~~)~~.

The amounts of street-purity drugs which circulated on the illegal market in Zagreb were calculated from the estimated annual consumption of pure drugs (expressed in kg/year), which were divided by the corresponding drug purity presented in Table S1.~~Annual consumption of so called street-purity illicit drugs was calculated considering the data on the average purity of the drugs seized in Croatia in investigated years (Table S1) and WBE-based estimates of pure drug consumption.~~

~~Apart from some exceptions~~Most of~~,~~ the correction factors used in the calculation of drug consumption were taken from the paper published by ~~Zuccato~~ Gracia-Lor et al. ~~et al.~~ (~~2008~~2016). The estimation of cocaine consumption was made by using the ~~later proposed~~ correction factor of 3.6 (Castiglioni et al., 2013), whereas heroin consumption was calculated from 6-AM mass loads, using a correction factor of 86.9 (van Nuijs et al. 2011).

2.5. Statistical evaluation

Statistical analysis of the data was performed using Sigma Plot 12.0 (Systat software Inc., SAD). Depending on data distribution, parametric (*t*-test, One-way ANOVA) and non-parametric tests (Mann-Whitney, Kruskal-Wallis test) were applied. In order to examine differences among multiple groups, One-way ANOVA and Kruskal-Wallis tests were used (with follow-up Holm-Sidak and Dunn's method post-hoc testing, respectively) while for testing the differences between two groups, *t*-test and Mann-Whitney test were used.

3. Results and discussion

3.1. Occurrence of drug biomarkers in municipal wastewater of the city of Zagreb

~~The study was performed in a city of Zagreb, with the population size of approximately 780000 inhabitants and 3.82 registered drug addicts/1000 inhabitants of age 15-64 (data for 2016, Katalinic and Huskic 2017).~~ The analyses included selected drug biomarkers which are excreted after the consumption of 6 illegal drugs (cannabis, heroin, cocaine, amphetamine, MDMA and methamphetamine) and methadone which is primarily used in the treatment of heroin users. The analyses performed between 2009 and 2016 showed that most of the investigated drug biomarkers were rather common constituents in the wastewater of the city of Zagreb (Table 2). The most frequently detected biomarkers were MOR, BE, THC-COOH and EDDP, which were determined in all analyzed wastewater samples (*n* = 270-282). Very high frequency of detection was obtained also for 6-AM (98%), M3G (97%), AMP (96%) and MDMA (99%; *n* = 282), whereas MAMP was the least frequently detected drug biomarker (83%). Regarding abundances, the highest average annual concentrations were determined for MOR (from 74 ± 29 ng/L to 294 ± 83 ng/L), BE (from 143 ± 34 ng/L to 273 ± 101 ng/L) and EDDP (from 121 ± 41 ng/L to 190 ± 67 ng/L), followed by AMP (from 7.5 ± 7.5 ng/L to 109 ± 58 ng/L) and MDMA (from 6.8 ± 7.7 ng/L to 92 ± 58 ng/L). The lowest concentrations were determined for MAMP (from 0.6 ± 0.6 ng/L to 1.4

± 1.8 ng/L), M3G (from 1.6 ± 2.2 ng/L to 9.9 ± 6.7 ng/L) and 6-AM (from 2.0 ± 2.4 ng/L to 12 ± 4.7 ng/L).

3.2. Drug consumption patterns

3.2.1. Workday/weekend drug consumption patterns

Possible differences in workday to weekend consumption patterns of individual drugs have been evaluated based on the ratios of weekend and workday daily mass loads of selected drug biomarkers for individual years (Fig.~~ure~~ 1). It was arbitrarily assumed that a ratio significantly different from 1.0 ± 0.2 was a confirmation of some specific weekday-related consumption pattern. It should be stressed that the ratio for MOR consumption was calculated from the corresponding mass loads of the total morphine (MOR_{tot}). The MOR_{tot} mass loads were obtained by summing up the daily mass loads of MOR and M3G (taking into account the molar ratio to MOR of 1.62).

Almost all ratios of the weekend and workday average daily mass load of stimulating drug biomarkers, BE (1.5 ± 0.3 to 1.7 ± 0.5), MDMA (2.3 ± 0.5 to 4.3 ± 3.6) and AMP (1.0 ± 1.0 to 2.2 ± 1.3), were significantly (*t*-test) different from 1.0 ± 0.2. By contrast, most of the ratios for MOR_{tot} (0.95 ± 0.4 to 1.1 ± 0.3), 6-AM (0.6 ± 0.8 to 1.2 ± 0.3), THC-COOH (0.9 ± 0.4 to 1.2 ± 0.4) and EDDP (0.9 ± 0.3 to 1.1 ± 0.3) indicated a rather uniform consumption of heroin, cannabis and methadone throughout the week. The observed weekend-related drug consumption patterns of stimulating drugs (MDMA, cocaine and AMP) documented in this study not only fully support the results obtained in a number of previous studies based on 7 consecutive days sampling scheme (e.g. Krizman et al., 2015; Ort et al., 2014a; Terzic et al., 2010; Thomas et al 2012), but also confirm the robustness of the applied whole-year sampling scheme to demonstrate the importance of weekday-weekend dynamics at long-term time scales.

3.2.2. The impact of holiday season on drug consumption patterns

~~The impact of holiday season on drug consumption patterns was investigated in two selected 15-days periods (21 December 2012 – 4 January 2013 and 20 December 2013 - 03 January 2014).~~ The results of ~~this~~ research dealing with the impact of holiday season on drug consumption patterns are presented in Fig. 2~~-~~, Fig. S1 and Fig. 3. In both periods, the 1st of January (New Year) was characterized by a significantly enhanced daily mass load~~s~~ of BE (224 g/day and 197 g/day), MDMA (62 g/day and 67 g/day) and AMP (42 g/day and 60 g/day), which confirmed an increased consumption of all major stimulating drugs in holiday seasons (Fig. 2). By contrast, the 25th of December (Christmas) was associated with an enhanced excretion of BE (166 g/day and 130 g/day) whereas the Christmas consumption of most amphetamine-type drugs (AMP and MDMA) was not clearly elevated. These results probably reflect the life-style differences of cocaine and amphetamine-type drug consumers within the investigated population. In both holiday season periods, a steady increase of MOR excretion towards Christmas was also observed. However this increase was not associated with the concomit~~t~~ant increase of 6-AM and therefore cannot be unequivocaly related to the enhanced consumption of heroin. Furthermore, unlike for stimulating drugs, ~~a~~ holiday-related consumption patterns could not be established for the remaining investigated drugs, such as cannabis and EDDP (Fig.

S1). The comparison of the average mass loads during the two holiday ~~-~~season periods with the average weekend and workday mass loads in the corresponding years (Fig. 3) confirmed a significantly higher consumption ($p < 0.05$) of stimulating drugs (BE, MDMA, AMP) during the weekend (n = 19-24) and holiday ~~-~~season period (n = 14-15) as compared to workday periods (n = 19-22). The average mass loads of stimulating drugs during Christmas holiday season were 2 – 3.9-fold higher ~~then~~ than during the average weekday and 1.2 – 1.9-fold higher than during the average weekend of the corresponding year. This is in a good agreement with previous studies which indicated the enhanced consumption of stimulating drugs during the holidays, festivals, tourist seasons etc. (e.g. Krizman et al., 2016; Lai et al., 2013a; Lai et al. 2013b, van Nuijs et al., 2009) and underlines the ability of the applied WBE approach to address the problem of relative contributions of special events to the overal drug consumption in a particular yearly period.

3.2.3. The seasonal differences in drug consumption patterns

~~The most frequently used sampling strategy in WBE studies is based on one-week sampling scheme which includes collection of wastewater samples over a period of 7 consecutive days (e.g. Ort et al., 2014a; Thomas et al., 2012; Zuccato et al., 2016).~~ In this study, we compared the average daily mass loads determined in the city of Zagreb in 2 different one-week periods, early spring and summer, in 2009 and 2013. The results of this comparison are presented in Fig. 4. In both investigated years, the average summer mass loads of most of the investigated drug biomarkers were lower than those determined in early spring (Fig. 4). However the observed differences were statisticaly significant ($p < 0.05$; Mann-Whitney test) only for drug biomarkers which exhibit lower intra-week variability (e.g. MOR_{tot}, THC-COOH and EDDP) whereas they were not significant for the biomarkers of stimulating drugs (BE, MDMA), probably due to the comparatively higher intra-week variability. The lower average daily mass loads determined in summer are ~~very~~ most likely associated with a ~~seasonal changes in population number, which in the large continental cities, like city of Zagreb, can be characterized by a pronounced~~disbalanced ~~decrease of residential population due to~~ outward and inward population ~~summer tourist~~ migrations during the summer vacation season. Namely, the contribution of tourists to the total city population is rather negligible throughout the year (<1%, data from Zagreb Tourist Board), whereas a significant percentage of residential population might be out of town during the peak of summer season. Unfortunately, this assumption cannot be confirmed since ~~the precise~~ the official data ~~on the~~related to the ~~seasonal changes~~outward migrations ~~in~~ of the city population ~~size~~ were not available. Another possible factor which might have caused the observed differences in spring and summer mass loads is faster in-sewer drug biomarker degradation at higher temperatures (e.g. Devault et al., 2017). However, the model experiments which were performed with the wastewater from the city of Zagreb at 10°C and 20°C, indicated rather higher stability of all urinary biomarkers included in this research at the both temperature conditions (Senta et al., 2016.). Since the in-sewer wastewater residence time in Zagreb is relatively short (<5 h) and a typical wastewater temperature in March and July/August is 12°C and 20.5°C, respectively, it is not very likely that the observed seasonal mass load diferences were primarily caused by faster in-sewer degradation in summer. Although the reasons for the observed seasonal differences of the average mass loads are not yet fully understood, they indicated that the total drug consumption might be underestimated if extrapolated from the average daily mass loads determined in summer.~~Nevertheless, the observed seasonal differences of the average mass loads indicated that the total drug consumption might be underestimated if extrapolated from the average daily mass loads determined in summer.~~

3.3. Impact of sampling ~~strategy~~ scheme on the estimation of drug consumption in multiannual studies

Most of the previously conducted multiannual WBE studies ~~was~~ were based on relatively short one-week sampling periods (e.g. Kankaanpää et al., 2016; Mastroianni et al., 2017, Ort et al., 2014a; Zuccato et al. 2016), which, due to the possible week-to-week variability of daily mass loads, may be associated with ~~a~~ a potential error in tracking the drug consumption on ~~the~~ an annual basis. In this study, a comparison was made between the representative average daily mass loads of selected drug biomarkers obtained by applying two different sampling ~~strategies~~schemes: one-week sampling scheme (March/April 2012 – 2016) and whole-year sampling scheme (Sundays and Tuesdays; sampled either ~~bimonthly~~fortnightly in 2012-2014 or monthly in 2015-2016). Based on the extended scheme of the whole-year sampling carried out in 2013 and 2014, which included ~~bimonthly~~fortnightly sampling (n = 48), it was shown that the reduction of the sample number to half (monthly sampling; n = 24) did not significantly affect the estimate of the mass loads (t-test; $p < 0.05$).

The representative daily mass loads of individual drug biomarkers determined by applying the one-week and the whole-year sampling scheme are presented in Fig. 5. Apart from some occassional exceptions, the application of the whole-year sampling scheme was, in principle, associated with somewhat higher day-to-day variability of daily mass loads -than the one-week sampling scheme, which is probably a result of higher intra-annual variability of drug consumption. The amphetamine-type drugs (MAMP, AMP and MDMA) exhibited the strongest day-to-day variability within the both sampling schemes, which is most probably associated with a rather irregular consumption pattern of these drugs, characterized by enhanced weekend and holiday consumption rates. Furthermore, the one-week sampling scheme was occasionally associated with relatively high day-to-day variability of AMP and MDMA. The statistical analysis of the data exhibited a significant difference ($p < 0.05$) between the representative mass loads of AMP obtained by the two applied sampling strategies schemes in all investigated years. By contrast, the differences for other investigated drug biomarkers were found to be significant ($p < 0.05$) only occasionally. The performed comparison indicated that, in the large cities like city of Zagreb, the one-week sampling scheme may provide a rather reliable base for the estimate of the annual consumption of most of the classical illicit drugs, assuming that the sampling periods exclude special events. Previous study by Ort at al. (2014b) has shown that the variability of drug consumption in smaller communities (<10 000 inhabitants) is extremely high, requiring very high sampling frequency to achieve the proper estimate of drug consumption. It was estimated that the average annual consumption calculated from 1-week sampling was subject to approximately 60% relative error. In contrast, our study suggests that intra-annual varibilities in larger cities can be significantly smaller allowing detection of relatively small changes (20%) of the drug consumption among different years. Nevertheless, although some previous studies, addressing the issue of multiannual changes, demonstrated the applicability of one-week sampling scheme (Mastroianni et al., 2017; Ort et al. 2014a, Zuccato et al., 2016), our data show that such a scheme is insufficiently reliable for the drugs exhibiting high day-to-day and intra-annual variability, even in case of larger cities like the city of Zagreb-.

3.4. Multiannual trends in drug consumption patterns and comparison with available epidemiological data

The back-calculations of drug consumption were ~~made~~ based on representative daily mass loads determined for all samples collected within each investigated year, with the exception of those collected during the Christmas-New Year holiday seasons. The consumption was calculated for heroin, cocaine, AMP, MDMA, cannabis (THC) and methadone (MTHD). The results expressed in mg/day/1000 inhabitants of age 15-64 are presented in Fig. 6, whereas the results expressed in other units (e.g. mg/day/1000 inhabitants, doses/day/1000 inhabitants, g/day, kg/year, kg/year of street purity drug) are given in ~~Electronic~~ Supplementary Material (Table S2). The highest illicit drug consumption rate was determined for cannabis (from ~~6153~~ 7368 ± ~~1835~~ 2197 mg/day/1000 inhabitants 15-64 years to ~~16322~~ 19544 ± ~~3862~~ 4624 mg/day/1000 inhabitants 15-64 years), followed by heroin (from 107 ± 104 mg/day/1000 inhabitants 15-64 years to 712 ± 193 mg/day/1000 inhabitants 15-64 years), cocaine (from 249 ± 27 mg/day/1000 inhabitants 15-64 years to 699 ± 121 mg/day/1000 inhabitants 15-64 years), MDMA (from ~~6.0~~17 ± ~~2.6~~7.5 mg/day/1000 inhabitants 15-64 years to ~~88~~ 259 ± ~~24~~ 69 mg/day/1000 inhabitants 15-64 years) and AMP (from ~~16~~ 13 ± ~~10~~ 8.8 mg/day/1000 inhabitants 15-64 years to ~~251~~ 213 ± ~~72~~ 61 mg/day/100 inhabitants 15-64 years). The estimated consumption rate of the therapeutic opioid methadone was in the range from 280 ± 26 mg/day/1000 inhabitants 15-64 years to 393 ± 61 mg/day/1000 inhabitants 15-64 years. ~~Collectively~~ In principle, the determined drug consumption patterns and rates were rather similar to those determined in some other Mediterranean countries, like Spain and Italy (Mastroianni et al., 2017; Zuccato et al., 2016), although some differences regarding the prevalence of individual drugs as well as regarding the temporal trends were observed. For example, cannabis and cocaine were the most prevalently consumed illicit drugs in Barcelona (Spain) and investigated Italian cities, whereas a heroin consumption was reported to be much lower (Mastroianni et al., 2017; Zuccato et al., 2016). ~~, the determined drug consumption rates are of similar order of magnitude as in some other WBE studies (e.g. Mastroianni et al., 2017; Zuccato et al., 2016) although there were some differences regarding the prevalence of individual drugs as well as regarding temporal trends.~~

In our study, all investigated illicit drugs, except heroin, exhibited a significant increase ($p < 0.05$) of the consumption rates over the investigated 8-year period (Fig. 6 and 7, Table S2). In 2016, the average consumption rate of pure MDMA, AMP, THC (cannabis) and cocaine, were 15-fold, 16-fold, 3-fold and 2-fold higher ~~then~~ than in 2009, respectively. The multiannual consumption patterns of pure AMP and MDMA were characterized by a rather continuing increase of their consumption rates (Fig. 6) over the whole investigated time period, whereas the consumption of THC (cannabis) was characterized by a significant increase in 2009-2014 period ($p < 0.05$, 3-fold increase), and rather stable consumption rate in 2014-2016 period. By contrast, the consumption rate of pure heroin dropped significantly ($p < 0.05$; 5-7-fold) between 2009 and 2011-2012 period, and kept at significantly lower level until 2016 ($p < 0.05$). However, a significant ($p < 0.05$) 2-3-fold increase in pure heroin consumption was recorded between 2011/2012 and 2016, which indicated a gradual recovery of heroin market in that period. Interestingly, a reduction of heroin consumption in the period 2010-2012 was reported for Italy as well (Zuccato et al., 2016).

Based on the estimated amounts of consumed drugs and the official data on ~~the~~ purity of seized drugs ~~in the same period~~provided by the Office for Combating Narcotic Drug Abuse of the Government of the Republic of Croatia (see Table S1), we calculated the amounts of the street-purity drugs which circulated on the illegal market in Zagreb in the corresponding years (Table S2). It should be stressed that the street-drug purity of investigated drugs (heroin, amphetamine, MDMA, cocaine) exhibited a pronounced temporal variability (Table S2). The amounts of the most prevalent drugs present on the illegal market in Zagreb were as follows: from 211 to 565 kg/year of heroin, from 157 to 323 kg/year of cocaine, from ~~52~~ 44 to ~~364~~ 309 kg/year of amphetamine, from ~~3.5~~14 to ~~43~~ 127 kg/year of MDMA and from ~~19086~~ 22853 to ~~45089~~ 53988 kg/year of cannabis.

Consequently, the observed multiannual trends in the consumption of pure drugs are probably not impacted exclusively by the changes in drug consumption prevalence but also by the changes in the street drug purity. In this context, it is interesting to note that a significant drop in the heroin consumption rate between 2009 and 2011/2012 was associated with a concomit~~t~~ant decrease of heroin street-drug purity (from 21.5% to 8.4%) and an increase in the consumption of the sub~~p~~stitution therap~~h~~y drug methadone (40%), which then kept a rather stable consumption rate in the subsequent period (2013-2016). The average number of consumed methadone doses estimated in this study (e.g. 3.1 ± 0.4 doses/day/1000 inhabitants in 2015; 80 mg/dose) were in a rather good agreement with the amount of that drug prescibed in the city of Zagreb in 2015 (11.76 DDD/TSD; DDD = 25 mg; 3.7 doses/day/1000 inhabitants for the average dose of 80 mg/L) (Draganic et al., 2017), which confirmed a reliability of WBE approach for tracking the changes of the illicit drug consumption patterns.

The trends in population normalized number of addicts treated due to consumption of different types of drugs did not, however, reflect the multiannual drug consumption trends determined in this study (Fig. 7), probably due to a rather long time-gap between the initial drug consumption and the involvement of the consumers in the treatment.

Furthermore, the drug consumption trends which were determined in the present study were only partialy in agreement with the results of general population surveys performed in Croatia in 2011 and 2015, which indicated a significant increase only in the consumption of cannabis (2.9% last-month prevalence in 2011; 5.0% last-month prevalence in 2016) (Glavak Tkalic et al., 2013; Glavak Tkalic et al., 2016), whereas the differences in the consumption prevalence of other illicit drugs were not found to be significant. Our study suggests that the outcome of national population surveys on drug consumption is not necessarily representative for larger cities. Given the fact that the city of Zagreb represents approximately 20% of the whole Croatian population, the drug consumption trends determined in this study imply the need for specific surveys focusing on larger cities. Moreover, the trends observed in the city of Zagreb might be ~~indicative~~ an indication of some trends developing at the national level.

5. Conclusion

The seveneight-year monitoring period of drug consumption patterns in the city of Zagreb, Croatia, using wastewater-based epidemiology revealed several temporal variability patterns, including weekday-weekend dynamics, holiday season effects and multiannual trends. In agreement with the literature, the enhanced consumption of stimulating drugs was systematically observed during weekends and Christmas holiday season. In addition, a significant multiannual increase of cocaine (1.6-fold), THC (2.7-fold), amphetamine (16-fold) and MDMA (15-fold) consumption with a concomitant decrease (2.3-fold) of the consumption of heroin was observed during the investigated 8-year period (2009-2016). All these variabilities should be taken into account to get a representative estimate of the average annual consumption for comparison of different years. The whole-year sampling The whole-year sampling strategyscheme showed a clear advantage over the seven-consecutive-dayone-week sampling scheme, especially for drugs showing enhanced day-to-day and intra-annual variability. which has been recently applied to study multiannual trends. Moreover, it was shown that tThe errors associated with day-to-day and intra-annual variability of BE (<20%) for large citiesdetermined in the city of Zagreb (>500 000 inhabitants) study were are much smaller than from those for thereported for small communities (,Ort et al. 20914b), which indicated enhanced robustness of the estimates obtained for large sized cities. Our data suggest that large sized cities can provide providing a basis for the a reliable detection of relatively small changes in drug consumption over a multi-year period. ~~Moreover~~Consequently, t~~, t~~he trends observed in the larger cities could be used as an early warning of the trends developing at the national level.

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Figure captions

Fig.ure 1. Ratios of weekend and workday average mass loads of selected urinary drug biomarkers (MOR_{tot}, 6-AM, AMP, MDMA, BE, THC-COOH, EDDP) determined in the period from 2009 to 2016. Error bars represent standard deviations. Horizontal lines represent arbitrarily assumed weekend to workday mass load ratio of 1.0 ± 0.2.

Fig.ure 2. Mass loads of BE, MDMA and AMP in two different Christmas-New Year holiday periods: A) 2012/2013 and B) 2013/2014.

Fig.ure 3. Average mass loads of selected drug biomarkers determined on workdays, weekend and during two Christmas-New Year periods: A) 2012/2013 and B) 2013/2014. Error bars represent standard deviations.

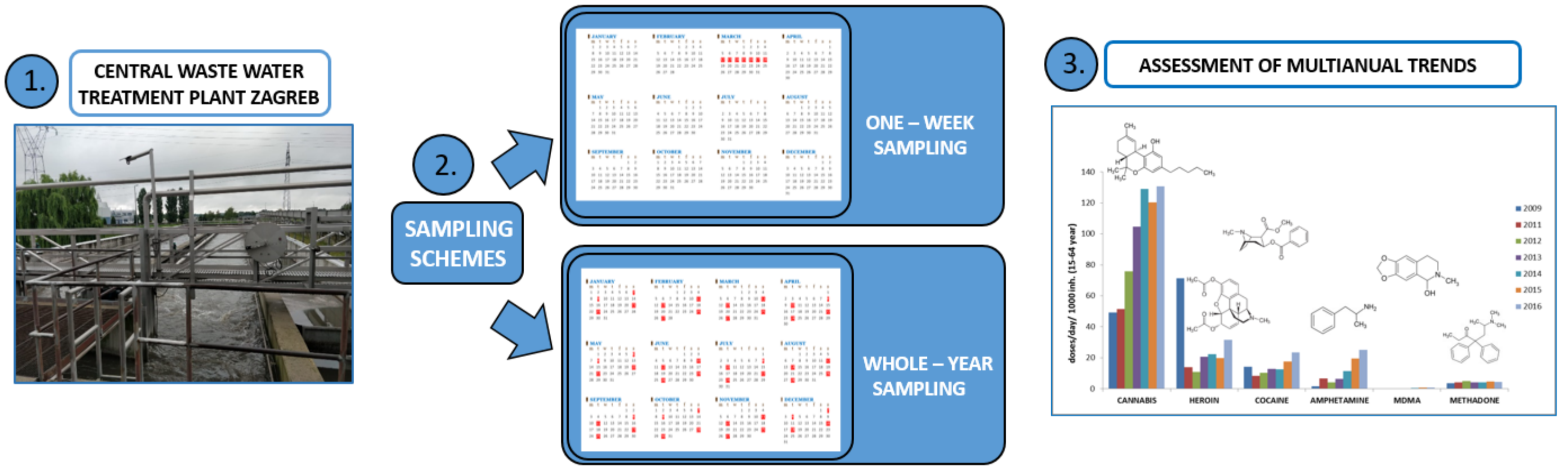
Fig.ure 4. Variability of average mass loads of selected urinary drug biomarkers in Zagreb during the spring and summer sampling week in A) 2009 and B) 2013. Error bars represent standard deviations.

Fig.ure 5. Impact of the selected sampling strategy schemes (whole-year and one-week monitoring) on the determination of representative mass loads. Error bars represent standard deviations.

Fig.ure 6. Consumption of cocaine, heroin, MDMA, amphetamine, THC and methadone in the city of Zagreb in the period 2009-2016. Error bars represent standard deviations.

Fig.~~ure~~ 7. Comparison of estimated drug consumption in the city of Zagreb with available epidemiological data for Croatia in the period of 2009-2016. Stimulants in the epidemiological figure include amphetamine-type drugs. Opiates include heroin and morphine.

**Graphical Abstract*



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

Highlights

- Drug consumption patterns were studied using wastewater-based epidemiology
- The 8-year study was performed in a large European city
- Comparison of one-week and whole-year sampling strategies was made
- Significant multiannual drug consumption changes were determined
- A comparison with epidemiological data was performed

Long-term monitoring of drug consumption patterns in a large-sized European city using wastewater-based epidemiology: Comparison of two sampling schemes for the assessment of multiannual trends

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Abstract

A comprehensive study aimed at monitoring of temporal variability of illicit drugs (heroin, cocaine, amphetamine, MDMA, methamphetamine and cannabis) and therapeutic opiate methadone in a large-sized European city using wastewater-based epidemiology (WBE) was conducted in the city of Zagreb, Croatia, during an 8-year period (2009-2016). The study addressed the impact of different sampling schemes on the assessment of temporal drug consumption patterns, in particular multiannual consumption trends and documented the possible errors associated with the one-week sampling scheme. The highest drug consumption prevalence was determined for cannabis (from 59 ± 18 to 156 ± 37 doses/day/1000 inhabitants 15-64 years), followed by heroin (from 11 ± 10 to 71 ± 19 doses/day/1000 inhabitants 15-64 years), cocaine (from 8.3 ± 0.9 to 23 ± 4.0 doses/day/1000 inhabitants 15-64 years) and amphetamine (from 1.3 ± 0.9 to 21 ± 6.1 doses/day/1000 inhabitants 15-64 years) whereas the consumption of MDMA was comparatively lower (from 0.18 ± 0.08 to 2.7 doses ± 0.7 doses/day/1000 inhabitants 15-64 years). The drug consumption patterns were characterized by clearly enhanced weekend and Christmas season consumption of stimulating drugs (cocaine, MDMA and amphetamine) and somewhat lower summer consumption of almost all drugs. Pronounced multiannual consumption trends were determined for most of the illicit drugs. The investigated 8-year period was characterized by a marked increase of the consumption of pure cocaine (1.6-fold), THC (2.7-fold), amphetamine (16-fold) and MDMA (15-fold) and a concomitant decrease (2.3-fold) of the consumption of pure heroin. The heroin consumption decrease was associated with an increase of methadone consumption (1.4-fold), which can be linked to its use in the heroin substitution therapy. The estimated number of average

methadone doses consumed in the city of Zagreb was in a good agreement with the prescription data on treated opioid addicts in Croatia.

Keywords: illicit drugs, opioids, multiannual trends, wastewater-based epidemiology, Zagreb, LC-MS/MS

1. Introduction

Abuse of illicit drugs has become a major global problem with numerous negative consequences including increase in crime rate, negative impacts on public health, economic damage as well as costs of treatment of drug addicts (EMCDDA, 2009). Consequently, knowing the extent and patterns of drug abuse is very important for planning timely and effective actions to mitigate these problems. The official data about illicit drug consumption usually include the information about the amount and purity of seized drugs, number of treated drug addicts and general population survey data, whose frequency in different countries may be rather different. In recent years, wastewater-based epidemiology (WBE) has been used as a complementary approach for the estimation of drug consumption across the world (e.g. Bijlsma et al., 2016; Bones et al., 2007; Huerta-Fontela et al., 2008; Kahn et al., 2014; Kankaanpää et al., 2014; Kasprzyk-Hordern et al., 2009; Irvine et al., 2011; Lai et al., 2013a, 2016; Metcalfe et al., 2010; Postigo et al., 2010; Terzic et al., 2010; van Nuijs et al., 2009; Zuccato et al., 2008).

The main advantages of the WBE approach are objectivity and suitability for near-real-time monitoring. In order to improve and expand the WBE approach, several publications addressed the problem of uncertainties associated with sample collection (Ort et al., 2010), sample stability (McCall et al., 2016; van Nuijs, 2012; Senta et al., 2014) as well as back-calculation of drug consumption (Castiglioni et al., 2013; Gracia-Lor et al., 2016; Lai et al., 2011). A number of studies have already demonstrated the potential of WBE to provide information about the spatial (Been et al., 2016; Bijlsma et al., 2016; Kankaanpää et al., 2016; Nefau et al., 2013) and temporal (Bade et al., 2018; Been et al., 2016; Lai et al., 2016; Mastroianni et al., 2017; Tscharke at al., 2016) drug consumption patterns, including large international comparative studies (Ort et al., 2014a; Thomas et al., 2012), which showed a pronounced regional and temporal varibility of drug abuse accross the Europe. In several studies, the potential of this approach as a complementary tool to support epidemiological and seizure data (Baz-Lomba et al., 2016; Been et al., 2016; Zuccato et al., 2016) was demonstrated. The WBE approach was also successfully applied to study the differences in drug consumption patterns between the large and small cities (Krizman et al., 2016; van Nuijs et al., 2009), with a clear indication that large cities represent communities with significantly enhanced drug consumption and, consequently, are very suitable for the investigation of the drug consumption patterns.

Regarding temporal variability, a significant emphasis of existing studies was on short-term consumption variability, especially regarding so-called recreational stimulating drugs. A number of WBE studies performed in different countries confirmed an enhanced consumption of stimulating illicit drugs during the weekend (e.g. Krizman et al., 2016; Terzic et al., 2010; Thomas et al., 2012), large sport events (Gerritry et al., 2011), music festivals (Bijlsma et al., 2014; Jiang et al., 2015; Lai et al., 2013b; Mackuľak et al., 2014) and the peak of tourist season in the vaccation areas (Krizman et al., 2016; Lai et al., 2013c). In contrast, only few reports addressed the issue of multiannual changes in drug consumption patterns within the selected population (e.g. Kankaanpää et al., 2016; Mastroianni et al., 2017; Ort et al., 2014a; Tscharke at al., 2016; Zuccato et al. 2016). Most of the published multiannual studies were based on the comparison of one-week wastewater sampling campaigns in a given time-period (Kankaanpää et al., 2016; Mastroianni et al., 2017; Ort et al., 2014a; Zuccato et al. 2016.). In such cases, possible week-to-week variability during the particular year was not taken into account, which might increase the uncertainties related to the annual consumption estimates. In order to get a more accurate estimate, representative of average annual drug consumption, a recent study by Ort et al. (2014b) recommended the use of stratified annual sampling to minimize the errors associated with day-to-day varibility. The importance of sampling scheme for the assessment of consumption was also discussed in Humphries et al. (2016).

In this study we investigated the multiannual trends in the consumption of 6 illicit drugs (cannabis, cocaine, heroin, MDMA, amphetamine, methamphetamine) and one therapeutic opioid (methadone) in the city of Zagreb in the period 2009-2016, by applying two different sampling schemes (one-week sampling scheme and a whole-year sampling scheme). The city of Zagreb is the capital and the largest Croatian city, representing almost 20% of Croatia's population. Furthermore, an initial WBE study conducted in Zagreb (Terzic et al., 2010) indicated specific drug consumption patterns which were different from those reported for most of the other European cities, in particular regarding comparatively higher prevalence of heroin consumption and lower prevalence of cocaine and amphetamine drug consumption.

The specific goals of the present study included: a) long-term study of the weekday-related drug consumption patterns; b) impact of the holiday season on drug consumption patterns; c) seasonal changes in drug consumption patterns; d) testing different sampling schemes for the assessment of multiannual trends; e) tracking the multiannual changes of the drug consumption over a period of 8 years and comparison with the available epidemiological data.

2. Materials and methods

2.1. Selection of target compounds

The selection of target compounds was based on the available data on drug consumption patterns in Croatia (Glavak Tkalic et al., 2013) and in the city of Zagreb (Krizman et al., 2016; Terzic et al., 2010). Selected analytes included morphine (MOR), morphine-3-glucuronide (M3G) and 6-acetylmorphine (6-AM) as principal heroin-derived substances as well as benzoylecgonine (BE), amphetamine (AMP), methamphetamine (MAMP), 3,4-methylendioximethamphetamine (MDMA), 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH) and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) as principal biomarkers of cocaine, amphetamine, methamphetamine, MDMA, cannabis and methadone consumption, respectively.

2.2. Chemicals and materials

Standard solutions of all target analytes (1 g/L) and their deuterated analogues (0.1 g/L) were purchased from Lipomed AG (Switzerland). Mixed standard solutions of the analytes and their deuterated analogues, used as surrogate standards, were prepared in methanol (MeOH) at concentrations of 10 mg/L and 2 mg/L, respectively, and kept in the dark at −20 °C. Aqueous ammonia solution (NH_3, 25%) and LC-MS grade MeOH were purchased from Merck AG (Darmstadt, Germany). Acetic acid (CH_3COOH), also LC-MS grade, formic acid (HCOOH) and phosphoric acid (H_3PO_4) were purchased from Fluka (Switzerland). Milli-Q water was obtained by purifying with an Elix-Mili-Q-system (Millipore, Bedford, USA). Oasis MCX cartridges (150 mg / 6 mL) were purchased from Waters (Milford, MA, SAD) whereas Strata NH_2 (200 mg / 3 mL) cartridges as well as HPLC columns used for the chromatographic separation (Synergi Polar; 4 µm, 150 mm × 3 mm, Kinetex PFP; 2.6 µm, 100 mm × 2.1 mm) were purchased from

Phenomenex (Torrance, California, USA). Glass-fiber filters (GF/C) were purchased from Whatman (USA).

2.3. Wastewater sampling and analysis

The 24-h composite samples (from 8 a.m. of the previous day to 8 a.m. of the sample collection day) of untreated wastewater were collected at the inlet of the central WWTP of the city of Zagreb in the period 2009-2016, except in 2010. All collected samples were time-proportional, with the sampling time interval of 15 min. A total number of 282 samples, having an average pH of 7.6 ± 0.2, was collected. Depending on the specific research goals, different sampling schemes were applied to cover both short-time and long-term variability: one-week sample scheme, a whole-year sampling-scheme and Christmas season sampling scheme.

All investigated years included at least one one-week sampling period (25 March - 2 April 2009; 26 August - 3 September 2009, 9-15 March 2011, 17-24 March 2012, 6-12 March 2013, 24 July - 31 August 2013, 11-18 March 2014, 17-23 March 2015, 9-15 March 2016).

In addition, in 2009 and further throughout the period 2012-2016, samples were also collected over the whole year, two to four times per month, and uniformly covered all seasons (whole-year sampling scheme). In principle, a whole-year sampling scheme included a collection of equal number of weekend (Sunday) and weekday (Tuesday) samples. The total number of samples collected within one whole-year sampling scheme varied from 21 to 46. Special time-periods such as Christmas holiday season and major festivals were avoided within the one-week and whole-year sampling schemes. Christmas season sampling scheme included two Christmas holiday seasons in the period: 21 December 2012 – 4 January 2013 (*n*=15) and 20 December

2013 – 3 January 2014 (*n*=14). The samples collected within the one-week sampling scheme and the Christmas holiday sampling scheme were frozen immediately after collection and kept frozen until analyses, whereas all other samples were processed within a few hours after collection. Since the study covers a rather long time-period, some of the data, resulting from the sampling campaigns described above, were partially used in previously published studies (e.g. Krizman et al., 2016; Ort et al., 2014b; Terzic et al., 2010; Thomas et al., 2014).

The sample preparation and LC-MS/MS analysis were performed by applying already published and validated analytical method (Senta et al., 2013). The performance of the method was repetitively confirmed in 6 international intercalibration studies performed during the period 2011-2016 (van Nuijs et al., 2018). Briefly, samples of wastewater (125 mL) were spiked with surrogate standards (120 ng/L) and after equilibration filtered using GF/C filters. After filtration, samples were enriched on Oasis MCX cartridges. The basic drugs were eluted with 6 mL of 0.5% NH_3 in MeOH whereas THC-COOH was eluted with methanol and additionally cleaned-up using Strata NH_2 cartridges. These two fractions were analyzed separately by triple-quadrupole liquid chromatography tandem mass spectrometry (Quantum AM, Thermo Electron, USA). Chromatographic separation of basic drug biomarkers was performed using a gradient elution on Synergy 4µ POLAR-RP 80 Å column (Phenomenex, 150 x 3 mm), whereas for the analyses of THC-COOH, Kinetex 2.6 µm PFP 100 Å (Phenomenex, 100 x 2.1 mm) column was used. Eluents used for the separation of basic analytes included 0.1% acetic acid in H_2O (*v*/*v*) and 0.1% acetic acid in MeOH (*v*/*v*), whereas THC-COOH analyses were performed using H_2O and MeOH as eluents. THC-COOH was analyzed in negative ionization mode (NI) whereas the analyses of all other analytes were performed in positive ionization mode (PI). Identification and quantification was performed using two characteristic transitions for each analyzed compound (MRM mode). Quantitation of all analytes was performed using corresponding deuterated internal standards for all analytes.

2.4. Estimation of drug consumption

Estimation of drug consumption was performed as described earlier by Krizman et al. (2016), applying the methodology originally proposed by Zuccato et al. (2008). The representative average mass loads (X_{rp}) and their corresponding standard deviations (S_{RP}) used for the assessment of drug consumption were calculated using the following equations:

$$XRP = \frac{5}{7} x\,(workday) + \frac{2}{7}\, x\,(weekend)$$

$$SRP = \sqrt{\left(\frac{5}{7} S\,(workday)\right)^2 + \left(\frac{2}{7} S\,(weekend)\right)^2}$$

in which X (workday), S (workday), X (weekend) and S (weekend) represent the average values and standard deviations of workday and weekend daily mass loads. The concentration equal to the half of the detection limit was applied in all cases when the analyzed urinary biomarkers were not detectable.

The number of inhabitants as well as the number of inhabitants of age 15-64 years, served by the WWTP, was based on 2011 Census data. The normalized consumption of individual drugs, expressed as the number of doses per 1000 inhabitants, was calculated using the corresponding average dose size listed in Table 1.

The amounts of street-purity drugs which circulated on the illegal market in Zagreb were calculated from the estimated annual consumption of pure drugs (expressed in kg/year), which were divided by the corresponding drug purity presented in Table S1.

Most of the correction factors used in the calculation of drug consumption were taken from the paper published by Gracia-Lor et al. (2016). The estimation of cocaine consumption was made by using the correction factor of 3.6 (Castiglioni et al., 2013), whereas heroin consumption was calculated from 6-AM mass loads, using a correction factor of 86.9 (van Nuijs et al. 2011).

2.5. Statistical evaluation

Statistical analysis of the data was performed using Sigma Plot 12.0 (Systat software Inc., SAD). Depending on data distribution, parametric (*t*-test, One-way ANOVA) and non-parametric tests (Mann-Whitney, Kruskal-Wallis test) were applied. In order to examine differences among multiple groups, One-way ANOVA and Kruskal-Wallis tests were used (with follow-up Holm-Sidak and Dunn's method post-hoc testing, respectively) while for testing the differences between two groups, *t*-test and Mann-Whitney test were used.

3. Results and discussion

3.1. Occurrence of drug biomarkers in municipal wastewater of the city of Zagreb

The analyses included selected drug biomarkers which are excreted after the consumption of 6 illegal drugs (cannabis, heroin, cocaine, amphetamine, MDMA and methamphetamine) and methadone which is primarily used in the treatment of heroin users. The analyses performed between 2009 and 2016 showed that most of the investigated drug biomarkers were rather common constituents in the wastewater of the city of Zagreb (Table 2). The most frequently detected biomarkers were MOR, BE, THC-COOH and EDDP, which were determined in all analyzed wastewater samples (n = 270-282). Very high frequency of detection was obtained also for 6-AM (98%), M3G (97%), AMP (96%) and MDMA (99%; n = 282), whereas MAMP was the least frequently detected drug biomarker (83%). Regarding abundances, the highest average annual concentrations were determined for MOR (from 74 ± 29 ng/L to 294 ± 83 ng/L), BE (from 143 ± 34 ng/L to 273 ± 101 ng/L) and EDDP (from 121 ± 41 ng/L to 190 ± 67 ng/L), followed by AMP (from 7.5 ± 7.5 ng/L to 109 ± 58 ng/L) and MDMA (from 6.8 ± 7.7 ng/L to 92 ± 58 ng/L). The lowest concentrations were determined for MAMP (from 0.6 ± 0.6 ng/L to 1.4 ± 1.8 ng/L), M3G (from 1.6 ± 2.2 ng/L to 9.9 ± 6.7 ng/L) and 6-AM (from 2.0 ± 2.4 ng/L to 12 ± 4.7 ng/L).

3.2. Drug consumption patterns

3.2.1. Workday/weekend drug consumption patterns

Possible differences in workday to weekend consumption patterns of individual drugs have been evaluated based on the ratios of weekend and workday daily mass loads of selected drug biomarkers for individual years (Fig. 1). It was arbitrarily assumed that a ratio significantly different from 1.0 ± 0.2 was a confirmation of some specific weekday-related consumption pattern. It should be stressed that the ratio for MOR consumption was calculated from the corresponding mass loads of the total morphine (MOR_{tot}). The MOR_{tot} mass loads were obtained by summing up the daily mass loads of MOR and M3G (taking into account the molar ratio to MOR of 1.62).

Almost all ratios of the weekend and workday average daily mass load of stimulating drug biomarkers, BE (1.5 ± 0.3 to 1.7 ± 0.5), MDMA (2.3 ± 0.5 to 4.3 ± 3.6) and AMP (1.0 ± 1.0 to 2.2 ± 1.3), were significantly (*t*-test) different from 1.0 ± 0.2. By contrast, most of the ratios for MOR_{tot} (0.95 ± 0.4 to 1.1 ± 0.3), 6-AM (0.6 ± 0.8 to 1.2 ± 0.3), THC-COOH (0.9 ± 0.4 to 1.2 ± 0.4) and EDDP (0.9 ± 0.3 to 1.1 ± 0.3) indicated a rather uniform consumption of heroin, cannabis and methadone throughout the week. The observed weekend-related drug consumption patterns of stimulating drugs (MDMA, cocaine and AMP) documented in this study not only fully support the results obtained in a number of previous studies based on 7 consecutive days sampling scheme (e.g. Krizman et al., 2015; Ort et al., 2014a; Terzic et al., 2010; Thomas et al 2012), but also confirm the robustness of the applied whole-year sampling scheme to demonstrate the importance of weekday-weekend dynamics at long-term time scales.

3.2.2. The impact of holiday season on drug consumption patterns

The results of research dealing with the impact of holiday season on drug consumption patterns are presented in Fig. 2, Fig. S1 and Fig. 3. In both periods, the 1st of January (New Year) was characterized by a significantly enhanced daily mass load of BE (224 g/day and 197 g/day), MDMA (62 g/day and 67 g/day) and AMP (42 g/day and 60 g/day), which confirmed an increased consumption of all major stimulating drugs in holiday seasons (Fig. 2). By contrast, the 25th of December (Christmas) was associated with an enhanced excretion of BE (166 g/day and

130 g/day) whereas the Christmas consumption of most amphetamine-type drugs (AMP and MDMA) was not clearly elevated. These results probably reflect the life-style differences of cocaine and amphetamine-type drug consumers within the investigated population. In both holiday season periods, a steady increase of MOR excretion towards Christmas was also observed. However this increase was not associated with the concomitant increase of 6-AM and therefore cannot be unequivocaly related to the enhanced consumption of heroin. Furthermore, unlike for stimulating drugs, holiday-related consumption patterns could not be established for the remaining investigated drugs, such as cannabis and EDDP (Fig. S1). The comparison of the average mass loads during the two holiday season periods with the average weekend and workday mass loads in the corresponding years (Fig. 3) confirmed a significantly higher consumption ($p < 0.05$) of stimulating drugs (BE, MDMA, AMP) during the weekend (n = 19-24) and holiday season period (n = 14-15) as compared to workday periods (n = 19-22). The average mass loads of stimulating drugs during Christmas holiday season were 2 – 3.9-fold higher than during the average weekday and 1.2 – 1.9-fold higher than during the average weekend of the corresponding year. This is in a good agreement with previous studies which indicated the enhanced consumption of stimulating drugs during the holidays, festivals, tourist seasons etc. (e.g. Krizman et al., 2016; Lai et al., 2013a; Lai et al. 2013b, van Nuijs et al., 2009) and underlines the ability of the applied WBE approach to address the problem of relative contributions of special events to the overal drug consumption in a particular yearly period.

3.2.3. The seasonal differences in drug consumption patterns

In this study, we compared the average daily mass loads determined in the city of Zagreb in 2 different one-week periods, early spring and summer, in 2009 and 2013. The results of this comparison are presented in Fig. 4. In both investigated years, the average summer mass loads of most of the investigated drug biomarkers were lower than those determined in early spring (Fig. 4). However the observed differences were statisticaly significant (p < 0.05; Mann-Whitney test) only for drug biomarkers which exhibit lower intra-week variability (e.g. MOR_{tot}, THC-COOH and EDDP) whereas they were not significant for the biomarkers of stimulating drugs (BE, MDMA), probably due to the comparatively higher intra-week variability. The lower average daily mass loads determined in summer are most likely associated with a disbalanced outward and inward population migrations during the summer vacation season. Namely, the contribution of tourists to the total city population is rather negligible throughout the year (<1%, data from Zagreb Tourist Board), whereas a significant percentage of residential population might be out of town during the peak of summer season. Unfortunately, this assumption cannot be confirmed since the official data related to the outward migrations of the city population were not available. Another possible factor which might have caused the observed differences in spring and summer mass loads is faster in-sewer drug biomarker degradation at higher temperatures (e.g. Devault et al., 2017). However, the model experiments which were performed with the wastewater from the city of Zagreb at 10^{o}C and 20^{o}C, indicated rather higher stability of all urinary biomarkers included in this research at the both temperature conditions (Senta et al., 2016.). Since the in-sewer wastewater residence time in Zagreb is relatively short (<5 h) and a typical wastewater temperature in March and July/August is 12^{o}C and 20.5^{o}C, respectively, it is not very likely that the observed seasonal mass load diferences were primarily caused by faster in-sewer degradation in summer. Although the reasons for the observed seasonal differences of the average mass loads are not yet fully understood, they indicated that the total drug consumption might be underestimated if extrapolated from the average daily mass loads determined in summer.

3.3. Impact of sampling scheme on the estimation of drug consumption in multiannual studies

Most of the previously conducted multiannual WBE studies were based on relatively short one-week sampling periods (e.g. Kankaanpää et al., 2016; Mastroianni et al., 2017, Ort et al., 2014a; Zuccato et al. 2016), which, due to the possible week-to-week variability of daily mass loads, may be associated with a potential error in tracking the drug consumption on an annual basis. In this study, a comparison was made between the representative average daily mass loads of selected drug biomarkers obtained by applying two different sampling schemes: one-week sampling scheme (March/April 2012 – 2016) and whole-year sampling scheme (Sundays and Tuesdays; sampled either fortnightly in 2012-2014 or monthly in 2015-2016). Based on the extended scheme of the whole-year sampling carried out in 2013 and 2014, which included fortnightly sampling (n = 48), it was shown that the reduction of the sample number to half (monthly sampling; n = 24) did not significantly affect the estimate of the mass loads (t-test; $p < 0.05$).

The representative daily mass loads of individual drug biomarkers determined by applying the one-week and the whole-year sampling scheme are presented in Fig. 5. Apart from some occasional exceptions, the application of the whole-year sampling scheme was, in principle, associated with somewhat higher day-to-day variability of daily mass loads than the one-week sampling scheme, which is probably a result of higher intra-annual variability of drug consumption. The amphetamine-type drugs (MAMP, AMP and MDMA) exhibited the strongest day-to-day variability within the both sampling schemes, which is most probably associated with a rather irregular consumption pattern of these drugs, characterized by enhanced weekend and holiday consumption rates. Furthermore, the one-week sampling scheme was occasionally associated with relatively high day-to-day variability of AMP and MDMA. The statistical analysis of the data exhibited a significant difference ($p < 0.05$) between the representative mass loads of AMP obtained by the two applied sampling schemes in all investigated years. By contrast, the differences for other investigated drug biomarkers were found to be significant ($p < 0.05$) only occasionally. Previous study by Ort at al. (2014b) has shown that the variability of drug consumption in smaller communities (<10 000 inhabitants) is extremely high, requiring very high sampling frequency to achieve the proper estimate of drug consumption. It was estimated that the average annual consumption calculated from 1-week sampling was subject to approximately 60% relative error. In contrast, our study suggests that intra-annual varibilities in larger cities can be significantly smaller allowing detection of relatively small changes (20%) of the drug consumption among different years. Nevertheless, although some previous studies, addressing the issue of multiannual changes, demonstrated the applicability of one-week sampling scheme (Mastroianni et al., 2017; Ort et al. 2014a, Zuccato et al., 2016), our data show that such a scheme is insufficiently reliable for the drugs exhibiting high day-to-day and intra-annual variability, even in case of larger cities like the city of Zagreb.

3.4. Multiannual trends in drug consumption patterns and comparison with available epidemiological data

The back-calculations of drug consumption were based on representative daily mass loads determined for all samples collected within each investigated year, with the exception of those collected during the Christmas-New Year holiday seasons. The consumption was calculated for heroin, cocaine, AMP, MDMA, cannabis (THC) and methadone (MTHD). The results expressed in mg/day/1000 inhabitants of age 15-64 are presented in Fig. 6, whereas the results expressed in other units (e.g. mg/day/1000 inhabitants, doses/day/1000 inhabitants, g/day, kg/year, kg/year of street purity drug) are given in Supplementary Material (Table S2). The highest illicit drug consumption rate was determined for cannabis (from 7368 ± 2197 mg/day/1000 inhabitants 15-64 years to 19544 ± 4624 mg/day/1000 inhabitants 15-64 years), followed by heroin (from 107 ± 104 mg/day/1000 inhabitants 15-64 years to 712 ± 193 mg/day/1000 inhabitants 15-64 years), cocaine (from 249 ± 27 mg/day/1000 inhabitants 15-64 years to 699 ± 121 mg/day/1000 inhabitants 15-64 years), MDMA (from 17 ± 7.5 mg/day/1000 inhabitants 15-64 years to 259 ± 69 mg/day/1000 inhabitants 15-64 years) and AMP (from 13 ± 8.8 mg/day/1000 inhabitants 15-64 years to 213 ± 61 mg/day/100 inhabitants 15-64 years). The estimated consumption rate of the therapeutic opioid methadone was in the range from 280 ± 26 mg/day/1000 inhabitants 15-64 years to 393 ± 61 mg/day/1000 inhabitants 15-64 years. In principle, the determined drug consumption patterns and rates were rather similar to those determined in some other Mediterranean countries, like Spain and Italy (Mastroianni et al., 2017; Zuccato et al., 2016), although some differences regarding the prevalence of individual drugs as well as regarding the temporal trends were observed. For example, cannabis and cocaine were the most prevalently consumed illicit drugs in Barcelona (Spain) and investigated Italian cities, whereas a heroin consumption was reported to be much lower (Mastroianni et al., 2017; Zuccato et al., 2016).

In our study, all investigated illicit drugs, except heroin, exhibited a significant increase ($p < 0.05$) of the consumption rates over the investigated 8-year period (Fig. 6 and 7, Table S2). In 2016, the average consumption rate of pure MDMA, AMP, THC (cannabis) and cocaine, were 15-fold, 16-fold, 3-fold and 2-fold higher than in 2009, respectively. The multiannual consumption patterns of pure AMP and MDMA were characterized by a rather continuing increase of their consumption rates (Fig. 6) over the whole investigated time period, whereas the consumption of THC (cannabis) was characterized by a significant increase in 2009-2014 period ($p < 0.05$, 3-fold increase), and rather stable consumption rate in 2014-2016 period. By contrast, the consumption rate of pure heroin dropped significantly ($p < 0.05$; 5-7-fold) between 2009 and 2011-2012 period, and kept at significantly lower level until 2016 ($p < 0.05$). However, a significant ($p < 0.05$) 2-3-fold increase in pure heroin consumption was recorded between 2011/2012 and 2016, which indicated a gradual recovery of heroin market in that period. Interestingly, a reduction of heroin consumption in the period 2010-2012 was reported for Italy as well (Zuccato et al., 2016).

Based on the estimated amounts of consumed drugs and the official data on purity of seized drugs provided by the Office for Combating Narcotic Drug Abuse of the Government of the Republic of Croatia (see Table S1), we calculated the amounts of the street-purity drugs which circulated on the illegal market in Zagreb in the corresponding years (Table S2). It should be stressed that the street-drug purity of investigated drugs (heroin, amphetamine, MDMA, cocaine) exhibited a pronounced temporal variability (Table S2). The amounts of the most prevalent drugs present on the illegal market in Zagreb were as follows: from 211 to 565 kg/year of heroin, from 157 to 323 kg/year of cocaine, from 44 to 309 kg/year of amphetamine, from 14 to 127 kg/year of MDMA and from 22853 to 53988 kg/year of cannabis.

Consequently, the observed multiannual trends in the consumption of pure drugs are probably not impacted exclusively by the changes in drug consumption prevalence but also by the changes in the street drug purity. In this context, it is interesting to note that a significant drop in the heroin consumption rate between 2009 and 2011/2012 was associated with a concomitant decrease of heroin street-drug purity (from 21.5% to 8.4%) and an increase in the consumption of the substitution therapy drug methadone (40%), which then kept a rather stable consumption rate in the subsequent period (2013-2016). The average number of consumed methadone doses estimated in this study (e.g. 3.1 ± 0.4 doses/day/1000 inhabitants in 2015; 80 mg/dose) were in a rather good agreement with the amount of that drug prescibed in the city of Zagreb in 2015 (11.76 DDD/TSD; DDD = 25 mg; 3.7 doses/day/1000 inhabitants for the average dose of 80 mg/L) (Draganic et al., 2017), which confirmed a reliability of WBE approach for tracking the changes of the illicit drug consumption patterns.

The trends in population normalized number of addicts treated due to consumption of different types of drugs did not, however, reflect the multiannual drug consumption trends determined in this study (Fig. 7), probably due to a rather long time-gap between the initial drug consumption and the involvement of the consumers in the treatment.

Furthermore, the drug consumption trends which were determined in the present study were only partialy in agreement with the results of general population surveys performed in Croatia in 2011 and 2015, which indicated a significant increase only in the consumption of cannabis (2.9% last-month prevalence in 2011; 5.0% last-month prevalence in 2016) (Glavak Tkalic et al., 2013; Glavak Tkalic et al., 2016), whereas the differences in the consumption prevalence of other illicit drugs were not found to be significant. Our study suggests that the outcome of national population surveys on drug consumption is not necessarily representative for larger cities. Given the fact that the city of Zagreb represents approximately 20% of the whole Croatian population, the drug consumption trends determined in this study imply the need for specific surveys focusing on larger cities. Moreover, the trends observed in the city of Zagreb might be an indication of some trends developing at the national level.

5. Conclusion

The eight-year monitoring period of drug consumption patterns in the city of Zagreb, Croatia, using wastewater-based epidemiology revealed several temporal variability patterns, including weekday-weekend dynamics, holiday season effects and multiannual trends. In agreement with the literature, the enhanced consumption of stimulating drugs was systematically observed during weekends and Christmas holiday season. In addition, a significant multiannual increase of cocaine (1.6-fold), THC (2.7-fold), amphetamine (16-fold) and MDMA (15-fold) consumption with a concomitant decrease (2.3-fold) of the consumption of heroin was observed during the investigated 8-year period (2009-2016). The whole-year sampling scheme showed a clear advantage over the one-week sampling scheme, especially for drugs showing enhanced day-to-day and intra-annual variability. The errors associated with day-to-day and intra-annual variability of BE (<20%) determined in the city of Zagreb (>500 000 inhabitants) study were much smaller from those reported for small communities (Ort et al. 2014b), which indicated enhanced robustness of the estimates obtained for large sized cities. Our data suggest that large sized cities can provide a basis for a reliable detection of relatively small changes in drug consumption over a multi-year period. Consequently, the trends observed in the larger cities could be used as an early warning of the trends developing at the national level.

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Figure captions

Fig. 1. Ratios of weekend and workday average mass loads of selected urinary drug biomarkers (MOR_{tot}, 6-AM, AMP, MDMA, BE, THC-COOH, EDDP) determined in the period from 2009 to 2016. Error bars represent standard deviations. Horizontal lines represent arbitrarily assumed weekend to workday mass load ratio of 1.0 ± 0.2.

Fig. 2. Mass loads of BE, MDMA and AMP in two different Christmas-New Year holiday periods: A) 2012/2013 and B) 2013/2014.

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Fig. 4. Variability of average mass loads of selected urinary drug biomarkers in Zagreb during the spring and summer sampling week in A) 2009 and B) 2013. Error bars represent standard deviations.

Fig. 5. Impact of the selected sampling schemes (whole-year and one-week monitoring) on the determination of representative mass loads. Error bars represent standard deviations.

Fig. 6. Consumption of cocaine, heroin, MDMA, amphetamine, THC and methadone in the city of Zagreb in the period 2009-2016. Error bars represent standard deviations.

Fig. 7. Comparison of estimated drug consumption in the city of Zagreb with available epidemiological data for Croatia in the period of 2009-2016. Stimulants in the epidemiological figure include amphetamine-type drugs. Opiates include heroin and morphine.

Table 1

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Table 1. Selected drug biomarkers and data used for estimation of drug consumption.

Drug	Biomarker for estimation of consumption	Percentage of drug doses excreted as drug biomarker	Molar ratio	Correction factor	Dose (mg)
Heroin	6-AM	1.3	1.13	86.9[a]	10[d]
Cocaine	BE	29	1.05	3.6[b]	30[d]
Amphetamine	AMP	36	1.00	2.8[c]	10[c]
MDMA	MDMA	22.5	1.00	4.4[c]	97[d]
THC (Cannabis)	THC-COOH	0.5	0.91	182[c]	125[c]
Methadone	EDDP	25	1.12	3.6	80[e]

[a]van Nuijs et al., 2011; [b]Castiglioni et al., 2013; [c]Gracia-Lor et al., 2016; [d]Office for Combating Narcotic Drug Abuse of the Government of the Republic of Croatia, data for 2013; [e]Croatian Institute of Public Health, data for Zagreb for 2010.

Table 2

Table 2. Mass loads of urinary biomarkers (MOR_{tot}, MOR, M3G, 6-AM, MAMP, AMP, MDMA, BE, MDMA, THC-COOH, EDDP) in raw wastewater of the city of Zagreb (Croatia) in the period 2009 - 2016.

Urinary drug biomarker	Year	*n*[a]	FD[b]	Concentration range (ng/L)	Average ± SD (ng/L)	Mass load range (g/day)	Average ± SD (g/day)
MOR_{tot}	2009	39	100	161 - 476	294 ± 83	45 -106	75 ± 15
	2011	7	100	130 - 160	142 ± 10.8	30 - 36	32 ± 2.4
	2012	54	100	26 - 183	95 ± 37	11 - 61	27 ± 12
	2013	72	100	33 - 167	90 ± 32	17 - 50	28 ± 6.8
	2014	54	100	25 - 129	80 ± 28	17 - 62	28 ± 7.5
	2015	30	100	45 - 144	94 ± 23	16 - 39	30 ± 5.2
	2016	26	100	49 - 147	97 ± 22	18 - 44	35 ± 6.2
MOR	2009	39	100	160 - 476	294 ± 83	45 - 106	75 ± 15
	2011	7	100	109 - 135	120 ± 9.3	25 - 31	27 ± 2.1
	2012	54	100	19 - 183	94 ± 37	11 - 61	27 ± 12
	2013	72	100	26 - 166	86 ± 32	15 - 50	27 ± 7.0
	2014	54	100	22 - 127	74 ± 29	13 - 61	26 ± 7.3
	2015	30	100	41 - 141	91 ± 22	16 - 38	29 ± 5.0
	2016	26	100	42 - 143	91 ± 23	17 - 41	32 ± 5.7
M3G	2009	0	NA	NA	NA	NA	NA
	2011	7	100	3.3 - 5.5	4.7 ± 0.7	0.7 - 1.3	1.1 ± 0.2
	2012	54	85	< 0.2 – 10.5	1.6 ± 2.2	< 0.03 – 6.8	0.5 ± 1.1
	2013	72	100	< 0.3 - 19	6.6 ± 4.9	< 0.1 – 8.9	2.3 ± 2.0
	2014	54	100	< 0.3 -29	8.5 ± 6.7	< 0.1 -15	3.4 ± 3.3
	2015	30	100	< 0.3 - 16	3.9 ± 4.2	< 0.1 -6.5	1.2 ± 1.5
	2016	26	100	< 0.3 -24	9.9 ± 6.7	< 0.1 -11	3.9 ± 3.0
6-AM	2009	39	100	3.3 - 28	12 ± 4.7	0.7 - 6.0	3.1 ± 1.2
	2011	7	100	2.3 – 4.2	3.3 ± 0.6	0.5 - 0.96	0.8 ± 0.1
	2012	54	91	< 0.1 - 16	2.0 ± 2.4	< 0.01 - 3.7	0.5 ± 0.6
	2013	72	100	0.1 – 14	3.1 ± 1.9	0.1 - 3	1.1 ± 0.5
	2014	54	100	0.1 – 7.0	3.1 ± 1.04	0.1 - 2.3	1.2 ± 0.4
	2015	30	93	< 0.1 – 7.6	3.4 ± 1.8	< 0.04 - 1.9	1.1 ± 0.5
	2016	26	100	2.2 - 16	5.0 ± 2.9	0.7 - 4.1	1.7 ± 0.7
MAMP	2009	0	NA	NA	NA	NA	NA
	2011	0	NA	NA	NA	NA	NA
	2012	54	83	< 0.2 – 4.0	0.7 ± 0.9	< 0.1 - 0.96	0.2 ± 0.2
	2013	72	78	< 0.2 – 3.8	1.1± 1.0	< 0.1 - 2	0.4 ± 0.5
	2014	54	78	< 0.2 – 2.8	0.63 ± 0.66	< 0.1 - 1.9	0.25 ± 0.3
	2015	30	100	< 0.4 – 5.9	1.4 ± 1.8	< 0.1 - 1.7	0.4 ± 0.5
	2016	26	89	< 0.2 - 12	1.3 ± 2.3	< 0.1 – 3.7	0.5 ± 0.7
AMP	2009	39	72	< 1.3 - 35	7.5 ± 7.5	< 0.3 - 7.6	1.9 ± 1.8
	2011	7	100	32 - 62	42 ± 10.3	7.2 -13	9.5 ± 2.1
	2012	54	100	7.2 - 58	27 ± 15	2.3 - 17	7.5 ± 4.1
	2013	72	100	6.3 - 235	45 ± 38	2.7 - 63	13 ± 8.4
	2014	54	100	14 - 149	51 ± 26	6.1 - 74	18 ± 12
	2015	30	100	34 - 320	100 ± 70	12 -111	32 ± 23
	2016	26	100	25 - 295	109 ± 58	15 - 89	38 ± 19
MDMA	2009	39	79	< 1.1 - 33	6.8 ± 7.7	< 0.2 - 7.4	1.7 ± 1.7
	2011	7	100	5.3 - 16	9.4 ± 4.6	1.2 - 3.6	2.1 ± 1.0
	2012	54	98	< 0.1 - 96	26 ± 22	< 0.03 - 21	7.1 ± 4.9
	2013	72	100	3.4 - 260	30 ± 40	1.8 - 62	8.5 ± 8.7

	2014	54	100	8.0 - 133	38 ± 30	3.4 - 67	15 ± 12
	2015	30	100	23 - 316	91 ± 68	7.6 - 92	28 ± 19
	2016	26	100	18 - 215	92 ± 58	8.9 - 80	32 ± 20
BE	2009	39	100	89 - 325	186 ± 59	27 - 77	47 ± 12
	2011	7	100	100 - 189	143 ± 34	22 - 43	32 ± 7.6
	2012	54	100	52 - 497	196 ± 94	24 -166	56 ± 29
	2013	72	100	57 - 769	203 ± 125	31 - 224	60 ± 27
	2014	54	100	35 - 399	150 ± 66	24 - 197	57 ± 29
	2015	30	100	114 - 474	236 ± 96	45 - 125	75 ± 24
	2016	26	100	92 - 520	273 ± 101	52 - 173	97 ± 32
THC-COOH	2009	30	100	21 - 128	60 ± 23	7.3 - 31	16 ± 5.5
	2011	7	100	71 - 100	87 ± 10.4	16 -22	20 ± 2.2
	2012	54	100	34 - 183	107 ± 36	18 -52	30 ± 7.4
	2013	72	100	44 - 214	133 ± 43	16 - 74	42 ± 11
	2014	54	100	38 - 312	137 ± 54	19 - 117	49 ± 16
	2015	30	100	52 - 309	141 ± 58	15 - 88	45 ± 17
	2016	24	100	60 - 363	156 ± 66	32 - 105	54 ± 14
EDDP	2009	27	100	71 - 156	128 ± 20	24 - 38	30 ± 3.6
	2011	7	100	177 - 196	184 ± 6.5	40 - 45	42 ± 1.8
	2012	54	100	61 - 330	190 ± 67	25 - 69	52 ± 10.4
	2013	72	100	60 - 220	140 ± 43	31 - 67	43 ± 7.9
	2014	54	100	44 - 220	121 ± 41	29 - 92	43 ± 11
	2015	30	100	85 - 205	145 ± 24	25 - 67	47 ± 8.9
	2016	26	100	67 - 194	128 ± 34	26 - 60	45 ± 7.2

[a]Number of analyzed samples; [b]Frequency of detection; NA – not applicable

Figure 1

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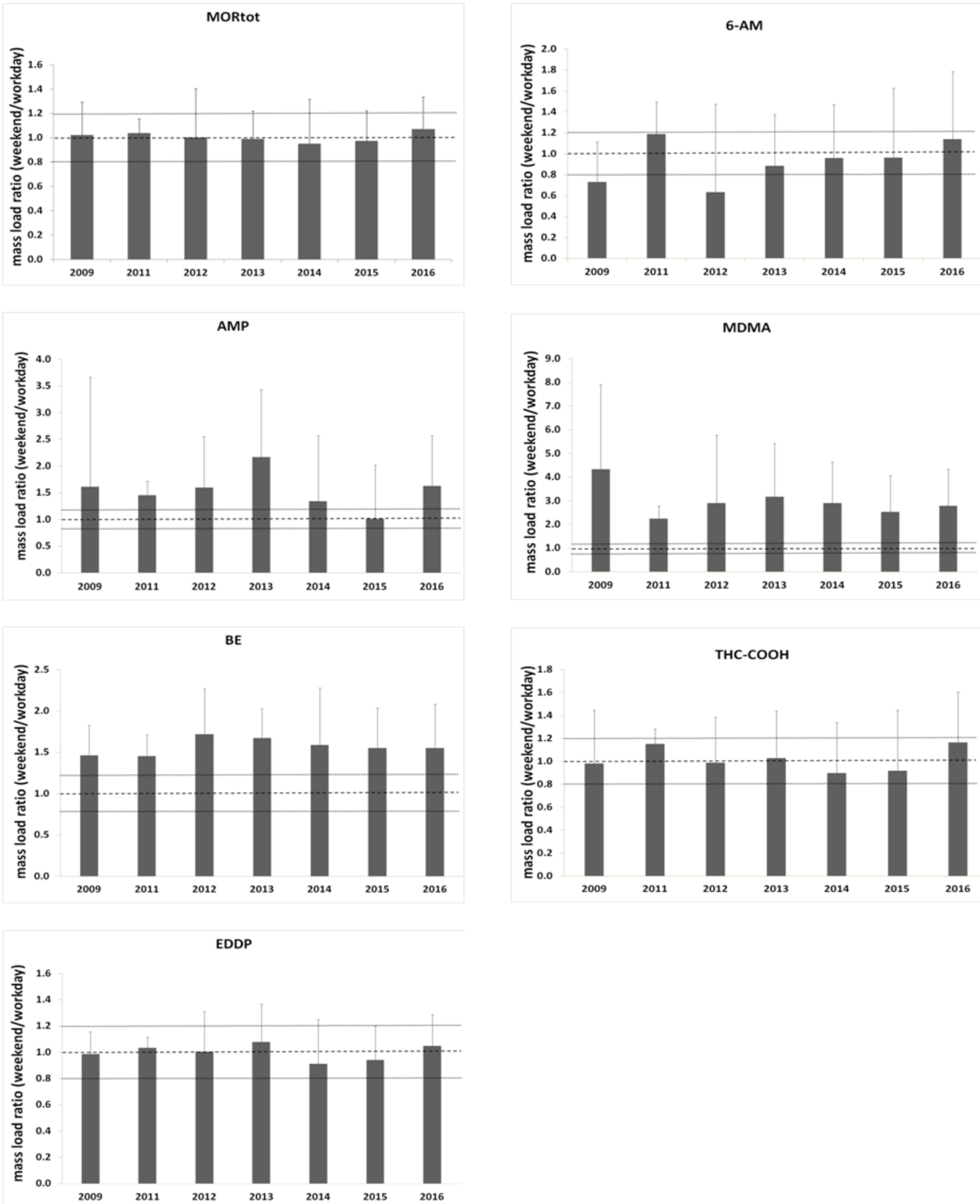

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Figure 2
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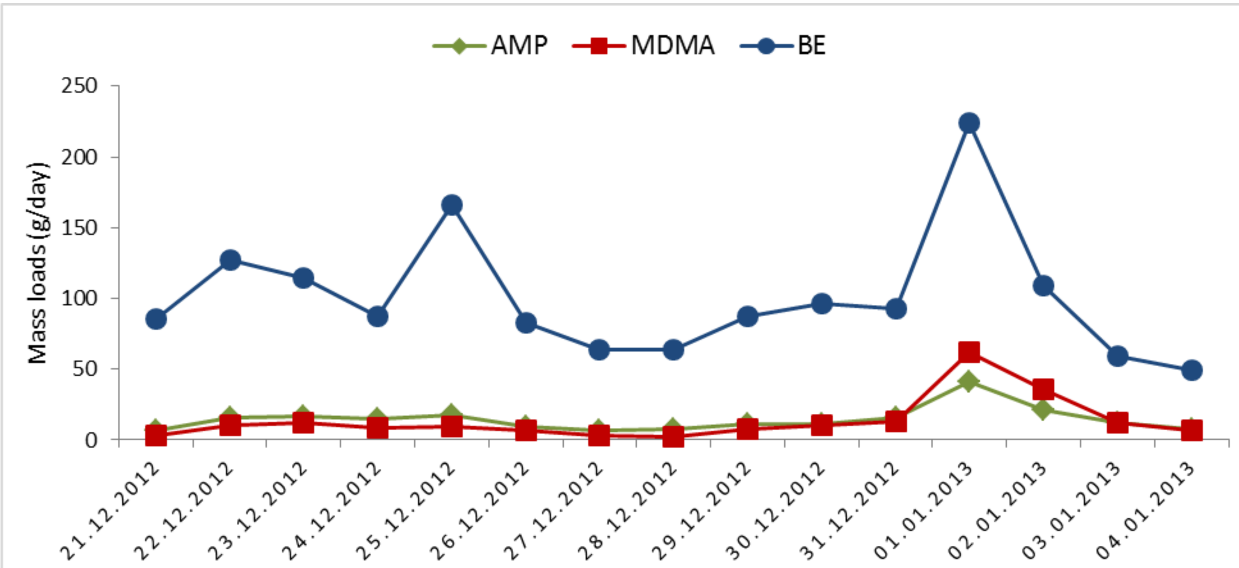
A)



B)

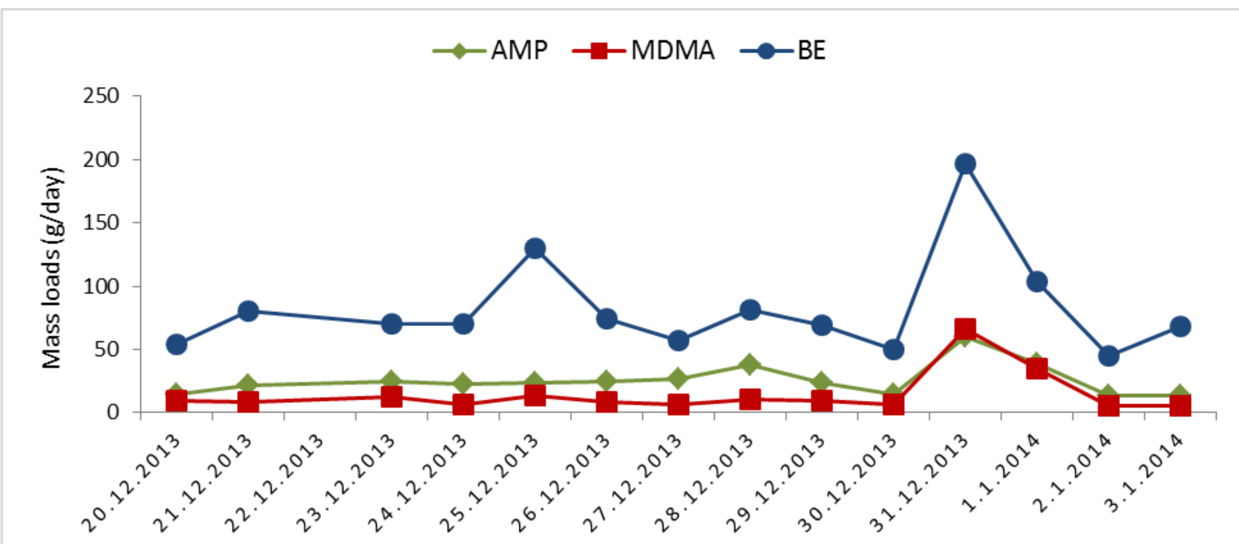


Fig. 2. Mass loads of BE, MDMA and AMP in two different Christmas-New Year holiday periods: A) 2012/2013 and B) 2013/2014.

Figure 3
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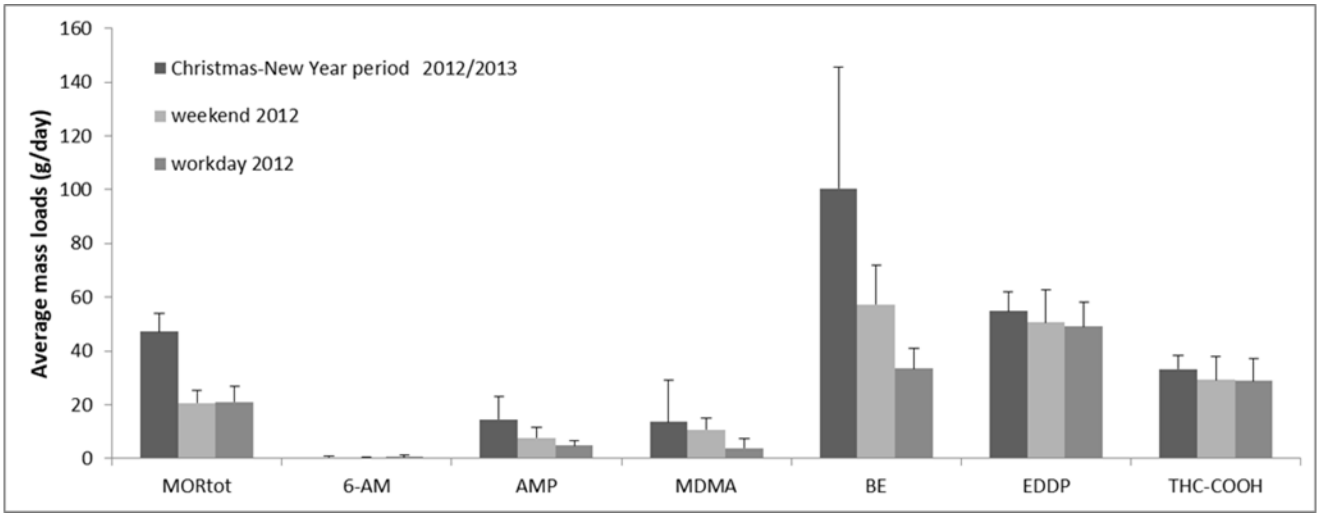
(A)



(B)

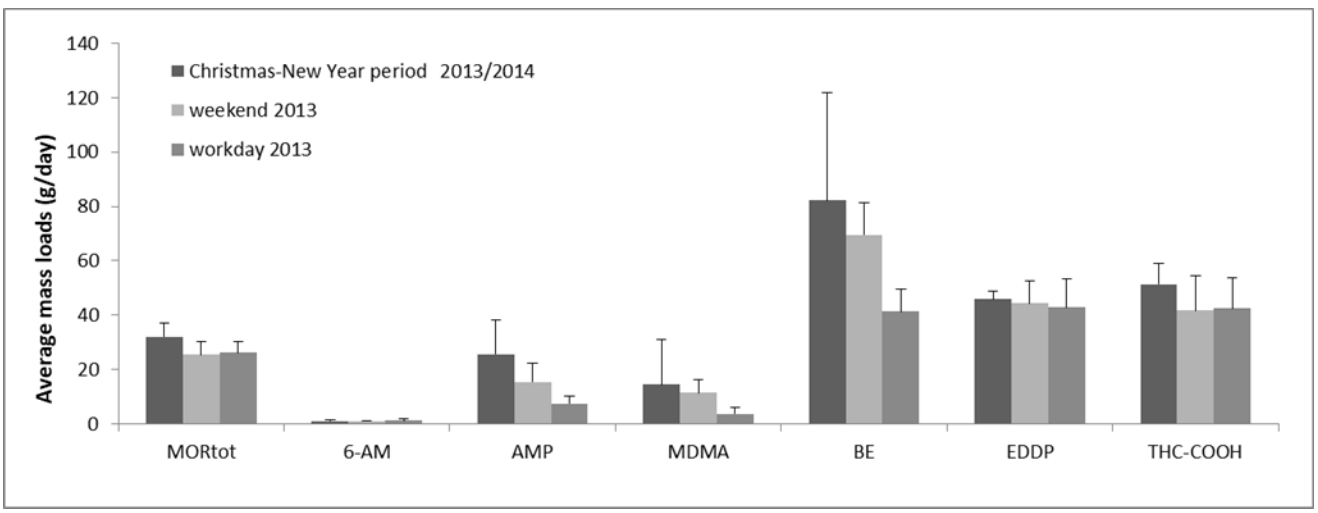


Fig. 3. Average mass loads of selected drug biomarkers determined on workdays, weekend and during two Christmas-New Year periods: A) 2012/2013 and B) 2013/2014. Error bars represent standard deviations.

Figure 4
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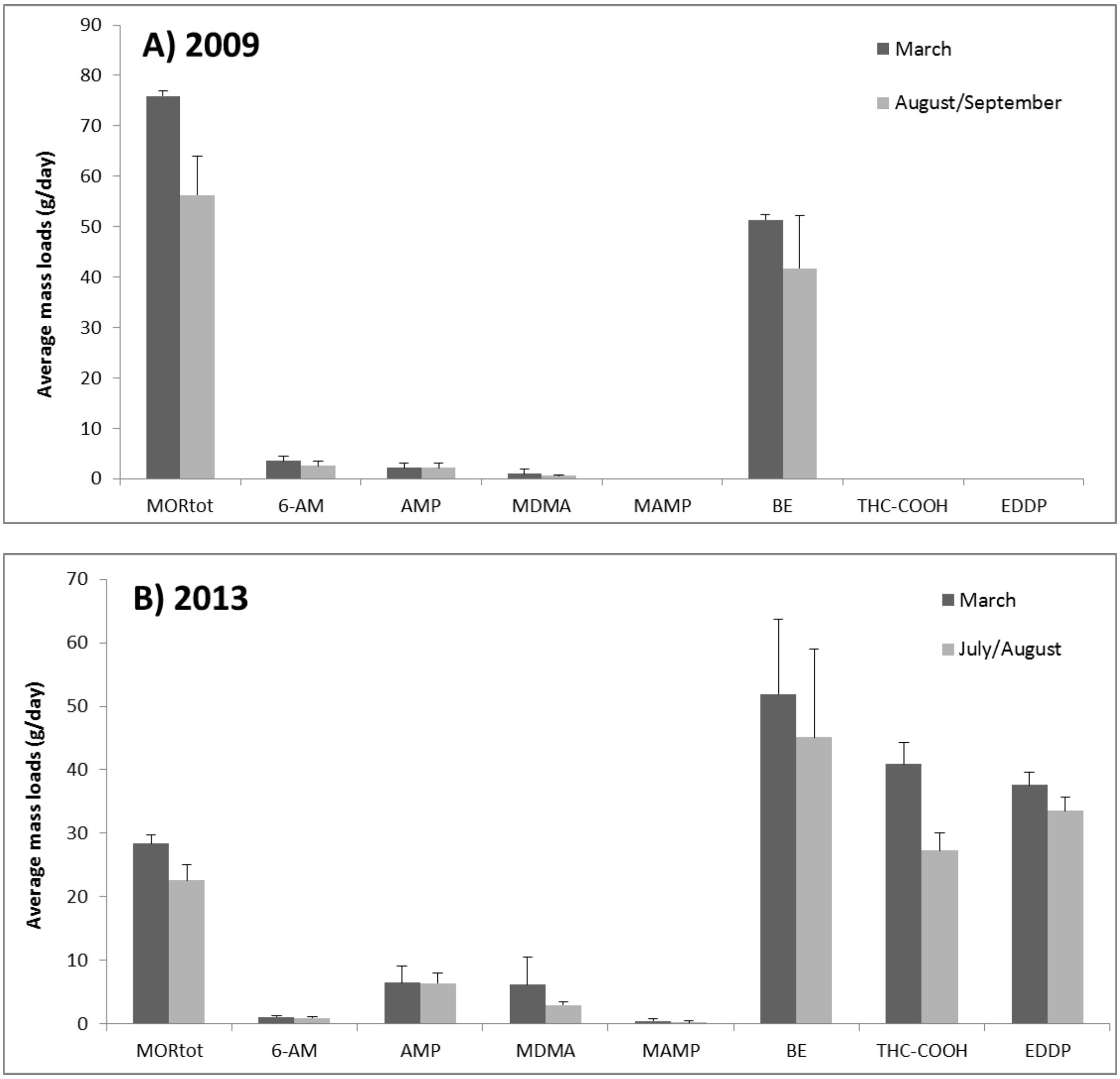

Fig. 4. Variability of average mass loads of selected urinary drug biomarkers in Zagreb during the spring and summer sampling week in A) 2009 and B) 2013. Error bars represent standard deviations.

Figure 5

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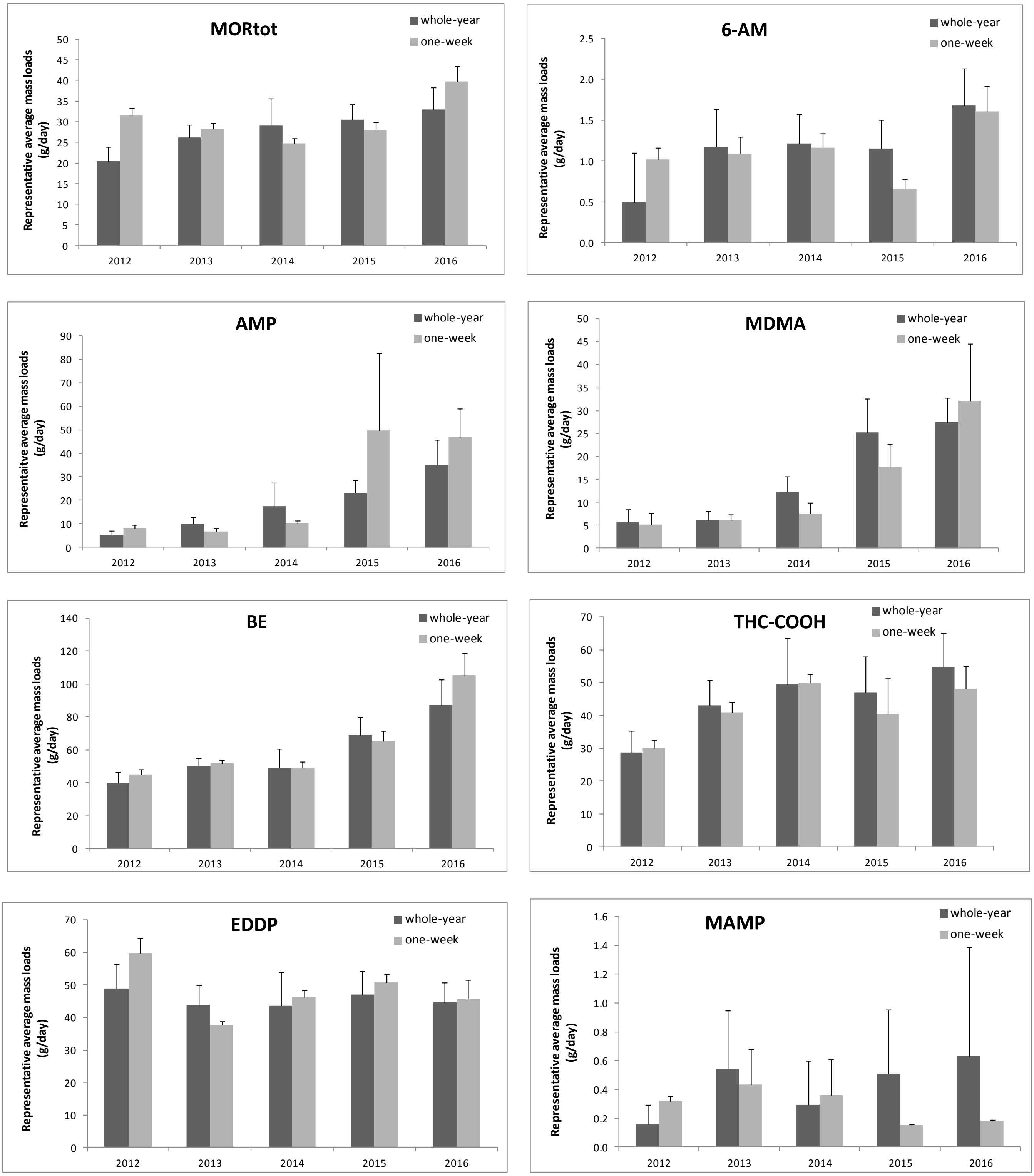

Figure 5. Impact of the selected sampling strategy (whole-year and one-week monitoring) on the determination of representative mass loads.

Figure 6

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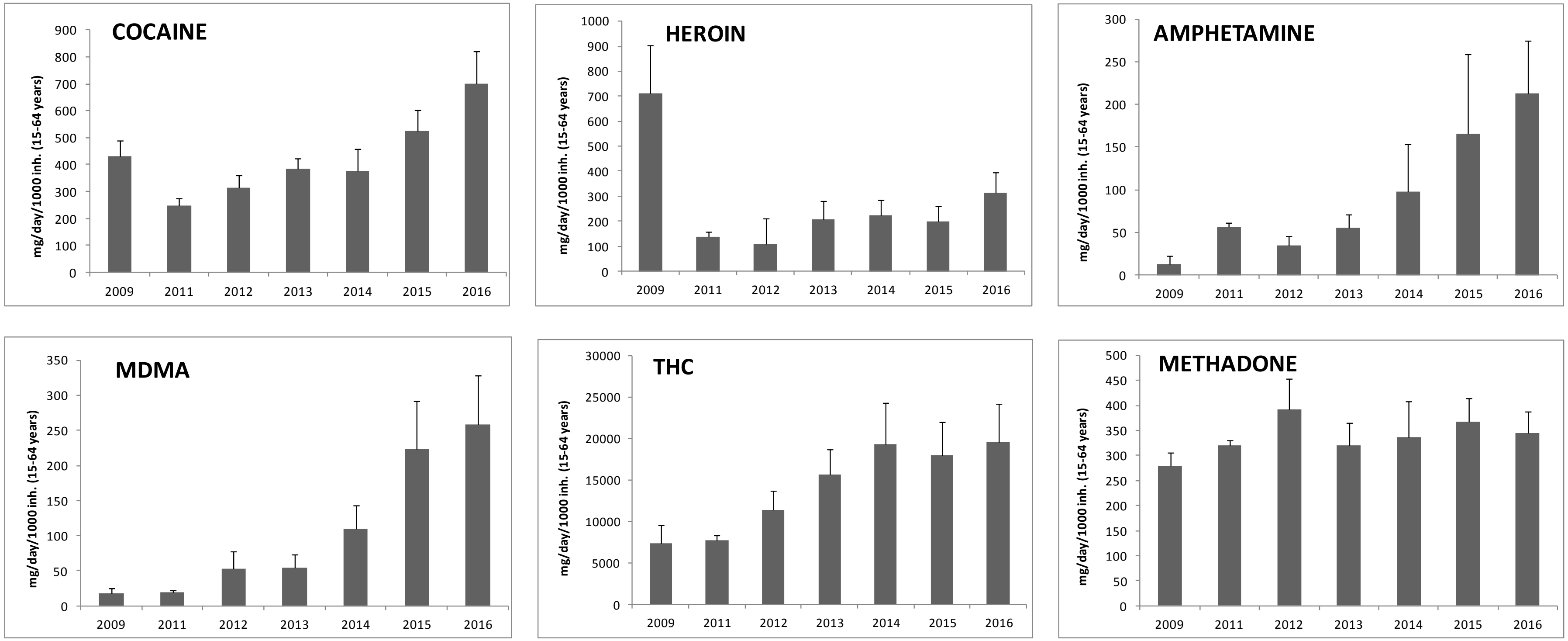

Fig. 6. Consumption of cocaine, heroin, MDMA, amphetamine, THC and methadone in the city of Zagreb in the period 2009-2016. Error bars represent standard deviations.

Figure 7
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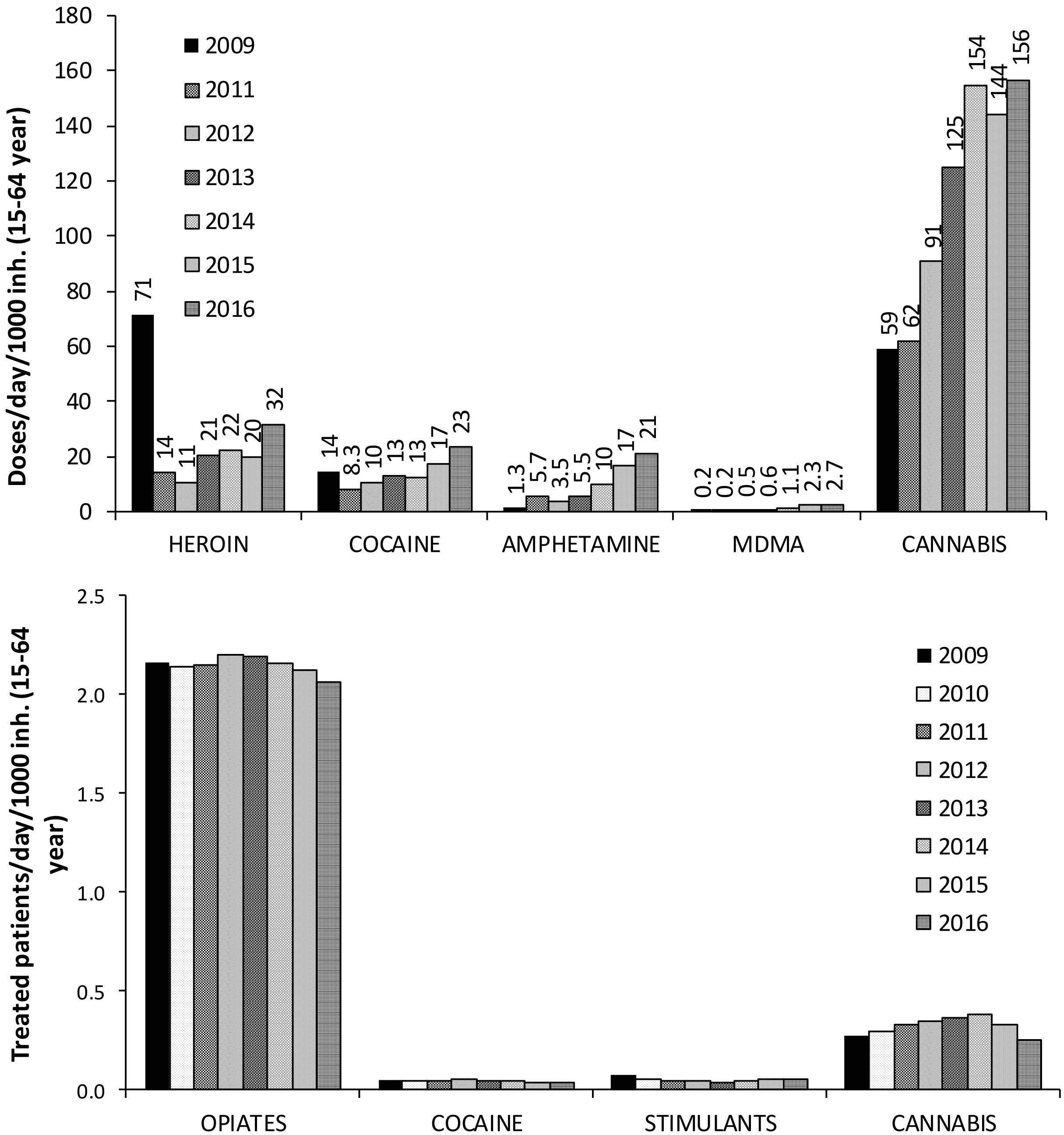

Fig. 7. Comparison of estimated drug consumption in the city of Zagreb with available epidemiological data for Croatia in the period of 2009-2016. Stimulants in the epidemiological figure include amphetamine-type drugs. Opiates include heroin and morphine.