



DELIVERABLE 4.1.-4.2

D4.1 Report on application of a) best sampling techniques; b) specific analytical methods; c) refined calculation methods for sewage biomarkers

D4.2 Report on testing of novel sewage epidemiology applications

D4.3 Report on comparison of health and lifestyle profiles of different communities

D4.4 Report on identification of new drugs of abuse in sewage

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PROJECT COORDINATOR: Dr Barbara Kasprzyk-Hordern, University of Bath

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Europe-wide sewage epidemiology monitoring

The main aim of WP4 was to conduct a Europe-wide monitoring campaign to apply the *sewage epidemiology* approach in a Europe-wide sampling campaign. A sampling campaign was organized in 2015, in the countries involved in Sewprof and included large cities and smaller conurbations typical of Europe. The sampling procedures optimized in WP1 were adopted to collect urban wastewater samples from the selected sites with both high frequency, short-term, active-, and long-term (weeks) passive-sampling techniques deployed. In WP4 Sewprof Partners tested their developed approaches using real samples, collected from across Europe, with the analytical or calculation assessment techniques developed in WP2 and 3.

The samples collected were for the first time distributed and analysed for the sewage biomarkers of human health and human exposure to environmental and food toxicants (identified in WP2). The exchange of samples was performed between participants in order to produce a complete characterization of samples for established and emerging illicit drugs, health, disease and environmental factors to provide a unique insight at the European scale. The presence of new substances identified in WP3 was monitored in wastewater using the highly specific analytical techniques developed in WP2. The community specific sewage biomarker fingerprints allowed for both the quantitative and qualitative assessment of factors influencing health and lifestyle and allowed comparison with more traditional epidemiological techniques. Information about the eventual transformation of target compounds in wastewater was shared during this phase of the project and was included in the calculation methods to estimate community health and lifestyle.

This report summarizes the deliverables related to WP4. Deliverables D4.1 and D4.2 consist of previously published reports and peer-reviewed manuscripts and, therefore, they are summarized in this report only very succinctly.

D4.1 Report on application of a) best sampling techniques; b) specific analytical methods; c) refined calculation methods for sewage biomarkers

This report summarises 16 papers:

- a. The best sampling techniques available for WBE are available via a best practice document ([POD on ww](#)) and a questionnaire for characterising sewer networks available as supplementary material of an Open Access paper (<http://doi.org/10.1111/add.12405>). Additional details on sampling are available here <http://www.futuremedicine.com/doi/pdf/10.4155/ebo.13.476>.
- b. Specific analytical methods have been developed for a number of different biomarkers:
 - i. Illicit drugs (Bade et al., 2015)
 - ii. Alcohol (Ryu et al. 2016)
 - iii. Tobacco (Senta et al., 2015)
 - iv. New psychoactive substances (Baz-Lomba et al., 2016; Bade et al., 2015; González-Mariño et al., 2016)
 - v. Pharmaceuticals (Bade et al., 2015; Baz-Lomba et al., 2016; Causanilles et al., 2016)
 - vi. Oxidative stress (Ryu et al., 2016)
 - vii. Pesticides (Rousis et al., 2016)
 - viii. Caffeine (Senta et al., 2016)
 - ix. Chiral drugs (Castrignano et al. 2016)
 - x. Chiral antibiotics (Castrignano et al. 2017a, under preparation)
- c. Refined calculation methods for sewage biomarkers are presented in Paper 1 - Gracia-Lor et al. 2016

Paper 1: Gracia-Lor, E., Zuccato, E., Castiglioni, S. (2016) Refining correction factors for back-calculation of illicit drug use. *Sci. Total Environ.* <http://dx.doi.org/10.1016/j.scitotenv.2016.09.179>

The estimation of illicit drugs use through wastewater analysis has become an important issue in the last few years due to their large worldwide consumption, which results in economic, social and health costs. The amounts of urinary biomarkers of illicit drugs (selected drugs or their metabolites) measured in wastewater are used to back-calculate the consumption of a particular drug by the population and to monitor temporal and spatial trends of illicit drug use in a community. The reliability of back-calculation depends on different factors, one being the accuracy of correction factors. A wide range of correction factors have been used in different studies and some biases must be expected when comparing results. Most of the correction factors were developed several years ago, so they need to be updated to include the latest information on pharmacokinetics. Moreover, new comprehensive methods to treat data should be adopted. The goal of this study is to refine current correction factors for back-calculation of the most widely used illicit drugs: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA) and tetrahydrocannabinol (THC). The mean percentages of excretion of the parent drugs and their metabolites were calculated for each route of administration, utilizing all accessible pharmacokinetic studies in the literature. This allowed to select the most suitable drug target residue and a refined correction factor was obtained for each substance considering the most frequent route of administration. The refined correction factors we propose can be used in wastewater based epidemiology to standardize the back-calculation of these drugs. These results can be included in the best practice protocol currently adopted in EU studies in order to reduce uncertainty and improve the comparability of results.

Publications

Bade, R., Rousis, N.I., Bijlsma, L., Gracia-Lor, E., Castiglioni, S., Sancho, J.V., Hernandez, F. (2015) Screening of pharmaceuticals and illicit drugs in wastewater and surface waters of Spain and Italy by high resolution mass spectrometry using UHPLC-QTOF MS and LC-LTQ-Orbitrap MS. *Anal. Bioanal. Chem.* 407, 8979-8988. (DOI: 10.1007/s00216-015-9063-x)

Baz-Lomba, J., Reid, M. J. & Thomas, K. V. (2016) Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF. *Anal. Chim. Acta* 914, 81–90.

Castrignanò E., Lubben A., Kasprzyk-Hordern B. (2016). "Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry", *J Chromatogr A*, 1438:84-99 (DOI: 10.1016/j.chroma.2016.02.0150).

Castrignanò E., et al. (2017) "Multi-residue stereoisomeric analysis of human and veterinary chiral drugs in wastewater using chiral liquid chromatography coupled with tandem mass spectrometry"; under preparation

Causanilles, A., Emke, E., de Voogt, P. (2016) Determination of phosphodiesterase type V inhibitors in wastewater by direct injection followed by liquid chromatography coupled to tandem mass spectrometry. *Sci. Total Environ.* 565, 140-147. <http://dx.doi.org/10.1016/j.scitotenv.2016.04.158>

González-Mariño, I., Gracia-Lor, E., Rousis, N.I., Castrignanò, E., Thomas, K.V., Quintana, J.B., Kasprzyk-Hordern, B., Zuccato, E., Castiglioni, S. (2016). "Wastewater-Based Epidemiology To Monitor Synthetic Cathinones Use in Different European Countries", *Environ. Sci. Technol.* 50:10089–10096. (DOI: 10.1021/acs.est.6b02644).

Gracia-Lor, E., Zuccato, E., Castiglioni, S. (2016) Refining correction factors for back-calculation of illicit drug use. *Sci. Total Environ.* <http://dx.doi.org/10.1016/j.scitotenv.2016.09.179>

Rousis, N.I., Zuccato, E., Castiglioni, S. (2016) Monitoring population exposure to pesticides based on liquid chromatography-tandem mass spectrometry measurement of their urinary metabolites in urban wastewater: A novel biomonitoring approach. *Sci. Total Environ.* 571, 1349-1357.

Ryu, Y. et al. (2016) Comparative measurement and quantitative risk assessment of alcohol consumption through wastewater-based epidemiology: An international study in 20 cities. *Sci. Total Environ.* 565, 977-983.

Ryu, Y., Reid, M. J. & Thomas, K. V. (2016) Liquid chromatography–high resolution mass spectrometry with immunoaffinity clean-up for the determination of the oxidative stress biomarker 8-iso-prostaglandin F2 α in wastewater. *J. Chromatogr. A* 1409, 146-151.

Senta, I., Gracia-Lor, E., Borsotti, A., Zuccato, E., Castiglioni, S. (2015) Wastewater analysis to monitor use of caffeine and nicotine and evaluation of their metabolites as biomarkers for population size assessment Authors: Journal: Water Research 74, 23-33.

D4.2 Report on testing of novel *sewage epidemiology* applications

This report summarizes a multi-city testing of the novel wastewater-based epidemiology tools developed in SEWPROF. The final deliverable encompasses 8 publications on the application of the following novel wastewater biomarkers in wastewater from a number of European cities. These biomarkers include a number of studies utilising pharmaceuticals and their metabolites (Papers 2 & 9), legalised drugs such as alcohol and nicotine (Papers 3 & 5), new psychoactive substances (Paper 4), pesticide exposure (Paper 6) and the measurement of community oxidative stress levels through the measurement of an F2-isoprostane (Paper 5). What the work clearly shows is that wastewater-based epidemiology has huge potential beyond its traditional application in for assessing trends in illicit drug use.

Paper 2: J.A. Baz-Lomba, Stefania Salvatore, Emma Gracia-Lor, Richard Bade, Sara Castiglioni, Erika Castrignanò, Ana Causanilles, Felix Hernandez, Barbara Kasprzyk-Hordern, Juliet Kinyua, Ann-Kathrin McCall, Alexander van Nuijs, Christoph Ort, Benedek G. Plósz, Pedram Ramin, Malcolm Reid, Nikolaos I. Rousis, Yeonsuk Ryua, Pim de Voogt, Jorgen Bramness, Kevin Thomas, "Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities" BMC Public Health 16 (2016) 1035.

This study compared and correlated the consumption estimates of pharmaceuticals, illicit drugs, alcohol, nicotine and caffeine from wastewater analysis and other sources of information. Wastewater samples were collected in 2015 from 8 different European cities over a one week period, representing a population of approximately 5 million people. Published pharmaceutical sale, illicit drug seizure and alcohol, tobacco and caffeine use data were used for the comparison. High agreement was found between wastewater and other data sources for pharmaceuticals and cocaine, whereas amphetamines, alcohol and caffeine showed a moderate correlation. methamphetamine and 3,4- methylenedioxymethamphetamine (MDMA) and nicotine did not correlate with other sources of data. Most of the poor correlations were explained as part of the uncertainties related with the use estimates and were improved with other complementary sources of data. This work confirms the promising future of WBE as a complementary approach to obtain a more accurate picture of substance use situation within different communities. Our findings suggest further improvements to reduce the uncertainties associated with both sources of information in order to make the data more comparable.

Paper 3: Ryu, Y., Barceló, D., Barron, L.P., Bijlsma, L., Castiglioni, S., de Voogt, P., Emke, E., Hernández, F., Lai, F.Y., Lopes, A., de Alda, M.L., Mastroianni, N., Munro, K., O'Brien, J., Ort, C., Plósz, B.G., Reid, M.J., Yargeau, V., Thomas, K.V. (2016). "Comparative measurement and quantitative risk assessment of alcohol consumption through wastewater-based epidemiology: An international study in 20 cities", Sci. Total Environ. 565, 977–983. (DOI: 10.1016/j.scitotenv.2016.04.138).

This paper presented the measurement of alcohol consumption in 20 cities across 11 countries through the use of wastewater-based epidemiology (WBE), and reports the application of these data for the risk assessment of alcohol on a population scale using the margin of exposure (MOE) approach. Raw 24-h composite wastewater samples were collected over a one-week period from 20 cities following a common protocol. For each sample a specific and stable alcohol consumption biomarker, ethyl sulfate (EtS) was determined by liquid chromatography coupled to tandem mass spectrometry. The EtS concentrations were used for estimation of per capita alcohol consumption in each city, which was further compared with international reports and applied for risk assessment by MOE. The average per capita consumption in 20 cities ranged between 6.4 and 44.3L/day/1000 inhabitants. An increase in alcohol consumption during the weekend occurred in all cities, however the level of this increase was found to differ. In contrast to conventional data (sales statistics and interviews), WBE revealed geographical differences in the level and pattern of actual alcohol consumption at an inter-city level. All the sampled cities were in the "high risk" category (MOE<10) and the average MOE for the whole population studied was 2.5. These results allowed direct comparisons of alcohol consumption levels, patterns and risks among the cities. This study shows

that WBE can provide timely and complementary information on alcohol use and alcohol associated risks in terms of exposure at the community level.

Paper 4: Richard Bade; Lubertus Bijlsma; Juan V Sancho; J.A Baz-Lomba; Sara Castiglioni; Erika Castrignanò; Ana Causanilles; Emma Gracia-Lor; Barbara Kasprzyk-Hordern ; Juliet Kinyua; Ann-Kathrin McCall; Alexander L. N. van Nuijs; Christoph Ort; Benedek G Plósz; Pedram Ramin; Nikolaos I Rousis; Yeonsuk Ryu; Kevin V Thomas; Pim de Voogt; Ettore Zuccato; Felix Hernandez (2016). "Liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in influent wastewater of eight European cities" *Chemosphere* (submitted).

This paper tested several European locations for new psychoactive substances.

Paper 5: Yeonsuk Ryu, Emma Garcia-Lor, Richard Bade, J.A. Baz-Lomba, Jørgen G. Bramness, Sara Castiglioni, Erika Castrignanò, Ana Causanilles, Adrian Covaci, Pim de Voogt, Felix Hernandez, Barbara Kasprzyk-Hordern, Juliet Kinyua, Ann-Kathrin McCall, Christoph Ort, Benedek G. Plósz¹⁰, Pedram Ramin¹⁰, Nikolaos I. Rousis³, Malcolm J. Reid¹, and Kevin V. Thomas (2016). "Increased levels of the oxidative stress biomarker 8-isoprostaglandin F_{2α} in a city's wastewater related to tobacco use". *Scientific reports* (submitted).

In this work, 8-iso-prostaglandin F_{2α} (8-iso-PGF_{2α}) was analysed in wastewater samples collected from 4 Norwegian and 7 other European cities in 2014 and 2015. Using the same samples, biomarkers of alcohol (ethyl sulfate) and tobacco (trans-3'-hydroxycotinine) use were also analysed to investigate any possible correlation between 8-iso-PGF_{2α} and the consumption of the two drugs.

Paper 6: Rousis, N.I., Gracia-Lor, E., Zuccato, E., Bade, R., Baz-Lomba, J.A., Castrignanò, E., Causanilles, A., Covaci, A., de Voogt, P., Hernández, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.-K., Ort, C., Plósz, B.G., Ramin, P., Ryu, Y., Thomas K.V., Castiglioni, S. "Assessment of pesticide metabolite levels in influent wastewater of 8 European cities by wastewater-based epidemiology". (In preparation).

This paper will investigate public exposure to pesticides using WBE.

Paper 7: Castrignanò, E. et al. Enantiomeric profiling of illicit drugs in a pan-European study (in preparation)

This paper will investigate enantiomeric profiling in estimating chiral drug use across Europe.

Paper 8: Castrignanò, E. et al., Enantiomeric profiling of quinolones and monitoring of resistance genes in European wastewaters (in preparation)

This paper will investigate enantiomeric profiling in estimating quinolones use versus antimicrobial resistance across Europe.

Paper 9: Causanilles, A. et al., 2016. Application of wastewater-based epidemiology to assess erectile dysfunction pharmaceuticals usage in 10 European cities (in preparation).

This paper will investigate application of WBE in the assessment of the usage of erectile dysfunction pharmaceuticals in Europe.

Paper 10: Gracia-Lor, E., Rousis, N.I., Zuccato E., Bade, R., Baz-Lomba, J.A., Castrignanò, E., Causanilles, A., Covaci, A., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A-K, Ort, C., Plósz, B.G., Ramin, P., Ryu, Y., Thomas,

K.V., de Voogt, P., Castiglioni, S., 2016. Caffeine intake estimation through the analysis of caffeine metabolites in wastewater (in preparation).

This paper verifies caffeine intake in different European locations.

Publications

1. Bade, R. et al., 2016. Ultra-high performance liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in wastewater.
2. Baz-Lomba, J.A. et al., 2016. Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities. *BMC Public Health*, 16, 1035. (IF: 3.00 ; DOI: 10.1186/s12889-016-3686-5)
3. Causanilles, A. et al., 2016. Application of wastewater-based epidemiology to assess erectile dysfunction pharmaceuticals usage in 10 European cities.
4. Gracia-Lor, A., et al., 2016. Caffeine intake estimation through the analysis of caffeine metabolites in wastewater.
5. Rousis, N.I., et al., 2016. Assessment of pesticide metabolite in influent wastewater from 8 European countries by wastewater-based epidemiology.
6. Ryu, Y., et al., 2016. Increased levels of the oxidative stress biomarker 8-isoprostaglandin F2 α in wastewater associated with tobacco use. Submitted to *Scientific Reports*.
7. Ryu, Y. et al. (2016) Comparative measurement and quantitative risk assessment of alcohol consumption through wastewater-based epidemiology: An international study in 20 cities. *Sci. Total Environ.* 565, 977-983.
8. Castrignanò, E. 2016. Enantiomeric profiling of chiral drug biomarkers in wastewater- a European study.
9. Castrignanò, E. 2016. Wastewater profiling for antibiotics and resistance genes in Europe.

D4.3 Report on comparison of health and lifestyle profiles of different communities

Comparison of health and lifestyle profiles in different communities via utilisation of several characteristic biomarkers has been undertaken in the following papers:

Paper 1. J.A. Baz-Lomba, Stefania Salvatore, Emma Gracia-Lor, Richard Bade, Sara Castiglioni, Erika Castrignanò, Ana Causanilles, Felix Hernandez, Barbara Kasprzyk-Hordern, Juliet Kinyua, Ann-Kathrin McCall, Alexander van Nuijs, Christoph Ort, Benedek G. Plósz, Pedram Ramin, Malcolm Reid, Nikolaos I. Rousis, Yeonsuk Ryua, Pim de Voogt, Jorgen Bramness, Kevin Thomas, "Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities" *BMC Public Health* 2016 (accepted).

This study compared and correlated the consumption estimates of pharmaceuticals, illicit drugs, alcohol, nicotine and caffeine from wastewater analysis and other sources of information. Wastewater samples were collected in 2015 from 8 different European cities over a one week period, representing a population of approximately 5 million people. Published pharmaceutical sale, illicit drug seizure and alcohol, tobacco and caffeine use data were used for the comparison. High agreement was found between wastewater and other data sources for pharmaceuticals and cocaine, whereas amphetamines, alcohol and caffeine showed a moderate correlation. methamphetamine and 3,4- methylenedioxymethamphetamine (MDMA) and nicotine did not correlate with other sources of data. Most of the poor correlations were explained as part of the uncertainties related with the use estimates and were improved with other complementary sources of data. This work confirms the promising future of WBE as a complementary approach to obtain a more accurate picture of substance use situation within different communities. Our findings suggest further improvements to reduce the uncertainties associated with both sources of information in order to make the data more comparable.

Paper 2. Yeonsuk Ryu, Emma Garcia-Lor, Richard Bade, **J.A. Baz-Lomba**, Jørgen G. Bramness, Sara Castiglioni, Erika Castrignanò, Ana Causanilles, Adrian Covaci, Pim de Voogt, Felix Hernandez, Barbara Kasprzyk-Hordern, Juliet Kinyua, Ann-Kathrin McCall, Christoph Ort, Benedek G. Plósz, Pedram Ramin, Nikolaos I. Rousis, Malcolm J. Reid, and Kevin V. Thomas (2016). “Increased levels of the oxidative stress biomarker 8-isoprostaglandin F_{2α} in a city’s wastewater related to tobacco use”. Scientific reports (under review).

In this work, 8-iso-prostaglandin F_{2α} (8-iso-PGF_{2α}) was analysed in wastewater samples collected from 4 Norwegian and 7 other European cities in 2014 and 2015. Using the same samples, biomarkers of alcohol (ethyl sulfate) and tobacco (trans-3'-hydroxycotinine) use were also analysed to investigate any possible correlation between 8-iso-PGF_{2α} and the consumption of the two drugs.

Paper 3: Gatidou G, Kinyua J, van Nuijs A.L.N., Gracia-Lor E, Castiglioni S, Covaci A, Stasinakis, A. S. (2016). “Drugs of abuse and alcohol consumption among different groups of population on the Greek Island of Lesbos through sewage-based epidemiology”, *Science of The Total Environment* 563:633-40.

The occurrence of 22 drugs of abuse, their metabolites, and the alcohol metabolite ethyl sulphate was investigated in raw sewage samples collected during the non-touristic season from three sewage treatment plants (STPs), which serve different sizes and types of population in the Greek island of Lesbos. Using the sewage-based epidemiology approach, the consumption of these substances was estimated. Five target analytes, cocaine (COC), benzoylecgonine (BE), 3,4-methylenedioxymethamphetamine (MDMA), 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) and ethyl sulphate (EtS) were detected at concentrations above their limit of quantification, whereas the rest eighteen target compounds were not detected. THC-COOH was detected in most of the samples with concentrations ranging between < 20 and 90 ng L⁻¹, followed by EtS (range < 1700–12,243 ng L⁻¹). COC, BE, and MDMA were present only in the STP that serves Mytilene (the main city of the island), at mean concentrations of 3.9 ng L⁻¹ for COC (95% CI: 1.7–6.1), 9.4 ng L⁻¹ for BE (95% CI: – 1.6–23) and 3.2 ng L⁻¹ for MDMA (95% CI: 1.2–5.1). Back-calculations to an amount of used substance indicated more intense use of drugs among city population than rural and University population with average values of 9.5 and 1.2 mg day⁻¹ per 1000 inhabitants for COC (95% CI: – 1.43–20.4) and MDMA (95% CI: 0.52–1.85), respectively, and 2.8 g day⁻¹ per 1000 inhabitants for tetrahydrocannabinol (THC) (95% CI: 2.4–3.1), the active ingredient of cannabis. Alcohol consumption was observed to be higher in the city population (5.4 mL pure alcohol per day per inhabitant) than in the rural population (3.4 mL pure alcohol per day per inhabitant), but the difference was not statistically significant. Consumption of THC differed significantly among the three STPs.

Paper 4: Senta, I., Gracia-Lor, E., Borsotti, A., Zuccato, E., Castiglioni S. (2015). “Wastewater analysis to monitor use of caffeine and nicotine and evaluation of their metabolites as biomarkers for population size assessment”, *Water Research* 74: 23-33 (DOI: 10.1016/j.watres.2015.02.002)

The use of caffeine, nicotine and some major metabolites was investigated by wastewater analysis in 13 sewage treatment plants (STPs) across Italy, and their suitability was tested as qualitative and quantitative biomarkers for assessing population size and dynamics. A specific analytical method based on mass spectrometry was developed and validated in raw urban wastewater, and included two caffeine metabolites, 1-methylxanthine and 7-methylxanthine, never reported in wastewater before. All these compounds were found widely at the µg/L level. Mass loads, calculated by multiplying concentrations by the wastewater daily flow rate and normalized to the population served by each plant, were used to compare the profiles from different cities. Some regional differences were observed in the mass loads, especially for nicotine metabolites, which were significantly higher in the south than in the center and north of Italy, reflecting smoking prevalences from population surveys. There were no significant weekly trends, although the mean mass loads of caffeine and its metabolites were slightly lower during the weekend. Most caffeine and nicotine metabolites fulfilled the requirements for an ideal biomarker for the assessment of population size, i.e. being easily detectable in wastewater, stable in sewage and during sampling, and reflecting human metabolism. Nicotine metabolites were tested as quantitative biomarkers to estimate population size and the results agreed well with census data. Caffeine and its metabolites were

confirmed as good qualitative biomarkers, but additional information is needed on the caffeine metabolism in relation to the multiple sources of its main metabolites. This exploratory study opens the way to the routine use of nicotine metabolites for estimating population size and dynamics.

Paper 5: Stefania Salvatore, Jo Røislien, J.A. Baz-Lomba, Jørgen G. Bramness, “Functional principal component analysis (FPCA) for analysing wastewater data as a method of investigating whether medicinal drugs are abused” *Pharmacoepidemiology and Drug Safety* (submitted).

D4.4 Report on identification of new drugs of abuse in sewage

Specific methods were developed to enable identification of new drugs of abuse in sewage (see D4.1). These methods were applied in different European locations and in the Europe-wide monitoring study. Example papers include:

Paper 1. J.A. Baz-Lomba, Malcolm Reid, Kevin V. Thomas “Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF”. *Analytica Chimica Acta* 2016, 914, 81-90 (DOI: 10.1016/j.aca.2016.01.056).

The quantification of illicit drug and pharmaceutical residues in sewage has been shown to be a valuable tool that complements existing approaches in monitoring the patterns and trends of drug use. The present work delineates the development of a novel analytical tool and dynamic workflow for the analysis of a wide range of substances in sewage-based samples. The validated method can simultaneously quantify 51 target psychoactive substances and pharmaceuticals in sewage-based samples using an off-line automated solid phase extraction (SPE-DEX) method, using Oasis HLB disks, followed by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF) in MS(e). Quantification and matrix effect corrections were overcome with the use of 25 isotopic labeled internal standards (ILIS). Recoveries were generally greater than 60% and the limits of quantification were in the low nanogram-per-liter range (0.4-187 ng L⁻¹). The emergence of new psychoactive substances (NPS) on the drug scene poses a specific analytical challenge since their market is highly dynamic with new compounds continuously entering the market. Suspect screening using high-resolution mass spectrometry (HRMS) simultaneously allowed the unequivocal identification of NPS based on a mass accuracy criteria of 5 ppm (of the molecular ion and at least two fragments) and retention time (2.5% tolerance) using the UNIFI screening platform. Applying MS(e) data against a suspect screening database of over 1000 drugs and metabolites, this method becomes a broad and reliable tool to detect and confirm NPS occurrence. This was demonstrated through the HRMS analysis of three different sewage-based sample types; influent wastewater, passive sampler extracts and pooled urine samples resulting in the concurrent quantification of known psychoactive substances and the identification of NPS and pharmaceuticals.

Paper 2. M.J. Reid, J.A. Baz-Lomba, Y. Ryu, K.V. Thomas “Using biomarkers in wastewater to monitor community drug use: A conceptual approach for dealing with new psychoactive substances” *Science of the Total Environment* 487 (2014) 651 (DOI: 10.1016/j.scitotenv.2013.12.057)

Data obtained from the analysis of wastewater from large-scale sewage treatment plants has been successfully applied to study trends in the use of classical illicit drugs such as cocaine, but the dynamic nature of the new psychoactive substances (NPS) market presents a unique set of challenges to epidemiologists. In an attempt to overcome some of the challenges, this paper presents a framework whereby a collection of tools and alternative data-sources can be used to support the design and implementation of wastewater-based studies on NPS use. Within this framework the most likely and most suitable biomarkers for a given NPS are predicted via in-silico metabolism, biotransformation and sorption models. Subsequent detection and confirmation of the biomarkers in samples of wastewater are addressed via high-resolution mass spectrometry (HRMS). The proposed framework is applied to a set of test substances including synthetic cannabinoids and cathinones. In general, the in-silico models predict that transformation via N-dealkylation and hydroxylation is likely for these compounds, and that adsorption is expected to be significant for cannabinoids in wastewater. Screening via HRMS is discussed with

examples from the literature, and common-fragment searching and mass-defect filtering are successfully performed on test samples such that spectral noise is removed to leave only the information that is most likely to be related to the NPS biomarkers. HRMS screening is also applied to a set of pissoir-sourced wastewater samples and a total of 48 pharmaceuticals and drugs including 1-(2-methoxyphenyl)piperazine (oMeOPP) are identified. The framework outlined in this paper can provide an excellent means of maximizing the chances of success when identifying and detecting biomarkers of NPS in wastewater.

Paper 3. Kinyua, J., Covaci, A., Maho, W., McCall, A.-K., Neels, H., van Nuijs, A. L. N.(2015). “ Sewage-based epidemiology in monitoring the use of new psychoactive substances: Validation and application of an analytical method using LC-MS/MS” *Drug Testing and Analysis*, 7: 812-818. (DOI: 10.1002/dta.1777)

Sewage-based epidemiology (SBE) employs the analysis of sewage to detect and quantify drug use within a community. While SBE has been applied repeatedly for the estimation of classical illicit drugs, only few studies investigated new psychoactive substances (NPS). These compounds mimic effects of illicit drugs by introducing slight modifications to chemical structures of controlled illicit drugs. We describe the optimization, validation, and application of an analytical method using liquid chromatography coupled to positive electrospray tandem mass spectrometry (LC-ESI-MS/MS) for the determination of seven NPS in sewage: methoxetamine (MXE), butylone, ethylone, methylone, methiopropamine (MPA), 4-methoxymethamphetamine (PMMA), and 4-methoxyamphetamine (PMA). Sample preparation was performed using solid-phase extraction (SPE) with Oasis MCX cartridges. The LC separation was done with a HILIC (150 x 3 mm, 5 µm) column which ensured good resolution of the analytes with a total run time of 19 min. The lower limit of quantification (LLOQ) was between 0.5 and 5 ng/L for all compounds. The method was validated by evaluating the following parameters: sensitivity, selectivity, linearity, accuracy, precision, recoveries and matrix effects. The method was applied on sewage samples collected from sewage treatment plants in Belgium and Switzerland in which all investigated compounds were detected, except MPA and PMA. Furthermore, a consistent presence of MXE has been observed in most of the sewage samples at levels higher than LLOQ.

Paper 4. Lai, F. Y., Erratico, C., Kinyua, J., Mueller, J. F., Covaci, A., van Nuijs, A. L. N.(2015). “ Liquid chromatography-quadrupole time-of-flight mass spectrometry for screening in vitro drug metabolites in humans: investigation on seven phenethylamine-based designer drugs”, *Journal of Pharmaceutical and Biomedical Analysis* 114: 355-375.

Phenethylamine-based designer drugs are prevalent within the new psychoactive substance market. Characterisation of their metabolites is important in order to identify suitable biomarkers which can be used for better monitoring their consumption. Careful design of in vitro metabolism experiments using subcellular liver fractions will assist in obtaining reliable outcomes for such purposes. The objective of this study was to stepwise investigate the in vitro human metabolism of seven phenethylamine-based designer drugs using individual families of enzymes. This included para-methoxyamphetamine, para-methoxymethamphetamine, 4-methylthioamphetamine, N-methyl-benzodioxolylbutanamine, benzodioxolylbutanamine, 5-(2-aminopropyl) benzofuran and 6-(2-aminopropyl) benzofuran. Identification and structural elucidation of the metabolites was performed using liquid chromatography-quadrupole-time-of-flight mass spectrometry. The targeted drugs were mainly metabolised by cytochrome P450 enzymes via O-dealkylation as the major pathway, followed by N-dealkylation, oxidation of unsubstituted C atoms and deamination (to a small extent). These drugs were largely free from Phase II metabolism. Only a limited number of metabolites were found which was consistent with the existing literature for other phenethylamine-based drugs. Also, the metabolism of most of the targeted drugs progressed at slow rate. The reproducibility of the identified metabolites was assessed through examining formation patterns using different incubation times, substrate and enzyme concentrations. Completion of the work has led to a set of metabolites which are representative for specific detection of these drugs in intoxicated individuals and also for meaningful evaluation of their use in communities by wastewater-based drug epidemiology.

Paper 5. Kinyua, J., Negreira, N., Miserez, B., Causanilles, A., Emke, E.; Gremeaux, L., de Voogt, P., Ramsey, J., Covaci, A., van Nuijs, A. L. N.(2016). Qualitative screening of new psychoactive substances in pooled urine samples from Belgium and United Kingdom. (<http://dx.doi.org/10.1016/j.scitotenv.2016.08.124> In press.)

Concerns about new psychoactive substances (NPS) are increasing due to the rising frequency of serious intoxications. Analysis of biological fluids (urine) is necessary to get reliable information about the use of these substances. However, it is a challenging task due to the lack of analytical standards and the dynamic character of the NPS market. In the present work, a qualitative screening of NPS was carried out in 23 pooled urine samples collected from a city center in the UK and festivals in the UK and Belgium. The analytical method was based on data-independent acquisition mode using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. An in-house library was used with > 1500 entries corresponding to NPS, classical drugs and metabolites. All samples contained 53 and 28 compounds of interest from the UK and Belgium respectively. Of the different compounds detected, about 70% were confirmed using retention time and product ions while the remaining compounds were identified using elucidated fragmentation pathways. The highest numbers of NPS identified in both countries were from the cathinone and phenylethylamine families, with a higher number being detected in samples from the festival in the UK. Moreover, several cathinone metabolites in human urine were detected and identified. The screening method proved useful to detect a large number of compounds and determine the use of NPS.

Paper 6. Negreira, N., Kinyua, J., De Brabanter, N., Maudens, K., van Nuijs, A. L. N.(2016). Identification of in vitro and in vivo human metabolites of the new psychoactive substance nitracaine by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. *Analytical and Bioanalytical Chemistry* 408: 5221-5229. (doi:10.1007/s00216-016-9616-7)

The purpose of this work was to investigate the in vitro metabolism of nitracaine, a new psychoactive substance, using human liver microsome incubations, to evaluate the cytochrome P450 (CYP) enzyme isoforms responsible for the phase-I metabolism and to compare the information from the in vitro experiments with data resulting from an authentic user's urine sample. Accurate mass spectra of metabolites were obtained using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) and were used in the structural identification of metabolites. Two major and three minor phase-I metabolites were identified from the in vitro experiments. The observed phase-I metabolites were formed through N-deethylation, N,N-deethylation, N-hydroxylation, and de-esterification, with CYP2B6 and CYP2C19 being the main enzymes catalyzing their formation. One glucuronidated product was identified in the phase-II metabolism experiments. All of these metabolites are reported for the first time in this study except the N-deethylation product. All the in vitro metabolites except the minor N,N-deethylation product were also present in the human urine sample, thus demonstrating the reliability of the in vitro experiments in the prediction of the in vivo metabolism of nitracaine. In addition to the metabolites, three transformation products (p-nitrobenzoic acid, p-aminobenzoic acid, and 3-(diethylamino)-2,2-dimethylpropan-1-ol) were identified, as well as several glucuronides and glutamine derived of them.

Paper 7. Mardal, M.; Meyer, M.R. (2014) Studies on the microbial biotransformation of the novel psychoactive substance methylenedioxypropylvalerone (MDPV) in wastewater by means of liquid chromatography-high resolution mass spectrometry/mass spectrometry. *The Science of the total environment* 2014;493:588-95.

Sewage profiling as a mean to estimate consumption of drugs of abuse is gaining increasing attention. However, only scarce data are available so far on the impact of microbial biotransformation on the presence and hence detectability of drugs of abuse and their metabolites in wastewater (WW) samples. The aim of this work was therefore to study the biotransformation pathways of the novel psychoactive substance 3,4-methylenedioxypropylvalerone (MPDV) in WW by incubating it, based on the OECD guideline 314 A. MDPV was incubated (100 µg/L) for 10d at 22 °C in WW from a local WW treatment plant. Furthermore, urine and feces collected from rats administered 20mg MDPV/kg BW were incubated correspondingly. Samples were worked-up either by centrifugation/filtration and solid-phase (HXC) extraction or QuEChERS. High resolution (HR) mass

spectra (MS) were recorded using an Orbitrap mass spectrometer. All products were identified via their HR-MS(2) spectra and chromatographic properties. The observed biotransformations in WW were: demethylenation and subsequent O-methylation, hydroxylation at the phenyl part, hydroxylation at the pyrrolidine part with subsequent methylation or oxidation, N-demethylation, and hydroxylation at the alkyl part as well as combination of them. In total, 12 biotransformation products were identified after 10 days of incubation. Three of these biotransformation products were previously reported to be also rat and human metabolites. No additional MDPV biotransformation products could be found after incubating the rat urine and feces samples. Instead, the urinary phase II glucuronides were nearly completely cleaved after one day of WW incubation. The presented study indicates that demethylenyl-methyl MDPV, the most abundant metabolite in human urine, should be the best indicator in WW to estimate its use.

Paper 8. Mardal, M.; Gracia-Lor, E.; Leibnitz, S.; Castiglioni, S.; Meyer, M.R (2016) Toxicokinetics of new psychoactive substances: plasma protein binding, metabolic stability, and human phase I metabolism of the synthetic cannabinoid WIN 55,212-2 studied using in vitro tools and LC-HR-MS/MS. Drug testing and analysis (e-pub ahead of print)

Paper 9. Mardal, M.; Miserez, B.; Bade, R.; Portoles, T.; Bischoff, M.; Hernandez, F.; Meyer, M.R. (2016) 3-Fluorophenmetrazine, a fluorinated analogue of phenmetrazine: Studies on in vivo metabolism in rat and human, in vitro metabolism in human CYP isoenzymes and microbial biotransformation in *Pseudomonas Putida* and wastewater using GC and LC coupled to (HR)-MS techniques. Journal of pharmaceutical and biomedical analysis 2016;128:485-95.

Wastewater-based epidemiology (WBE) as means to estimate illicit drug and new psychoactive substance (NPS) consumption with spatial and temporal resolution is gaining increasing attention. In order to evaluate a given NPS using WBE, in vivo metabolism and microbial biotransformation of excretion products and unchanged compounds need evaluation. The aims of this study were to identify in vivo phase I and II metabolites of the NPS 3-fluorophenmetrazine (3-FPM) in human and rat urine and study the in vitro contribution of Cytochrome P450 (CYP) isoenzymes in phase I metabolism. Additionally, to study microbial biotransformation products (MBPs) of 3-FPM from incubations in wastewater and in a wastewater isolated *Pseudomonas Putida* strain. To these aims gas chromatography and liquid chromatography coupled to mass spectrometry were applied. Metabolites and MBPs were isolated from urine and microbial incubations after solid phase extraction and precipitation with or without enzymatic conjugate cleaving. The main transformation pathways were N-oxidation, aryl hydroxylation and subsequent O-methylation, alkyl hydroxylation, oxidation, and degradation of the ethyl-bridge yielding the O/N-bis-dealkylated metabolite, combinations thereof and further glucuronidation or sulfations. The main excretion products in the human urine sample were the unchanged compound and the N-oxide, and the main MBPs were the N-oxide and hydroxylation with subsequent oxidations on the alpha-methyl position. Based on these findings, the proposed strategy for WBE analysis of 3-FPM is quantitative determination of unchanged 3-FPM together with qualitative verification of a number of selected metabolites to verify consumption and rule out discharge.

Paper 10. Gonzalez-Mariño, I., Gracia-Lor, E., Bagnati R., Martins, C.P.B., Zuccato, E., Castiglioni S. (2016). "Screening New Psychoactive Substances in urban wastewater using high resolution mass spectrometry", Anal. Bioanal. Chem. 408(16):4297-309 (DOI: 10.1007/s00216-016-9521-0)

Analysis of drug residues in urban wastewater could complement epidemiological studies in detecting the use of new psychoactive substances (NPS), a continuously changing group of drugs hard to monitor by classical methods. We initially selected 52 NPS potentially used in Italy based on seizure data and consumption alerts provided by the Antidrug Police Department and the National Early Warning System. Using a linear ion trap-Orbitrap high resolution mass spectrometer, we designed a suspect screening and a target method approach and compared them for the analysis of 24 h wastewater samples collected at the treatment plant influents of four Italian cities. This highlighted the main limitations of these two approaches, so we could propose requirements for future research. A library of MS/MS spectra of 16 synthetic cathinones and 19 synthetic cannabinoids, for which

analytical standards were acquired, was built at different collision energies and is available on request. The stability of synthetic cannabinoids was studied in analytical standards and wastewater, identifying the best analytical conditions for future studies. To the best of our knowledge, these are the first stability data on NPS. Few suspects were identified in Italian wastewater samples, in accordance with recent epidemiological data reporting a very low prevalence of use of NPS in Italy. This study outlines an analytical approach for NPS identification and measurement in urban wastewater and for estimating their use in the population.

Paper 11. González-Mariño, I., Gracia-Lor, E., Rousis, N.I., Castrignanò, E., Thomas, K.V., Quintana, J.B., Kasprzyk-Hordern, B., Zuccato, E., Castiglioni, S. (2016). "Wastewater-Based Epidemiology To Monitor Synthetic Cathinones Use in Different European Countries", *Environ. Sci. Technol.* 50:10089–10096. (DOI: 10.1021/acs.est.6b02644).

Synthetic cathinones are among the most consumed new psychoactive substances (NPS), but their increasing number and interchangeable market make it difficult to estimate the real size of their consumption. Wastewater-based epidemiology (WBE) through the analysis of metabolic residues of these substances in urban wastewater can provide this information. This study applied WBE for the first time to investigate the presence of 17 synthetic cathinones in four European countries. A method based on solid-phase extraction and liquid chromatography coupled to tandem mass spectrometry was developed, validated, and used to quantify the target analytes. Seven substances were found, with mephedrone and methcathinone being the most frequently detected and none of the analytes being found in Norway. Population-normalized loads were used to evaluate the pattern of use, which indicated a higher consumption in the U.K., followed by Spain and Italy, in line with the European prevalence data from population surveys. In the U.K., where an entire week was investigated, an increase of the loads was found during the weekend, indicating a preferential use in recreational contexts. This study demonstrated that WBE can be a useful additional tool to monitor the use of NPS in a population.

Paper 12. B. Miserez, O. Ayrton, J. Ramsey, Analysis of purity and cutting agents in street mephedrone samples from South Wales, *Forensic Toxicology*, 32 (2014) 305-310

Paper 13. L. Bijlsma, B. Miserez, M. Ibanez, C. Vincent, E. Guillaumon, J. Ramsey, F. Hernandez, Identification and characterization of a novel cathinone derivative 1-(2,3-dihydro-1H-inden-5-yl)-2-phenyl-2-(pyrrolidin-1-yl)-ethanone seized by customs in Jersey, *Forensic Toxicology*, 34 (2016), 144-150

Paper 14. L. Brockbals, M. Karlsen, J. Ramsey, B. Miserez, Single injection quantification of cocaine using multiple isotopically labeled internal standards, *Forensic Toxicol.* (2016) doi: 10.1007/S11419-016-0328-7

Paper 15. A. Frinculescu, C.L. Lyall, J. Ramsey, B. Miserez, Variation in commercial smoking mixtures containing third-generation synthetic cannabinoids, *Drug Test. Anal.* (2016) doi: 10.1002/dta.1975

Several papers are still in progress:

Paper 16. Richard Bade; Lubertus Bijlsma; Juan V Sancho; J.A Baz-Lomba; Sara Castiglioni; Erika Castrignanò; Ana Causanilles; Emma Gracia-Lor; Barbara Kasprzyk-Hordern ; Juliet Kinyua; Ann-Kathrin McCall; Alexander L. N. van Nuijs; Christoph Ort; Benedek G Plósz; Pedram Ramin; Nikolaos I Rousis; Yeonsuk Ryu; Kevin V Thomas; Pim de Voogt; Ettore Zuccato; Felix Hernandez (2016). "Liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in influent wastewater of eight European cities" *Chemosphere (submitted)*.

Paper 17. J.A. Baz-Lomba, Ana Causanilles, Malcolm Reid, Erik Emke, Pim de Voogt, Kevin Thomas "Non-target screening of pooled urine samples: the quest for NPS" (*work in progress*).

Paper 18. Causanilles, A., Kinyua, J., van Nuijs, A.L.N., Covaci, A., Emke, E., de Voogt, P. “Qualitative screening of New Psychoactive Substances in wastewater from a Dutch festival event using liquid chromatography coupled to high-resolution mass spectrometry” (*writing in progress*).

Paper 19. Bade, R., Bijlsma, L., Sancho, J. V., Baz-Lomba, J. A., Castiglioni, S., Castrignanò, E., Causanilles, A., Gracia-Lor, E., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.-K., van Nuijs, A.L.N., Ort, C., Plósz, B. G., Ramin, P., Rousis, N. I., Ryu, Y., Thomas, K., de Voogt, P., Zuccato, E., Hernández, F., “Ultra-high performance liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in wastewater” (2016) *Chemosphere (submitted)*.

Paper 20. Mardal, M.; Bischoff, M.; Ibáñez, M.; Ruffing, U.; Hernández, F.; Meyer, M.R. (2016) Microbial biotransformation of five pyrrolidinophenone-type psychoactive substances in wastewater and a wastewater-isolated pseudomonas putida strain 2016 (*submitted*).

Paper 21. Castrignano, E.; Mardal, M.; Rydevik, A.; Miserez, B.; Ramsey, J.; Shine, T.; Dan Pantos, G.; Meyer, M.R.; Kasprzyk-Hordern, B. A new approach towards biomarker selection in estimation of human exposure to chiral drugs with limited metabolism data: a case study of mephedrone 2016. (*in preparation*) .

Paper 22. Kinyua, J., Boogaerts, T, Negreira, N., McCall, A.-K., Ort, C., Covaci, A., van Nuijs A. L. N. (2016). “Target and suspect screening of new psychoactive substances in influent sewage from festivals and holidays in Switzerland and Belgium” (*in preparation*).

Paper 23. Kinyua, J., Negreira, N., McCall, A.-K., Ort, C., Covaci, A., van Nuijs A. L. N. (2016). “Investigating formation of transformation products during in sewers stability studies of NPS in the presence of biofilm” (*in preparation*).

Oslo, 30/09/2016

Dr. Kevin Thomas, leader WP4