WP2 - Qualitative and quantitative analysis of new psychoactive substances (NPS) in Europe, with focus on synthetic opioids and prescriptions opioids

Deliverable 8

Report on stability of biomarkers in wastewater

A suitable biomarker should be sufficiently stable in wastewater (WW) during the transport (in-sewer stability) from the input (i.e. toilet) to the sampling point and during sampling, storage and analysis (in-sample stability) (McCall et al., 2016). Consequently, in-sample and/or in-sewer stability studies are needed to check the suitability of a biomarkers for wastewater-based epidemiology (WBE). This report reports the in-sample stability of new psychoactive substances (NPS) and prescription opioids biomarkers assessed within the EuSeMe project.

NEW PSYCHOACTIVE SUBSTANCES (NPS)

The stability of 63 NPS biomarkers (fentanyl analogues and other NPS) in WW was assessed to take into account for any potential loss during sampling and storage (in-sample stability). WW samples were therefore kept (a) at room temperature for 48 h in order to mimic the residence time in the sewer (48 h was taken as a worst case, since residence time is normally lower than 24 h); (b) refrigerated at +4 °C for 24 h to mimic sampling conditions; and (c) frozen at -20ºC for 3 months to mimic the normal storage conditions until analysis (3 months was taken as a worst case, as WW samples were normally analysed within 2-4 weeks). The stability during freeze-thaw cycles was also evaluated.

Stability experiments

An influent WW sample (4 L) was spiked with a mixture of NPS at a concentration of 100 ng/L to ensure that spiked biomarkers were present in higher amounts compared to the "blank sample" (aliquot of the same sample without spiking). This time point was considered as t=0 h. Different aliquots (250 mL) were prepared to performed the stability tests previously mentioned (a), (b) and (c). All tests were performed in triplicate. For experiments at room temperature and +4ºC, smaller aliquots (50 mL) were taken at 6 h, 24 h and 48 h and analysed according to the analytical method described in Deliverable 7. Briefly, WW samples were vacuum-filtered, solid phase extracted by SPE using Oasis® MCX cartridges (150 mg, 6 cc) and analysed by liquid-chromatography tandem mass spectrometry (LC-MS/MS). In the case of experiments performed with samples at -20ºC, samples were initially frozen and aliquots were taken after 1 week, 1 month and 3 months for analysis, with a freeze-thaw cycle between time points.

In-sample stability

The in-sample stability of fentanyl analogues and other NPS at room temperature, +4ºC and -20ºC is reported in Table 1 and Table 2, respectively.

Fentanyl analogues. In general, metabolites showed higher stability than parent compounds. In-sample transformation lower than 20% were observed for almost all norfentanyl analogues and carboxy metabolites after 48 h at room temperature, 48 h at +4ºC and 3 months at -20ºC. However, losses higher than 50% of fentanyl analogues (except beta-hydroxyfentanyl and beta-hydroxythiofentanyl) were observed after 6 hours at room temperature and +4ºC, and after 1 week

at -20ºC. Metabolites 4-ANPP and 4F-ANPP showed some discrepancies in results with an in-sample transformation of +40 until +200% after 48h at room temperature, indicating that these compounds can be generated in the sewer (e.g. biotransformation of precursors). This result need further investigation to understand the potential sources. Both metabolites showed on the contrary low stability at +4ºC and -20ºC with losses between 50 and 90%.

Other NPS. Different behaviour was observed depending on NPS categories, being the most stable synthetic cathinones and arylcyclohexylamines (ketamine analogues). Almost all compounds were stable for 24-h at room temperature with less than 20% of in-sample transformation. Some exceptions were the synthetic cannabinoid JWH-122, synthetic cathinones naphyrone, pentedrone and 4-FMC, the synthetic opioid isotonitazene and the tryptamine 5-MeO-MIPT with more than 40% of transformation. NBOMe phenetylamines showed a loss of 40-60% after 48h at room temperature. A similar behaviour was observed for samples stored refrigerated at +4ºC, although the percentage of transformation was lower in comparison to that observed at room temperature.

Regarding the storage at -20ºC, the vast majority of NPS biomarkers remained stable for 1-month, except the synthetic cathinones pentylone and ethylone, the NBOMe phenetylamines, and the synthetic cannabinoids CumylPeGLaCONE and MDMB-CHMICA with more than 30% of transformation after 1-week (one freeze-thaw cycle). Higher transformation percentages (50-80%) were observed in the case of JWH-122 (synthetic cannabinoid), isotonitazene (synthetic opioid) and 5-MeO-MIPT (tryptamine).

Table 1. In-sample transformation of fentanyl analogues and metabolites at room temperature, refrigerated at +4ºC and at -20ºC									
NPS	**Room temperature**			**Temp = +4ºC**			**Temp = -20ºC**		
	6h	**24h**	**48h**	**6h**	**24h**	**48h**	**1 week**	**1 month**	**3 months**
Fentanyl	-55	-60	-85	-54	-57	-56	-56	-53	-44
Norfentanyl	-1.6	2.3	4.8	0.88	-1.0	1.63	3.8	8.4	-1.6
Acetylfentanyl	-43	-53	-77	-43	-50	-51	-46	-46	-41
Acetylnorfentanyl	-4.8	-16	-16	-4.0	-15	-15	-5.9	8.1	-5.0
Alfentanil	-8.3	-15	-27	-7.3	-13	-16	-12	-8.0	-8.4
Butyrylfentanyl	-66	-70	-81	-65	-66	-63	-64	-62	-54
Butyrylfentanyl carboxy met	-1.1	-5.6	-7.5	2.8	-2.9	-3.6	-2.9	5.4	-6.9
Butyrylnorfentanyl	-7.8	3.5	6.3	-7.2	-4.3	0.29	1.7	-1.2	-18
Carfentanil	-64	-70	-84	-65	-66	-62	-52	-53	-41
Cyclopropylfentanyl	-60	-64	-82	-57	-58	-56	-56	-59	-40
Phenylacetylfentanyl	2.8	-25	-31	1.8	-25	-28	-17	-16	-34
Beta-hydroxyfentanyl	-16	-6.9	-28	-11	-10	-5.3	-3.9	-4.7	-4.5
beta-hydroxythiofentanyl	-17	-23	-34	-18	-17	-15	-16	-21	-16
trans-3-methyl norfentanyl	-9.4	-10	-6.3	-13	-11	-8	-12	-5.8	-15
cis-3-methyl norfentanyl	-4.6	5.5	11	-2.8	3.4	6.9	6.0	0.52	-27
4-ANPP	-28	36	236	-49	-39	-56	-28	-98	-100
4-F-ANPP	-27	32	273	-50	-44	-53	-32	-80	-75
Metoxyacetylnorfentanyl	-4.9	-12	-16	-3.9	-9.3	-13	-6.6	3.3	-7.2
Valerylfentanyl carboxy met	-6.8	1.1	-1.3	-10	0.61	4.3	5.2	8.9	0.25
Furanylnorfentanyl	-8.0	7.8	5.5	-6.2	7.6	3.7	1.7	2.3	-5.5
Ocfentanil	-58	-61	-85	-60	-53	-56	-57	-54	-44

Table 2. In-sample transformation of other NPS biomarkers at room temperature, refrigerated at +4ºC and at -20ºC

NPS	Room temperature			Temp = +4ºC			Temp = -20ºC		
	6h	24h	48h	6h	24h	48h	1 week	1 month	3 months
alpha-PVP	-1.0	-3.9	-11	4.4	-8.2	-9.7	-4.3	-6.3	-4.4
4-Cl-alpha-PPP	-4.8	-12	-29	1.0	-12	-21	-10	-8.0	-13
PMA	-2.5	-18	-20	3.6	-17	-17	-16	-20	-45
PMMA	-0.83	-14	-15	0.67	-15	-17	-10	-10.0	-7.8
Methoxethamine	2.2	-9.0	-13	1.6	-15	-15	-9.3	-6.6	-10
5-MeO-MiPT	-30	-57	-63	-27	-60	-57	-61	-75	-86
Methcathinone	-7.1	-45	-61	1.2	-30	-29	-11	-11	-56
4,4-DMAR	-4.8	-43	-42	-2.4	-45	-44	-49	-43	-35
3-MMC	-5.6	-9.5	-32	3.4	1.8	-2.9	6.9	-18	-32
Ethcathinone	-1.7	-24	-43	6.3	-13	-13	0.14	-24	-51
Methedrone	0.44	-7.5	-19	0.30	-13	-13	-3.2	-38	-49
Dimethylcathinone	-0.80	-25	-25	0.66	-29	-26	-15	-11	-21
4-FMC	-0.90	-62	-72	3.0	-47	-48	-35	-52	-73
3,4-DMMC	-6.3	-17	-28	-1.3	-13	-11	-5.4	-3.1	-27
4-MEC	-3.6	-20	-26	-0.22	-16	-13	-15	-16	-31
Buphedrone	-2.5	-26	-46	4.4	-17	-19	2.2	-22	-46
Pentedrone	-2.5	-42	-50	4.7	-18	-20	-13	-10	-32
Methylone	-4.6	-8.9	-19	-2.1	-5.2	-5.0	-0.79	-3.0	-29
Ethylone	-9.2	-19	-28	-10	-24	-24	-34	-29	-32
Butylone	0.38	-25	-30	-0.21	-24	-23	-17	-12	-16
Pentylone	-2.6	-13	-16	-1.1	-16	-14	0.88	-12	-25
1-Naphyrone	-51	-64	-85	-42	-46	-53	-52	-71	-68
Naphyrone	-64	-69	-84	-62	-61	-67	-70	-74	-65
MDPV	-13	-13	-28	-8.9	-11	-12	-4.0	-0.09	-17
NEDPA	2.1	-63	-62	4.0	-71	-66	-58	-66	-63
6-APB	-5.6	-4.2	-8.1	1.2	-10	-10	-0.13	-8.8	-23
5-MeO-DMT	-19	-21	-33	-11	-25	-23	-18	-68	-76
NN-DMT	-8.1	4.5	-10	-1.5	4.4	-0.72	11	-49	-51
Ethylphenidate	-5.8	-24	-43	5.0	-9.1	-11	-5.2	-12	-24

2-FDCK	5.3	23	8.7	7.0	16.5	8.9	7.3	23	8.2
25-B-NBOMe	-4.1	-24	-40	-6.0	-31	-34	-32	-51	-60
25-C-NBOMe	-4.6	-24	-39	3.1	-31	-30	-37	-39	-63
25-I-NBOMe	-6.1	-26	-49	-6.5	-36	-35	-40	-61	-73
JWH 122	20	-63	-89	13	-46	-77	-81	-85	-93
CumylPeGLaCONe	19	18	4.2	16	15	-24	-38	-63	-76
AB-CHMINACA	-21	-6.5	-51	-27	-12	-35	-34	-20	-49
ADB-FUBINACA	-26	-6.8	-55	-24	-10	-19	-2.0	-14	-47
MDMB-CHMICA	16	1.9	-16	11	5.1	-29	-38	-64	-83
5-fluoropentyl-3-pyr	22	-19	-23	24	-27	-38	-11	3.2	-6.2
25iPNBOMe	-5.7	-6.8	-79	-11	-28	-77	-28	-67	-72
Isotonitazene	-4.9	-73	-89	-7.8	-67	-70	-63	-65	-79

PRESCRIPTION OPIOIDS

Stability experiments of prescription opioids were performed by the University of Antwerp (UA) as recently published by Boogaerts et al., 2021. In-sample stability of twenty parent compounds (P) and metabolites (M) at room temperature was investigated.

Fentanyl (P)
Norfentanyl (M)
Buprenorphine (P)
Norbuprenorphine (M)
Hydromorphone (P)
Naloxone (P)
Hydrocodone (P)
Oxycodone (P)
Oxymorphone (P, M)
Noroxycodone (M)
Cis-tramadol (P)
O-desmethyl tramadol (M)
Codeine (P)
Dehydrocodeine (P, M)
Morphine (P)
Normorphine (M)
Methadone (P)
EDDP (M)
Tilidine (P)
Nortilidine (M)

Stability experiments

A large WW pool was made by subsampling different influent WW sources. This influent WW pool was subsequently divided in 3 aliquots of 1000 mL, including a non-spiked 'control' influent WW sample and two spiked influent WW samples. P and M compounds were spiked separately in different pools at high concentrations (250–1000 ng/L) to ensure that spiked biomarkers were present in substantially higher amounts compared to control samples. During the experiment, the aliquots were placed at room temperature, and on a magnetic stirrer (300 rpm) to simulate sewer currents (Fig. 1). The time point the aliquots were spiked was considered the as t=0 h and at specific

time intervals (2 h, 6 h, 10 h, and 24 h) two aliquots of 100 mL were taken from each sample and prepared according to an established SPE-LC-MS/MS protocol (Boogaerts et al., 2021).

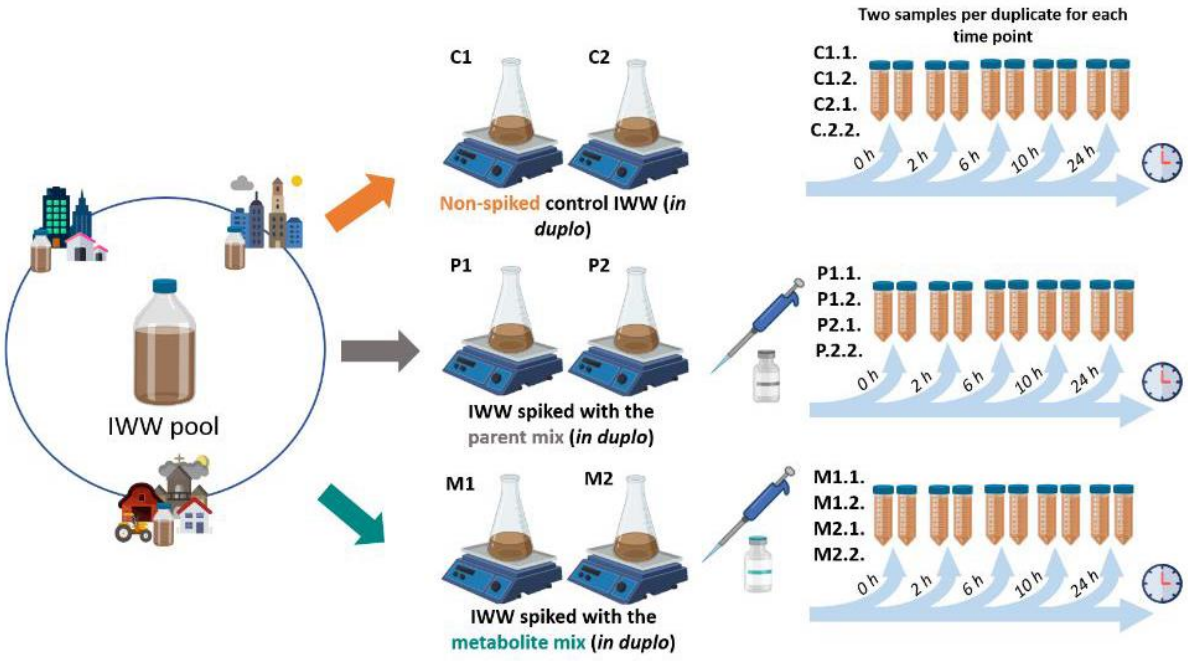

Fig 1. Experimental set-up for the benchtop stability tests (from Boogaerts et al., 2021).

In-sample stability

Almost all compounds (16 out of 20) showed high stability (20% transformation) over 24 h. Stability of buprenorphine, normorphine, fentanyl and EDDP was only medium, with more than 20% but less than 40% in-sample transformation. However, their metabolites showed high stability, without a parallel increase of norbuprenorphine and norfentanyl concentrations in the pooled influent WW samples. This means that buprenorphine and fentanyl are not degraded in norbuprenorphine and norfentanyl in wastewater, thus proving the suitability of these metabolites as alternative biomarker to the parent compounds.

The study conducted by UA did not evaluate in-sewer degradation in the presence of a biofilm under gravity or rising main sewer conditions or in a pilot sewer study, which could lead to additional uncertainty. Moreover, they did not investigate in-sample stability at − 20 ◦C. However, McCall et al. indicate that most of the opioid compounds (e.g. morphine, oxycodone, fentanyl) are sufficiently stable during sample storage at − 20 ◦C (McCall et al. 2016). The stability of biomarkers can depend also from sorption to solid particulate matter, which would potentially lead to uncertainty in back-estimating biomarker population-normalized mass loads. Baker et al. found that the average proportion of solid particulate matter was >10% with regard to methadone, EDDP and fentanyl, but was acceptable for most of the other opioid biomarkers (e.g. norcodeine, tramadol) (Baker et al. 2011).

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