Final report of PD_17 OTKA 124161 (2017-2020)

1. Environmental analytics

The release of pharmacologically active compounds (PhACs) into aquatic ecosystems poses an environmental risk resulting in a chronic exposure of non-target organisms. A great variety of PhACs, of generally low concentrations, and the complicated sample preparation, makes circumstantial the accurate detection and quantification. Additionally, there is little information published about the spatiotemporal variation of the PhAC load in a larger catchment area utilised for touristic purposes. In addition to the natural biotic and abiotic changes, the seasonal variation of tourism also has a dramatic impact on water quality and the natural ecosystem in larger catchment areas. Therefore, we developed a reliable solid-phase extraction (SPE)-supercritical fluid chromatography tandem mass spectrometry (SFC-MS/MS) method for simultaneous multi-residue analysis of drugs to reveal the spatiotemporal changes in the PhAC contaminations in the waters of an important touristic region, the catchment area of the largest shallow lake in Central Europe, Lake Balaton (Hungary). The environmental application of the developed method revealed 69 out of the traced 134 chemical compounds, including 15 PhACs, which were detected from natural waters for the first time. Wastewater treatment plant (WWTP) loads have a major role in the PhAC contamination of the studied area; at the same time, the mass tourism-induced PhAC contamination was also detectable. Furthermore, the impact of tourism was indicated by elevated concentrations of recreational substances (e.g., caffeine and illicit drugs) in the touristic season affecting the water quality of this important summer holiday destination [1-2].

Furthermore, another study presents the application of quantitative screening technique to water samples collected after large music festival, where likely users of recreational drugs gather and consequently higher residual concentrations of ordinary and new psychoactive substances are expected. Our data strengthen the view that residues of drugs of abuse have become widespread surface water contaminants following local mass events such as music festivals. The presence of the parent drugs and their metabolites collected over three monitored festival years suggested that consumption of these drugs remained consistent inasmuch as cocaine and ecstasy were the most popular drugs. Most of the illicit drugs retain their pharmacological activities having a potential adverse impact on the wildlife. We have no information on whether the contamination is derived

from a direct or indirect load, due to the inappropriate wastewater drainage from the festival area or toilet infrastructure conditions. One must bear in mind that in the festival area the contamination burden of IDs is expected to be much higher because terrain sources (including fixed and mobile toilets, bushes) were disregarded. Moreover, any accumulation by the sediment of the lake is also unknown, therefore measured drug concentrations could be much lower and cannot serve as an estimation of total drug consumption because the usage level may be underestimated. The survey strategy used cannot estimate the actual drug consumption level but it provides valuable information on whether these drugs are definitely present during the festival period. The overall results of this study however reliably complements traditional questionnaire surveys from such events and have additional advantages of giving direct evidence of drug abuse and being more objective while avoiding major ethical issues [3].

2. Risk assessment of the drug contaminations

The presence of pharmacologically active compounds (PhACs) in surface waters poses an environmental risk of chronic exposure to nontarget organisms, which is a well-established and serious concern worldwide. Our aim was to determine thetemporal changes in ecological risk quotient (RQ) based on the concentrations of 42 PhACs from six sampling sites on seven sampling dates in the water of a freshwater lake in Central Europe preferentially visited by tourists. Our hypothesis was that the environmental risk increases during the summer holiday season due to the influence of tourists. Different experimental toxicological threshold concentrations and seasonal measured environmental concentrations of 16 PhACs were applied to ecological risk assessment. RQs of 4 dominant PhACs (diclofenac, estrone [E1], estradiol [E2], and caffeine) indicated high ecological risk (RQ > 1) for freshwater ecosystems. Additionally, our results confirmed the assumptions that the high tourist season had a significant impact on the calculated RQ; however, these results are mainly due to the concentration and temporal change of particular PhACs, including diclofenac (5.3-419.4 ng/L), E1 (0.1-5.5 ng/L), and E2 (0.1-19.6 ng/L). The seasonal dependent highest RQs changed as follows: 9.80 (June 2017; E2), 1.23 (August 2017; E1), 0.43 (November 2017; E1), 0.51 (April 2018; E1), 5.58 (June 2018, diclofenac), 39.50 (August 2018; diclofenac), and 30.60 (October 2018; diclofenac). Furthermore, the fluctuation of summed measured environmental concentration, the highest RQ values in the months investigated, and toxic

units suggested the possibility of harmful effects on aquatic ecosystems in the summer tourist season. Caffeine, citalopram, diclofenac, E1, E2, E3, and EE2 presented at least a medium risk at least once during the whole period of investigation in Lake Balaton, the largest shallow lake in Central Europe, based on MAX RQ results. There is a real need for ongoing water quality monitoring and repeated toxicological testing for PhACs to ensure the real risk levels are understood. Besides, during our work, we found several discrepancies in raw ecotoxicological data; therefore, we propose to develop a unified predicted no effect concentrations database, including data regarding habitats, endpoints, and compounds, ensuring reliable and comparable results for ecological risk assessment [4].

3. Effect of drug contaminations

Based on drug monitoring results, *Lymnaea stagnalis* as aquatic model animal was exposed to chronic psychoactive drug treatments (carbamazepine, citalopram, alprazolam) to reveal what kind of alterations are induced in the *Lymnaea* CNS at system levels. Effects to locomotion activity were tested examining the synergistic effects of the active substances. The antidepressant CIT did not present any detectable effect. The anxiolytic ALP decreased the locomotion activity in the chronic exposure but this effect was disappeared after cleaning period. The antiepileptic CBZ using environmentally relevant concentration already decreased the locomotion activity of snails after first treatment week but this was also eliminated during cleaning period. However, CBZ-ALP combination caused a prolonged effect in locomotion activity, since this effect was also observed after the 21 days long cleaning period.

Furthermore, study of a complex adaptive behavioral process such as associative learning and memory was conducted. To this, food-reward classical conditioning with amyl acetate and gamma nonalactone (as conditioning stimuli) as well as sucrose (as unconditional stimulus) were applied. The long-time memory (LTM) formation was significantly attenuated by 1 μg/L CBZ and ALP due to 21 days chronic treatment and not improved even after 21 days cleaning period. The CIT had no effect to the LTM formation. Interesting observation was that no remarkable acts were present due to the combined drug treatments. Furthermore, comparing the learning scores after treatments (21st day of treatments) with the learning scores of recall experiment, respectively, the

learning scores significantly decreased in the control, CIT and mix groups while it significantly increased in the CBZ group.

Based on our results we conclude that the environmentally psychoactive contaminations could affect the behavioural activity of non-targeted organisms.

The single neuron analysis by capillary microsampling combined off-line nanoESI MS method have been optimizing to *Lymnaea stagnalis* neurons (Figure 1). The analysis of identified target neurons in the three experimental groups was also launched.

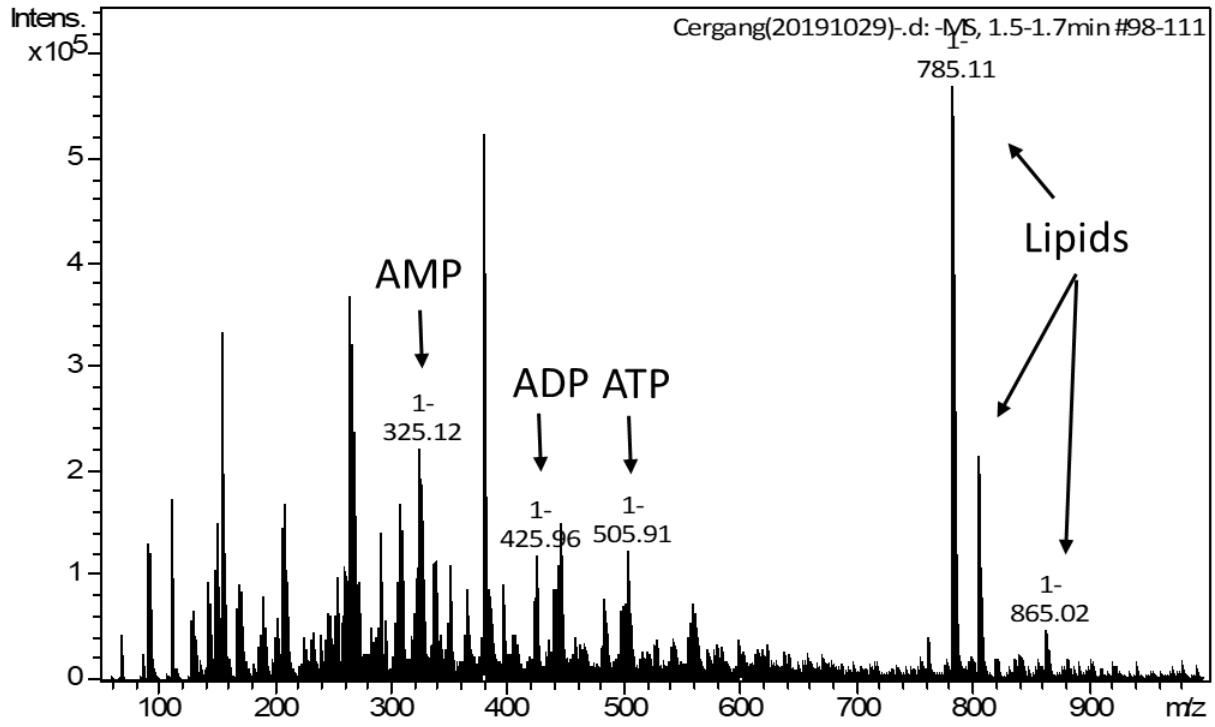

Figure 1 *Representative mass spectra of single neuron derived from cerebral ganglia of Lymnaea stagnalis*

But, based on the identified component list (Table 1) we realized that the number of identifiable component is limited due to the strongly limited sample volume. To the realistic investigation of effect of psychoactive drugs, application of a more sensitive instrumentation could be required.

ID	m/z_{meas}	I	I_{rel} (%)	Name	Ion Formula	m/z_{calc}	Δm (mDa)	HMDB Link
1	303,07	130589	2,2	arachidonic acid with interference	[C20H32O2-H]-	303,233	-163	http://www.hmdb.ca/metabolites/HMDB0001043
2	181,97	112193	1,9	carnitine	[C7H15NO3+Na-2H]-	182,0799	-110	http://www.hmdb.ca/metabolites/HMDB0000062
3	791,17	90552	1,5	EEEEEE	[C30H44N6O19-H]-	791,258	-88	Local database
4	164,00	81978	1,4	phenylalanine	[C9H11NO2-H]-	164,0717	-72	http://www.hmdb.ca/metabolites/HMDB0000159
5	662,15	637013	10,8	EEEEE	[C25H37N5O16-H]-	662,216	-66	Local database
6	611,09	662735	11,2	oxidized glutathione	[C20H32N6O12S2-H]-	611,145	-55	http://www.hmdb.ca/metabolites/HMDB0003337
7	606,02	585207	9,9	UDP-N-acetyl hexoseamine	[C17H27N3O17P2-H]-	606,074	-54	http://www.hmdb.ca/metabolites/HMDB0000290
8	533,13	3083622	52,2	EEEE	[C20H30N4O13-H]-	533,173	-43	Local database
9	203,04	76420	1,3	tryptophan	[C11H12N2O2-H]-	203,0826	-43	http://www.hmdb.ca/metabolites/HMDB0000929
10	187,03	353223	6,0	acetylglutamine	[C7H12N2O4-H]-	187,072	-42	http://www.hmdb.ca/metabolites/HMDB0006029
11	227,16	380076	6,4	myristic acid with interference	[C14H28O2-H]-	227,2017	-42	http://www.hmdb.ca/metabolites/HMDB0000806
12	534,14	775349	13,1	EEEE ^{13}C isotope peak	[C19^{13}CH30N4O13-H]-	534,173	-33	Local database
13	158,03	80410	1,4	tyramine	[C8H11NO+Na-H]-	158,0587	-29	http://www.hmdb.ca/metabolites/HMDB0000306
14	325,19	5543484	93,8	hydroxydesogestrel in background	[C22H30O2-H]-	325,217	-27	http://www.hmdb.ca/metabolites/HMDB0060719
15	297,16	1828335	30,9	norethindrone in background	[C20H26O2-H]-	297,186	-26	http://www.hmdb.ca/metabolites/HMDB0014855
16	404,11	390173	6,6	EEE	[C15H23N3O10-H]-	404,131	-21	Local database
17	255,22	1533772	26,0	palmitic acid with interference	[C16H32O2-H]-	255,233	-13	http://www.hmdb.ca/metabolites/HMDB0000220
18	156,02	186640	3,2	adenine with interference	[C5H5N5+Na-2H]-	156,029	-9	http://www.hmdb.ca/metabolites/HMDB0000034
19	565,04	93677	1,6	UDP-hexose	[C15H24N2O17P2-H]-	565,048	-8	http://www.hmdb.ca/metabolites/HMDB0000286
20	215,01	510704	8,6	glucuronic acid	[C6H10O7+Na-2H]-	215,017	-7	http://www.hmdb.ca/metabolites/HMDB0000127
21	283,26	409640	6,9	stearic acid with interference	[C18H36O2-H]-	283,2643	-4	http://www.hmdb.ca/metabolites/HMDB0000827
22	346,06	322586	5,5	AMP	[C10H15N5O7P-H]-	346,056	4	http://www.hmdb.ca/metabolites/HMDB0000045
23	311,17	5908069	100,0	hydroxyethinylestradiol In background	[C20H24O3-H]-	311,165	5	http://www.hmdb.ca/metabolites/HMDB0061027
24	306,09	104643	1,8	glutathione	[C10H17N3O6S-H]-	306,076	14	http://www.hmdb.ca/metabolites/HMDB0000125
25	243,10	107483	1,8	biotin with interference	[C10H16N2O3S-H]-	243,0803	20	http://www.hmdb.ca/metabolites/HMDB0000030
26	173,03	252758	4,3	xanthine with interference	[C5H4N4O2+Na-2H]-	173,0081	22	http://www.hmdb.ca/metabolites/HMDB0000292
27	157,04	99679	1,7	ascorbic acid	[C6H8O6-H2O-H]-	157,0137	26	http://www.hmdb.ca/metabolites/HMDB0000044
28	243,10	107483	1,8	uridine with interference	[C9H12N2O6-H]-	243,062	38	http://www.hmdb.ca/metabolites/HMDB0000296
29	662,15	637013	10,8	NAD	[C21H27N7O14P2-H]-	662,102	48	http://www.hmdb.ca/metabolites/HMDB0000902
30	266,14	429459	7,3	adenosine with interference	[C10H13N5O4-H]-	266,0895	50	http://www.hmdb.ca/metabolites/HMDB0000050
31	266,14	429459	7,3	deoxyguanosine with interference	[C10H13N5O4-H]-	266,0895	50	http://www.hmdb.ca/metabolites/HMDB0000085
32	242,15	98379	1,7	cytidine with interference	[C9H13N3O5-H]-	242,0782	72	http://www.hmdb.ca/metabolites/HMDB0000089
33	304,14	84110	1,4	guanosine	[C10H13N5O5+Na-2H]-	304,066	74	http://www.hmdb.ca/metabolites/HMDB0000133
34	267,15	310787	5,3	inosine with interference	[C10H12N4O5-H]-	267,0735	76	http://www.hmdb.ca/metabolites/HMDB0000195
35	259,10	95839	1,6	hexose phosphate	[C6H13O9P-H]-	259,022	78	http://www.hmdb.ca/metabolites/HMDB0001401
36	322,13	82426	1,4	CMP	[C9H14N3O8P-H]-	322,045	85	http://www.hmdb.ca/metabolites/HMDB0000095
37	241,18	334111	5,7	thymidine with interference	[C10H14N2O5-H]-	241,083	97	http://www.hmdb.ca/metabolites/HMDB0000273
38	323,14	210976	3,6	UMP	[C9H13N2O9P-H]-	323,029	111	http://www.hmdb.ca/metabolites/HMDB0000288
39	506,10	76252	1,3	ATP	[C10H16N5O13P3-H]-	505,989	111	http://www.hmdb.ca/metabolites/HMDB0000538
40	328,17	76425	1,3	glutathione	[C10H16N3O6S+Na-2H]-	328,058	112	http://www.hmdb.ca/metabolites/HMDB0000125
41	579,19	77750	1,3	UDP glucoronic acid	[C15H22N2O18P2-H]-	579,027	163	http://www.hmdb.ca/metabolites/HMDB0000935
42	442,21	135984	2,3	GDP	[C10H15N5O11P2-H]-	442,017	193	http://www.hmdb.ca/metabolites/HMDB0001201

Table1 Identified component from the Lymnaea stagnalis single neuron.

The results about the environmental psychoactive drug screening and environmental risk assessment of the detected psychoactive drug contaminations were published in SCI and other journals (**2 indicators**):

[1] Maász Gábor, Zrínyi Zita, Molnár Éva, Takács Péter, Pirger Zsolt (2018) “Tisztább, mint az átlag” - folytadódnak a gyógyszermaradvány felmérések a Balatonban https://www.bli.okologia.mta.hu/node/10758

[2] Maasz Gabor, Mayer Matyas, Zrinyi Zita, Molnar Eva, Kuzma Monika, Fodor Istvan, Pirger Zsolt, Takács, Péter (2019) Spatiotemporal variations of pharmacologically active compounds in

surface waters of a summer holiday destination. Science of Total Environment, 677:545-555 (IF:5.589, **D1**)

[3] Gabor Maasz, Eva Molnar, Matyas Mayer, Monika Kuzma, Péter Takács, Zita Zrinyi, Zsolt Pirger, Tibor Kiss (2020) Risk assessment of illicit drugs on the aquatic environment after a major music festival, *Environmental Toxicology and Chemistry* (IF:3.267, **Q1**)
under review (minor revision)

[4] Eva Molnar, Gabor Maasz, Zsolt Pirger (2020) Environmental risk assessment of pharmaceuticals at a seasonal holiday destination in the largest freshwater shallow lake in Central Europe. *Environmental Science and Pollution Research* (IF:3.054, **Q1**)
https://doi.org/10.1007/s11356-020-09747-4

The results about drug screening, ecological risk assessment and investigation of direct effect of psychoactive compounds on invertebrate neurons were presented at 5 domestic and 6 international conferences (**11 indicators**):

1) Éva Molnár, István Fodor, Péter Takács, Zita Zrínyi, Mátyás Mayer, Zsolt Pirger, Gábor Maász: Analytical measurement of active pharmaceutical ingredients in Lake Balaton and its catchment area. Környezettoxikológiai Munkabizottság előadóülése, MTA VEAB székház (Veszprém), 30.05.2018. (Oral presentation)

2) Molnár E., Fodor I., Takács P., Zrinyi Z., Kuzma M., Mayer M., Pirger Z., Maász G.: The environmental impact of summer social events on the largest shallow lake in central Europe. 40th International Conference on Environmental and Food Monitoring (ISEAC-40), Santiago de Compostela (Spain), 19-22.06.2018. (Poster presentation)

3) Gabor Maasz: Investigation of direct effect of psychoactive compounds on invertebrate neurons in real time, EFMC-YMCS, Ljubjana (Slovenia), 2018.09.06-07. (Oral presentation)

4) Molnár Éva, Fodor István, Takács Péter, Zrínyi Zita, Kuzma Mónika, Mayer Mátyás, Pirger Zsolt, Maász Gábor: Gyógyszerhatóanyag koncentrációk felmérése a Balatonban és annak vízgyűjtő területén a szezonális hatások figyelembevételével. LX. Hidrobiológus Napok, Tihany (Hungary), 2018.10.03-06. (Oral presentation)

5) Maász Gábor, Molnár Éva, Kuzma Mónika, Mayer Mátyás, Zrínyi Zita, Fodor István, Takács Péter, Pirger Zsolt: „Tisztább, mint az átlag”- folytatódnak a gyógyszermaradvány felmérések a Balatonban, Fiatal analitikusok XXVI. Előadóülése, Budapest (Hungary), 2018.11.12. (Oral presentation)

6) Maász Gábor: „Tisztább, mint az átlag”- gyógyszermaradvány felmérések a Balatonban ‚Magyar Tudomány Ünnepe 2018, Kutatóhelyek Tárt Kapukkal- Határtalan tudomány- Ökológia a tudománykommunikáció fókuszában, Budapest (Hungary), 2018.11.20. (Oral presentation)

7) Maász Gábor, Máyer Mátyás, Zrínyi Zita, Molnár Éva, Kuzma Mónika, Fodor István, Pirger Zsolt, Takács Péter: Gyógyszerhatóanyag maradványok vízminőségi és ökológiai kockázatának vizsgálata, 20. Kolozsvári Biológus Napok, Kolozsvár (Románia), 2019.04.12-13. (Oral presentation)

8) Gábor Maász, Istvan Fodor, Eva Molnar, Zita Zrínyi, Reka Svigruha, Richard Udvardi, Zita Laszlo, Tibor Kiss, Zsolt Pirger: Monitoring of environmental psychoactive drug contaminations and investigation of the induced neuronal changes, 1st Symposium on Invertebrate Neuroscience, Tihany (Hungary), 2019.08.13-17. (Poster presentation)

9) Gabor Maasz: Investigation of water pollution and environmental risk occured by pharmaceutically active compounds, 2nd International Environmental Chemistry Congress, Anatalya (Turkey), 2019.10.31-11.03. (plenary lecture as oral presentation)

10) Maász Gábor, Fodor István, Molnár Éva, Zrínyi Zita, Svigruha Réka, Kiss Tibor és Pirger Zsolt: A környezetből kimutatható pszichoaktív hatóanyag szennyezések felmérése és az általuk indukált neuronális változások vizsgálata a nagy mocsári csiga (Lymnaea stagnalis) központi idegrendszerében, IX. Ökotoxikológiai Konferencia, Budapest, Hungary, 2019.11.22. (poster presentatoin)

11) Gabor Maasz, Istvan Fodor, Eva Molnar, Zita Zrínyi, Reka Svigruha, Richard Udvardi, Zita Laszlo, Tibor Kiss, Zsolt Pirger: Monitoring of environmental psychoactive drug contaminations and investigation of the induced neuronal changes, IBRO Workshop, Szeged, Hungary, 29-30 January, 2020.01.29-30. (poster presentation)