Selection of chemical biomarkers for the risk assessment of exposure to food contaminants using wastewater samples analysis (LZP-2020/2-0128)

Wastewater-based epidemiology (WBE) is a novel approach to sampling and analysing chemical substances in wastewater (WW) samples to measure a population's consumption of or exposure to chemicals. This methodology was proposed for the first time as a potential tool to assess the use of illicit drugs and misused therapeutic drugs within a community in 2001 (Daughton, 2001). The major advantage of WBE is that it enables estimation of chemical exposure or consumption as a function of time, providing crucial information on the relation between compounds loads in WW, public health, and socioeconomic status for specific sewer sheds. WBE could provide the analysis of temporal trends, therefore gathering valuable information about population chemical consumption or exposure over time, such as changes in drug use in response to government interventions (Choi et al., 2019). In addition, the spatial assessment can provide insights into differences in population lifestyles or behaviour, such as the prevalence of illicit drug consumption in different cities and regions (Van Hal, 2019). Recently several studies were undertaken to extend the application areas of WBE, such as evaluation of human exposure to food contaminants (biomarkers of exposure to pesticides, polyfluoroalkyl substances (PFAS) and mycotoxins) and even fruit and vegetable consumption considering biomarkers of vitamin and lignan consumption (Been et al., 2017, Gracia-Lor et al., 2018, Bowes and Halden, 2019). Despite the latest development, several open scientific issues remain that need to be developed in order to successfully apply WBE in new areas (Gracia-Lor, 2020), including insufficient sensitivity and missing information on the stability of selected biomarkers in WW treatment plants (WWTP).

The project implemented by a team of scientists of the University of Latvia led by Prof V Bartkevics created new knowledge in relation to the application of WBE for the assessment of human exposure to food contaminants (PFAS and mycotoxins) and fruit, meat and vegetable consumption considering the content of relevant biomarkers in WW samples collected from WWTP in Latvia. The targeted determination of mycotoxins biomarkers was performed to evaluate human health status and indicate potential impacts and risks. Studies conducted within the project on the stability of biomarkers in WWTP will facilitate the applicability of WBE in new areas related to risk assessment activities.

The main outputs/outcomes of the project included:

Mass spectrometry-based methods (FT-ICR-MS, Orbitrap, QqQ) were used to assess human exposure to food contaminants (PFAS and mycotoxins) and dynamics of fruit and vegetable consumption considering the content of relevant biomarkers in WWs. The project contributed to the knowledge of public health, food safety by monitoring sewage for PFAS in several cities in Latvia and for mycotoxins in the WWTP of Riga.

The results of the project were summarised in four research papers. The following scientific topics were reviewed:

1) The WBE-based approach was employed for exploring human exposure to selected mycotoxins. This is the first study tracking human exposure to mycotoxins using the population size biomarker 5-hydroxyindoleacetic acid (5-HIAA). A sensitive analytical methodology was developed to achieve reliable quantification of deoxynivalenol, enniatins, and beauvericin in wastewater (WW) samples. The applicability of the method was evaluated by testing 29 WW samples collected at WW treatment plants in Latvia.
   Publication (open access journal, impact factor 4.546): Berzina, Z.; Pavlenko, R.; Jansons, M.; Bartkiene, E.; Neilands, R.; Pugajeva, I.; Bartkevics, V. Application of Wastewater-Based Epidemiology for Tracking Human Exposure to Deoxynivalenol and Enniatins. Toxins. 2022, 14, 91. https://doi.org/10.3390/toxins14020091
2) The review paper was prepared regarding the applications of nano-LC separation methods coupled with mass spectrometry for the analysis of food and environmental (including WW) samples. An assessment of sample preparation methods and analytical performance was provided, along with a comparison to other, more established analytical techniques. Three main groups of compounds that are crucial for food safety assessment were considered in this review: pharmaceuticals (including antibiotics), pesticides,

and mycotoxins. Recent practical applications of the nano-LC method in the determination of these compounds were discussed.
Publication (impact factor 6.535): Fedorenko, D.; Bartkevics, V. Recent Applications of Nano-Liquid Chromatography in Food Safety and Environmental Monitoring: A Review. Crit Rev Anal Chem. 2021 Aug 15:1-25. https://doi.org/10.1080/10408347.2021.1938968

3) This review paper summarised available information on the distribution of PFASs and their levels in different food, with a special interest in data from Europe. The current legislation and estimated dietary intake by the general population are described since this data could be useful in WW monitoring programmes. A critical evaluation of performance characteristics of the reviewed analytical methodologies revealed the insufficient sensitivity of quantification procedures for accurate risk assessment according to the guidelines proposed by EFSA. Therefore, the WW monitoring could be a good alternative to the existing risk assessment approaches.
Publication (impact factor 7.086): Pasecnaja, E.; Bartkevics, V.; Zacs D. Occurrence of selected per- and polyfluorinated alkyl substances (PFASs) in food available on the European market - A review on levels and human exposure assessment. Chemosphere. 2022 Jan; 287 (Pt4):132378. https://doi.org/10.1016/j.chemosphere.2021.132378
4) The paper overviewed occurrence data of 17 perfluoroalkyl substances (PFAS) (including 10 perfluorinated carboxylic acids and 7 perfluorinated sulfonic acids) in WW samples collected from 43 wastewater treatment plants (WWTPs) located in different cities in Latvia. Samples were collected in the period June-July 2021. Extraction and clean-up of the samples were performed using solid-phase extraction (SPE) on a weak-anion SPE phase. Observed extracts were analysed using high-performance liquid chromatography coupled with Orbitrap high-resolution mass spectrometry (HPLC-Orbitrap-MS) on the content of selected PFAS representatives. Information will be useful for human and environmental risk assessment studies.
Publication was submitted to Data in Brief journal (CiteScore 1.7) and now is being reviewed: Zacs, D.; Pasecnaja, E.; Bartkevics, V. Data on occurrence of perfluoroalkyl substances in influents and effluents collected from different wastewater treatment plants in Latvia. Data in Brief (submitted in 2021)

Outcomes of the project implementation

The project activities were implemented in the frame of four work packages (WPs).

WP1 "Development and implementation of analytical methods"

Literature-based analysis of the current state of instrumental techniques and the assessment of the occurrence data was done. Three scientific papers have been prepared and published in the frame of WP1:

- Fedorenko, D.; Bartkevics, V. Recent Applications of Nano-Liquid Chromatography in Food Safety and Environmental Monitoring: A Review. Crit Rev Anal Chem. 2021 Aug 15:1-25;
- Pasecnaja, E.; Bartkevics, V.; Zacs D. Occurrence of selected per- and polyfluorinated alkyl substances (PFASs) in food available on the European market - A review on levels and human exposure assessment. Chemosphere. 2022 Jan; 287 (Pt4):132378.
- Berzina, Z.; Pavlenko, R.; Jansons, M.; Bartkiene, E.; Neilands, R.; Pugajeva, I.; Bartkevics, V. Application of Wastewater-Based Epidemiology for Tracking Human Exposure to Deoxynivalenol and Enniatins. Toxins. 2022, 14, 91.

The paper published in the *Toxins* journal was especially important for the project objectives. The estimated total daily intake for enniatins was in the range of 1.8–27.6 µg/day per person. Free deoxynivalenol (DON) was determined in all analysed WW samples. Based on the average 5-HIAA excretion level and the determined 5-HIAA content in the samples, the intake of DON by the human population of Riga was estimated at 325 ng/kg b.w. day. These figures correlated with exposure levels observed in other scientific projects:

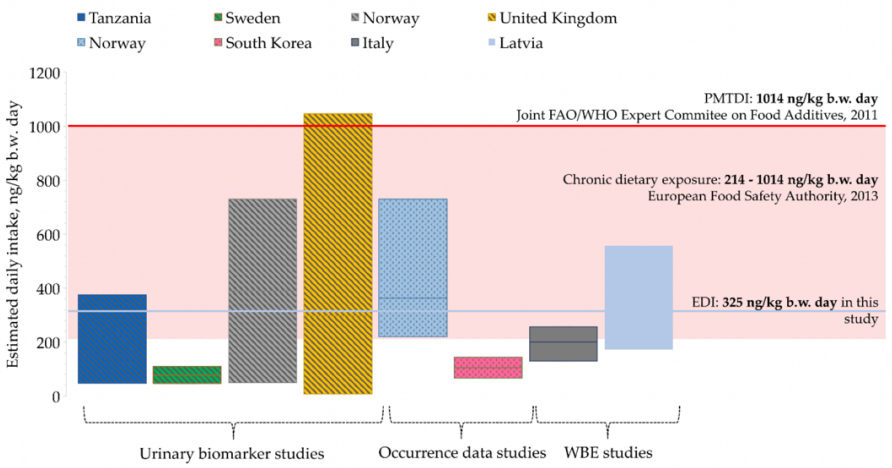
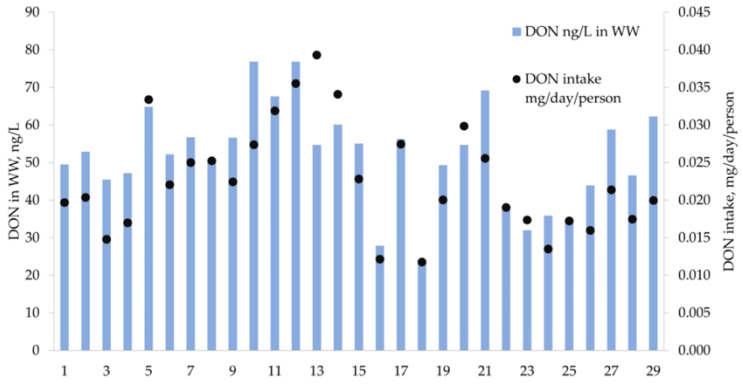


Fig.1. Comparison of the estimated daily intake of DON with other studies and with the reported health-based guidance values.

WP2 Method application for occurrence studies of biomarkers of food related contaminants and food consumption in WWs

Instrumental methods developed in WP1 were applied for the assessment of biomarkers in real samples collected regularly from WWTP in Riga, Latvia (for mycotoxins and food consumption biomarkers) and from other WWTP in difference cities in Latvia (PFAS). The analysis of 5-HIAA was used as a marker of population load. Differences in the content of selected biomarkers are given in Figure 2 (data for biomarkers of fruit and meat consumptions are not published yet).

A



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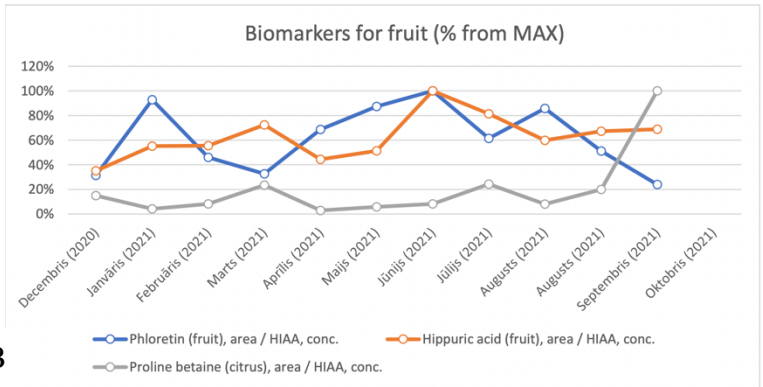


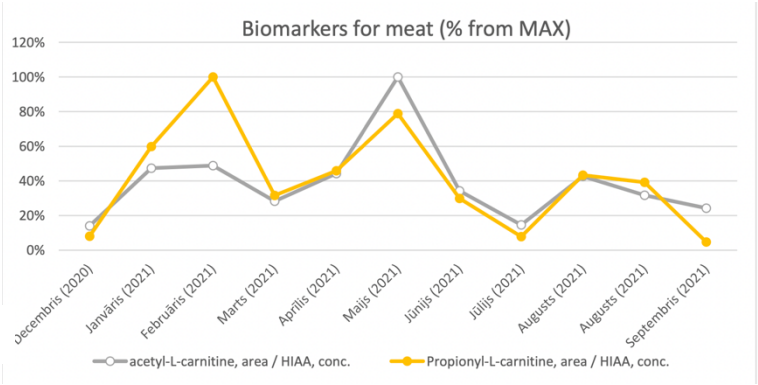
C 

Fig.2. Changes in the content of different biomarkers (A – DON, B – fruit consumption, C – meat consumption) in WW sampled from December 2020 to September 2021 (meat and fruit) and 29 days (DON)

WP3 "Stability of biomarkers related to food consumption and contaminants in WWs"

The fate of recalcitrant micropollutants in wastewaters (WW) was studied under laboratory conditions, using municipal WW, sewage-derived microbial community, and DON as a model of the most resistant and prominent mycotoxins. The main tested factors, which were supposed to influence the DON removal from WW, were: the nutrient/enzyme amendment, bioaugmentation, temperature, and initial concentration of DON with 300 ng/L to be the lowest concentration. For bioaugmentation the microbial biomass has been obtained from the sewage sludge by sonication and further cells concentration. This type of inoculum is considered to represent the real biodiversity of sewage microbiome harbored by flocs and biofilms.

The preliminary experiments on comparing the different sources of microbial consortia (e.g., sludges from municipal and industrial WWTPs, historically contaminated lakes, wheat-derived endophytes) did not show any considerable differences in DON removal after 7 days of incubation at the initial DON concentration of 300 ng/L.

The main batch experiments were performed in 300 mL columns with 100 mL WW, at 23 °C for 7 days with periodical shaking, in triplicate. In the presence of 300 ng/L DON, the results showed a slight increase of DON removal in the set with microbial consortium, as compared to the non-amended set, i.e., 8.4 % and 5.7 %, respectively. Nevertheless, these differences were not statistically significant. The addition of enzymes (laccase and cellulase) did not stimulate the DON degradation (Fig.3A). The addition of nutrient amendments, as well as incubation at 4 °C did not influence the DON degradation under tested conditions (data not shown).

The next series of experiments with different DON concentrations, i.e., in the decimal increasing range from 300 ng/L to 300,000 ng/L, showed a considerable increase of DON removal with increasing initial DON concentrations (from 25 ng/L to 39,140 ng/L in the sets with 300 and 300,000 ng/L, respectively) (Fig.3B). However, for all tested DON concentrations, the removal in % remained in the range from 8% to 10%. Biodegradation of DON was recently studied by (Chlebicz and Śliżewska, 2020). Authors reported about DON removal for 24h up to 19-39% with *Lactobacillus* spp. and 22-43 % with yeast *Saccharomyces cerevisiae*, respectively. Importantly, the initial concentration was 100 mg/L DON. Therefore, the data on DON degradation are not comparable if initial DON concentrations differ substantially. In case of our experiments, the tested concentration range of DON was close to DON appearance in the real WW (i.e., <300 ng/L). To support the data on DON biodegradation at initial DON concentrations above 3,000 ng/L, the changes of microbial enzyme activity have been revealed. Specifically, the microbial fluorescein diacetate hydrolysis and dehydrogenase activity were significantly ($p<0.05$) higher in the sets with DON concentrations ≥3,000 ng/L, comparing with those in the presence of 300 ng/L DON. These observations demonstrated a microbial physiological response towards DON. Most probably, stimulation of microbial enzyme activity by DON could serve as additional marker for optimization of biodegradation conditions in further studies.

The obtained data confirmed the project hypothesis on the stability of DON in ng/L concentrations for its serving as a biomarker.

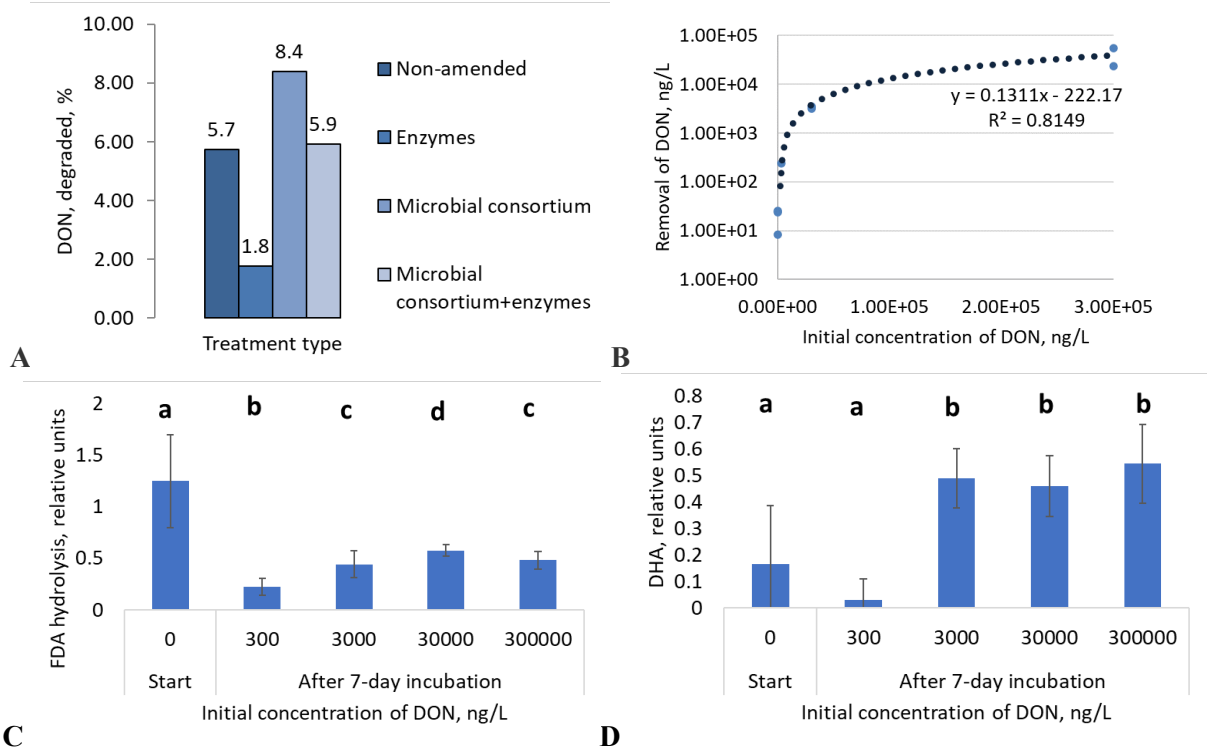


Fig.3. Removal of deoxynivalenol (DON) and microbial enzyme activity in municipal wastewater after the 7-day batch experiment under laboratory conditions. A – effect of different treatment types on DON removal with the initial DON concentration of 300 ng/L; B – effect of different initial DON concentrations on DON removal (300; 3,000; 30,000, and 300,000 ng/L DON); C - fluorescein diacetate (FDA) hydrolysis by microorganisms; D – dehydrogenase (DHA) activity of microorganisms.

WP4 "Selection of new biomarkers based on non-targeted screening"

Some changes were introduced to the initially planned implementation of non-targeted screening for the structure identification of potential biomarkers in analysed WWs. Instead of this approach, the suspect screening procedure was implemented for the following list of compounds (see Table No.6) using the Orbitrap HRMS technique. All compounds except for TMAO (a biomarker of fish consumption) were revealed in WW samples. The dynamics of food consumption biomarkers is given in Fig 2B and 2C obtained within WP2.

Table No. 6

Compound	**Biomarker for**	**Formula**	**[M+H]+**	**[M-H]-**	**Analytical column and ionisation**
2-Py	Vitamin B6	C7H8N2O2	153.0659	151.0513	C18+
4-Py	Vitamin B6	C7H8N2O2	153.0659	151.0513	C18+
alpha-CEHC	Vitamin E	C16H22O4	279.1591	277.1445	C18-
Enterodiol	Fiber	C18H22O4	303.1591	301.1445	C18+
Enterolactone	Fiber	C18H18O4	299.1278	297.1132	C18+
Proline betaine	Citrus	C7H13NO2	144.1019	142.0874	HILIC (+/-)
4-Pyridoxic acid	Vitamin B6	C8H9NO4	184.0604	182.0459	HILIC (-)
Hippuric acid	Fruit	C9H9NO3	180.0655	178.0510	HILIC (-)
Phloretin	Fruit	C15H14O5	275.0914	273.0768	C18-
DHPPA	Wholegraines	C9H10O4	183.0652	181.0506	C18-
TMAO	Meat, fish	C3H9NO	76.0757	74.0611	HILIC (+)
CMPF	Meat, fish	C12H16O5	241.1071	239.0925	HILIC (-)
3-methylhistidine	Meat	C7H11N3O2	170.0924	168.0779	HILIC (+/-)
L-Anserine	Meat	C10H16N4O3	241.1295	239.1150	HILIC (+/-)
L-Carnosine	Meat	C9H14N4O3	227.1139	225.0993	HILIC (+/-)
Acetyl-L-carnitine	Meat	C9H17NO4	204.1230	202.1085	HILIC (+/-)

Propionyl-L-carnitine	Meat	C10H19NO4 (C10H20NO4+)	218.1387	216.1241	HILIC (+/-)

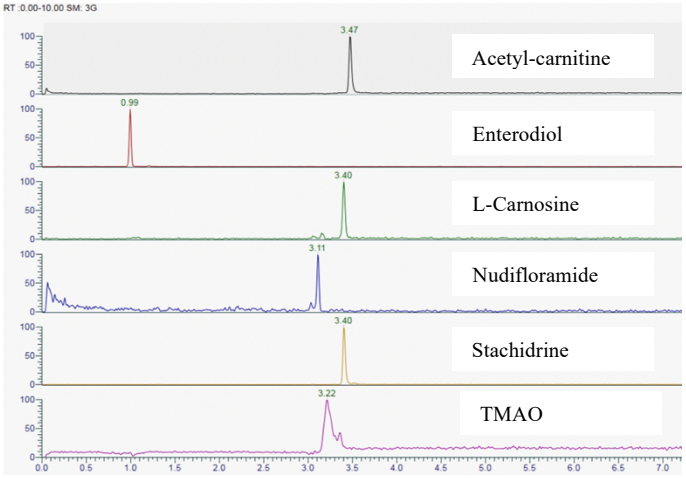


Fig 4. “dilute&shoot” HPLC-MS analysis of WW sample with spiked amount of 250 ng/L

Further experiments with the analysis of these food biomarkers in WW samples indicated that the “dilute&shoot” approach could be used for the determination of targeted compounds applying HPLC-QqQ-MS/MS technique (data no published). This makes this direction of scientific research especially promising since the analysis could be combined with the determination of other biomarkers (i.e. population size, selected pharmaceuticals and life-habits indicators).