Working Group “YELLOW”
Environmental pollution monitoring and adverse effects of chemicals used in food production
(FoCUS – Food Chemicals Used Safely)

ABSTRACT

This project addresses the need to assess the environmental and health risks of chemicals used in food production, since their release into the environment may lead to different ecological effects. The occurrence and effects of selected chemicals on wildlife and humans will be addressed to provide data sets, which are necessary for scientifically-based risk assessment. Special emphasis will be put on the combined effects of environmentally relevant mixtures. A combination of state-of-the-art methods will be applied to predict synergistic and/or additive effects of combined exposure. Development and implementation of new technologies for waste treatment and re-use of food industry by-products by converting them into value-added items will also be a significant task. The project will also generate new knowledge on environmental risks and well-defined adverse effects, and will provide evidence-based arguments for future recommendations and regulations.

KEYWORDS

Food production
Chemicals
Toxicity
Waste management
Sustainable development

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SOCIAL CHALLENGES IDENTIFIED

- Food security, sustainable agriculture and forestry, marine, maritime and inland water research and bioeconomy
- Health, demographic change and wellbeing

DANUBE RIVER BASIN CHALLENGES IDENTIFIED

- No more risk from toxic chemicals
- Healthy and sustainable river system

BACKGROUND

PROBLEM DEFINITION

The food industry sector (including agriculture) has a crucial role in the European Union (EU) economy, employing over 48 million people and representing more than one fifth of the EU’s total workforce. It generates the equivalent to almost 6% of the EU’s total GDP, with close to 17 million different enterprises operating in the “food chain”. The food and beverages processing sector has a turnover close to EUR 1 trillion making it the largest manufacturing sector in the EU economy, also being the leading employer in the EU, with 4.1 million staff, representing 15% of the total employment in the manufacturing sector.

The impacts of food industry and agriculture on freshwater systems are numerous, and the problem of river pollution resulting from agriculture and food processing industry is a subject of extensive research. However, the problem with food industry and agriculture is that it introduces a number of novel and alien substances, which are being released into the environment, therefore emphasizing the importance of the constant development of new monitoring methods. Even though, in an ideal world, it would be possible to take an individual agricultural/industrial activity, and precisely measure its effects on a certain species and its traits, this level of precision is not always possible in practice. Determining realistic environmental concentrations of chemicals in flowing water bodies is extremely difficult, as routine monitoring may miss peak concentration periods. Also, there are still significant knowledge gaps with regards to the effects that chemical mixtures have on the river systems, primarily on aquatic wildlife, but also on humans who depend on these water sources. Furthermore, the toxicity of substances used varies depending on the
species, its genotype, life stage and size, as well as on natural stressors affecting the species, such as food shortage, oxygen depletion and the pressure of potential predators. The current analysis of environmental status are mostly based on physicochemical methods, which do not provide enough information about many-sided interactions between different compounds which influence toxicity, thus the crucial task for permanent environmental monitoring would be to include bioassays. Therefore, toxicity studies should be based upon physicochemical analyses, biomonitoring and standardized biotests using several different organisms on different trophic levels of biological organization, ranging from bacteria to vertebrates.

This project would use the combined approach, evaluating the effects of both agriculture and food processing industry, as its aim is to assess the overall impact of food production on the environment. Substances resulting from both of these sources are simultaneously present in water systems, and have a synergistic effect on the biota living in the watersheds which are thus contaminated. Therefore, the advantage of such an approach would be to provide the relevant institutions with more realistic results of the actual state of the environment.

As it is said to be ‘the most international river basin in the world’, the Danube River Basin (DRB) has been subjected to significant pollution over the course of a long period of time, and also nowadays due to the poorly developed infrastructure and outdated environmentally unfriendly agricultural practices applied in certain parts of the basin. As the European Commission’s Water Framework Directive (EU Directive 2008/105/EC) requires that all aquatic systems in Europe are to be restored to ‘good ecological quality’ by 2015, this has completely changed the perspective on agriculture and its effects on the water systems. One of the main tasks assigned to the Danube Regional Project, in the preparation for the Danube Basin Analysis, was to identify significant point and diffuse sources of pollution. Since the analysis of point source pollution requires the existence of a complete inventory of point sources with data of high quality covering the whole catchment area, including the most significant sub-catchments. The data obtained as a result of this project would provide the future assessments with a starting point from which the valuable data on the most important pollution sources in the region could be drawn.

This project would be developed in accordance with the general objective of food law stating that “food law shall pursue one or more of the general objectives of a high level of protection of human life and health and the protection of consumers’ interests, including fair practices in food trade, taking account of, where appropriate, the protection of animal health and welfare, plant health and the environment” (General principles and requirements of Food Law, Regulation 178/2002, art. 5).
The DRB represents the foundation of the Black Sea Ecological Network, and as such its sustainable development is of the outmost importance for the social-economic wellbeing and environmentally sound development of the countries forming part of this river basin.

This project will be implemented in the Danube River region of the Pannonian Basin and Carpathian-Balkanian Basin which include Bosnia and Herzegovina, Bulgaria, Croatia, Hungary, Romania, Serbia, Slovakia and Ukraine.

The project results and findings can also be extended onto the entire Danube region since environmental pollution, regular monitoring and waste management issues are of high relevance for the entire DRB. Specifically, in addition to the formal project objectives an extra added value of this international collaboration will be the dissemination of the project findings onto the entire region and the creation of an international ecological network which will share data from the studies conducted in the DRB area.

The project will significantly increase the awareness of agricultural workers, food production industry and selected authorities and stakeholders on the issues of environmental contamination and negative impacts of chemical pollution, and provide evidence-based arguments for future recommendations and regulations, while in that manner promoting the ecological and sustainable development of the selected parts of the DRB, with the possible extension onto the entire Danube region and other regions of interests.

The areas of expertise required for the project implementation will be geography, chemistry, biology, toxicology, public health, biomedicine, ecology, civil engineering, policy, economics and public relations.

PROJECT GOAL AND OBJECTIVES

GOAL

The overall project goal is to prevent water and soil pollution in the DRB, particularly of the Pannonian Basin and Carpathian-Balkanian Basin (including Bosnia and Herzegovina, Bulgaria, Croatia, Hungary, Romania, Serbia, Slovakia and Ukraine), coming from the chemicals used in the entire chain of food production from agriculture to food industry, and consequently their adverse effects. Furthermore, the development and implementation of the new technologies for the prevention of chemical contamination, appropriate waste management and re-use of food production by-products will be done.
OBJECTIVES

1. To determine the occurrence of chemicals used in food production, their metabolites and transformation products in water and soil systems in the DRB particularly in the Pannonian Basin and Carpathian-Balkanian Basin.

2. To evaluate a set of well-defined adverse effects of the chemicals used in food production on different plant and animal experimental models and compare the data to those obtained in human experimental models.

3. To evaluate combined effects of mixtures of chemicals used in food production, their excreted metabolites and transformation products which are formed in the environment.

4. To develop and implement new technologies for waste treatment and re-use of food industry production by-products in order to convert them into value added items.

5. To develop and implement guidelines and new regulations regarding the use and disposal of certain chemicals used in food production, based on the project results, in order to prevent negative effects that certain chemicals have on the environment, wildlife and human health if used extensively and/or disposed inappropriately.

Fig. 1. Map of the Danube River Basin with the selected study area of the Pannonian Basin and Carpathian-Balkanian Basin which includes Bosnia and Herzegovina, Bulgaria, Croatia, Hungary, Romania, Serbia, Slovakia and Ukraine.
Fig. 2. Land use in the selected study area of the Pannonian Basin and Carpathian-Balkanian Basin as part of the greater Danube River catchment area

Fig. 3. Position of the agricultural sites and industry in the selected study area of the Pannonian Basin and Carpathian-Balkanian Basin as part of the greater Danube River catchment area
PROJECT ACTIVITIES IN REGARD TO SELECTED OBJECTIVES
(WORK PACKAGES)

WP1: PROJECT MANAGEMENT

In order to implement the project successfully and meet our overall objective, a solid management foundation needs to be established from the beginning. The first phase will be setting up the procedures and methodologies which will help us ensure the timely delivery of the results, the coordination of all included activities in regard to the milestones, and the connection between inputs and outputs. This step refers to the management of the consortium (WP1) when all partners will meet in order to set up a clear framework for workshops and the final conference, communications, meetings, intermediary reports, progress monitoring and evaluation, create and agree upon abovementioned procedures, and signing of the agreement with all the partners regarding the planned activities for their engagement in the implementation of certain WP.

Also, there is a need to establish the communication methods in order to monitor/track the progress of all partners especially with respect to the overall project milestones. If necessary, more intense communication will be possible for certain partners working on interdependent WPs, according to their work requirements.

WP1 will include the following activities: 1) leader team establishment, 2) kick-off meeting, 3) setting up of the project governance structures and procedures, 4) workshops with experts in which they will be asked to give best practices, 5) collecting data and information, in this respect, all the partners will have to develop a concrete plan with their own milestones in order to ensure the implementation and delivery of the expected results within the set time-frame, 6) workshops and intermediary reports after major deliverables, 7) ensuring legal and financial administration of the project, 8) ensuring monitoring and evaluation of all included activities, 9) providing the assessment reports and indicators, 10) development of a dissemination plan, especially in regard to the policy recommendations, after collection, analyses and interpretation of the data, further steps will be to elaborate the policy recommendations, write reports, contribute to dissemination materials and send articles for publication, 11) the final conference with stakeholders (state agencies, industries related to food production, food processing, small and medium enterprises (SMEs), farmers, policy-makers).
WP2: Spatial trends and occurrence of chemicals used in food production in water and soil systems using chemical analysis and bioassays

The objective of WP2 is to establish the spatial trends, occurrence and persistence of chemicals used in the entire chain of food production from agriculture to food industry in water and soil samples from the DRB particularly in the Pannonian Basin and Carpathian-Balkanian Basin. Detailed distribution of agricultural fields and food industry in the selected area will be mapped using Geographic Information Systems (GIS), while tracing of the chemicals will be done by chemical analytical methods and bioassays.

Within WP2 analytical chemistry methods and bioassays for the determination of chemicals used in food production, their excreted metabolites and transformation products will be developed, validated and applied to environmental samples. Chemicals of interest are pesticides (insecticides, herbicides, fungicides, rodenticides), pharmaceuticals (antibiotics), hormones (xenoestrogens), food additives (colorants, conservants, aromas) and chemicals derived from packaging materials (polypropylene, polystyrene, polyvinyl chloride (PVC), polyethylene terephthalate (PET), microplastics). Since plastic materials present a serious environmental hazard, with significant levels of accumulation in virtually all habitats, with large rivers acting as major pathways for plastic litter, special emphasis will be put on the assessment of the adverse effects of microplastics. After determining occurrence of chemicals of interest they will be classified according to their chemical structure and/or mechanisms of action.

To evaluate possible contamination of the surrounding surface waters, ground water and soil systems, samples will be collected around the hot-spots of agricultural and food industry production and analysed with the tools of analytical chemistry using high-performance liquid chromatography (HPLC) and MALDI-TOF mass spectrometry. Moreover, biomonitoring studies of watercourses using transplants of the aquatic moss (*Rhynchostegium riparioides* Hedw.) will be conducted as well. Mosses are excellent bio-accumulators of trace elements and can solve problems with detection limits of instruments, namely the concentrations of certain chemicals in water which are often below the instrumental limit or rapidly change in space and time. This will also enable the development, validation and future application of suitable methods for analysis of environmental samples.

Another objective of WP2 is to identify metabolites and transformation products of selected chemicals used in food production, and to chemically and toxicologically characterize the selected environmental samples. This will be done and simulated in laboratory conditions where the formation of stable transfor-
Information products will be followed. Following the identification and adaptation of analytical methods for metabolites and the characterisation of transformational products, their presence will be determined in the environment.

**WP3: Effects of Chemicals Used in Food Production in Plant and Animal Experimental Models**

The objectives of the WP3 are detailed toxicological studies on plant and animal experimental models. Results of single chemical exposure will provide data necessary for risk assessment of complex mixtures in the framework of the WP5.

Acute and chronic toxicity of the chemicals used in food industry will be done on algae and plants. The toxicity for algae will be determined by growth inhibition using freshwater green algae (*Pseudokirchneriella subcapitata*) as indicator species. The adverse effects in higher plants will be determined by measuring growth retardation, DNA damage and micronuclei (MNI) induction in root tips of common onion (*Allium cepa*) and duckweed (*Lemna minor*).

Toxic effects will be further evaluated on invertebrate and vertebrate animal experimental models. This will be done on a series of aquatic and terrestrial animal experimental models. The short- and long-term effects of chemicals used in food production will be done on several species of aquatic organisms such as crustacean (*Daphnia magna*) and fresh mussel (*Unio pictorum*) species as representatives of invertebrate model organisms. Fresh mussels are commonly found in the Danube River and can be used as bioindicators of exposure due to their ability to filter and accumulate harmful substances. Studies on mussels will be performed on hemolymph cells.

Tests will also be done on terrestrial organisms to evaluate the soil exposure. Effects of chemicals used in food production will be studied on earthworms (*Lumbricus terrestris*), which will provide a notable contribution to the field of terrestrial ecotoxicology. Furthermore, studies will be done on honey bees (*Apis mellifera*) as they are also of great importance from an agricultural perspective since they contribute actively to the pollination of cultivated crops and wild vegetation, making food production possible. Their sensitivity and vulnerability to chemically-induced impacts should be studied in order to reduce undesirable side effects of harmful substances on the environment to a minimum and demonstrate the importance of making these chemicals (primarily pesticides) as selective as possible.

Additionally, studies will be done on zebra fish (*Danio rerio*), a species widely used as a model in toxicity and genotoxicity studies. Zebra fish study
will be done on two generations to show the impact on the offspring. Studies will be conducted on blood, liver, gill and gonad cells, and in embryos, larvae, juvenile and adult stage to obtain the results regarding developmental toxicity as well. Studies will also be done on Wistar rats (Rattus norvegicus) commonly used in laboratory studies, to assess sensitivity to different chemical agents and/or naturally occurring substances, thus obtaining the results which can later on be extrapolated to human exposure, and connected with the results from WP4.

The adverse effects on animal experimental model will be determined by measuring DNA and chromosomal damage. Induction of DNA damage after exposure to chemicals used in food production will be measured by using comet assay and γ-H2AX assay. Further chromosomal disturbances will be assessed by using cytokinesis-block micronucleus (CBMN) assay measuring the induction of MNi, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs).

In addition, the mechanisms of selected chemically induced genotoxicity will be evaluated by examining the gene expression patterns of selected genes involved in metabolism, DNA damage responsive genes, genes involved in oxidative stress and apoptosis using real-time quantitative PCR (QRT-PCR). This will provide the relationship between transcriptome alterations and observed toxicological effects.

Moreover, histopathology will be included in the assessment of adverse effects on animal models for the selected chemicals since application of histopathology can be extended as an additional instrument or endpoint in toxicity studies, as histopathological organ and tissue changes can provide pivotal diagnostic and mechanistic features.

Due to the fact that not all of the methods used in WP3 are listed in the guidelines for evaluation of toxic exposure the specific aim of WP3 will be the standardization and optimization of the conducted methods/biomarkers for further biomonitoring studies.

**WP4: Effects of Chemicals Used in Food Industry on Different Model Cell Lines in Regard to Human Exposure**

The objective of the WP4 will be detailed toxicological characterization of chemicals used in food industry on different human cell lines. Results of single chemical exposure will provide data necessary for risk assessment of complex mixtures in the framework of the WP5.

For the human impact, studies will be conducted in vitro on primary human blood cells (human peripheral blood lymphocytes, HPBLs) as these cells are
sensitive biomarkers of exposure and human hepatoma HepG2 cells derived from the liver tissue. The studies on human hepatoma cells will enable the prediction of the level of hepatotoxicity caused by chemical exposure.

The effects of selected chemicals on different model cell lines will be assessed at the level of cyto- and genotoxic responses. Induction of DNA damage as a result of exposure to chemicals used in food production will be measured by using comet assay and $\gamma$-H2AX assay. Further chromosomal disturbances will be assessed by using CBMN assay measuring the induction of MNi, NPBs and NBUDs.

In addition, the mechanisms of genotoxicity induced by the selected chemicals will be evaluated through the examination of the gene expression patterns of selected genes involved in metabolism, DNA damage responsive genes, genes involved in oxidative stress and apoptosis using real-time quantitative PCR (QRT-PCR). This will show the relationship between transcriptome alterations and observed toxicological effects.

Due to the fact that not all of the methods used in WP4 are listed in the guidelines for evaluation of toxic exposure the specific aim of WP4 will be the standardization and optimization of the conducted methods/biomarkers for further biomonitoring studies.

WP5: Risk Assessment of Complex Mixtures, Metabolites and Transformation Products

The objective of WP5 will be to identify most commonly present chemicals used in food production, either coming from agriculture or actual food processing industry. Furthermore, the aim will be to identify specific metabolites and transformation products of selected chemicals used in food production. This will enable selection of model compounds to be included in the studies aiming to evaluate possible synergistic, additive and/or antagonistic effects of chemicals used in food production in mixtures and their relative contribution to observed toxicity of real environmental samples. This will also be done using the data on single chemical exposure based on the results obtained in WP3 and WP4.

The chemicals used in food production which will be included in analytical chemistry and toxicological studies regarding exposure to mixtures of chemicals will be selected according to the available literature about the presence of certain chemicals used in food industry and the data obtained in WP2. Based on those data, defined mixtures of chemicals will be prepared. The mixtures will be tested for toxicity, genotoxicity and changes in gene expression profiles which will be compared to the changes in profiles induced by single
compounds in order to explore the possibility for predicting the synergistic, additive and/or antagonistic effects of the mixtures.

The isobologram analysis method will be used for the assessment of mixtures of chemicals to determine the extent of their synergism using the results of single agent exposure and combination group.

**WP6: Development of New Technologies for Waste Treatment and Re-use of Food Industry Production By-products**

The objective of the WP6 will be to develop and implement new technologies for waste treatment and re-use of food industry production by-products by converting them into value-added items. This will be done in accordance with the results obtained in the WP2 to WP5.

In the framework of WP6, an introduction of SMEs will be included, with the special emphasis on regional SMEs aiming to promote regional development. The SMEs will be expected to introduce new technologies and put them into practice for the development of filtering systems necessary in the food production industry in order to minimize the environmental exposure to the chemicals used in food production proven to be an ecological and health burden. Moreover, the development and synthesis of novel chemicals or the use of naturally occurring substituents instead of chemicals used so far in food production will be one of the objectives of WP6.

**WP7: Dissemination**

The information generated in the framework of the FoCUS project will be disseminated mainly towards previously identified national and supranational authorities and businesses involved in the field of food production, food processing and agriculture, in order for them to be more sustainability oriented. Part of the authorities at EU level involved in the process are DG Agriculture and Rural Development, DG Environment, DG Enterprise and Industry, DG Research and Innovation, and DG SANCO (Directorate General for Health and Consumers). From the national level, the national authorities responsible for agriculture, industry and environmental issues (ministries and chambers of industry) will be involved.

The final conference will be organized and a final report will be delivered to the European Commission and to the stakeholders. Through this final report, the integrated review of the results of the research and the policy proposal
will be presented. The promotion of the results through mass media, project website and networking will be managed. The educational purposes of the project will be covered through scientific events and research papers, available through the scientific databases (Open Access).

**Fig. 4. The organization of the project organised into 7 technical work packages (WP1 to WP7)**
SPECIFIC RESULTS AND CONTRIBUTIONS OF THE PROJECT

• Contribution to improved risk assessment for plants, animals, humans and ecosystems for chemicals used in food production

• Contribution to the protection of wildlife and citizens from adverse effects through the strengthening of national mechanisms for surveillance and response to health threats

• Contribution to legal framework amendment in regard to the environmental and health safety norms

• Contribution to Water Framework Directive, European Environmental Protection Agency and REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals)

• Contribution to relevant EU and national bodies, policies and strategies


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