EXTERNAL SCIENTIFIC REPORT

A foresight study on emerging technologies: State of the art of omics technologies and potential applications in food and feed safety[1]

REPORT 1

Review on the state of art of omics technologies in risk assessment related to food and feed safety

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ABSTRACT

This report consists of a review of the available scientific evidence on the state of art of omics technologies with a particular focus on applications in the area of food and feed safety. The report gives a description of the different types of omics technologies with respect to recent progresses made on the application of these techniques in chemical and microbiological risk assessment. Hereto, case studies were selected in order to cover different areas under the remit of EFSA. In this review each case study has been investigated with respect to its hazard, experimental methodologies, (statistical) data analysis and its link to food/feed safety risk assessment. Finally, the added value of omics technologies as compared to classical risk assessment and current limitations of omics technologies for their application in chemical and microbiological food safety risk assessment has been discussed. This investigation show that most of the studies on chemical agents focus on mode of action identification and a search for biomarkers using transcriptomics. As for microbial food safety, omics have not yet been successfully applied often. When applied, they never target the host, but focus on aspects of the pathogen, e.g. in detection or source attribution.

KEY WORDS

Omics, food safety, risk assessment, mode of action, biomarker, new methods.

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SUMMARY

This report consists of a review of the available scientific evidence on the state of art of omics technologies with a particular focus on applications in the area of food and feed safety.

The report gives a description of the different types of omics technologies with respect to recent progresses made on the application of these techniques in chemical and microbiological risk assessment. Hereto, 61 case studies were selected in order to cover different areas under the remit of EFSA. In this review, the selected agent in each case study has been investigated with respect to: its hazard (to human or the environment); experimental methodologies (e.g. omics technique, test model, experimental conditions, classical effect identifiers); (statistical) data analysis (e.g. methods to significantly identify differentially expression of genes/proteins/metabolites/… compared to a control); and aspects of risk assessment (e.g. hazard identification, exposure assessment, risk characterisation, dose-response assessment, biomarker identification, mode of action identification). The report lists current international projects in the field of omics related to food and feed safety. Finally, the added value of omics technologies as compared to classical risk assessment and current limitations of omics technologies for their application in chemical and microbiological food safety risk assessment has been discussed.

Case study investigations show that most of the studies on chemical agents focus on mode of action identification and a search for biomarkers using transcriptomics. Although these are biologically relevant parameters for hazard characterization, statistically significant causal relationships cannot be drawn. Experimental set-up and (statistical) data analysis need to be aligned between studies in order to make the step to a public health risk estimate possible. For chemicals, the studies identified provide useful information for hazard identifications, i.e. clarification of the mode of action. None of the studies, however, applied multiple exposure doses including doses near to actual exposure levels. Therefore, the value of these studies for a full risk assessment is very limited. Omics have not yet been consistently applied to microbial food safety. When applied, they normally target the host, but focus on aspects of the pathogen, e.g. in detection or source attribution.

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BACKGROUND AS PROVIDED BY EFSA

A key objective of EFSA is the evaluation of new methodologies and technologies for risk assessment applied to food and feed safety (EFSA, 2009a). These may present complex methodological challenges for risk assessment as well as opportunities for emerging risk identification, impacting EFSA's mid- to long-term work.

Firstly, new and emerging technologies can raise questions relating to information gaps (*e.g.* reliability and interpretation of the results, data quality standards) and methodological uncertainties (*e.g.* integration of new knowledge in the existing risk assessment framework). Secondly, scientific and technical innovation is a well established driver of change linked to the food and feed chain, with potential implications, in the case of omics, for the identification of emerging risks (EFSA, 2009a; EU, 2002). For example, new analytical methods can improve our capability of identifying new hazards, new or increased exposures, or groups of the population whom may be more susceptible to certain environmental contaminants.

In the post-genomic era, the scientific community is now witnessing major advances in Omics technologies (*e.g.* genomics, proteomics, metabonomics, toxicogenomics, etc.)(Herrero *et al*., 2011; Kean, 2011). Omics technologies are firmly established as research tools, and are gaining credibility also in risk assessment, particularly toxicology, as they may offer certain advantages over traditional approaches (Aardema and MacGregor, 2002; Borner *et al*., 2011; Chassy, 2010; Knasmuller *et al*., 2008). Compared to traditional methods, omics technologies appear to combine the benefits of relative simplicity, sensitivity and speed of generating information, potentially reducing the need for animal testing.

Emerging risks have been defined by the scientific committee of EFSA as follows "an emerging risk to human, animal and/or plant health is understood as a risk resulting from a newly identified hazard to which a significant exposure may occur or from an unexpected new or increased significant exposure and/or susceptibility to a known hazard". A growing body of evidence is now becoming available on the application of omics for the identification of new hazards and emerging risks, including toxicological screening and prediction of chemical substances, and the identification of new and reliable biomarkers. Omics technologies have already been applied to the food and feed safety area, for example in the identification of biomarkers (Kussmann *et al*., 2006; Oozeer *et al*. 2010), the elucidation of mechanisms of action of toxic chemicals, the identification and screening of new and emerging contaminants in food (Lancova *et al*., 2011), the evaluation of nutritional health claims (Bagchi *et al*., 2010; Fukuda *et al*., 2011; Knasmuller *et al*., 2008), the safety assessment and evaluation of substantial equivalence of GMOs (Chassy, 2010; EFSA Panel on GMO, 2010; Ricroch *et al*., 2011), the detection and characterisation of foodborne pathogens (Fratamico, 2008), and the investigation of the association between diet and cancer risk (Ross, 2011).

Whilst, omics may have major implications for EFSA's scientific activities, current methodological and analytical uncertainties do not yet allow the identification of how and to what extent Omics technologies can be integrated within the current risk assessment framework, and to which extent they can be fully exploited for emerging risk identification.

At an international level, risk assessment bodies, including US-EPA, WHO, and OECD are currently starting to consider the integration of omics in their risk assessment frameworks, mainly in the field of mechanistic toxicology (EPA, 2004; OECD, 2011).

Thus, this procurement aims to critically review the state of the art of omics technologies applied to food and feed safety, in order to understand possible future implications for risk assessment and emerging risk identification in the areas under EFSA's remit.

TERMS OF REFERENCE AS PROVIDED BY EFSA

A review of the available scientific evidence on the state of art of Omics technologies. The objective of this review will be to address recent (*e.g.* last 5-10 years) advances of omics technologies with a particular focus on applications in the food and feed safety area.

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1. Introduction and Objectives

This procurement aims to critically review the state of the art of omics technologies applied to food and feed safety, in order to understand current and possible future implications for risk assessment and emerging risk identification in the areas under EFSA's remit. The main objectives of the contract resulting from the present procurement include a review of the scientific literature on the state of the art of omics technologies applied to risk assessment, with a focus on food and feed safety and a foresight study on the potential future developments of omics technologies and possible implications for risk assessment in food and feed safety.

This report describes deliverable 1 of the assignment, which consists of a critical review of the available scientific evidence on the state of art of omics technologies in the area of food and feed safety. This review will form the basis to understand possible future implications for risk assessment and emerging risk identification in the areas under EFSA's remit. The report gives a description of the different types of omics technologies with respect to recent progresses made on the application of these techniques in chemical and microbiological risk assessment. Hereto, 61 case-studies were selected in order to cover different areas under the remit of EFSA. In this review, the selected agent in each case study has been investigated with respect to: its hazard (to human or the environment); experimental methodologies (e.g. omics technique, test model, experimental conditions, classical effect identifiers); (statistical) data analysis (e.g. methods to significantly identify differentially expression of genes/proteins/metaboles/… compared to a control); and aspects of risk assessment (e.g. hazard identification, exposure assessment, risk characterisation, dose-response assessment, biomarker identification, mode of action identification). The report also lists current international projects in the field of omics related to food and feed safety. Finally, the added value of omics technologies as compared to classical risk assessment is discussed and current limitations of omics technologies for their application in chemical and microbiological food safety risk assessment are summarized.

1.1. General introduction to the application of omics techniques

Omics is a suffix indicating "a totality of some sort" (the Oxford English Dictionary), which in biology is used for very large-scale data collection and analysis, i.e. measuring/profiling a large number of variables simultaneously. Omics allows to study the mode of action of compounds or to obtain more insight in processes involved in diseases (Dulin *et al.*, 2013). Omics technologies can be divided in three main categories: genomics, proteomics, and metabolomics/metabonomics (Gehlenborg *et al.*, 2010).

Genomics techniques are used to analyse the structure and function of genomes by sequencing large sets of DNA within a cell (including genetic polymorphisms), profiling whole genome mRNA and miRNA expression (transcriptomics), and profiling epigenetic modifications on the genetic material (epigenomics)(Paoloni-Giacobino, 2011). High-throughput sequencing (or next-generation sequencing [NGS]) technologies are now available producing thousands or millions of sequences at once. Proteomics deals with cell and tissue-wide protein expression. Thus, while genomics is mainly concerned with gene expression, proteomics analyses the protein products of the genes. Examples of proteomics techniques are two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) for protein separation and identification, respectively (Hodgson, 2012).

Metabolomics is concerned with the identification and quantification of all the metabolites in a biological system (cell, tissue, organ, organism). Profiling metabolites in blood and urine is also referred to as metabonomics. Metabonomics broadly aims to measure the global, dynamic metabolic response of living systems to biological stimuli or genetic manipulation. The focus is on understanding systemic change through time in complex multicellular systems. Metabolomics seeks an analytical description of complex biological samples, and aims to characterize and quantify all the small molecules in such a sample. In practice, the terms are often used interchangeably, and the analytical

and modelling procedures are the same (Nicholson and Lindon, 2008). Most commonly used metabolomics techniques are liquid chromatography–mass spectrometry (LC-MS), gas chromatography–mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) (Nicholson and Lindon, 2008).

Omics is also being applied on exploring the genome-wide influence of nutrition, looking into the functional effects of nutrients on the genome, patterns of gene expression and metabolic responses in response to dietary interventions" (Stenne *et al.*, 2013). The omics approaches have several unifying features that include:

- Generation of very large data sets in response to interrogation of an extensive system
- Absence of simple interpretations (particularly in relation to cause and effect) because of inherent complexity

Omics technologies offer the possibility to assess the effects of potential toxic components on many parameters including thousands of mRNAs, proteins, metabolites, imprinting of genes, alternative splicing of mRNAs and mutations. One major promise of these techniques is that they will further increase our knowledge about toxic mechanisms of actions on basis of which the hazard and potentially the risk of a compound can be assessed. The effects on gene expression alterations precede clinical effects. Therefore, mechanisms of action can be detected after short exposure times of animals or even with use of *in vitro* techniques that result in a reduction of both test animals and suffering of animals. To correlate omics results to hazard identification, two approaches are being used: 1) via biomarkers, and 2) by translation into mechanisms of actions (Currie, 2012; Hartung *et al.*, 2012).

For the biomarker approach, knowledge of the functions of the genes or metabolites is not essential. In general, biomarkers are detected by exposure of animals or cell systems to a large number of toxic model compounds for which often the mode of actions are known. In the case of transcriptomics, this provides gene signatures specific for certain compounds and for mode of action shared by groups of compounds. In the fields of proteomics and metabolomics, proteins or metabolites can be used to identify biomarkers of toxic effects. Examples of this approach that have been commercially exploited are databases of transcriptomics responses of the liver and kidneys of rats to a large number of liver- or kidney toxicants (Ganter *et al.*, 2005; Natsoulis *et al.*, 2008). Expression data of unknown compounds can be loaded in this database after which the toxicity is predicted on basis of similarity to mRNA expression of the model compounds. *In vitro*, the so called Connectivity Map enables a comparison of the mRNA response of the breast cancer cell line MCF7 to an unknown compound to that of 1300 small compounds (Lamb *et al.*, 2006).

For the second approach, identification of the mode of action, knowledge about the functions of the affected genes is required. In the case of transcriptomics, groups of genes of which the mRNA expression is affected by a treatment are analysed on overrepresentation in pathways, processes or organelles. Examples of processes are cell proliferation, differentiation, oxidative stress, apoptosis, cholesterol synthesis, endoplasmatic reticulum (ER) stress, inflammation and DNA damage response. It is also possible to detect more specific effects, for example, (in)activation of transcription factors like NFAT, estrogens receptor, NFkB, or T cell antigen receptor (D'Eustachio, 2011; Katika *et al.*, 2011). The results on affected processes are certainly informative but this approach has also disadvantages. The main problem is that presently the functions of a relatively large proportion of the genes are not yet known, making biological interpretation for these genes impossible (Wang *et al.*, 2011). Obviously, these genes will not be included in gene sets representing pathways and processes and will therefore be disregarded for biological interpretation. Genes with unknown function will, however, be included in the biomarker approach. In addition, many processes will have an overlap in genes. For example, many DNA repair genes will be present both in the gene set "DNA repair" and in the gene set "cell cycle". Many genes up-regulated by ER stress are also up-regulated by other stress responses. The biological interpretation of pathway analysis should thus be done with caution and be confirmed by biochemical or cytological experiments (de Mello *et al.*, 2012; Katika *et al.*, 2012).

In short, omics techniques contribute strongly to hazard identification since these techniques put forward hypotheses on modes of action that direct the choice for confirmation experiments. For risk assessment, exposure limits have to be determined for e.g. NOAEL and LOAEL. In many cases, omics techniques will detect effects, for example on mRNA expression, of exposure levels lower than the NOAEL (Heneweer *et al.*, 2007). This implies that omics techniques have the advantage of being very sensitive but also the potential drawback of being too sensitive. Therefore, "phenotypic anchoring" will be important to link induction or repression levels of genes to clinical manifestation, and thus for its application in risk assessment.

1.2. Omics techniques applied in food and feed safety

At present, transcriptomics is the most frequently used omics technique used for food and feed safety since this is the most developed omics technology (Hartung and McBride, 2011). Most of these studies aim at hazard identification. Either cells *in vitro* or animals are exposed to doses of toxicants that are much higher than actual exposure levels (National Research Council (NRC), 2013). Results of these studies are informative about the modes of action of these toxicants, but do not add to risk assessment evaluations (National Research Council (NRC), 2013). Examples of transcriptomics studies aiming to risk assessment have been published by (Thomas *et al*., 2011; Thomas *et al*., 2013). In these studies, rats were treated with carcinogenic compounds and target organs were analyzed for traditional histological and organ weight changes and transcriptional changes using microarrays. Benchmark dose methods were used to identify non-cancer and cancer points of departure both for the traditional and the transcriptional changes. The main finding was that the benchmark dose for transcriptional changes of the most sensitive pathways did not occur at significantly lower doses than that of traditional parameters. However, the transcriptional changes could be detected much earlier (from day 5 onwards) than the traditional parameters (the rodent carcinogenesis bioassay takes in general two years) (Thomas *et al*., 2013).

Transcriptomics techniques have also been applied in toxicology to investigate the interaction between the genome and adverse biological effects induced by toxic compounds (Gatzidou *et al.*, 2007; van Vliet, 2011). Besides mechanistic information, gene expression profiling has been used to predict toxicity or to classify chemicals into different toxicity classes (Maggioli *et al.*, 2006). Because changed mRNA levels do not necessarily reflect the toxicity responses of a system, transcriptomics studies are often complemented by proteomics (Kikkawa *et al.*, 2005; Kikkawa *et al.*, 2006; Gao *et al.*, 2009) and metabolomics (Robertson, 2005).

Metabolomics is expected to have a strong role in the identification of the potential targets of toxicants (Ramirez *et al.*, 2013). It can give information on modes of action and target organs (Ramirez *et al.*, 2013). It can also detect the effects of endocrine disruptors, for example on steroid hormone synthesis (Rijk *et al.*, 2012).

Proteomics is for example applied to blood samples or urine of humans exposed to lead, cadmium and arsenic resulting in the finding of biomarkers that can be used to detect toxic effects on organs and progression of disease (Kossowska *et al.*, 2013). Proteomics has also been used to detect modes of action of toxicants using *in vitro* systems to detect effects on either protein levels or on phosphorylation of proteins (Osman *et al.*, 2010; Osman and van Loveren, 2012; Van Summeren *et al.*, 2012).

In addition, mode of actions of toxicants can include effects on miRNAs which are short non-coding RNAs that negatively regulate gene expression at the post-transcriptional level (Koturbash *et al.*, 2012).

1.2.1. Chemical risk assessment – the contribution of omics information

Chemical risk assessment, in relation to food and feed, is a well established process. Risk assessment includes four steps: 1) hazard identification and characterization, 2) dose-response assessment, 3) exposure assessment, and 4) risk characterization (Meek, 2011). In the majority of cases information relating to the effects of exposure to particular chemicals is collected, reviewed by experts and then used to support decisions. The decisions are often regulatory and prescribe thresholds, and limits, for the exposure of particular populations in particular scenarios (involving patterns of consumption etc.). Although this process is robust, and has operated effectively, there are several issues that drive a search for improvements.

Firstly, it is increasingly apparent that most of the information that is relevant to a particular exposure is subject to un-quantified uncertainties. That is, much of the information relevant to estimate human risk (e.g. dose-response data) is based on evidence from *animal models* and most of the information used for threshold quantification is extrapolated from evidence on relatively *high dose effects*. In addition, increasingly, the process of chemical risk assessment is challenged by scientific developments; these include the increased sensitivity of measurement techniques and an awareness of missing knowledge concerning the molecular mechanism of action of harmful substances. These shortcomings are compounded by an overwhelming complexity, driven by consumer demand for increased variety and quality, so that new chemicals of concern, and groups or mixtures, used in agriculture or processing or packaging or preservation arise continuously. In a recent report Thomas *et al*. (2012) indicated that only 1100 of 30,000 chemicals that are widely used in commercial operations had published risk assessments in the US Environmental Protection Agencies integrated risk information system.

Routine chemical risk assessment operates without inputs from omics based methodology although, over a substantial period, much genomics, proteomics and metabolomics research has been performed (and supported) on the basis of a relationship with safety assessment. It is widely believed that, in most cases, toxicity cannot occur without alterations at the transcription, protein or metabolite level but, despite progression in this field, it remains unclear how useful chemical safety information can be collected and harnessed from omics results.

The role of omics data, and particularly transcriptomics in chemical risk assessment has been reviewed in a series of reports and statements by the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (e.g. COT, 2011; 2012). COT reports summarize the status quo, highlight the challenges for interpretation and identify possible roles for omics in relation to chemical risk assessments. The COT survey conducted in late 2011 showed that the population of omics based reports that can contribute new findings to risk assessment is very sparse. A review of the evidence of the UK COT concluded that often the experimental design of omics studies precluded a role in risk assessment. Considerations of the mode of action of chemicals, qualitative risk assessments and generation of hypotheses were identified as the most likely outcomes for omics based studies relating to safety. The COT lists seven roles for omics in risk assessment;

- Aiding the assessment of toxicological mode of action (MOA)
- Informing on inter-species variability and extrapolations
- Modeling low-dose effects (with omics changes as possible biomarkers of overt effects that would occur at higher doses)
- Identification of biomarkers that can be measured more easily than, or in advance of pathology
- Interpreting or facilitating extrapolation between similar chemical structures (read-across)
- Candidate molecule comparison and selection (drug discovery)

- Aiding the development of *in vitro* models to replace animal models.

The UK COT has, additionally, collaborated with the US Food and Drugs administration and the US Environmental Protection Agency with regard to their experience with omics in the field of risk assessment (COT, 2011). An extensive EPA report (EPA, 2009a) describes an approach for evaluating omics data for use in risk assessment and includes an extended case study relating to dibutyl phthalate. The EPA report is, currently, the most complete examination of the relationship between omics and chemical risk assessment and it proposes some principles for a systematic approach which include examination of genomic and toxicity data together, defining a set of questions to direct the evaluation and performing new analyses of genomic data when necessary. The EPA report does not provide guide lines for incorporating genomic data into risk assessment and advocates a weight of evidence approach (i.e. non normative) in relation to decision support. The UK and US reviews identify significant challenges associated with quantification of risks based on omics datasets (e.g. Ein-Dor *et al*., 2006).

The availability of omics technologies potentially has a great impact on the safety analysis of food components. At present, the most often used tool is transcriptomics which is presently commonly done with the use of microarrays, but it is expected to be replaced by next generation sequencing in the near future due to the rapid decrease in prices (presently below €500 per sample). Transcriptomics enables to detect the response of cells or tissues to chemicals on the mRNA expression levels of all genes. Basically, two goals can be fulfilled: 1) insight in the mechanism of action of chemicals at the cellular and molecular level, and 2) the identification of possible biomarkers for toxic actions which may lead to bioassays. To obtain insight in the mode of action of chemicals, a biological interpretation of the mRNA expression alterations is essential, which requires knowledge about the functions of the affected gene products. As indicated before, knowledge on the function is desirable but not essential for biomarkers. When a gene repeatedly responds to a set of known toxic compounds and not to control compounds, the gene product (mRNA, protein, metabolite) can be successfully used in a bioassay.

Other omics tools used for toxicological analyses are microRNA profiling, proteomics and metabolomics. Examples of toxicological studies using these techniques will be mentioned in the review. Since at present the vast majority of omics studies used transcriptomics, the main focus of this review will be on transcriptomics studies.

1.2.2. Microbiological risk assessment – the contribution of omics information

Omics techniques in relation to microbiology are usually meant to indicate techniques applied to the microorganisms, not the hosts, or the foodstuffs they affect. In addition to the well-known omics, interactomics that are utilized to study the molecular interactions within the cell (Zhang *et al*., 2010), and fluxomics that focus on the dynamics of molecules (Kohlstedt *et al*., 2010), are also applied to microbes. Within microbiology the whole set of omics is primarily employed to understand how the living cells operate as a system. Up till now the potential of omics techniques for microbiological food safety has often been discussed, but in practice it has not been applied yet to this aim. Depending on one's definition of omics, the possible exception is genomics, as DNA sequencing has been used for taxonomic and tracing purposes.

The gold standard for food microbiology remains the ability to predict the safety and quality of the food on the plate of the consumer based on measurements during the production stages (Brul *et al*., 2008; Havelaar *et al*., 2010). For over a decade systems microbiology on the basis of omics has been predicted to fulfil this promise (Kay and Wren, 2009). Practical problems, however, have prevented the actual implementation, as the amount of data on the physiology of the organism under study, needed to make accurate predictions, is more than is available for any relevant species. The question that must be answered is how the organism is likely to behave in a certain matrix. Metabolomics can

in principle be utilized to furnish such part of a prediction, but this has only been achieved in a limited number of cases (Brul *et al*., 2008; Kohlstedt *et al*., 2010).

Two types of questions that are often asked within food microbiology can be addressed by omics. Genomics can be, and have been, used to 1) identify the species contaminating a foodstuff (O'Flaherty and Klaenhammer, 2011) or to verify the taxonomic status of an organism added as a processing aid (Pearson *et al*. 2009) and 2) to detect the presence of virulence factors or other genes of interest for food safety (Fang *et al*. 2010). Genomics, in particular Whole Genome Sequencing (WGS), can be used for both aims simultaneously during outbreak detection. To reveal a diffuse outbreak, relationships between strains recovered from patients have to be established to a level that allows to discern a common source. For this purpose WGS is particularly suitable.

In the field of microbiology the whole set of omics approaches have been used to understand how bacterial cells function as a living unit (Brul *et al*., 2008). The newly defined field of "foodomics" is defined as a new discipline that uses mass spectrometry to study food and nutrition, including the effect of microbes on the quality of foodstuffs, but not so much on safety (Herrero *et al*. 2011). Not only genomics, but also proteomics are being used to screen for foodborne pathogens (D'Alessandro and Zolla, 2012). Comparative genome analysis is used to investigate how pathogens adapt to a particular supply in specific foodstuffs (Bhagwat and Bhagwat, 2008). Integrated pan-genome comparisons can be used for source attribution during outbreaks based on the interaction of the accessory genome and the environment (Laing *et al*., 2011). It must be noted that however promising these developments may be, they have not been realized in everyday food microbiology to this date.

2. Achievements of omics technologies

Not only EFSA but also other organisations, such as FAO/WHO, OECD that discuss and develop guidelines for food and feed safety assessment want to have insight in the usefulness of new genomics technologies. This interest rises from the need for refinement-, reduction-, and replacement alternatives for animal testing (e.g. the 28-day and 90-day repeated dose studies with rodents currently performed according to OECD guidelines 407 and 408) not only from an ethical and economical point of view, but also to increase the predictivity of current tests.

Toxiconomics is the integration of omics technologies, bioinformatics methods and toxicology to study the response of the genome to hazardous substances. In a 2007 publication of the U.S. National Research Council of the National Academies (Toxicity Testing in the 21st Century: A Vision and a Strategy), the potential relevance of toxiconomics for risk assessment was already considered. In 2009, the U.S. EPA (U.S. EPA, 2009b) outlined, using dibutyl phthalate as case study, a framework for the use of toxiconomics in risk assessment.

It is now generally considered that in relation to chemical hazard/risk assessment toxiconomics provide tools to:

- understand mechanisms of toxicity/toxicological mode of action (MOA),
- determine QSARs (facilitate extrapolation between similar chemical structures),
- reduce the uncertainties with respect to extrapolation within and between species (inter/intra-species extrapolation, effects of susceptible populations), high to low dose, short-term to long-term,
- identify (early, sensitive, specific) biomarkers of exposure and effect (toxicity); early means before visible toxicity (pathology) occurs; sensitive means a biomarker for a visible effect that only would occur at higher doses,

- develop cost-effective alternative toxicity test methods, particularly *in vitro* systems that can refine, reduce, and replace animal experimentation (the 3 R's concept of "alternatives" of (Russell W.M.S., 1959).

Despite all the promises of toxiconomics, it is recognized that various issues have to be addressed before it can be applied for regulatory and risk assessment purposes.

Toxicogenomics is currently considered being useful in qualitative assessment (hazard identification), to generate hypotheses on MOA, and to guide the design of follow-up experiments. Most of the toxiconomics studies performed so far are not designed for the purpose of qualitative RA. So, a proper design of a toxiconomics study (e.g. the use of a range of doses to support dose-response modelling) is crucial to be able to perform quantitative RA.

For food microbiology few concrete achievements of omics technologies can be listed. This is likely to change soon, because WGS will most probably become a standard technique for identifying strains (Fang *et al*., 2010). The price of WGS is going down so rapidly that it has become a feasible technique, and a reference databank for foodborne pathogens is under construction by the FDA. The implementation of other omics techniques is more complex and therefore bound to require more time and effort. The main potential in addition to establishing relationships between strains for omics techniques lies in the prediction of the behaviour of strains in a given matrix. In particular, quite some research on the physiology of food pathogens will have to be performed before this potential can be fully realized (Brul *et al*., 2011). At the moment correlations between proteomics or transcriptomics and physiology have not yet been established.

3. Materials and Methods

For papers on the application of omics in chemical risk assessment, searches were performed covering the last 10 years in PubMed and Web of Science during May-December 2012. The syntax of the search string was as follows: (component) AND (transcriptom* OR proteom* OR metabolom* OR metabonom* OR microarray* OR omics). Up to 436 hits (cadmium) were found, but for most other compounds less than 150 hits. Our search string was quite general, therefore omics papers on plant development were also retrieved for some components. The earliest literature found dated from about 2000, but most contributions originated from 2005 and later. Because omics technology is rapidly developing, preference was given to the most recent papers. In addition, *in vivo* research had preference over *in vitro* research. Far more papers on transcriptomics than on metabolomics or proteomics were found.

Relatively few paper on the application of omics in food microbiology have been published. Hence, the search strategy was encompassing rather than selective. Initial searches using obvious search terms such as "*omics/genomics/transcriptomics" combined with "food microbiology/microbial food safety" yielded primarily review articles. The most recent of these reviews have been used to search for original papers. Subsequently, additional searches were performed using terms derived for the titles of both the review and the original articles. This yield very many papers that were mostly irrelevant to the topic and a selection was made based on reading parts of the articles. Given the scarcity of articles on omics in food microbiology, all relevant articles have been included.

4. Results

4.1. Contaminants

4.1.1. Cadmium

Hazard

Cadmium is a natural environmental element that has no known role for humans. Cadmium can be found in some food and water and accumulates in animals and plants. Cadmium is toxic, particularly in kidneys, but is also classified as a human carcinogen. Cadmium is also believed to induce genomic instability by inducing an increase in reactive oxygen species that damage DNA, and is associated with an increase in epigenetic changes (Filipic, 2012).

Cadmium levels in food and water are monitored and regulated by the EU. High urinary concentrations of cadmium are often the first signs of over exposure. A scientific opinion from EFSA (EFSA, 2009b) established a tolerable weekly intake ~2.5µg/kg bw (from the diet) and concluded that the current exposure to Cadmium at the population level should be reduced. The scientific opinion indicates that there are uncertainties associated with bioavailability of Cadmium and that urinary beta-2-microglobulin is a useful biomarker for kidney disorder.

The references identified includes 18 studies corresponding with Cadmium (Sanchez BC *et al.*, 2011; Wang B *et al.*, 2012; Ling X-P, 2009; Garrett SH, 2011; Espinoza HM, 2012; ZU *et al.*, 2012; Liu *et al.*, 2012; Lu *et al.*, 2012; Pierron *et al.*, 2011; Fabbri *et al.*, 2012; Zhang *et al.*, 2011; Hispard F et a, 2011; Benton *et al.*, 2011; Bakshi S *et al.*, 2008; Hossain *et al.*, 2012; Thompson EL *et al.*, 2012; Permenter MG *et al.*, 2011; Yu *et al.*, 2010). The omics studies include, dominantly, transcriptomics and proteomics and experiments with cell systems and animal experiments including fish, shellfish, rats and mice. The reports deals with liver tissues, gills and human proximal tubule cells. One study used human urine samples. In general the animal experimental conditions correspond with high doses of cadmium. In the case of fish and cell systems experimental conditions are difficult to compare with human exposures.

Results from omics experiments

Some of the transcriptomics investigations identify very large numbers (>1000) of individual gene expression changes in response to exposure to Cadmium exposure whereas proteome changes are less pronounced (typically 20 differentially expressed proteins). The gene lists are difficult to interpret, but there are several instances of up-regulated heat shock proteins. These results indicate a systematic pathway analysis and changes in pathways associated with energy metabolism, oxidative stress and protein synthesis are consistently identified.

Risk assessment

One study concerning the transcriptomics response of normal prostate epithelial cells (Bakshi *et al.*, 2008) fits many criteria in relation to risk assessment and a genomic study (Benton *et al.*, 2011) clearly maps differential expression onto pathway information. The omics studies are related equally to mode of action or biomarker identification.

- A transcriptomics investigation by Bakshi *et al*. (2008) links changes in cell growth with changes in gene expression for normal prostate epithelial cells exposed to low doses of Cadmium and identifies a transient over expression of TNF as part of the mode of action leading to cancer;

- A genomic approach, Benton *et al.* (2011), using lymphoblastoid cells establishes the influence of Cadmium exposure on important cellular pathways;
- Analysis of transcriptional response (Sanchez *et al*., 2010), in fish exposed to Cadmium, was used to explore novel biomarkers for mechanisms of toxic action, but cannot clearly quantify associated uncertainties;

Omics reports do not clearly address issues surrounding bioavailability of Cadmium, but, in combination with systems biology, have advanced an understanding of possible routes for carcinogenesis.

4.1.2. Mycotoxins: Aflatoxin B1

Hazard

Aflatoxins (Aflatoxin B1 is a major form) are produced by the fungus Aspergillus flavus and may occur in wheat, corn, barley and nuts (Woloshuk and Shim, 2013). The Scientific Committee for Food concluded that Aflatoxins are genotoxic carcinogens: only a zero level of exposure will result in no risk (EC, 1996). From many reports on risk assessment, it can be concluded that even very low levels of exposure to aflatoxins, i.e. 1 ng/kg bw per day still contribute to the risk of liver cancer (EFSA, 2007).

The following references were identified on Aflatoxin B1, using transcriptomics or metabolomics approaches (Doktorova *et al.*, 2012; Austin *et al.*, 2012; Ellinger-Ziegelbauer *et al.*, 2004; Josse *et al.*, 2012; Zhang *et al.*, 2011). Experiments were performed with mice (1) rats (3), primary rat hepatocytes (1) and primary human hepatocytes (1). All but one study report on effects on liver, one study reports effects on spermatogenesis in rats. The metabolomics study was performed with rat plasma, urine and liver after oral exposure.

Results of the omics experiments

Between 60 and 270 differentially expressed genes were reported. Affected genes in liver belonged to various pathways, e.g. DNA and mitochondrial damage, apoptosis, inflammation. In vivo and *in vitro* changes in gene expression (rat liver and primary rat hepatocytes) were comparable. Gene expression in human hepatocytes revealed that effects can be measured already at an early stage (24 h) which shows promises as biomarkers for genotoxic effects. The metabolic profile of plasma and urine in rats may predict liver damage (steatosis) by AFB1. AFB1 has been shown to disrupt spermatogenesis in mice, probably involving Renin.

Risk assessment

These omics data identify potential modes of action of AFB1 in liver and testis. However, implications for risk assessment cannot be drawn, because exposures followed a non-physiological route or were extremely high (up to 0.24 mg AFB1/kg bw/day). EFSA states that the benchmark dose lower limit for an extra 10% risk ($BMDL_{10}$) is 0.17 µg AFB1/kg bw/day (EFSA, 2007).

4.1.3. Mycotoxins: Deoxynivalenol

Hazard

Deoxynivalenol (DON) is a mycotoxin produced by several Fusarium species and is often detected in grains. Because of its high abundance, there has been a large interest in the effects of DON in animals and humans. There is no evidence for teratogenicity and genotoxicity of DON and its metabolites. Products of animal origin do not contribute significantly to human exposure (EFSA, 2004). DON is known to be immunosuppressive at high concentrations and immunostimulatory at low concentrations (van Kol *et al*., 2011). The Tolerable Daily Intake (TDI) of DON in humans is 1 µg/kg bw/day (EFSA, 2004).

The studies identified on DON used transcriptomics and proteomics approaches (Kinser S *et al.* 2004; Nielsen C *et al.*, 2009; Nogueira da Costa *et al.*, 2011; Osman *et al.* 2010; van Kol *et al.*, 2011). Experiments were performed with mice (2), human immune cells (1), human Hep-G2 cells (1), and mouse EL4 cells (1). The *in vivo* mouse experiments investigated the effects on thymus and spleen.

Results of the omics experiments

In murine thymus, differential gene expression was time dependent, and disappeared after 24 h. However, at high dose the effects persisted for at least 24h. Gene sets related to proliferation, mitochondria and ribosomes were affects. In murine spleen about 100 out of 1200 genes involved in leukocyte function, immunity and inflammation were significantly changed. In Hep-G2 cells up to 800 genes were differentially regulated, a 10-fold increase in dose increased the number of genes involved three times. Most of the up-regulated genes coded for transcription factors, and the major MAPK-kinase pathway was mainly involved. DON changed phosphoprotein expression in human immune cells. These proteins are involved mainly in signal transduction. In mouse thymoma cells DON affected a total of 30 proteins involved in fatty acid and glucose metabolism, protein degradation, IgE binding, and repression of a number of transcription factors.

Risk assessment

These omics data identify potential modes of action of DON in thymus, spleen and liver. Implications for risk assessment cannot be made, because exposures were extremely high (up to 25 mg DON/kg bw/day). Human exposure to DON was estimated to be close to the TDI of 1 µg/kg bw/day (EFSA, 2004).

4.1.4. Mycotoxins: Ochratoxin A toxin

Hazard

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species of the genera Penicillium and Aspergillus. Contamination of food commodities, including cereals and cereal products, pulses, coffee, beer, grape juice, dry vine fruits and wine as well as cacao products, nuts and spices, has been reported from all over the world. OTA has been found to be a potent renal toxin in all of the animal species tested (EFSA, 2006). In addition, OTA induces genotoxic effects that are most likely caused by oxidative stress due to the generation of free radicals. These effects may finally lead to kidney and liver tumours as observed in animal studies with rodents. The TWI has been estimated to 120 ng per kg body weight (EFSA 2006).

The following references were identified corresponding with OTA (Mantle *et al*., 2011; Yoon *et al*., 2009; Marin-Kuan *et al*. 2006; Luhe *et al*., 2003; Jennings *et al*., 2012), involving metabolomics, proteomics and transcriptomics studies.

Results from omics experiments

One study (Mantle *et al*., 2011) studied changes in metabolites in urine after giving a dose of 6.25 mg OTA which is equal to half of the acute LD_{50}, making this study irrelevant for risk assessment. For the proteomics study (Yoon *et al*., 2009), mouse hippocampal HT22 cells and human neuroblastoma SH-SY5Ywere exposed *in vitro* for 24h of treatment with OTA (100 µM). The findings on affected proteins support the identification of mode of actions, but do not allow to draw definitive conclusions on the risk assessment.

Two transcriptomics studies on liver and kidney (reported in one publication, (Marin-Kuan *et al*. 2006)) exposed male Fischer rats orally to OTA for up to 2 years. The levels of OTA were first 300 µg OTA/kg bw, and was held at 100 µg/rat after animals reached 333 g. These exposure levels are more than 1000-fold higher than tolerable weekly (TWI) intake for humans. Therefore, this study is of interest for hazard identification and identification of modes of action, but does not allow drawing conclusions about risk assessment. Jennings *et al*. (2012) applied transcriptomics on a range of *in vitro* models: three human renal proximal tubular models (human primary, RPTEC/TERT1 and HK-2 cells) and two rat renal proximal tubular models (rat primary and NRK-52E cells). The results are interesting for mode of action identification. Due to the *in vitro* approach and relative high doses (0.3 to 5 µM), the relevance for risk assessment is very low.

In another transcriptomics study (Luhe *et al*., 2003), rats were exposed to 1 or 10 mg/kg/day for 24 or 72h followed by microarray analysis on the kidney. The doses applied are 10,000 to 100,000-fold higher than the TWI making this study irrelevant for risk assessment.

Risk assessment

This study is of interest for hazard identification and identification of modes of action, but does not allow drawing firm conclusions about risk assessment.

As described above, the reports have low relevance to risk assessment due to application of *in vitro* models or exposure to levels exceeding the TWI level with 1,000 or more.

4.1.5. Persistent organic pollutants: organotins

Hazard

Organotins are chemical compounds containing tin and hydrocarbon substituents. One organotin bis(tri-n-butyltin)oxide (TBTO) has been widely used as biocide in wood preservatives and antifouling paints (De Waal *et al*., 1997). Human exposure to organotins mainly occurs via consumption of meat and fish products (Inadera, 2006). Immunosuppression is the most prominent endpoint of organotin toxicity in rodents (Snoeij *et al*., 1988). Next to TBTO, dibutyltin (DBT) and triphenyltin (TPT) belong to the most toxic organotins. Organotins are immunotoxic and exert endocrine disrupting effects (EFSA Contaminant Panel, 2004). A no observed adverse effect level (NOAEL) for immunotoxicity of 0.025 mg/kg bw/day was identified for TBT oxide from chronic feeding studies. By applying a safety factor of 100, a group TDI of 0.25 µg/kg bw for TBT, DBT and TPT compounds was established.

The references corresponding with organotins focussed on effects of TBTO on transcriptomics in immune cells (four studies) or neuronal cells (Katika *et al*.., 2011; Suzuki and Ishido, 2011; van Kol *et al*.., 2012; Baken *et al*.., 2006; Baken *et al*.., 2008). Two studies are performed *in vivo*. Three studies are performed *in vitro* or *ex vivo*.

Results from omics experiments

Within the three *in vitro* experiments (Katika *et al*., 2011; Suzuki and Ishido, 2011; van Kol *et al*., 2012), either human Jurkat cells, primary mouse thymocytes or rat mesencephalic neural stem cells were exposed to relatively high doses of TBTO (>100-fold higher than the TDI). These experiments were informative for unravelling the mode of action of TBTO. Within the two *in vivo* experiments (Baken *et al*., 2006; Baken *et al*., 2008), rats and mice were exposed to a maximum tolerated doses of TBTO.

Risk assessment

These experiments were informative for unravelling the mode of action, but they are not informative for a full risk assessment.

4.1.6. Marine biotoxins: Ciguatoxin

Hazard

Ciguatoxins (CTX) bind to voltage sensitive sodium channels, forcing the cells that are normally resting to open them. Ciguatoxins induce a wide variety of symptoms: gastrointestinal, such as vomiting, diarrhoea and nausea; neurological, such as tingling or itching; and cardiovascular, such as hypotension and bradicardia (EFSA Panel on Contaminants in the Food, 2010). Exposure of humans occur via eating fish. Recently CTX-group toxins were identified for the first time in fish in Europe and CTX-group toxins are therefore considered as an emerging risk for Europe (EFSA Panel on Contaminants in the Food, 2010; Paredes *et al*., 2011). Due to the very limited quantitative data both in experimental animals as well as related to human intoxications, the EFSA CONTAM Panel concluded that the establishment of an oral Acute reference dose (ARfD) was not possible. Based on case reports on human intoxications it appears that a concentration of 0.01 µg CTX equivalents/kg fish is expected not to exert effects in sensitive individuals when consuming a single fish meal (EFSA Panel on Contaminants in the Food, 2010).

Two studies were identified for CTX: each *in vivo* mouse study assessing effects on transcriptomics in either the brain (Ryan *et al*. 2010) or on blood cells (Ryan *et al*. 2007).

Results from omics experiments

A sub-lethal dose of the potent marine neurotoxin was given, causing hundreds of genes being affected.

Risk assessment

Due to the exposure to a very high (sub-lethal) dose, no firm conclusions on risk assessment can be drawn.

4.1.7. Marine biotoxins: Domoic acid

Hazard

Domoic Acid (DA) causes Amnesic Shellfish Poisoning. It causes brain damage, which consists in alteration of the memory function among other neurological symptoms such as hallucinations and confusion (Grant *et al*., 2010). Also vomiting, cramping, coma and death are included in the non-neurological symptoms that DA can cause (Grant *et al*., 2010). The toxin is produced by diatoms of the genus Pseudo-nitschia and by marine red algae of the genus Chondria. There is a wide range of

vectors: blue mussels, anchovies or crabs. These toxins have been found in several places all over the world (Paredes *et al*. 2011). The CONTAM Panel of EFSA established an ARfD of 30 µg DA/kg bw by applying the overall uncertainty factor of 30 to the LOAEL of 0.9 mg/kg bw (EFSA Contam Panel 2009).

Two studies were identified for DA: one *in vivo* mouse and one *in vivo* zebra fish study (Lefebvre KA *et al.*, 2009; Ryan JC et , 2005). In both studies, effects on transcriptomics in the brain were assessed. DA was given intra-peritoneal.

Results from omics experiments

In both studies hundreds of genes were affected. In the zebrafish genes involved in apoptosis were up-regulated while genes involved in protein synthesis were down-regulated. In the mouse, genes involved in growth arrest and DNA damage, and in inflammatory response were up-regulated.

Risk assessment

The lowest dose the zebrafishes and the mice were exposed to was 0.47 mg/kg and 1 mg/kg, respectively. This dose is around the LOAEL of 0.9 mg/kg assessed by EFSA. However, the zebrafishes were exposed intra-peritoneally making this exposure difficult to compare to human oral exposure.

4.1.8. Estrogens

Hazard

Estrogens are crucial for the development and function of the reproductive organs in females. Post-menopausal estrogens therapy of women increases the risk for breast cancer (Marjoribanks *et al*., 2012). Exposures of males to estrogens might affect sexual development, sex drive and fertility (Tilghman *et al*., 2010). At present, there is no regulation about maximal levels of natural estrogens in e.g. milk or meat (EFSA, 2010a).

The following studies were identified on estrogens and transcriptomics. Each of these concern *in vivo* studies. Three studies assessed estrogenic effects on the uterus of either the rat (Heneweer *et al*. 2007; Kato *et al*. 2004) or mouse (Newbold *et al*. 2007). One study assessed the effects of ethinyl estradiol on the rat liver (Kato *et al*. 2004), and the fifth study assessed the effects of 17β-estradiol on gene expression in serotonin neurons in rhesus monkeys (Bethea and Reddy 2012).

Results from omics experiments

Heneweer *et al*. (2007) treated immature female rats (PND21) orally with a range of doses of ethinyl estradiol: 0, 0.03, 0.1, 0.3, 1 or 10 µg/kg bw. Based on classical parameters for utero-trophic response, the first effects become apparent at doses of 1 and 10 µg/kg bw for uterus weight and uterine epithelial cell height, respectively. Effects on gene expression in the uterus, however, are already observed from the lowest dose of 0.03 µg/kg bw onwards, indicating effects on gene expression to be at least 33-fold more sensitive than that on classical parameters.

Kato *et al*. (2004) treated 7-weeks old rats, both males and females, for 28 days orally by adding ethinyl estradiol at concentrations of 0 (control), 0.01, 0.1, and 1.0 ppm in the feed. The lowest dose of 0.01 ppm resulted to an exposure of 0.6 µg/kg/day. Effects on mRNA expression in the liver was assessed with small size (3776 genes) microarrays. Interestingly, the lowest dose of 0.6 µg/kg/day affected a relatively large number of genes in the liver: In males, 38 and 87 genes were up- and down-

regulated, respectively. In females, 95 and 27 genes were up- and down-regulated, respectively. Detection of changes in classical parameters like oestrous cyclicity was only possible at the high dose of 1.0 ppm.

Newbold *et al*. (2007) treated female mouse pups on days 1–5 PND with diethylstilbestrol (DES) by subcutaneous injection at doses of 1, 10, or 1000 µg/kg/d. Pups were sacrificed by CO2 asphyxiation at 19 d of age, prior to puberty. Previous studies demonstrated that treatment to 1000 µg/kg/d induce uterine adenocarcinoma following neonatal treatment. Tumour incidence was dose-dependent reaching >90% by 18 mo following neonatal treatment with 1000 µg/kg/d of DES. Treatment to 1 µg/kg/d DES caused uterine adenocarcinoma at 12 mo of age in 19% of the treated mice. Microarray analysis demonstrated that significant alterations of gene expression could be detected at the level of 1 µg/kg/d DES already.

Naciff *et al*. (2007) treated immature female rats subcutaneously with a single dose of 10 µg/kg 17α-ethinyl estradiol. Effects on transcriptomics of the uterus was assessed after 1, 2, 8, 24, 48, 72, or 96 h. The treatments affected thousands of genes.

Bethea and Reddy (2012) treated ovariectomized rhesus macaques with placebo or estradiol via Silastic implants for 1 month resulting in estradiol levels of 136.5 ± 15 pg/ml (in blood serum). The number of rhesus macaques was limited to two per group for the microarray analysis. Using laser-capture, serotonin neurons (tryptophan hydroxylase (TPH)-positive) neurons were captured from the midbrain and subjected to transcriptomics (microarray) analysis. More than 10,000 probes (number of genes not mentioned) were detected to be affected. Effects on pathways included up-regulation of cell adhesion and synapse assembly.

Risk assessment

The treatments with relatively low dosed as done by (Heneweer *et al*., 2007; Kato *et al*., 2004; Newbold *et al*., 2007) is interesting since these exposures are more nearby actual exposures. These studies show that estrogens induce alterations of gene expression levels at a much lower exposure level than that classical parameters like uterine weight or oestrous cyclicity are affected. An important challenge for the future is to assess whether these alterations in gene expression reflect adverse effects or are adaptations that do not affect the functionality of the endocrine systems or liver.

The study of Naciff *et al*. (2007) used a too high dose of ethinyl estradiol to draw conclusions about risk assessment. The study of Bethea and Reddy (2012) was done in relation to hormone replacement therapy for post-menopausal women. It is difficult to conclude how the estradiol levels in the rhesus macaques relate to that in humans, and whether the observed effects are positive or negative.

4.1.9. Arsenic

Hazard

Arsenic is an environmental toxicant, and exposure usually occurs via drinking water. Long-term exposure to arsenic is causally related to a variety of dermal symptoms (arsenicosis), peripheral neuropathy, encephalopathy, bronchitis, pulmonary fibrosis, hepato-splenomegaly, hypertension, peripheral vascular disease, atherosclerosis, cancer (lung, bladder, other organs) and diabetes mellitus (Bolt, 2012). WHO and European Union have set an upper limit of 10 µg/l arsenic in drinking water (Zhao, 2010).

The following studies were identified on arsenic using transcriptomics or proteomics approaches (Robinson *et al.*, 2010; Hong *et al.*, 2009; Boellmann *et al.*, 2010; Kozul *et al.*, 2009; Zhao *et al.*, 2010). Experiments were performed with mice (4). The mouse studies focused on effects in lung (2),

brain and whole embryos, after exposure to high doses of arsenic. In one human study proteomics of blood was applied after long-term exposure to various levels of arsenic in drinking water.

Results of the omics experiments

In lung, high level arsenic exposure induced methylation of a number of genes that play a functional role in cancer-related effects of arsenic. Also in lung, exposure to low level arsenic differentially regulated genes involved in immune response. In brain, very high level exposure affected gene expression of the mitochondrial respiratory chain, leading to reduced brain activity. As a result, arsenic may impair cognitive function. In embryos during the neurulation phase, arsenic affected a multitude of genes involved in cell cycle regulation, glutathione, sugar and sterol metabolism and RNA processing. These changes point to a teratogenic potential of arsenic. Proteomics of chronic arsenic human exposure revealed twenty proteins that potentially can be used as biomarkers for risk of arsenic induced skin lesions.

Risk assessment

Exposure to levels which may occur in drinking water in Europe (around 0.1 PPB) revealed the potential of arsenic to increase the risk of respiratory infections.

4.1.10. Lead

Hazard

Food is the major source of human exposure to lead. The central nervous system is the main target organ for lead toxicity. In adults, lead-associated neurotoxicity was found to affect central information processing and short-term verbal memory, to cause psychiatric symptoms and to impair manual dexterity. The developing brain is more vulnerable to the neurotoxicity of lead than the mature brain. Lead accumulates in the body and due to its long half-life in the body, chronic toxicity of lead is of most concern when considering the potential risk to human health. At relatively low blood levels, associations were found with increased systolic blood pressure and kidney disease. Inorganic lead was classified by IARC as probably carcinogenic to humans. There is no recommended tolerable intake level, because evidence of thresholds for a number of critical health effects is lacking (EFSA, 2012a).

The following studies were identified for lead using transcriptomics and proteomics approaches (Birdsall *et al.*, 2010; Kasten-Jolly J *et al.*, 2011; Kossowska *et al.*, 2010; Witzmann *et al.*, 1999; Zheng *et al.*, 2011). Experiments were performed with mice (2) and rats (1). In two human studies proteomics of blood was applied after occupational and low-level environmental exposure. The animal studies focused on effects in liver, brain and kidney, after exposure to very high doses of lead in the high mg/kg bw/day range.

Results of the omics experiments

Effects on gene expression in liver (Cyp and mitochondria) were only found at the highest doses that induced clinical effects on liver enzymes. In the developing brain, lead increased the expression of signal transduction and transcription factors, indicative for neuro inflammation. In the kidney, lead exposure altered the expression of 78 proteins in the cortex, and of 16 proteins in the medulla. Of these, 22 proteins could be identified. High occupational exposure to lead, revealed 6 differentially expressed proteins of which the biological function is not clear. Low-level exposure in children showed regulation of a number of proteins relevant for cardiovascular disease (CVD).

Risk assessment

Implications for risk assessment cannot be made, because exposures were extremely high in the animal studies (up to 200 mg Pb/kg bw/day). The proteomic study with low-level exposure showed associations with proteins involved in CVD.

4.1.11. Dioxins, such as 2,3,7,8-tetrachlorodibenzo-p-diossina (TCDD)

Hazard

Dioxins induce metabolic disorders and a wasting syndrome that may lead to reproductive toxicity and endocrine dysfunction, neurodevelopmental toxicity, immunosuppression, carcinogenicity and teratogenicity. In humans, high TCDD exposure resulted mainly in chloracne and hepatic alterations. Mixed exposure to dioxins and PCBs also seems to exert developmental and reproductive toxicities in humans. Exposure at low levels in humans has been suggested to affect hormone homeostasis, endometriosis, delayed male puberal onset, reproduction, and cognition, but evidence is not compelling. The TWI for dioxins and PCBs has been set at 14 pg TEQ/kg bw/week (EFSA, 2011a).

The following studies were identified on dioxins, all using transcriptomics approaches (Kopec *et al.*, 2010; Kopec *et al.*, 2011; Magre *et al.*, 2012; Nault *et al.*, 2013; Ovando *et al.*, 2010). Experiments were performed with rats (2), and mice (3). All experiments were performed with high to very high doses. Effects on liver (4) en testis were measured.

Results of the omics experiments

Effects on liver were measured in mice (3) and rats (1). In the mouse liver experiments exposures ranged from 1 to 7 days, whereas doses ranged from 0.001 to 300 ppb. Regulated genes ranged from 200 to 1400. Regulated pathways differed between the various mouse studies and related to Ah receptor gene battery, trafficking and transport, cell adhesion, cell signalling, development and differentiation, xenobiotic, lipid, steroid and glucose metabolism, cell cycle and cell death, immune and inflammatory response, oxidation/reduction, and cytokine production. In rat liver, up to 400 genes were regulated, and related pathways overlapped with those of the mouse experiments: Ah receptor battery, trafficking and transport, cell signalling, development and differentiation. The experiments in rat testis revealed 160 regulated genes, relating to immune and defence response, chemotaxis, chemokine activity, and response to steroid hormone stimulus.

Risk assessment

Implications for risk assessment cannot be made, because exposures were high or extremely high. These ‘omics studies increase knowledge on potential modes of action of high dioxin levels.

4.1.12. Mycotoxins: Zearalenone

Hazard

Zearalenone is a non-steroidal estrogenic mycotoxin produced by several Fusarium species, and is found worldwide in cereal crops. Zearalenone appears to bind to oestrogen receptors and can result in hormonal changes (Richard, 2007). The TDI of zearalenone for humans is 0.25 µg/kg bw/day (EFSA, 2011b).

The following studies were identified for zearalenone, using transcriptomics and proteomics approaches (Busk *et al.*, 2012; Heneweer *et al.*, 2007; Lopez-Casas, 2012; Parveen *et al.*, 2009; Updike *et al.*, 2007). Experiments were performed with mice (1) rats (1), primary human breast cells (1), a human breast cancer cell line (MCF-7) (1), and a human adrenal gland carcinoma cell line (H295R) (1). The *in vivo* studies looked at effects on uterus and developing testis, whereas *in vitro* studies involved breast cells and adrenal gland cells.

Results of the omics experiments

Zearalenone affected up to 60 genes in mouse testis, without a clear dose or timing effect. Most important pathways involved were Nrf2, protein ubiquination, and mitochondrial dysfunction. In rat uterus, only doses ≥ 1 mg/kg bw/day affected gene expression. At this level of exposure, severe damage of the uterine epithelial occurred. Regulated pathways involved were inflammation, complement activation, and water homeostasis. Regulation by zearalenone was much lower than by estradiol. In breast cells, all zearalenone metabolites had very similar gene expression profiles. These genes were involved in signalling, proliferation, transcription and transport. Proteomics in human primary breast cells revealed increased production (5-fold) of protein disulfide isomerase, a protein which is known to be up-regulated by cancer-causing agents. Up to 14 proteins were regulated by zearalenone and it α and β metabolites. However, the patterns showed differences between these three compounds.

Risk assessment

Risks of effects on testis are not clear, because much higher doses were used than the TDI. Doses up to the tolerable daily dose did not affect gene expression in the uterus. It appeared that zearalenone had a much lower estrogenic potency than estradiol.

4.1.13. Pyrrolizidine alkaloids

Hazard

Pyrrolizidine alkaloids (PA) are a group of naturally occurring alkaloids exclusively produced by plants. Based on the present knowledge of metabolism, activation, DNA adduct-formation, genotoxicity and carcinogenicity, 1,2-unsaturated PAs may act as genotoxic carcinogens in humans (EFSA Panel on Contaminants in the Food Chain, 2011). Because 1,2-unsaturated PAs are genotoxic and carcinogenic, the CONTAM Panel concluded that it was not appropriate to establish a TDI, and decided to apply the Margin of Exposure (MOE) approach. A $BMDL_{10}$ for excess cancer risk of 70 µg/kg bw per day was calculated for induction of liver haemangiosarcomas by one of the most toxic PAs, i.e. lasiocarpine, in male rats and used as reference point for comparison with the estimated dietary exposure. The EFSA Contam Panel concluded that there is a possible health concern for those toddlers and children who are high consumers of honey.

Of all PAs riddelliine is the best studied using omics techniques both as an individual phytochemical and in the context of the pyrrolizidine alkaloid-containing plant, comfrey (Symphytum officinale). The following studies were identified on riddelliine. Both omics studies were performed with livers of female Big Blue transgenic rats upon oral gavage at a dose of 1 mg/kg body weight riddelliine 5 days a week for 12 weeks. One study (Wei *et al*., 2007) focussed on changes in gene expression and the other (Chen *et al*., 2012) on alterations in microRNA (miRNA) expression profiles.

Results of the omics experiments

Mei *et al* (2007) studied gene expression in livers of female Big Blue transgenic rats upon oral gavage at a dose of 1 mg/kg body weight riddelliine 5 days a week for 12 weeks. Whole genome expression microarray analysis of hepatic samples showed that 919 genes were differentially regulated by the riddelliine treatment (429 were up-regulated and 490 were down-regulated). Pathway analysis revealed that these genes were involved in cancer, tissue development, cell death, cell-to-cell signalling , and cellular growth and proliferation.

This study of Mei *et al* (2007) was extended by Chen *et al* (2012) who explored the effect of riddelliine treatment on microRNA (miRNA) expression in rat liver. They found that the expression of 47 miRNA expression was significantly altered by riddelliine treatment (38 were up-regulated and 9 were down-regulated). Functional analysis of these differentially expressed miRNAs by riddelliine revealed that these miRNAs were involved in liver carcinogenicity, liver proliferation, liver necrosis/cell death, hepato-cellular carcinoma, liver hepato-megaly, liver inflammation and liver fibrosis.

Risk assessment

It was demonstrated that the changes in the transcriptome were reflecting previously observed *in vivo* effects of riddelliine including liver abnormalities, alteration of metabolizing genes, injury of endothelial cells, and cancer development. All of the effects noted in the two studies occurred in the absence of tumours indicating that they may represent early changes related to liver carcinogenesis. Implications for risk assessment, other than risk identification, are difficult to made, since the Big Blue rats were treated with only one high dose of riddelliine (1 mg riddelliine/kg bw/day).

4.1.14. Tropane alkaloids

Hazard

Tropane alkaloids (TAs) are a class of alkaloids that contain a tropane ring in their chemical structure and occur naturally in many members of the plant family Solanaceae. The mechanism of action of tropane alkaloids relates to their competitive antagonism at muscarinic acetylcholine receptors, preventing the binding of acetylcholine. The most important natural tropane alkaloids are hyoscyamine and scopolamine. High concentrations of these alkaloids have been found particularly in Datura stramonium and Datura ferox, as well as in Datura innoxia. Datura plants are toxic for animals if ingested in larger amounts. Their seeds, which contain significant amounts of hyoscyamine and scopolamine, can be found as botanical impurities in feed materials, particularly in soybean and linseed products. Tropane alkaloids are mainly a problem for feed safety. After ingestion, tropane alkaloids are rapidly biotransformed or excreted. Therefore, it is unlikely that residues of tropane alkaloids in edible tissues, milk and eggs constitute a risk for consumers (EFSA Panel on Contaminants in the Food Chain, 2008a).

The following studies were identified on the TA scopolamine. Both studies (Hsieh *et al*. 2003; Brouillette *et al* 2007) deal with hippocampal gene expression profiling in scopolamine-treated rats and are aimed at the establishment of molecular and cellular mechanisms that underlie the neuropharmacological effect (memory impairment) of scopolamine.

Hsieh *et al*. (2003) studied hippocampal gene expression in anesthetized Sprague–Dawley rats 30 min after injection of a high dose (10 mg/kg/bw) of scopolamine in the lateral ventricle. They used cross-species hybridization with human cDNA microarrays containing a relatively low number of genes (9600 human cDNAs) followed by semi-quantitative RT-PCR using primer pairs against rat orthologs.

Brouillette *et al* (2007) performed gene expression analysis on the hippocampus of Long–Evans rats treated i.p. with 0.4 mg/kg/bw scopolamine daily during 4 days. Gene expression was assessed 6 h

after the last injection at day 4 using rat whole genome microarrays and quantitative real-time RT-PCR. In addition, spatial memory of the rats was determined using a hippocampus-dependent behavioural test.

Results of the omics experiments

In the rat hippocampal gene expression study of Hsieh *et al*. (2003), 48 genes were selected for further analysis and 28 of these were validated by RT-PCR. Fifteen of the genes were associated with muscarinic receptor signalling pathways, whereas others were associated with novel pathways including apoptosis, cytoskeleton reconstruction, protein trafficking, and cell differentiation.

In the study of Brouillette *et al* (2007) a total of 31 genes were found to have altered expression levels upon scopolamine treatment. Of these 17 were up-regulated and 14 down-regulated. Some of these genes were already known to be associated with memory processes (Homer1, $GABA_B$ receptor, early growth response 1, prodynorphin, VGF nerve growth factor inducible) and others represented novel candidate genes possibly involved in cognition (including calcium/calmodulin-dependent protein kinase kinase 2, dual specificity phosphatase 5 and 6, glycophorin C).

Risk assessment

In both studies, rats were treated with relatively high doses of scopolamine, either via i.p. injection (Brouillette *et al.*, 2007) or injection in the lateral ventricle (Hsieh *et al*. 2003). Both studies are performed to get insight into the mechanisms underlying scopolamine-induced amnesia, but do not add to aspects of risk assessment already known.

4.1.15. Acrylamide

Hazard

Acrylamide is a chemical compound that appears to increase its concentration in a wide variety of food during high temperature cooking, possibly as a result of the Maillard reaction or 'browning' process. Acrylamide was identified as a major concern in relation to consumption of baked and fried starchy foods by a Swedish report in 2002 (Lipworth *et al*. 2012) and is believed to be a neurotoxin and carcinogenic (bowel, bladder, kidney) for humans.

EFSA systematically monitors the levels of acrylamide in foods to assess exposures (EFSA, 2012b). In 2008 EFSA organised a scientific colloquium concerning exposures and carcinogenicity for acrylamide (EFSA, 2008a). The Joint FAO/WHO expert committee on food additives include risk assessment for acrylamide in their reports (JECFA, 2011) and note that benchmark doses for tumour formation in rats are a few mg/kg bw/day, that margins of exposure lead to concerns in relation to human health and that biomarkers for acrylamide exposure are poor.

The following studies were identified on acrylamide, including eight rat based studies (Barber and LoPachin, 2004; Barber *et al.*, 2007; Cha *et al.*, 2009; El-Sayyad *et al.*, 2011; Feng *et al.*, 2011; Hasegawa e al, 2008; Hochstenbach *et al.*, 2010; Lampen *et al.*, 2009; Mei *et al.*, 2008; Oshida *et al.*, 2011; Schwend *et al.*, 2009; Seale *et al.*, 2012; Sun *et al.*, 2010; Wang *et al.*, 2010). Four cell based studies involve a variety of human cell types. Proteomics and transcriptomics are the dominant omics techniques. The proteomics studies by Feng *et al*. (2011) and Barber *et al*. (2007) correspond with observations of protein modifications. Many of the studies involve acrylamide doses ~10 mg/kg bw/day.

Results from omics experiments

Transcriptomics studies report differential expression of several hundred genes, but there are no discernible patterns in the corresponding gene lists. Proteomics studies identify changes in expression for a wide variety of proteins as well as some protein modifications. None of the reports includes a systematic pathway analysis and no single pathway change is regularly identified as a response to acrylamide exposures although neuronal and detoxification pathways dominate.

Risk assessment

The reports provide some information on the analysis of results. Most of the studies identified investigate the neurotoxic mode of action following acrylamide exposure and the role of biomarkers.

- A report by Seale *et al*. (2012) identifies differentially expressed genes not previously associated with acrylamide neurotoxicity (e.g. Mylpf involved in muscle contraction) that might be developed into biomarkers
- A report by Barber *et al*. (2007) using isotope affinity tagging indicates a progressive formation of protein (cysteine) adducts causing nerve terminal damage that is distinct from changes in protein abundance
- A report by Feng *et al*. (2011) uses proteomics to discover protein-acrylamide (or glycidamide) adducts that are biomarkers of exposure to high-dose acrylamide but it is difficult to translate the results to food like exposures

Omics experiments support an established mode of action for acrylamide but, currently, are relatively poor at establishing quantitative descriptions. Metabolomics studies indicate the potential to find more informative biomarkers for acrylamide exposure.

4.1.16. Natural plant products contaminants: Glucosinolate indole-3-carbinol

Hazard

This case study deals with the natural plant product glucosinolate indole-3-carbinol (I3C). Indole-3-carbinol is produced by the breakdown of the glucosinolate glucobrassicin, which can be found at relatively high levels in cruciferous vegetables such as broccoli, cabbage, cauliflower, Brussels' sprouts, collard greens and kale. I3C is also available in dietary supplements. Consumption of particularly broccoli is believed to protect against cancer due to the action of glucosinolates. On the other hand, glucosinolate-derived degradation products (like the acid condensation products DIM and ICZ) might also have undesirable effects, especially genotoxic activities (Latte *et al*., 2011).

The following studies were identified on I3C. The work of De Santi *et al*. (2013) is a follow-up of an earlier study that showed the inhibition of proliferation of MCF-7 and MDA-MB-231 breast cancer cell lines by the I3C cyclic tetrameric derivative CTet (Brandi *et al*., 2003). The study aimed to determine the mechanisms involved in the *in vitro* inhibition of proliferation of these cells among others by whole genome microarray analyse. Relevant in this work was that, *in vivo*, CTet administration was able to significantly inhibit the growth of MCF-7 xenotransplanted into nude mice, without adverse effect on body weight or on haematological parameters.

Results of the omics experiments

The overexpression of genes encoding p21/CDKN1A and GADD45A were identified as the main molecular events responsible for the anti-proliferative effects in MCF-7 and MDA-MB-231 cells treated with 6 and 12 µM CTet (for 24 hours). The inhibition of Akt activity, revealed in CTet-treated cells by immunoblotting, could be responsible for p21/CDKN1A overexpression.

Risk assessment

These omics data provide mechanistic insight into a potential beneficial effect of the I3C derivative CTet on breast cancer cells. However, no data are available with respect to exposure of normal cells to CTet. Thus, implications for risk assessment are limited.

4.1.17. Natural plant products contaminants: Gossypol

Hazard

This case study deals with the natural plant product contaminant gossypol. Gossypol is a yellow compound produced by the cotton plant that confers resistance to pests. Cottonseeds are by-products of cotton fiber production and are rich in oil and proteins and are therefore used for cottonseed oil production and as a feed supplement. Storage, steam and heat, and extrusion of oil reduce free gossypol concentrations, and commercial production of cottonseed meals with low levels of free gossypol is now achieved routinely with only 0.1-0.2% remaining as free gossypol. The two enantiomers of gossypol, (+)-gossypol and (-)-gossypol have markedly different biological effects. In comparison with (+)-gossypol, the (–)-enantiomer generally exhibits more pronounced effects. The main target organ of gossypol toxicity following repeated exposure to lower doses in rats and humans are the testes with reduced sperm motility, inhibited spermatogenesis and depressed sperm counts. No health-based guidance value (e.g. ADI, TDI) has been established for gossypol. The lowest oral doses inhibiting spermatogenesis in humans and monkeys were 0.1 and 0.35 mg/kg bw, respectively (EFSA Panel on Contaminants in the Food Chain, 2008b). A primary research paper in this field describes the effects of Gossypol on the transcriptome of two human cancer cell lines (Sikora *et al.*, 2008).

The following study was identified for gossypol (Sikora *et al*., 2008). DNA microarray analysis was done on two human head and neck squamous cell carcinoma (HNSCC) cell lines, UM-SCC-5 and Pt-R, upon treatment with (-)-gossypol to identify gene expression profiles that would give insight into (non-apoptotic) mechanisms of cell death induced by (-)-gossypol. Cell lines were exposed for 4, 8, 12, 24 hrs to 10 µM (-)-gossypol and the subjected to DNA microarray analysis.

Results of the omics experiments

A total of 5231 and 2045 changes in gene expression across the treatment time-course (0–24 hrs) were identified in UM-SCC-5 and Pt-R, respectively. Genes in the reactive oxygen species (ROS)/hypoxia pathway, including ATF3, BNIP3, BTG1, DDIT4, GADD45B, NOXA and VEGF, were highly up-regulated in response to (-)-gossypol in both cell lines.

Risk assessment

These omics data identify potential beneficial modes of action of (-)-gossypol on carcinoma cells. No data are available with respect to exposure of normal cells to this compound and therefore implications for risk assessment are limited.

4.1.18. Natural plant products contaminants: Ricin and Abrin

Hazard

Ricin is a toxic glycoprotein belonging to the type II group of ribosome inactivating proteins (type II RIP) found in the seeds (beans) of the castor oil plant (Ricinus communis L. (Euphorbiaceae)). A limited number of other plants in the same family contain type II RIPs, such as Abrus precatorius L. and Croton tiglium L. which contain abrin and crotin I, respectively. Following cell uptake by endocytosis, ricin causes acute cell death by inactivation of ribosomal RNA. Ricin acute symptoms in humans after intake of castor beans are hematemesis (vomiting containing blood), diarrhoea, haemorrhagic necroses in several organs, renal failure, circulatory collapse and death after 6 to 14 days with a fatal oral dose of about 1 mg/kg bw (5-10 castor beans). Exposure of animals would only be expected as a result of accidental contamination (EFSA Panel on Contaminants in the Food Chain, 2008c).

The following studies were identified on ricin and abrin. Horrix *et al.* (2011) have analysed the effect of ricin as well as of other type II RIPs, i.e. ripromoxin and volkensin, on the human adenocarcinoma cell lines MDA-MB-231 (breast) and HCT116 (colon) using micro-array, qRT-PCR and Western blot. Actually, only RNA from MDA-MB-231 cells exposed to different concentrations of riproximin-corresponding to IC25 and IC50 values-was used for micro-array analysis. In subsequent qRT-PCR and Western blot experiments also ricin and volkensin have been included.

Bhaskar *et al* (2012) have examined both dose and time-dependent transcriptional responses induced by abrin in the adult mouse brain. Swiss albino mice (two experimental groups of 6 animals each) were exposed to 1 (2.83 µg/kg) and 2 LD_{50} dose of abrin (5.66 µg/kg body weight) by intra-peritoneal route. Three animals from each group were killed at day 1 and day 2 to harvest brain tissue. Effects of abrin on gene expression profile of brain tissue were examined using mouse whole genome arrays.

Results of the omics experiments

Among the 2000 genes, significantly modulated in riproximin-treated MDA-MB-231 cells, those belonging to the Unfolded Protein Response (UPR) were distinctly altered in their expression (Horrix *et al*. 2011). UPR is a cellular mechanism activated in response to endoplasmic reticulum stress. The UPR-related genes that showed significantly increased expression include the UPR sensors ATF6, IRE1, and ATF3 and the cytosolic effectors GADD34, GADD45A, GADD45B, and GADD153. The UPR pathways were examined in more detail by performing qRT-PCR and Western blot experiments also including ricin and volkensin. These experiments confirmed that ripromoxin treatment leads to UPR and demonstrated that ripromoxin, ricin, and volkensin induced similar effects.

The whole genome microarray data of the mouse study with abrin revealed the significant regulation of various pathways like MAPK pathway, cytokine-cytokine receptor interaction, calcium signalling pathway, Jak-STAT signalling pathway and natural killer cell mediated toxicity (Bhaskar *et al* 2012). The comparison of differential gene expression at both the doses showed dose dependent effects of abrin toxicity. Real-time qRT-PCR analysis of selected genes supported the microarray data. The results were in agreement with histopathological analysis of the brain showing the induction of an immune and inflammatory response (infiltration of inflammatory cells).

Risk assessment

The *in vitro* omics data are obtained upon exposure of adenocarcinoma cell lines to type II RIPs and are informative with respect to the mode of action of these toxic proteins and identification of potential risks. They can not be used for other risk assessment steps (hazard characterization etc.).

The omics data from the *in vivo* study with abrin are obtained shortly (1 or 2 days) after i.p. injection of animals to relatively high concentrations of abrin (acute toxicity study). Changes in gene expression likely reflect neuroinflammation as a result of cell death rather than primary effects of abrin. This study provides useful information for hazard identification.

4.1.19. Perfluorooctanoic acid (PFOA) and Perfluorooctane sulfate (PFOS)

Hazard

Perfluorooctanoic acid (PFOA) and perfluorooctane sulphate (PFOS) are both perfluorinated chemicals found in the environment and in some foods such as fish. PFOS and PFOA both accumulate in the body and are known to have moderate acute toxicity in animals. Reviews relating to relevant human hazards and exposures include Lau *et al.* (2007) and the UK Health Protection Agency compendium of chemical hazards (HPA, 2009). In 2008 the EFSA (CONTAM Panel) published a Scientific Opinion on PFOS and PFOA (EFSA, 2008b) that identified 150 ng/kg bw/day as a TDI for PFOS and 1.5µg/kg bw/day as a TDI for PFOA. Both assessments include an uncertainty factor of 200. For both substances the most recent estimates of dietary exposure in Europe are smaller than the TDI values, EFSA (2012a).

The EFSA assessments conclude that PFOS and PFOA are unlikely to result in adverse effects in the general population. Based on evidence from experiments on rats and monkeys EFSA highlighted uncertainties associated with body burdens from environmental exposures, with the mode of action for potential carcinogenic effects and with potential developmental toxicity.

The following studies were identified on PFOS and PFOA (Bjork *et al*., 2008; Dorts *et al*., 2011; Hu *et al.*, 2005; Huang *et al.*, 2012; Rosen *et al*., 2008; Scharmach *et al*., 2012; Shi *et al.*, Wei *et al*., 2008; Wei *et al.*, 2009; Yeung *et al.*, 2007). The omics studies involve transcriptomics or proteomics. These studies report experiments with cell systems (hepatocytes) and animal experiments including fish, rats, mice and chicken. Most of the reports relate to liver tissues but three studies, corresponding with PFOS, concern embryos. In general the experimental conditions correspond with high doses of PFOA and PFOS in the mg/kg bw/day range.

Results of omics experiments

All of the omics investigations identify large numbers of individual expression changes in response to exposure to PFOA or PFOS. Transcriptomics investigations report between 100 and 1000 differentially expressed genes and proteomics studies report between 20 and 70 differentially expressed proteins and, in isolation, the gene/protein lists are difficult to evaluate in terms of risk assessment or decision making. Approximately half of the reports include some systematic pathway analysis. Elements of the lipid metabolism pathway are regularly identified as part of the omics response to PFOS or PFOA.

Risk assessment

Most of the omics studies identify investigation of the mode of action for PFOS and PFOA but two studies, concerning PFOA in fish, identify a search for biomarkers:

- Protein biomarkers identified by Wei *et al*. (2008) are insufficiently quantified to reduce uncertainty in environmental exposure estimates
- Several novel mechanisms are proposed in relation to toxicity and tumour promotion (e.g. Tilton *et al*. (2008) identify a significant effect of PFOA on oestrogen signalling and

Scharmach *et al* (2012) identify the HNF4 alpha transcription factor as a key element affected by PFOA) but there is little consensus and each requires further investigation to contribute to risk assessment

- Bjork *et al*. (2008) report that for rats the transcriptional responses do not identify mechanisms associated with *in utero* exposure to PFOS that are distinct from those reported for adults

A report of proteomics by Scharmach *et al*. (2012) satisfies many of the criteria for risk assessment, with particular emphasis on the analysis and interpretation of the omics results, but the conclusions are equivocal in relation to the identification of a mode of action for PFOA in relation to liver toxicity. Several of the reports indicate that mixtures of perfluoroalkylated substances could be considered as distinct hazards.

4.1.20. Melamine

Hazard

Melamine is a non-food organic chemical with high nitrogen content that is believed to cause kidney problems and cancer (Skinner *et al*. 2010). In 2007-8 melamine was used in economically motivated adulteration incidents involving pet food and milk. In 2010 EFSA published scientific opinion concerning melamine (and associated materials including cyanuric acid) contamination of food (EFSA, 2010b) that indicates a TDI ~0.2 mg/kg body weight and concluded that melamine from approved sources (such as migration from packaging but not adulteration) does not represent a risk to humans. The EFSA opinion identified uncertainty associated with dose-response models and with exposure (and co-exposure) estimates.

The following studies were identified, dominated by research from China (Camacho *et al.*, 2011; Duan *et al.*, 2011; Schnackenberg *et al.*, 2012; Shi *et al.*, 2010; Sun *et al.*, 2012; Wang *et al.*, 2010; Xie *et al.*, 2010). These studies include three experiments on rats and two involving human urine. Metabolomics methods are used in 4 of 5 studies. The animal experiments generally explore the effects of very high exposures (~100 mg/kg/day) that cause observable adverse health effects.

Results from omics experiments

Metabolomics investigations indicate relatively small numbers of expression changes in response to melamine exposures and the results do not show a consistent response. None of the reports include systematic analysis of pathways and many elements of metabolism are reported with dysfunction. A Chinese study, Duan *et al*. (2011), takes the form of a case control study to explore potential biomarkers (from metabolomics) for melamine exposure.

Risk assessment

Three of the omics studies are identified with the investigation of the mode of action, one with biomarker identification and one with dose-response assessment.

- An animal based metabolomics study, Xie *et al*. (2010), includes distinct responses from co-exposures involving melamine and cyanuric acid although the high doses restrict direct interpretation of risks

- A human study, Duan *et al*. (2011) successfully identifies potential markers for melamine exposure based on high performance analysis of urine samples and a model that might help with medical management

Metabolomics investigation show changes in behaviour at the lowest experimental doses of melamine in rats so that it is difficult to infer useful information about dose-response. There is some potential for improved markers, based on metabolomics, to reduce uncertainty in exposure estimates but it is unclear whether markers discovered in animal experiments will be relevant.

4.1.21. Furan

Hazard

Furan is produced during the high temperature heat degradation of some sugars and organic acids. Notably furan has been associated with some processed foods such as coffee, potato crisps and infant formula. Furan is known as a carcinogen in rodents and is treated as a possible hazard for humans (UK Food Standards Agency, 2012); furan levels in foods are monitored by the EU (EFSA, 2011c) but dietary exposures are difficult to establish.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2010) report concerns relating to Furan levels in foods and their effect on human health and particularly DNA damage and express uncertainty associated with mode of action or mitigation. The JECFA report indicates typical benchmark doses in the range of mg/kg bw/day.

The following studies were identified, using metabolomics, proteomics and transcriptomics approaches (Hamadeh *et al.*, 2004; Kellert *et al.*, Mally *et al.*, 2010; Moro *et al.*, 2012). All the reports involve experimental male rats and furan doses in excess of 1 mg/kg bw/day. Two reports correspond to liver tissues and two correspond with analysis of urine.

Results from omics experiments

A proteomics investigation identifies 83 differentially expressed proteins and a transcriptomics study identifies 100 differentially expressed genes. None of the studies includes a systematic pathway analysis although mitochondrial injury is identified by two reports.

Risk assessment

One metabolomics study identifies dose-response assessment, one identifies a search for biomarkers and the others concern mode of action.

- A metabolomics study primarily aimed at investigation of a dose-response relationship, Mally *et al*. (2010), established dominantly negative results in terms of metabolic changes in urine although there were some changes in serum concentrations of bile acids at the highest doses
- A metabolic profiling study, Kellert *et al*. (2008), identified unknown furan metabolites but did not establish strong evidence for biomarker identification

The omics reports indicate a potential for improved markers and hence reduced uncertainty associated with exposure but, currently, do not include strong quantifications. The omics reports include indicators for mode of action without strong conclusions.

4.2. Food ingredients and packaging

4.2.1. 2-Isopropyl thioxanthone (ITX)

Hazard

2-Isopropylthioxanthone (ITX) is commonly applied as a photoinitiator in printing industries and as such also used in UV-cured inks for food packaging materials. ITX can enter the food chain by the so-called set-off effect: following printing of carton-based packaging materials, these are stored on rolls and ink of the printed external side is transferred onto the internal surface. Consequently, ITX could be released into foods and beverages after packaging.

The following study was identified on effects of ITX on transcriptomics. This concerns an *in vitro* study (Peijnenburg *et al*. 2010) in which a rat liver cell line was exposed to ITX.

Results from omics experiments

Rat H4IIE hepatoma cells were exposed *in vitro* for 6 or 24 h to either 5 µM ITX or to 150 pM of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a very well-known agonist of the arylhydrocarbon receptor. TCDD up-regulated 54 genes and the vast majority of these genes were are also up-regulated by ITX. This is a strong indication that ITX activates the arylhydrocarbon receptor.

Risk assessment

This observation is of interest for identification of mode of action, but due to the *in vitro* approach the relevance for risk assessment is limited.

4.3. Food additives, Flavourings, Processing aids and Materials in contact with food

4.3.1. Phthalates assessed by EFSA

Hazard

Phthalates are a group of related organic chemicals commonly used in the plastics industry as plasticisers. The five phthalates most commonly used by industry are di-(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), di-isononyl phthalate (DINP), di-isodecyl phthalate (DIDP) and benzyl butyl phthalate (BBP). Food products can become contaminated with phthalates from a wide variety of sources, but there has been particular concern over migration from food packaging.

Since the early 1980's there have been concerns about the effect that phthalates have on human health. Most of the data on the health effects of phthalates comes from experiments exposing rats and mice to high levels of the chemicals for prolonged periods. Long-term health effects of phthalates may include changes in sperm production, adverse effects on fertility and birth defects. They have also been reported to cause kidney and liver damage. Phthalates may be potential carcinogens and also endocrine disruptors, and as such could affect reproductive development.

TDIs have been set for some individual phthalates. For the five most commonly used phthalates, the EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Foods (AFC) set TDI figures in 2005. The TDI for DEHP is 0.05 mg/kg bw; for BBP it is 0.5 mg/kg bw; for DBP it is 0.01 mg/kg bw; and for both DIDP and DINP it is 0.15 mg/kg bw. The US EPA has set an oral reference dose (RfD) of 0.02 mg/kg bw day for DEHP, 0.20 mg/kg bw day for BBP and

0.10 mg/kg bw day for DBP. It is generally considered that the levels of individual phthalates currently found in foods are not a significant concern for human health.

The following studies were identified on phthalates. One concerns the reanalysis of nine rat *in vivo* studies from the published literature that exposed rats to DBP during gestation and evaluated gene expression changes in testes or Wolffian ducts of male foetuses using microarrays (5) and/or qPCR (9) (Euling *et al*., 2011). A second study (Lehmann *et al.*, 2004) corresponds to a rat *in vivo* study with DBP of which the qRT-PCR data were reanalysed by Euling *et al* (2011). In a third study the concentration-dependent effects of mono(2-ethylhexyl) phthalate (MEHP) and monomethyl phthalate (MMP, less toxic) on the transcriptome of rat whole embryo cultures (WECs) were analysed and examined in relation with dysmorphogenesis (Robinson *et al*., 2012).

Results of the omics experiments

The *in vivo* data showed DBP-induced down-regulation of genes in the steroidogenesis pathway and lipid/sterol/cholesterol transport pathway as well as effects on immediate early gene/growth/differentiation, transcription, peroxisome proliferator-activated receptor signalling and apoptosis pathways in the testis.

The WEC transcriptome study revealed that MEHP was more potent than MMP in inducing gene expression changes (as well as changes on morphology). MEHP affected cholesterol/lipid/steroid metabolism and apoptosis pathways in a dose-dependent manner in relation with increased developmental toxicity in WEC.

Risk assessment

In the toxicogenomics studies on DBP dose-response data were lacking. Thus, these studies allow to hypothesize on modes and mechanisms of action of DBP regarding male reproductive development, but do not allow a quantitative risk assessment. The WEC study with MEHP and MMP allowed to perform a quantitative analysis (e.g. determination of BMD_{10} values) and has a potential value for hazard characterization.

4.3.2. Butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT)

Hazard

Butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA) are widely used EU authorised food additives with anti-oxidant properties (E321 and E320). Both materials are believed to have carcinogenic properties in animals and are associated with other adverse health effects including circulation or respiration problems and hypersensitivity (Weber, 2008). Recent EFSA opinions for BHT (EFSA, 2012c) and BHA (EFSA, 2011d) established Acceptable Daily Intake (ADI) of 0.25 and 1 mg/kg bw/day based on historical evidence in a rat models. Each estimate incorporates a safety factor of 100. Mechanisms for toxic or genotoxic effects of BHT and BHA are not well understood and both substances are believed to have interactions with other food additives.

The following studies were identified concerning BHT and BHA (Abdullah*et al.*, 2012; Makino*et al.*, 2009; Nair *et al.*, 2006; Rathaur *et al.*, 2011; Radonjic *et al.*, 2007; Stierum *et al.*, 2008; Stierum *et al.*, 2005). Some studies were carried out with rodent experiments and liver tissues and others used earth worms. The studies used proteomics and transcriptomics approaches. A majority of the experiments correspond with very high doses of BHT or BHA (e.g. 200 mg/kg bw).

Results from omics experiments

Proteomics experiments report 20-50 differentially expressed proteins. One of the transcriptomics reports identifies more than 1000 differentially expressed genes. The results consistently identify up-regulated Glutathione-S-Transferase. None of the reports includes a systematic investigation of metabolic pathways.

Risk assessment

Four of the omics studies investigate the mode of action, one hazard identification and one dose-response assessment.

- A transcriptomics investigation by Stierum *et al*. (2008) identified dose-dependent expression changes (involving phase I and phase II xenobiotic metabolism enzymes) in response to BHT, but does not lead to an estimate of a threshold dose or a clear dose-response relationship
- A study addressing the changes induced by mixtures of food additives including BHT, Radonjic *et al* (2007), concludes that toxicogenomics has potential for hazard identification
- A complex proteomics study involving knock-out mice, Abdullah *et al*. (2012), identified induction of defence proteins associated with the anti-oxidant response element as a component within the mode of action for BHA but does not indicate, clearly, the relative importance of this element and indicates little concordance with corresponding microarray studies
- A micro array expression profiling study involving knockout mice, Nair *et al*. (2006), identifies many genes that respond to BHA and highlights those with Nrf2 dependence but is not conclusive about mechanism of action

Omics studies clearly identify changes associated with exposures to BHA or BHT and, in terms of Nrf2 dependent genes, partially explore mechanism. There is no evidence that omics information reduces the uncertainty associated with threshold doses.

4.4. Feed

4.4.1. Copper

Hazard

Copper is an essential trace element and micro-nutrient for all animals and is authorised as a supplement for animal feed in the EU (EC 1334/2003). For cattle 35 mg copper/kg of total diet is a maximum acceptable supplement in the absence of veterinary prescription. In many areas forage diets are supplemented with copper (usually copper sulphate) to reduce the possibility of copper deficiency (for cattle, pigs). The role of copper antagonists (e.g. molybdenum) significantly affects the role of dietary copper (ACAF, 2010). Over exposure increases the risk of copper toxicity (accumulation in the animal liver leading to tissue degradation and subsequent release into the blood - both acute and chronic effects). Animal tissue, particularly liver, containing high levels of copper could, potentially, enter the food chain and represent a hazard.

In addition to its toxicity copper also has antimicrobial activity for some Gram positive bacteria. This activity within animal gut flora is believed to select for copper resistant strains and may represent an additional hazard. Copper resistance is associated with the trcB gene and is potentially correlated with

resistance to families of antibiotics (e.g. macrolides). Antimicrobial resistance genes have been found on the same conjugative plasmid as the copper resistance gene.

An opinion of the EC Scientific Committee for Animal Nutrition (EC, 2003) indicates that normal use of copper supplementation in animal feed does not represent a significant human hazard but that piglets and calves can increase human exposure. This conclusion is based on a TDI level ~0.05 - 0.5 mg/kg bw for dietary copper. The EC opinion suggested further information was required to assess the impact of co-selection of antimicrobial resistance in animals over exposed to dietary copper.

The studies identified include one specifically associated with the selection of resistant gut bacteria in pigs (Amachawadi *et al.*, 2011; Bundy *et al.*, 2008; Chen D and Chan K, 2009; Isani *et al.*, 2011; Santos *et al.*, 2010; Song *et al.*, 2009). The studies used six animal systems (three for fish) and two (hepatocyte) cell systems. Only one study was carried out with farm animals (piglets). A wide range of omics techniques are employed. The omics investigations generally explore the effects of very high copper exposures.

Results from omics experiments

Transcriptomics and proteomics investigations indicate large numbers of expression changes in response to copper exposures but there is very little overlap of the lists of differentially expressed elements and therefore little opportunity to consider risks. Three of the reports include some systematic analysis of pathways; disruption of lipid metabolism and protein biosysthesis pathways are indicated among others. Several of the reports indicate that, for the doses considered, there were no observable effects on health or wellbeing. A genomics investigation of faecal bacteria for piglets confirmed the increasing presence of *trcB* and *erm(B)* genes corresponding with elevated copper concentration in feed.

Risk assessment

Most of the omics studies identified investigate the mode of action for copper in liver tissues.

- A study involving a fish model, Santos *et al*. (2010), identifies a target for chemical toxicity, a switch to anaerobic respiration, not apparent from classical toxicology that could be considered as a hazard identification step for risk assessment
- A genomic study of resistant elements in faecal bacteria from copper fed piglets, Amachawadi *et al*. (2011), could contribute to the hazard characterization
- A study involving a non-model organism (earthworms), Bundy *et al*. (2008), was able to identify a no observed effect level for long term exposure to copper in soil but it is hard to extend this result to foodborne hazards

Although the results include some detailed information that quantifies the effect of exposure to copper in animal tissues, it is difficult to relate any of the responses to human health end points without further studies.

4.4.2. L-arginine

Hazard

L-Arginine is a common amino acid that is manufactured by the human body but is also available in animal and plant food sources. L-arginine is used as a dietary supplement in animal feed, to counteract

weaning stress and intestinal dysfunction, and sometimes in functional foods for consumption by humans. L-arginine has some medical uses but has been associated with increased mortality following heart attack. Adverse effects are difficult to identify e.g. Shao and Hatchcock, 2008.

There is very little evidence concerning hazards associated with L-arginine supplementation and most interest concerns claims for health benefit.

A few studies were identified for L-Arginine (He *et al.*, 2009; He *et al.*, 2011; Jobgen *et al.*, 2009). Two studies correspond to experiments with pigs and one with rats and each corresponds with a diet supplemented at ~1% wt:wt. In each case the response to L-Arginine supplementation is measured relative to responses associated with alternative alanine supplements.

Results from omics experiments

Metabolomics studies indicate between 10-40 changes in the metabolites in pig's blood associated with the L-arginine supplement but the results are not compelling and, in some cases, contradictory. A single transcriptomics study, in rats, indicates differential expression of ~40 genes.

Risk assessment

Two studies concerned with hazard identification and one with the mode of action of L-arginine. There is insufficient evidence to evaluate the impact of omics investigations on risk assessment for L-arginine.

4.5. GMO

Hazard

Genetically modified (GM) plants and derived food and feed might differ from their non-GM counterparts with respect to their potential impact on the environment, safety for humans and animals, and nutritional quality. Therefore, it is important to assess whether 1) consumption of GM food or feed is safe, and 2) the genetic modification does not induce unintended effects in plants causing unwanted changes of natural constituents (EFSA GMO Panel, 2008).

4.5.1. Comparison of GM and non-GM maize varieties

The GMO part includes three studies (Frank *et al*., 2012; Levandi *et al*., 2008; Manetti *et al*., 2006) in which transgenic maize is compared to conventional maize using metabolomics. A fourth study applied proteomics (Albo *et al*., 2007) and a fifth study (Barros *et al*., 2010) applied a combination of metabolomics, proteomics and transcriptomics to compare wild type to transgenic maize.

Results from omics experiments

Levandi *et al*. (2008) applied capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) to compare a limited number of metabolites (n = 27) in conventional varieties (Aristis, Tietar, and PR33P66 maize) with their corresponding transgenic lines (Aristis Bt, Tietar Bt, and PR33P66 Bt maize). L-carnitine and L-proline−betaine were found to diver in levels between the wild type and transgenic lines.

Frank *et al*. (2012) compared the metabolite profiles of genetically modified (GM) Bt-maize (DKC78-15B, TXP 138F) to that of Roundup Ready-maize (DKC78-35R) using GC-MS. The maize were grown together both in Germany and South Africa. The main conclusion is that the effect of environment (grown in Germany or South Africa) is much higher than the effect of genetic modification.

Manetti *et al*. (2006) compared the metabolite profiles of maize containing the transgene Cry1Ab to that of the wild type maize using NMR. The results indicate that expression of Cry1Ab protein induces metabolic variations involving osmolytes and branched amino acids.

Albo *et al*. (2007) used proteomics to compare maize hybrid flour to its corresponding transgenic version carrying the Cry1Ab gene. For this, two-dimensional gel electrophoresis coupled with mass spectrometry was used. A glucose and ribitol dehydrogenase spot was unique for transgenic maize, while an endochitinase A spot was unique for WT maize. Triosephosphate isomerase 1 and globulin-1 S were higher expressed while cytosolic 3-phosphoglycerate kinase and one spot of aldose reductase were lower expressed in transgenic maize than WT maize.

Barros *et al*. (2010) compared two genetically modified maize varieties (GM Bt, GM RR), each containing a single insert, to the near-isogenic non-GM variety CRN3505. For this, a combination of metabolomics, proteomics and transcriptomics was applied. A main finding was that the environment (location) and the season have more impact on metabolites, proteins and mRNAs than the transgene. For example, five proteins, 65 mRNAs and 15 metabolites were found to be differentially expressed between maize grown at different locations.

Risk assessment

For each of the five studies described above, rather subtle effects of the transgene on metabolites, proteins and/or mRNAs are detected. It remains, however, unanswered whether these alterations affect human or environmental safety. One approach proposed is that a genetically modified crop is safe to consume when the natural variation in omics results due to e.g. location or season is larger than the variation induced by the transgene. It is too early to know whether this approach is always valid. In addition, these studies mainly relate to hazard identification but not yet to full risk assessment.

4.5.2. Effect of transgene on transcriptomics difference between GM and non-GM rice

Four studies (Batista *et al*., 2008; Luo *et al*., 2009; Montero *et al*., 2011; Xue *et al*., 2012) compared transgenic rice to wild type rice.

Results from omics experiments

Batista *et al*. (2008) evaluated the extent of transcriptome modification occurring during rice improvement through transgenesis versus mutation breeding. Oligonucleotide microarrays were used to analyze gene expression in four different pools of four types of rice plants and respective controls: (i) a γ-irradiated stable mutant, (ii) the M1 generation of a 100-Gy γ-irradiated plant, (iii) a stable transgenic plant obtained for production of an anticancer antibody, and (iv) the T1 generation of a transgenic plant produced aiming for abiotic stress improvement, and all of the unmodified original genotypes as controls.

Main conclusions from this study included:

- GM plants showed fewer genetic alterations than mutagenized ones.

- The improvement of a plant variety through the acquisition of a transgene causes stress and thus has a broad impact on gene expression which maintains several generations after the breeding event.

Luo *et al*. (2009) applied mass spectrometry-based proteomics on endosperm of rice transgenic for human granulocyte-macrophage colony stimulation factor (hGM-CSF). The aim was to produce this protein for pharmaceutical use and thus not for food. 103 proteins displayed significant changes (p-value < 0.05) between the transgenic and the wild-type endosperm cells. Endogenous storage proteins and most carbohydrate metabolism-related proteins were down-regulated, while 26S proteasome-related proteins and chaperones were up-regulated in the transgenic rice endosperm.

Using transcriptomics, (Montero *et al*., 2011) compared the conventional rice var. Senia to the transgenic variant Senia-afp constitutively expressing the AFP antifungal protein. The two variants differed for around 0.40 % of the transcriptome. Only around half of the transcriptional unintended effects could be associated to the transgene itself. The other half is likely attributable to the process used to produce GM plants which have similarities to the effects of wounding.

Xue *et al*. (2012) applied proteomics (two-dimensional electrophoresis) on seed of two strains of transgenic rice (Bt rice and PEPC rice) and on each line's non GM counterpart. Fructose-bisphosphate aldolase was found to be up-regulated in both transgenic Bt Rice and PEPC Rice. 5-methyltetrahydropteroyltriglutamate-homocysteine methyl-transferase was up-regulated in transgenic Bt rice. Isocitrate lyase was down-regulated in transgenic Bt rice. Cyclin-dependent kinase B2-1 and Serpin-Z2B were up-regulated in transgenic PEPC rice. These proteins are involved in separate processes.

Risk assessment

The studies above do not allow drawing conclusions about human risk assessment since no interpretation on possible hazards to consumers can be made.

4.5.3. Comparison of GM and non-GM potatoes

One study (Catchpole *et al*. 2005) was identified comparing transgenic potatoes to wild type potatoes.

Results from omics experiments

Catchpole *et al*. (2005) performed metabolomics on the potato cultivar Désirée: non-GM vs. transgenic for either sucrose:sucrose 1-fructosyltransferase (SST) or fructan:fructan 1-fructosyltransferase (FFT). A main conclusion is that metabolic changes caused through conventional breeding techniques were at least of a comparable magnitude to those resulting as an unintended effect of genetic engineering techniques.

Risk assessment

The study above do not allow drawing conclusion about human risk assessment since no interpretation on possible hazards to consumers can be made.

4.5.4. Effect of transgene on consumption

Two studies assessed the effects of consuming GM food after consumption in either rats (Cao *et al*. 2012) or zebrafish (Sissener *et al*. 2010).

Results from omics experiments

Cao *et al*. (2012) fed rats for 90 days with either T1c-19 GM rice flour or its non-transgenic MH63. The rice made up 70% wt/wt of the diet. Metabolomics was performed on the urine, which did not detect significant differences between the GM rice diet and the non-GM rice diet. In addition, real-time polymerase chain reaction (RT-PCR) on the bacterial profiles in faces did also not detect differences between the two groups.

(Sissener *et al*. 2010) fed zebrafishes for 20 days to transgenic glyphosate-tolerant Roundup Ready® soya or Bt toxin-producing YieldGard® maize. As controls, zebrafishes were fed to non-transgenic maize cultivar Hi-II and soya cultivar A5403 (the maternal, near-isogenic lines). Effects on mRNA expression of HSP90α(36), DNA repair protein RAD51(37), SOD-1 and glutathione peroxidase-1(38) and four reference genes were assessed. Compared with fish fed non-GM maize, those fed GM maize had lower mRNA transcription levels in the liver of superoxide dismutase (SOD)-1 and a tendency for lower mRNA levels of heat shock protein 70. In addition, zebra fishes fed GM maize exhibited significantly better growth.

Risk assessment

About the study of Cao *et al.* (2012) the question remains whether toxic effects might be induced that cannot be detected in urine (with the presently used method). About the study of Sissener *et al*. (2010), the approach and findings are interesting. The number of genes studied is very limited and the dose given is extremely high. Therefore, both studies yield limited information for risk assessment.

4.6. Nutrition

4.6.1. Vitamin A

Hazard

Vitamin A or retinol is a naturally occurring substance that is needed for maintaining the light sensitive cells in the eye and has a role in growth for epithelial cells. Vitamin A, converted to retinoic acid, has a role in the regulation of gene transcription. Vitamin A is available from a variety of foods, but is most strongly associated with carrots (beta-carotene) and liver. Vitamin A is oil soluble and, therefore, difficult to excrete from the body. There is a prospective link between excess vitamin A and liver toxicity (Stickel *et al*., 2009).

Two studies were identified on vitamin A (Ross *et al.*, 2011; van Helden *et al.*, 2011). These correspond to transcriptomics experiments with rats and mice and doses that vary widely (one experiment uses a dose of 1500 IU /kg diet whereas a recommended daily dose is ~5000 IU).

Results from omics experiments

One of the omics studies reports many thousand differentially expressed genes without further interpretation and neither study expresses results in terms of metabolic pathways.

Risk assessment

The relevance of these reports for risk assessment concern the investigation of the mode of action of vitamin A.

There is insufficient evidence to evaluate the impact of omics investigations on risk assessment for vitamin A. Clearly vitamin A has many important roles in human nutrition so that targeted investigations that do not assess both risks and benefits are very difficult to interpret.

4.6.2. Lycopene (synthetic lycopene)

Hazard

Lycopene is a carotenoid found mainly in tomatoes, red fruits and some vegetables such as red carrots. Interest in lycopene metabolism and regulation is growing rapidly because studies have suggested an important role for lycopene in human health promotion, such as the prevention of a range of chronic diseases including prostate cancer. There is, however, apart from information of accumulation of lycopene in tomatoes little information about the molecular processes regulating lycopene accumulation in fruits other than tomatoes. A study with oranges provided a global picture of the gene expression changes when comparing a mutant with the wild type orange (Xu *et al*. 2009). Studies with breast cancer cells should provide more information on the effect of lycopene exposure on the expression of genes in these cells.

Three studies were identified for Lycopene (Chalabi *et al.*, 2007; Guo *et al.*, 2012; XU *et al.*, 2009).

The omics studies identified involve transcriptomics and genomics. Experiments were performed with the natural plant product (citrus fruit pulp, tomato) or with cell lines (breast cancer cells exposed to 10 µM Lycopene for 48h).

Results from omics experiments

All of the omics investigations identify large numbers of individual expression changes in response to Lycopene. Transcriptomics investigations report that 32 genes are differently expressed i.r.t. metabolic pathways in tomato and 3738 genes showed 2-fold expression difference in citrus fruit pulp compared to a wild type. The genomics study reports 391 genes involved with exposure of cell lines to lycopene solution medium.

The reports include some systematic pathway analysis. Elements of apoptosis and cell cycle pathways are regularly identified as part of the omics response to lycopene.

Transcriptomics analysis describe the use of Massively Parallel Signature Sequencing (MPSS) of citrus fruit pulp. Significance of the difference of signature frequency and transcript abundance is analysed using the *z*-score method. Sequences obtained from micro-array analysis that are associated with a False Discovery Rate of <0.05 and a fold change >2 were considered as representing differentially expressed genes in tomato.

Specific software is often used for micro-array data analysis, also in the genomics investigations reported here; for each gene a ration of signal intensities was calculated, Minus Add (MA) plots were produced for each array followed by hierarchical clustering and significant genes were clustered by the *t*-test.

Risk assessment

Most of the omics studies investigate the mode of action of lycopene, investigations with breast cancer cell lines were related to hazard identification.

4.6.3. Vitamin D

Hazard

Once foods were fortified with vitamin D and rickets appeared to have been conquered, many health care professionals thought the major problems resulting from vitamin D deficiency had been resolved. However, rickets can be considered the tip of the vitamin D-deficiency-iceberg. In fact, vitamin D deficiency remains common in children and adults. *In utero* and during childhood, vitamin D deficiency can cause growth retardation and skeletal deformities and may increase the risk of hip fracture later in life. Vitamin D deficiency in adults can precipitate or exacerbate osteopenia and osteoporosis, cause osteomalacia and muscle weakness, and increase the risk of fracture.

The discovery that most tissues and cells in the body have a vitamin D receptor and that several possess the enzymatic machinery to convert the primary circulating form of vitamin D, 25-hydroxyvitamin D, to the active form, 1,25-dihydroxyvitamin D, has provided new insights into the function of this vitamin. Of great interest is the role it can play in decreasing the risk of many chronic illnesses, including common cancers, autoimmune diseases, infectious diseases, and cardiovascular disease (Holick, 2007). Besides these disorders excessive body weight is a risk factor for cardiovascular diseases, diabetes mellitus type 2, metabolic bone disorders and especially abnormal vitamin D metabolism. Yet, recent developments in genomics and proteomics have provided new opportunities to identify molecular targets of vitamin D action (Fleet, 2004).

Threee studies were identified for vitamin D (Anic *et al.*, 2012; Karami *et al.*, 2009; Ramagopalan *et al.*, 2010). These are genomics studies consisting of one experiment using a lymphoblastoid cell system and two case-control studies. One case (n=622) – control (n=628) study consists of Caucasian adult glioma patients in the USA from who DNA samples were obtained by oral rinse. In the other, renal cell carcinoma case (n=777) and control (n=1,035), study genome DNA was extracted from whole blood buffy coat.

Results from omics experiments

All of the omics investigations identify large numbers of individual expression changes in response to vitamin D. The cell system study reports 229 differentially expressed genes and the case – control study with glioma patients report 6 genes and 15 rs-numbers. The renal cell carcinoma case – control study reports numerous up-regulated genes and metabolic pathways.

Massively parallel DNA sequencing (ChIP-seq) was used to identify genomic regions in lymphoblastoid cells in response to vitamin D, subsequently specific software was used to discover motifs and the Significance Analysis of Microarray (SAM) method was used to analyse gene expression levels.

Statistical analysis in the case – control studies consist of modelling risk associations using logistic regression with odds ratios (ORs) (including likelihood ratio testing), multinomial logistic regression to examine associations and Cox proportional hazard regression to evaluate influence of vitamin D on glioma survival.

Risk assessment

The report in which the cell system was used has limited relevance for risk assessment. The case – control studies have a higher relevance as they not only investigate the mode of action, but also include hazard identification in their analysis and result interpretation. Identification of genomic

regions exhibiting increased vitamin D receptor binding helps to understand the molecular basis of complex diseases.

4.6.4. Caffeine

Hazard

Assessment of the dietary intake of caffeine is important because of the possible adverse effects of high intakes, particularly in susceptible groups such as children, infants and pregnant women (Klebanoff *et al*. 1998). Accurate consumption data are required that can be used to inform risk assessment and contribute to the provision of good safety advice. One approach to the determination of the dietary intake of caffeine, and that of other dietary constituents, is to use a dietary questionnaire. However, it is difficult to produce adequate consumption data for caffeine-containing foods and beverages using questionnaire-based intake surveys. For example, although assumptions can be made about the number of cups of coffee or tea consumed per day, the strength of the drink is difficult to estimate accurately as it depends upon the choice of drink itself and the individual preference of the consumer.

Moreover, caffeine is one of the 30,000 existing chemicals that need toxicological evaluation as a result of new European legislation. To circumvent the use of approximately 1 – 2 million test animals, new techniques for assessing toxicity are under investigation. This case study gives articles on the detection of metabolites and articles on the implementation of new toxicity studies. For example, toxicogenomic-based approaches may improve the predictive ability of alternative tests for the assessment of compound-specific mechanistic responses (Robinson *et al*. 2010).

The following studies were identifie for caffeine (Crews *et al.*, 2001; Josse *et al.*, 2012; Robinson *et al.*, 2010). Embryotoxicant-specific transcriptomic responses in rat postimplantation whole-embryo culture. Toxicol Sci, 118, 675-685.

The omics studies involve metabolomics, transcriptomics and genomics. The metabolomics study consists of human volunteers who's urine was analysed with HPLC (chromatography) up to 36 h of urine collection after the intake of 50-200 mg caffeine tablets. The transcriptomics study uses rat embryos exposed to 200 mM caffeine with subsequent micro-array data analysis. Genomics investigations were performed in a case – control study in which blood samples were taken from the Costa Rican population over a one year period (2523 questionnaires were used to assess caffeine intake). Subjects who consumed < 100 mg caffeine d^{-1} were used as a control group. Genotyping with iPLEX Gold assay and MS-based detection was used to analyse the blood samples.

Linear regression was used in the metabolomics study to relate caffeine intake to caffeine metabolites. An all-effects model (ANOVA, F-test) was used to determine the significance of impact of genes in the transcriptomics study. Here, the p-values were corrected for multiple testing (FDR) and post-hoc Student's *t*-test was used to determine the significance of the response. In the case – control study, Chi-squared test and ANOVA with Tukey's post-hoc test was used for genotyping distribution.

Results from omics experiments

Metabolomics investigations report 5 differentially expressed metabolites, the transcriptomics study identifies 200 significantly altered genes and the genomics study identifies 4 SNPs.

Risk assessment

These reports are relevant for risk assessment. The omics studies investigate for biomarkers of exposure. Three metabolites, 1,7-dimethylxanthine, 1,7-dimethyluric acis and 1-methylxanthine are

suggested to be further studied as potential biomarkers for caffeine dietary intake. Despite inducing common morphological effects, the analysis of Robinson *et al.* (2010) suggests limited overlap in terms of toxicogenomics responses at the gene level and at the level of biological processes. Age and smoking are identified as being important effect modifiers in the genomics study.

4.6.5. Zinc

Hazard

Zinc is an essential trace element for all known organisms. It is required for a range of basic biological processes, such as metabolism of proteins, nucleic acids, carbohydrates, and lipids, and is involved in other processes such as immune response, neurotransmission, and cell signalling. An estimated 10% of all proteins contains Zn which is essential for their enzymatic activity. Many transcription factors bind Zn which is also needed for their proper functioning. Zinc has low toxicity, but high intakes may lead to impairment of iron and copper uptake (EFSA, 2012d).

The following studies were identified for zinc (Jackson *et al.*, 2009; Jing *et al.*, 2007; Lin *et.*, 2009; Sun *et al.*, 2006). All used transcriptomics approaches. Experiments were performed with rats (2), human prostate cells (normal (HPR-1), benign (BPH) and malignant hyperplasia (PC-3)) (1), and human intestinal cancer (Caco2) and placental cell lines (JAR). In the rat studies the effects of zinc deficient, adequate and overdose diets on liver and pituitary gland were compared.

Results of the omics experiments

In rat liver, affected genes related to growth, lipid and protein metabolism, stress and immune response, signalling, and antioxidant enzymes. Zinc deficiency impaired antioxidant functions, zinc overdose did not enhance or impair these antioxidant functions. Similar to the liver, in rat pituitary gland, genes involved in growth, lipid and protein metabolism, stress response, and signalling were affected, and in addition, genes regulating food intake. Zinc overdose had no effect on food intake.

In prostate cells up to 3500 genes were regulated by zinc. Expression patterns were cell-type specific. Regulated genes related to protein phosphorylation, cell differentiation, transcription factors, signal transduction, cell growth and apopotosis and transcriptional DNA binding. In intestinal cells, expression patterns also differed between the cell types. In addition, gene expression was dose-dependent and dependent on duration of exposure.

Risk assessment

High intakes of zinc, beyond adequacy, did not affect antioxidant enzymes, nor food intake in rats.

4.6.6. Fluoride

Hazard

Fluoride is not an essential trace element for humans, but is beneficial in the prevention of dental caries. Fluoride increases bone density, but excessive long term intake reduces bone strength and increases skeletal and dental fluorosis. Main sources of fluoride are drinking and mineral waters, fluoridated salts, dental health products such as toothpaste and fluoride tablets. Intake from food is generally low, except when food is prepared with fluoridated water or grown near fluoride emitting plants. Insecticides may contain fluoride. EFSA established an upper limit (UL) for fluoride of 0.12 mg/kg bw/day (EFSA, 2005a).

The following studies were identified for fluoride (Ge *et al.*, 2011; Sun *et al.*, 2011; Wu *et al.*, 2010; Wurtz *et al.*, 2008; Yan *et al.*, 2007). Four used transcriptomics approaches and one proteomics. Experiments were performed with rats (2), mice (1), primary cultured human ameloblasts, and mouse odontoblasts (MO6-G3). The animal studies involved effects on brain (cerebra), sperm, and incisor pulp tissue after exposure to high fluoride drinking water.

Results of the omics experiments

In incisor pulp tissue, 250 genes were differentially expressed. These genes related to cell proliferation and apoptosis, RNA splicing and transport, NFκB cascade, protein modification and skeletal development. In mouse sperm, about 100 genes were differentially expressed, genes that are involved in signal transduction, amino acid phosphorylation, oxidative stress, cell cycle, apoptosis, glycolysis, spermatogenesis and sperm capacitation. It was hypothesised that increased apoptosis caused the decreased fertility in the exposed mice. About 70 proteins in rat brain were differentially regulated. These proteins related to cellular signalling, energy and protein metabolism, and were hypothesised to cause neurotoxicity. No effects on osteogenesis-related genes (96) were found in the human ameloblasts, but an effect on DSPP expression, a marker of early ameloblast differentiation was apparent. At much higher concentrations (100-fold) in MO6-G3 cells, fluoride differentially expressed genes encoding for extracellular matrix proteins, membrane associated proteins, TNF-receptor 9, and a chemokine.

Risk assessment

Implications for full risk assessment cannot be made, because exposures were high in the animal studies (> 100 mg/l drinking water). Together with the *in vitro* studies, these omics studies increase knowledge on potential modes of action of high fluoride levels.

4.6.7. Iodine

Hazard

Iodine is an essential trace element for humans, and is a crucial constituent of thyroid hormones, which play a role in growth and development. Thyroid hormones regulate energy-yielding metabolism, thermogenesis, protein and enzyme synthesis, nitrogen retention, gluconeogenesis and pituitary gonadotropins. A wide spectrum of iodine deficiency disorders is known: goitre, hypothyroidism, and impaired mental functions. Most countries in the world, including several European countries have some degree of iodine deficiency disorders (EFSA, 2012e).

The following studies were identified for iodide which all used transcriptomics approaches (Kitagawa *et al.*, 2005; Stoddard *et al.*, 2008; Yamada *et al.*, 2006; Yamazaki *et al.*, 2010; Yamazaki *et al.*, 2003). Experiments were performed with human cultured thyroid follicles (3), a human breast cancer cell line (MCF-7), and the yeast *Saccharomyces cerevisiae*.

Results of the omics experiments

The *ex vivo* thyroid follicle studies all used follicles isolated from patients with Graves'disease, used similar concentrations of iodine, and exposures from 24 – 48 h. Differentially regulated genes ranged from 50 to 300. Remarkably, only little overlap of regulated pathways was noticeable between these experiments. Regulated genes related to angiogenesis, energy metabolism, thyroid hormone syntheses and release, stress and immune response, adhesion molecules, IFNγ signalling, TGFβ signalling, cell cycle, and inflammatory processes. In MCF-7 cells, a very high iodine exposure differentially

expressed >40 genes, involved in lipid, steroid metabolism, cell cycle, transcription and tRNA synthesis.

Risk assessment

Implications for risk assessment cannot be made from these *ex vivo/in vitro* studies; these omics studies will add to insight in the modes of action of high iodine levels.

4.6.8. Saponin

Hazard

Saponins are a large diverse group of glycosides found in various plant species. Their aglycone (glycoside-free) moiety may vary widely and consists of a triterpene skeleton (C30), which may be modified to a steroid skeleton (C27). Additional modifications include the incorporation of nitrogen, which will turn them into alkaloids such as solanine. Saponins can have an "anti-nutritional" effect and cause toxic effects like solanine, but have also been claimed to cause beneficial health effects. As an example, saponins, supposedly pregnane glycosides, in a specified plant extract have been implicated to help appetite control (EFSA, 2010c). Saponins have been included as an additional case-study on the state of omics technologies for nutrition research.

The following studies were identified for saponins of ginseng (ginsenosides) which used transcriptomics approaches in mice and colorectal cancer cells (Luo *et al.*, 2008; Song *et al.*, 2012).

Results of the omics experiments

In a mouse experiment, 900 genes were regulated by the ginseng extract. Pathways involved were cholesterol biosynthesis, fatty acid metabolism, cell adhesion, Toll-like receptors, and nucleotide and sugar metabolism. These pathways suggest that the ginseng extract counteracted the effects of a high fat diet. In the colon cancer cells (HT-116), 400 genes were differentially regulated by the ginseng extract. Pathways involved were Eph/Ephrin, cell division, PI3K/Akt, SAPK/JNK and TGFβ signalling.

Risk assessment

Implications for risk assessment could not be made, because there was no info on potential risks.

4.7. Pesticides

4.7.1. Azole-induced teratogenesis

Retinoic acid (RA), a metabolite of vitamin A, is involved in craniofacial morphogenesis in vertebrates with jaws. The importance of the control of the RA concentration and distribution in embryotic tissue is well known. Azoles inhibit the degradation of RA. Two studies report on dysmorphogenetic effects of azoles. Transforming growth factors (TGF's) are activated by RA. In the study by Di Renzo *et al* (2009), the expression of six genes encoding various TGF's was measured in rat embryos exposed to the azole derivative triadimefon (FON). Additionally, its effect was measured on expression of molecules involved in the modulation of cellular concentrations of RA (CRABPI). The expression of three TGF's decreased after exposure. Subsequent protein analysis showed that the observed changes in level of expression of these three TGF's only resulted in a lower protein

concentration of TGF-beta1. The expression of CRABPI was also decreased. Distribution of CRABPI positive cells was abnormal in FON-exposed embryos. It was concluded that two RA-related molecules are altered by FON-exposure.

In mice, control of RA is mediated by the family of CYP26 enzymes. In a study by Tiboni, Marotta and Carletti (2010), mice embryos were exposed to fluconazole. The impact of this exposure on the gene expression of CYP26 isoforms was investigated. Pregnant mice were orally administered with 700 mg/kg and embryos were collected. Expression of three genes encoding a CYP26 was measured, of which two of them were up-regulated. This finding was reported to support the idea that inhibition of the CYP26 system represents a mechanistic component of flucanozole-induced abnormal development.

4.7.2. (Tri-)azole-induced hepatocarcinogenesis

Conazoles have a variety of toxicological outcomes in mammals including carcinogenicity, reproductive toxicity and hepatotoxicity. In mammal systems, they both inhibit and induce cytochrome P450 (CYP) enzymes. In search for a common core of transcriptional responses to three different conazoles, mice were fed with diets containing various concentrations of these conazoles. Micro-array-based transcriptional analysis revealed 330 significantly altered probe sets common to conazoles, many of which showed strong dose-responses for cytochrome P450 (CYP), glutathione S-transferase, and oxidative stress genes. A subset of 80 genes were associated with cancer, and associated with xenobiotic metabolism, oxidative stress, cell signaling and cell proliferation. Increased cell proliferation was also observed as a toxicological effect. A common transforming growth factor was identified (Hester *et al.*, 2012).

In another study (Nesnow *et al.*, 2011) mice were fed with increasing doses of propiconazole and metabolomics changes in the liver were identified. These changes were interpreted with biochemical, transcriptomics and proteonomics findings from a previous study and related to changes associated with the carcinogenesis process. It is hypothesized that propiconazole activates a series of nuclear receptors (transcription factors) leading to CYP induction, oxidative stress, suppressed RA levels and consequently enhanced cell proliferation. Observed metabolic responses (oxidative stress and increases in the cholesterol synthesis pathway) in this study supported this hypothesis.

In a study on the origin of reactive oxygen species (ROS)(Nesnow *et al.*, 2011), propiconazole was found to increase CYP protein levels leading to increased ROS levels. Authors state that the hydroxyl radical is the major ROS. Superoxide dismutase (SOD) is the enzyme responsible for the detoxification of the hydroxyl radical. No differences were observed in level of expression of the gene encoding SOD or in SOD enzyme activity between controls and propiconazole treated AML12 immortalized mouse hepatocytes.

4.7.3. The effect of low-dose pesticide intake

The daphnid embryonic development can be divided into six different stages. Propiconazole interferes with the embryonic development of Daphnia and causes development abnormalities and embryonic death. Life stage-specific and reproduction-related genes were identified by subtractive hybridization (Soetaert *et al.*, 2006). The effects of propiconazole exposure on the expression of the identified genes were studied in a micro-array. A differentially expressed gene related to embryo development was identified. Such a gene might be an interesting marker for reproductive effects of chemicals like propiconazole. In response to propiconazole exposure significant differences in embryo abnormalities and reduced growth in the population were observed. However, at different concentrations and exposure periods different genes were affected, and not always in a dose-depending manner. Therefore

authors conclude that the use of gene expression profiles for toxicity screening purposes needs further research. Understanding of the long term exposure effects on a population level requires further investigation.

In a related study by Soetaert and co-workers (2007), effects of fenarimol were demonstrated on both gene expression and organismal level. An embryo development related gene was differentially expressed and more embryo abnormalities in the offspring were observed. This result suggests that transcription analysis using micro-arrays can be used for elucidation of mechanisms and for hazard characterization.

Fenarimol was also used in a study by Park *et al* (2011). They studied the effect of low dose maternal exposure on the reproductive performance in mouse offspring. Offspring of fenarimol exposed mice had increased body weight, increased number of pups, more ovarian follicles and enhanced sperm quality. Microarray analysis in the ovaries was performed and up-regulation (82 genes) and down-regulation (743 genes) was observed. Genes involved in the regulation of steroidogenesis were up-regulated (microarray) and expression levels (qRT-PCR) of three genes involved in an estrogenic response were increased. This increase was accompanied by elevated levels of their proteins. This transgenerational animal study implies that consumption of fenarimol-contaminated diets by mothers may possibly influence the reproductive functions of their offspring.

The application of an NMR-based metabonomic approach (Merhi *et al.*, 2010) led to the identification of gender-linked variations in the level of hepatic metabolites involved in oxidative stress and in the regulation of glucose metabolism after exposure of mice to a low-dose pesticide mixture.

4.7.4. Organophosphorus insecticides and neurotoxicity

Exposure of neonatal rats to chlorpyrifos or diazinon resulted in transcriptional changes in forebrain and brainstem in 20-25% of the genes involved in cell cycle and apoptosis pathways (Slotkin and Seidler, 2012). Changes in response to both organophosphorus compounds were in the same direction and in the same order of magnitude. The doses used were below the threshold for growth retardation and first signs of systemic toxicity. Transcriptional analysis of neuronotypic PC12 cells, both differentiated and undifferentiated, exposed to chlorpyrifos revealed that the effect was development stage depending (Slotkin and Seidler, 2010, 2012). Not all affected genes could be identified.

Oocyte cultures from fetal mice ovaries show a significant decrease in survival after exposure to malathion or diazinon (Bonilla *et al.*, 2008). Gene expression analysis after exposure identified changes in the level of expression of genes encoding proteins with roles in essential/general cell processes.

Dose-depending effects of exposure to chlorpyrifos (0,2,4,5,8,10,12 and 15 mg/kg/day) on gene and pathway level in maternal and fetal mouse brain were studied by Moreira *et al* (2010). The number of differentially expressed genes appeared to be dose-depending, being highest in a mid-dose group. Lower doses influenced expression level of ontology genes in the maternal brain, whereas in the fetal brain low doses of chlorpyrifos altered the expression of genes involved in nervous system development.

Chlorpyrifos and diazinon were administered to neonatal rats at one dose (chlorpyrifos) or two doses (diazinon). Using microarrays, expression of mRNA's encoding fibroblast growth factors and their receptors in the forebrain and brain stem was examined (Slotkin, Seidler and Fumagalli, 2007). Both similarities and disparities were observed in the responses to chlorpyrifos and diazinon. The effects were observed at doses below the threshold for any signs of systemic toxicity. Authors claim that the identification and characterization of neurotrophic factors involved in the developmental neurotoxicity of organophosphates may enable the design of intervention strategies that might prevent or offset neurodevelopmental damage in cases of known exposure.

4.8. Plant health

4.8.1. Plant pathogen interaction

Hazard

Numerous *Fusarium* species have been associated with Fusarium head blight (FHB) disease of wheat, barley and other small-grain cereals. FHB is of primary concern because some species commonly produce the mycotoxin deoxynivalenol (DON) which is harmful to human and animal health (Allwood *et al.* 2010; Bollina *et al.* 2011; Walter *et al.* 2010).

The following studies were identified for *Fusarium graminearum* and *Pseudomonas syringae* (Bollina *et al.*, 2011; Sghaier-Hammami *et al.*, 2012; Walter *et al.*, 2010). The omics studies involve matabolomics (7) or proteomics (1). These studies report experiments using the whole plant, with separate results for five barley genotypes varying in resistance as compared to a susceptible genotype, experiments using an *Arabidopsis thaliana* cell system, or experiments using a part of a *Arabidopsis thaliana* plant (leaf). Experimental conditions correspond with barley surface inoculation of 1500 *Fusarium graminearum* spores (up to 72 hours) and 10^9 *Pseudomonas syringae* cells ml^{-1} (up to 24 hours) applied to *Arabidopsis thaliana*.

Results from omics experiments

All of the omics investigations identify large numbers of individual expression changes in response to *Fusarium graminearum* and *Pseudomonas syringae*. Among 1,430 *Fusarium graminearum* resistance related metabolites, 115 were putatively identified. Pair-wise analysis (resistant vs susceptible genotype) using a *t*-test was used as a statistical tool.

The total metabolism change of plant cells was quantified using FT-IR spectroscopy for the exposure of *Arabidopsis thaliana* culture to *Pseudomonas syringae*. Principal Component Discriminant Function Analysis (PC-DFA) was use as a statistical tool to analyse the FT-IR results. Mass spectrometry analysis identified 147 expressed proteins for this plant-pathogen interaction system, where a web-based software NIA array analysis tool (based on ANOVA) was used.

A range of different pathways were identified involved in detoxification and oxidative stress defence. Visual signs of disease or cell death was used as the classical effect identifier.

Risk assessment

All reports have a limited relevance for risk assessment. Namely, Allwood *et al*. (2010) describes experiments in which concentrations of total metabolomes were measured instead of identifying individual metabolites. A realistic model/system and exposure/dose is used (from a risk assessment viewpoint) in the other studies.

The role of resistance-related and resistance-indicator metabolites/proteins on plant defence, and their use as potential biomarkers for plant resistance is the basis for the plant-pathogen interaction studies.

4.8.2. Plant responses to abiotic stresses

Hazard

Local cultivars with particular resistances to abiotic stresses have attracted a great deal of interest in the field of plant omics since they might represent a biological clue to the need of increasing food

productivity worldwide, especially in those areas where the climate makes it difficult to fulfil a self-sustaining plant production politics (D'Alessandro and Zolla, 2012). The following references have been identified on analyzing plant responses to abiotic stresses include (i) drought, (ii) flooding, (iii) salinity and osmotic stress, (iv) cold, (v) heat (vi) radioactive contamination and (vii) heavy metal exposure, *i.e.* Ashraf (2010), Charlton *et al.*(2008), D'Allesandro and Zolla (2012) and Rinalducci *et al.* (2011).

The following studies were identified for plant responses to abiotic stress (pea to drought; spring wheat, orange and tobacco to cold and potato to cold and salt stress)(Yan Jin *et al.*, 2011; Charlton *et al.*, 2008; Evers *et al.*, 2012; Long *et al.*, 2012; Rinalducci *et al.*, 2011). The omics studies involve metabolomics (1), proteomics (5) or transcriptomics (1). In the experiments whole plants were exposed to a specific abiotic stress, varying in time, i.e. 12 d. to drought stress, 50 min. to 42 d. to cold stress, and 3 or 8 d. to cold and salt stress.

Results from omics experiments

All of the omics investigations identify large numbers of individual expression changes in response to abiotic stress. The metabolomics study on pea to drought stress reports 12 differently expressed metabolites, the transcriptomics study on potato to cold and salt stress report 192 differentially expressed genes and the other studies, on proteomics, report between 30 and 164 differentially expressed proteins.

NMR spectroscopy was used to profile the pea leaf metabolome and different PCR methods in combination with different mass spectroscopy methods were used for RNA extraction and protein identification in the proteomics studies. Microarray analysis was used in the transcriptomics study on potato response to cold and salt stress.

The student's *t*-test is an often applied test statistic to report changes in expression of the experimental plant compared to a control. The study on pea response to drought reports the use of a Student's *t*-test in combination with Principal Component Analysis and Partial least squares linear discriminant analysis (PLS-LDA) to determine statistical difference between input variables, variance in the data matrix and subsequent presence of data trends.

A range of different pathways were identified which were involved in e.g. photosynthesis, protein metabolism and signal transduction.

Physiological response and biochemical tests were used as the classical effect identifier.

Risk assessment

Most of these reports had limited relevance for risk assessment. Some studies included a realistic model/system and exposure/dose is used which makes them more relevant for risk assessment.

The main contribution to risk assessment from these experiments is in unravelling molecular mechanisms to be applied for cultivar selection and future crop protection.

4.8.3. Biocontrol

Hazard

Conventional agriculture is heavily dependent on the application of chemical inputs to maintain consistent high yields. There is, however, a growing desire for alternatives to this system due to 1. Environmental protection, and 2. Human health considerations. Advances in omics technology and the publication of complete genome sequences of a number of plant-associative bacterial strains, has

facilitated investigations into the molecular basis underpinning the establishment of beneficial plant-microbe interactions (Mark *et al*. 2005).

The following studies were identified for biological control (Chen *et al.*, 2009; Feng *et al.*, 2012; Grinyer *et al.*, 2004). The omics studies involve transcriptomics, proteomics and genomics. The plant root (*Arabidopsis thaliana*) or potato dextrose broth was used as a model system in the transcriptomics and proteomics study respectively. The experimental conditions correspond to high doses (2 10^7 cfu ml^{-1} surface inoculation of *Ralstonia solanacearum* to the plant root or 8 10^8 *Trichoderma harzianum* spores to the potato broth). Software was used to compare the genomes of *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus pumilus*.

Results from omics experiments

Microarray analysis in the transcriptomic investigations report 1185 differentially expressed genes in *Arabidopsis thaliana* plants pre-inoculated with a *Ralstonia solanacearum* mutant strain. A statistical analysis was performed with the LIMMA package using an empirical Bayes linear modelling approach and *P*-values were adjusted using the Benjamini & Hochberg method for multiple comparisons.

A total of 25 protein spots were identified with LC MS/MS from a strain of *T. harzianum* that exhibits biological control properties in preventing fungal growth on potato crops. A whole-cell protein map of *T. harzianum* was used as a reference map for positive identification.

The antimicrobial action (antifungal, antibacterial and nematocidal activity) of *Bacillus amyloliquefaciens* was investigated by identifying 9 gene clusters involved in the synthesis of lipopeptides and polyketides using a software package (Mauve) for genome comparison. ABA-related pathways were identified during the transcriptomics investigations and visual signs of disease were used as a classical effect.

Risk assessment

Overall, these reports showed some relevance for risk assessment in the transcriptomics, proteomics (purpose to produce an initial protein reference map) and genomics study. *Bacillus amyloliquefaciens* has been shown to produce a vast array of secondary metabolites aimed to suppress competitive bacteria and fungi within the plant rhizospere.

4.8.4. Plant pest interaction

Hazard

In nature, plants are constantly surrounded by herbivorous insects that negatively influence plant health (Arimura *et al.* 2011). Aphids, caterpillars and the grapevine pest phylloxera are major pests of agriculture world-wide and included here as examples for the use of omics in plant-pest research.

The following studies were identified for plant pest interactions (Broekgaarden *et al.*, 2007; Ferry *et al.*, 2011; Lawo *et al.*, 2011). The omics studies involve proteomics (2), metabolomics (1) and transcriptomics (2). Four experiments used the whole plant (wheat and white cabbage) and one used a grapevine rootstock, respectively exposed to the aphid *Sitobion avenae*, the caterpillar *Pieris rapae* and Phylloxera. Experimental conditions correspond with plant exposure to 1 up to 960 insects in the range of 24 h. up to 8 days.

Results from omics experiments

All of the omics investigations identify large numbers of individual expression changes in response to the insects. Proteomics investigations upon wheat-aphid interaction report 500 protein spots, the metabolomics study after grapevine-phylloxera interaction reports 38 metabolites and transcriptomics investigations after white cabbage-caterpillar interaction report 398 differentially expressed genes.

MALDI-TOF MS & database searches were used for proteomics investigations, GC-MS for metabolomics analysis and micro-array analysis in the transcriptomics studies.

A Welch's *t*-test and student's *t*-test were used to investigate significant changes in expression. Genes were identified to be differentially expressed when they showed an expression ratio ≥ 2 fold or ≤ 0.5 fold.

A range of different pathways were identified upon insect pests attack, e.g. changes in metabolism and photosynthesis, changes in protein degradation, antioxidants and other stress responses.

Risk assessment

All reports have limited relevance for risk assessment. The goal of these studies was the understanding of the molecular mechanisms. The listed metabolites or genes are difficult to evaluate in terms of risk assessment. In addition, it has been discussed that apparently global transcriptional responses in two cultivars of the same plant species in response to insect feeding can differ dramatically.

4.8.5. Xenobiotics response

Hazard

Many plant species respond to herbivore attack by an increased formation of volatile organic compounds (upon a xenobiotic response (XR) that enhances the expression of detoxifying enzymes). Insights into omics responses might help in obtaining a more thorough insight in the mechanism underlying this response (Lawo *et al.* 2011; Skipsey *et al.* 2011).

The following studies were identified for xenobiotic response (Skipsey *et al.*, 2011; Smith *et al.*, 2004; Zhang *et al.*, 2007). Safeners coordinately induce the expression of multiple proteins and MRP transcripts involved in herbicide metabolism and detoxification in Triticum tauschii seedling tissues. Proteomics, 7, 1261-1278. Available from http://www.ncbi.nlm.nih.gov/pubmed/17380533.

The omics studies involve one transcriptomics and two proteomics investigations. *Arabidopsis thaliana* and *Triticum* tauschii seedlings were used as a plant model system, respectively exposed to the following herbicide safeners: 1-chloro-2,4-dinitrobenzene (CDNB), cloquintocet-mexyl and benoxacor. Experimental conditions correspond with plant exposure to 0.1 µM CDNB (24 h) up to 100 µM (4 d and 24 h) of the other safeners.

Results from omics experiments

Transcriptomics investigations identify 3 individual expression changes (GST induction) using RNA micro-array analysis. The proteomics investigations using LC-MS identify 29 cloquintocet-mexyl safener induced proteins and one benoxacor safener induced protein (AtGSTU19).

Cluster analysis, statistical significant changes in abundance between treatments and comparative quantification with expression of the gene of interest normalized against the mean of the housekeeping genes were statistical analysis techniques used in the transcriptomics study. Data were subjected to analysis of variance (ANOVA) and relative transcript levels were calculated to statistically compare the level of expression in different proteomics study samples

A range of different pathways were identified in the xenobiotic response of *triticum tauschii* seedlings, e.g. detoxification, protein biosynthesis and secondary metabolism.

Risk assessment

All reports have a limited relevance for risk assessment. The omics studies aimed at the identification of biomarkers and at the investigation of the mode of action.

4.9. Animal health and welfare

In the field of Animal Health and Welfare there is interest in using breeding based on genetics to improve animal health. The difference in genetic make-up is likely to affect the resistance to disease in cattle. At present, however, there is insufficient information on susceptibility of individual animals to disease, and the correlation between genetics and vulnerability to certain diseases. There is a potential for omics approaches to this problem, but at present there is not scientific basis for the application of omics techniques to animal health and welfare. It is therefore omitted from this report.

4.10. Biological hazards

Whole genome sequencing was chosen as the central theme for the case studies related to microbiological risk assessment. The potential use of whole genome sequencing of foodborne pathogens for assessment of microbiological food safety risks is analysed and considered in relation to the other omics techniques relevant for microbiological hazards. To illustrate the general principles, representative case studies have been selected to elaborate on the implementation of whole genome sequencing and other omics techniques in microbial risk assessment. Selection criteria included:

- Possibility to assess the potentials of using omics data for the identification of new hazards and emerging risks,
- Possibility to study uncertainty in methodology and data gaps for the potential application of omics data in risk assessment,
- Primary importance to the EFSA panels
- Trends and sources of zoonoses (EFSA, 2012f)

The case studies presented below are not examples of the past application of omics techniques for risk assessment, as these were not encountered in the scientific literature. Instead the case studies are to be considered as illustrations of prospective applications of whole genome sequencing and other omics techniques for microbial food safety.

The theory underlying genomic analysis of microbial food safety has been developed for many years, starting in the early years of the new millennium, illustrated by an early review of the potential of genomics for microbial food safety (Abee *et al*., 2004). A wide variety of applications, even including combating bioterrorism (Segerman, *et al*., 2011) has been suggested. Most emphasis is aimed at epidemiology within the public health setting, undertaking to detect and preferably prevent outbreaks (Svraka, *et al*., 2010, Cheung & Kwan, 2012, Grad, *et al*., 2012).

The following sections focus on:

- The use of whole genome sequencing for detection of outbreaks and source attribution,
- Prediction of virulence and other health related properties of foodborne microbes,
- The development of new diagnostic tests based on relevant (biological significant) genetic markers based on comparative analysis,

- Identify core or variable genes/regions enabling the pathogen to survive in particular environments (ecological niche),
- Identify core or variable genes/regions related to pathogenicity, like high-plasticity zones,
- The understanding of the origin and emergence of a pathogen,
- Determine the evolutionary relationship of pathogenic strains,
- Bridging the gap between the omics data and the experimental data on the dynamics of pathogens in the food and host,
- Managing of the huge amount of raw data, and
- To predict disease severity on the basis of genetic diversity at the individual and population level.

4.10.1. Whole genome sequencing and other omics techniques as a tools for promoting microbial food safety

The extremely rapid advances in sequencing techniques open up new avenues for promoting microbial food safety (Abee, *et al.*, 2004, Boxrud, 2010, Carrico, *et al.*, 2013). Now that whole genome sequencing (WGS) can be performed for just over € 100,- per strain with the expectation that the price will soon approach € 50,- it is becoming a cost-effective technique for large scale application. The advantages of WGS are considerable, because both taxonomic and functional information is becoming available at the same time (Sabat, *et al.*, 2013). The data can be used for more than one purpose. For example, WGS data gathered for outbreak detection can be analysed for the presence of virulence or antimicrobial resistance genes without additional costs. In addition, when WGS is applied large-scale older data based on e.g. PFGE will still be useful, because the genomic data predict the band pattern. Therefore, older databanks can still be used for analysis and comparisons. The major disadvantage is that it requires a considerable paradigm shift, including different data analysis and vastly different methodology for laboratories, and therefore considerable initial investments.

An omics approach based on WGS can be applied to all microorganisms and even to viruses, once the genetic information is linked to phenotypical data (Zhang, *et al.*, 2010). Often this type of data is already available, as many virulence and resistance genes have been identified for a large number of microorganisms. If this approach is followed on a wide scale and the data are deposited in databases that are made available to qualified researchers, epidemiological exploration can identify hitherto unknown genetic factors and connections between these. For example, in EHEC strains both toxin and adhesion genes must be present for the strain to be virulent. An epidemiological analysis can establish combinations of toxin and adhesion genes that are particularly virulent, or almost certainly innocent. In a later stage the analysis might be complemented with data on expression and metabolic activity. The combined genomics, proteomics and metabolomics could conceivably yield such a complete description of a microorganism that its growth and other characteristics can be estimated with sufficient accuracy to enable to predict the quality of a foodstuff on the plate of the consumer, based on measurements in the production stages (Brul, *et al.*, 2011). This would greatly improve the usefulness of microbial criteria and other tools used to ensure food safety.

At present a variety of techniques is used to classify microbes, serotyping being one of the most used. The disadvantage of this methodology is that it does not in itself provide information on the characteristics of the organism involved. This information is only indirectly available by linking old epidemiological data to the serovar status. Preventive action must then be based on shifts in the distribution of serovars, which do not necessarily correlate to the actual food safety risks. The advantage of WGS is that it can be used for taxonomic purposes, while at the same time revealing information on virulence factors and other relevant traits (Carrico, *et al.*, 2013).

Re-examining the EHEC outbreak of spring 2011 clearly shows the potential of WGS as a food safety tool. The genetic homology indicates the chance that the isolates from different patients and foodstuffs are from the same source and can even give an indication of when the isolates have originated from a common source. In combination with information on what the patients have eaten, where they have bought food, in which restaurants they have eaten, etc., the genetic information can reveal the common factors and thus the source of the outbreak. Had this methodology been available, detection of the common factor would have taken less time and the link to the Finnish patient who was infected by the same strain would have been made in a much earlier stage. The combination of two virulence factors, the stx2a gene and the intimin genes coding for enzymes that enable attachment to the intestinal epithelium, would have been discovered immediately. This would have facilitated the risk assessment (Sabat, *et al.*, 2013).

In general, for every organism a database in which sequence data are matched to the genome sequence will have to be established. These database will always be a work in progress, as new information can be expected to become available regularly. Once sequences are stored annotated and complemented with information on where and from which source the isolates were obtained, the data can be analysed for many different purposes. The data generated by WGS can be used simultaneously with information derived from other techniques, so that in-depth analysis can be accomplished with comparatively little effort. Again, older data acquired in a different way will still be relevant and can be incorporated in new investigations.

4.10.2. Typing for biological based target setting of *Salmonella* within Europe

Several opinions have been published by the EFSA Biohaz Panel (Anonymous 2009 and 2011) on serovar typing in relation to target setting in food for the most frequently isolated (top five) serotypes of *Salmonella*. In addition, an external scientific report is under development for evaluating targets in the turkey meat production. These analyses are all based on serovars selected from human cases in relation to the distribution of different serovars within the EU Member states. Subsequently, a virulence factor for 23 serovars has been defined based on the correlation between human cases and the occurrence of these types in the Member states. Genetic analysis of the serovars can elucidate the underlying causes for virulence. The case of typing of *Salmonella* with the aim of target setting is included because omics techniques have great potential to contribute to achieving this objective. This in turn, would facilitate the process of risk assessment considerably.

The limit of the past approach, as further explained below, is that the method strongly relies on historical epidemiological data, continuous monitoring of serotypes prevalence, notifying possible changes in the frequency distribution of serotypes (both quantitatively, i.e. in numbers, and qualitatively, i.e. in types). Complementing these data with genomic information will provide new insights.

Salmonella prevalence and serovar distribution, however, varies widely among the European Union Member States and over time (Huehn, *et al.*, 2010). In addition, it is not established whether virulence (factors or determinants) are serovar related. Risk based target setting would, therefore, benefit from a bottom-up approach. That is, if virulence (factors or determinants) would be established for individual strains of *Salmonella*, then predictions could be made of the impact of a variety of strains on public health. Several techniques are available to analyse *Salmonella* strains on a molecular level.

Several strains of *Salmonella* are in the process of being sequenced or have already been sequenced. This information has been used to develop whole genome microarrays to study the variation in genome content within *Salmonella* enterica subspecies enterica. However, the wealth of data from whole genome microarrays can be difficult to handle and may be of limited use because only on the DNA microarray represented strain-specific targets are considered. Consequently, arrays with a lower

number of targets, representing specific bacterial properties such as virulence genes (virulotyping), have been developed.

Relating virulotyping patterns to phenotypic behaviour in the food chain will enhance risk assessment. The advantage of this approach is that it could reveal clustering based on virulence (factors or determinants) rather than based on serovar properties. Behavioural knowledge based on the biology of strains will enhance targeted interventions.

Emerging risks can be observed at the beginning of the food chain rather than when established in the population. In this respect it is important to continue to review the literature that becomes available on virulotyping in relation to *Salmonella*. This will reveal the type of data that becomes available from these studies. Subsequently, one can explore if, and how, this data can be processed for risk assessment. Insight in the available information will be the basis for the foresight on how to use virulotyping of *Salmonella* in all steps of microbial risk assessment.

4.10.3. Use of genotypic diversity for hazard characterization: *Campylobacter*

Bacterial species have the ability to cope with the ongoing environmental changes encountered in nature. For this they possess a variety of adaptation mechanisms, among which genomic adaptation which depends on genetic diversity. Genetic diversity refers to any variation in the DNA of an organism at the nucleotide, gene, chromosome, or whole genome level. These changes can lead to changes in virulence, host range and survival in hostile environments.

This case is included because the progress made in genomic approaches offer tremendous opportunities to identify new risks due to modified *Campylobacter* strains. The main tool to study genetic diversity is comparative genomics, which, linked to knowledge of virulence factors, will provide valuable input for risk assessments.

Comparative genomics will enable investigators to unravel evolutionary relationships and pathogenicity of food-borne pathogens and reveal the genes involved in virulence, severity of disease, host specificity, ecological niche and mechanisms to adapt to particular nutrient supply in certain food.

Genome comparison can identify strain-, lineage- and niche-specific regions and track evolutionary process of loss, acquisition and variation of genetic information. Furthermore, comparison between the genome sequences of commensal bacteria and those of pathogenic bacteria can identify genes that are observed in host specificity and the mechanisms of host-microorganism interaction.

There has, for example, been significant interest in studying the core lipo-oligosaccharide (LOS) of *C. jejuni* as of its potential role in paralytic disorders. In reference to other studies, Habib and co-workers (Habib, *et al*., 2009) screened *C. jejuni* isolates from chicken meats and human cases of diarrhoea by PCR for five LOS classes. In addition, invasion potentials were tested in relation to the LOS classes. Results of this analysis were compared to PFGE and MLST data.

4.10.4. Source attribution: *Campylobacter*

Infection with *Campylobacter* is one of the leading causes of foodborne disease in Europe and throughout the developed world. Source attribution is an essential step in identifying targets for preventive measures. In the industrialized societies, outbreaks of *Campylobacter* are rare, but sporadic cases contribute to a considerable disease load (de Haan, *et al*., 2010). Up till now multilocus sequence typing has mostly been used to identify the strains involved in diseases and to trace them back to the origin (Strachan, *et al*., 2009, de Haan, *et al*., 2010).

The main reason for including this case study is the potential for omics information to contribute to source attribution. Up till now this kind of approaches have not yet been applied to source attribution, but it can easily be imagined how omics information can be used to investigate the distribution of virulent strains.

Tracing back the pathogen to the origin is key to attribution studies, if one wants to be certain about the source. A more overall relationship can be established by comparing strains isolated from patients with strains found in likely sources, such as chicken farms or cattle. This approach was used to calculate the disease load from chicken farms to their surroundings, cleverly using a period of absence of chickens following culling because of avian influenza (Friesema, *et al*., 2012).

Several methods that are genomics or closely related omics approaches have been compared for their ability to distinguish clusters and thereby outbreaks (Clark, *et al*., 2012). Comparative genomic fingerprinting (CGF) in case of need supplemented by analysis of the flaA gene short variable region, turned out to be the best technique for detecting clusters of related cases. Multilocus sequence typing (MLST) by itself was not sufficient, while pulse-field gel electrophoresis (PFGE) could at times incorrectly group together strains that were in fact not related.

The methodological problems outlined above can be overcome by relying on WSG to identify strains, establish relationships between them and perform an instant risk assessment based on the virulence factors that are present. Given the recently strongly reduced costs and the possibilities for automated analysis, WSG seems to be an avenue to be explored with priority. Other advantages of WSG, such as compatibility with older classification systems have been outlined above.

4.10.5. Detection of emerging pathogens: Viruses

The discrimination between infectious and non-infectious viral particles is notoriously difficult. It can be imagined that whole genome sequencing combined with statistical analysis will be a successful approach for predicting the ability of viral particles to infect. This case is included because the potential to solve this long-standing problem deserves to be investigated.

Attempts have been made to distinguish between these two states of norovirus, using RT-PCR preceded by binding to specific receptors, but this method turned out not to be fully discriminative (Li, *et al.*, 2011). A similar method was further developed using the binding to porcine gastric mucin to differentiate between infectious and non-infectious norovirus (Dancho, *et al.*, 2012). This method definitely has the potential to reduce false-positive RT-PCR outcomes when incorporated in the enrichment procedure. It is not clear, however, to which extent the separation is quantitatively reliable. Reverse genetics mediated recovery of the virus from samples may yield reliable data on the number of infectious particles (Arias, *et al.*, 2012a, Arias, *et al.*, 2012b), but also this technique needs to be further developed for large scale application.

4.10.6. Rapid detection in relation to safety assessment of diffuse outbreaks: *E. coli* O104:H4

The EHEC-crisis in spring 2011 has demonstrated very clearly the need for a system that can detect diffuse outbreaks in the earliest stages possible and ideally provide at the same time information needed for risk assessment. The rational for including this case study, is to explore the potential of whole genome sequencing to accelerate the detection of diffuse outbreaks and to apply it towards discovery of the source.

For example, if the first isolates of the O104:H4 strain would have been subjected to an analysis that included virulence genes, the risks associated with this strain would have been clear. Moreover, the

link to the Finnish patient who got infected with the same strain in Egypt in autumn 2009 would have been established as well.

In summary, detailed outbreak case study assessments, in which the most relevant and recent literature should be included, should be followed in close cooperation with EFSA (e.g. as in the provisionally subjects described above; toxicological risk assessment of single food chemicals/contaminants, safety assessment of products derived from (GG) crops and omics in rapid detection of diffuse outbreak in combination with risk assessment). Based on these outbreak studies, limitations and added values of omics approaches can be clearly identified. Forecast studies and future requirements for a full incorporation of omics in risk assessment can then be defined and further developed.

4.10.7. Identification of virulence factors for the screening of species for qualified presumption of safety (QPS)

The principle of QPS is to remove unnecessary burden during the authorisation process of microorganisms to be added into the food chain by considering taxonomic units instead of individual strains (EFSA, 2011e). Taxonomic units are eligible for the QPS list if they do not raise any safety concerns. Organisms belonging to taxonomic units on the QPS list do not need to undergo safety assessments before being used in food production. In the framework of a notification for QPS, existing safety concerns can be defined as specific qualifications, to be addressed in a summary assessment procedure. WGS can provide the detailed genetic information that is needed to predict the potential virulence or absence of it of strains under consideration for QPS status.

This case study is included in the overview, because whole genome sequencing has the potential to facilitate the efforts of EFSA authorising the QPS status of specific strains and to reduce the workload by simplifying the assessment.

Genomics, in particular WGS, has enormous potential in the QPS context. It can be used for taxonomy (Pearson, *et al*., 2009, Klenk & Goker, 2010) and for typing at the most detailed level (Boxrud, 2010). The limitations of WGS identified earlier (Hyytia-Trees, *et al*., 2007) are likely to be overcome as more information becomes available once WGS will be used on a larger scale for this purpose. The additional benefits that the use of WGS brings are that not only taxonomic data are generated, but that also information on virulence factors, resistance genes and other characteristics of the cell become available with little additional effort. As genes can transfer between bacterial species, the taxonomic unit to which a strain belongs may not always accurately predict its features, such as virulence, the ability to form toxins or antibiotic resistance.

It can be imagined that the QPS system will convert at some moment in the future from a taxonomy based system into a system based on genome information. The predictive value of taxonomy is limited, because genetic changes are always possible. Our knowledge of genomics at the level of individual genes is not yet so complete that predictions can be made with absolute certainty, but the continuously evolving body of knowledge steadily increases the accuracy of genome based forecasts. It would be logical if in due time genomics would be used for QPS instead of taxonomy.

5. The added value of omics technologies as compared to classical risk assessment methods

Presently, toxicological tests for risk assessment are based on exposures of test animals with very high doses, a method that is being used for several decades with relatively few adjustments. The new omics tools promise 1) to provide much more information about the action of chemicals, and 2) are able to detect effects at more realistic (lower) exposure doses. This review provides cases for this.

As to microbial food safety, omics have not yet been successfully applied often. When applied they never target the host, but instead focus on the pathogen. Mostly utilized for this purpose has been genomics. Genetic information on pathogenic strains allows not only to establish relationship between the strains, but also to predict some of the traits, based on the presence of virulence factors, resistance genes or other properties. The review focuses primarily on the use of whole genome sequencing for these aims, as the potential benefits of the use of WGS are immense and already genomics using partial sequences has shown the real benefits. The other omics techniques have obvious potential benefits, but these cannot be realized until considerable additional research has been concluded successfully.

6. International projects in the field of omics related to food and feed safety

Chemical risk assessment:

With the U.S. currently a program is running entitled the Next Generation of Risk Assessment (NexGen; http://www.epa.gov/risk/nexgen/). The aim is to improve current RA by integrating the outcome from projects such as ToxCast (http://actor.epa.gov/actor/faces/ToxCastDB/Home.jsp) and the use of new molecular biology approaches, including omics techniques, into the risk assessment process.

Safety assessment of herbal preparations (incl. TCM):

EU-funded FP7 Coordinated Action GP-TCM (Uzuner *et al*, 2010; 2012)

Microbiological risk assessment:

The 100K Pathogen Genome Project is a consortium that addresses food safety concerns by engaging world-wide partners to create a publicly available genetic database of the most common foodborne disease causing microbes (http://100kgenome.vetmed.ucdavis.edu/).

MIMOmics network of experts (http://www.mimomics.eu/). A collaborative project funded by the EU through the 7th FP (FP7/2007-2013).

7. Conclusions

7.1. Omics technologies in risk assessment for chemical substances

Risk assessment involves multiple components: hazard identification, hazard characterization (dose-response assessment), exposure assessment and risk characterization. In a 2007 publication of the U.S. National Research Council of the National Academies (U.S. National Research Council of the National Academies, 2007) (Toxicity Testing in the 21st Century: A Vision and a Strategy) the potential relevance of toxicogenomics for risk assessment was already considered. In 2009 the U.S. EPA outlined, using dibutyl phthalate (DBP) as a case study, a framework for the use of toxicogenomics in risk assessment and also identified a number of issues for future consideration. Since there were no dose-response genomic data for DBP, the case study focused on the qualitative application of genomic data to risk assessment. Actually, as is the case for DBP, the majority of omics studies on chemical compounds are lacking dose-response data. Therefore, these studies have limited relevance for quantitative risk assessment and do not provide insight into the issue at which exposure dose the first adverse effects are being induced.

Very few of the studies applied doses that were more nearby actual exposures. Examples are the exposures to estrogens published by Heneweer *et al*. (2007); Kato *et al*. (2004) and Newbold *et al*. (2007). Interestingly, these low doses did affect the mRNA expression levels of many genes in either the uterus or liver while effects on classical toxicological parameters could not be detected. This

indicates that mRNA expression profiling is a very sensitive technique. This also raises the next challenge: to which extent can alterations in mRNA expression be interpreted in relation to adverse effects. A limited activation of the oestrogen receptor in the uterus will lead to induction of oestrogen receptor target genes, but that does not necessarily has to result in disturbance of endocrine system. An important issue to address in the future, therefore, is to obtain more insight in the relation between alterations and magnitude of these alterations of expression of genes (or metabolites or proteins) in relation to adverse effects. Likewise, it will be important to derive insights in genes which change in expression due to an adaptive response. Alterations of expression of these adaptive genes should not automatically be interpreted as an adverse effect.

The biological interpretation of the changes of mRNAs, proteins or metabolites plays a crucial role in the potential of omics techniques to assist risk assessment. One has to interpret these changes into knowledge on pathways or processes that are affected. Thereafter, one has to determine through dose-response studies at which level of distortion of these pathways adverse effects are being induced, a process which is called "phenotypic anchoring". It should be noted that many omics studies do involve biological interpretation. In general, this leads to important information about mechanisms of action of compounds. For hazard identification, information about mechanisms of action is certainly very important. In almost all cases, however, the mechanisms of action are derived from high dose studies. As mentioned above, these studies do not allow conclusions on actual exposure levels. Recently, however, the first papers appeared dealing with the use of omics data for quantitative risk assessment (Thomas *et al* 2011; 2012).

In conclusion, all studies identified on chemical compounds have limited value for risk assessment. In almost all studies dose-response data were lacking and high doses have been used. Omics studies do have a potential for risk assessment due to their high sensitivity. This might enable detection of alterations of mRNAs, proteins or metabolites at levels close to the actual exposure levels. For the future, it will be very important to acquire knowledge about alterations and magnitude of these alterations of expression of genes (or metabolites or proteins) in relation to adverse and adaptive effects.

7.2. Omics technologies in risk assessment for GMO's

Several studies used omics methods to compare GM crops to their wild type parents. This was often combined with culture at different locations and environments. In each of these studies, effects of the transgenes on either mRNAs, proteins or metabolites have been found. A difficult issue is to interpret these effects in relation to safety of consumers, nutritional quality or impact on environment. In one study (Batista *et al*. 2008), the observed changes in mRNAs could be contributed to induction of stress to the plant. The consequences of this finding and those published by others remain unclear. Several authors compare the effects of culture at different locations to the effects of the transgene. They hypothesize that a transgene is not harmful when the variation in omics response induced by the transgene is smaller than that of different environments. This hypothesis, however, has not been supported yet by experiments, for example by creating and examining GMO plants of which harmful effects are obvious. An additional question is how many different factors, such as locations, soils, climates, fertilizers need to be assessed by omics technologies to obtain a representative view of all natural variations.

A second approach involves application of omics technology to assess effects on consumption of GMO food. This approach has been applied by using either rats (Cao *et al*. 2012) or zebrafishes (Sissener *et al*. 2010). Due to the limited number of parameters tested and application of very high doses, these studies yield very limited information about risk assessment.

7.3. Omics technologies in risk assessment for microbiological hazards

The review for the application of omics in microbiological risk assessment was not based on past applications as these were not found in primary scientific research papers. It focused on the application of WGS, which is promised to be a useful tool in linking genetic information to phenotypical data for the purpose of food safety risk assessment. Here it was investigated how WGS could be a helpful tool in relating virulence factors to serotyping for biological based target setting, enable us to unravel evolutionary relationships and pathogenicity of food borne microorganisms, source attribution to identify targets for preventive measures, predicting the ability of viral particles to infect, accelerate the detection of diffuse outbreaks and facilitate the efforts in authorising the QPS status of specific strains. The overall conclusion is that however promising the developments may be, WGS has not been implemented in everyday food safety risk assessment.

7.4. Current limitations of omics technologies for their application in risk assessment

The use of omics techniques vs classical risk assessment methods

As stated in the Introduction section, it is widely believed that the omics approaches can reveal patterns of response, for biological systems, that cannot be detected by classical methods. In this way omics can, potentially, expose new and more powerful modes for intervention in relation to the control of hazards associated with food and feed.

Reviewing relevant case-studies revealed the following considerations to put these beliefs into perspective.

- This interest in the application of omics techniques rises from the need for refinement-, reduction-, and replacement alternatives for animal testing not only from an ethical and economical point of view, but also to increase the predictive value of current tests.
- Indeed, the application of omics techniques can aid the development of *in vitro* models to replace animal models. An advantage is that the effects on gene expression alterations precede clinical effects. Therefore, mechanisms of action can be detected after short exposure times of animals or even with use of *in vitro* techniques.
- Much of the information relevant to estimate human risk (e.g. dose-response data) is based on evidence from animal models. Translating these results to a human health risk introduces uncertainty. The application of omics techniques might reduce this uncertainty as 1) insight in the mechanism of action at the cellular and molecular level is generated and 2) possible biomarkers for toxic actions might be identified which may lead to bioassays.
- Most of the information used for threshold quantification in classical risk assessments is extrapolated from evidence on relatively high dose effects. Omics techniques can reveal an 'adverse' effect at lower doses.

It is now generally considered that in relation to chemical hazard/risk assessment toxiconomics provide tools to:

- understand mechanisms of toxicity/toxicological mode of action (MOA),
- determine QSARs (facilitate extrapolation between similar chemical structures),
- reduce the uncertainties with respect to extrapolation within and between species,
- identify (early, sensitive, specific) biomarkers of exposure and effect,
- develop cost-effective alternative toxicity test methods, particularly *in vitro* systems that can refine, reduce, and replace animal experimentation.

Current limitations and contemplations

Despite all the promises of the different omics techniques, it is recognized from this review that various issues have to be addressed before it can be applied for regulatory and risk assessment purposes. Here, we summarize the most important limitations:

For the identification of the MOA, knowledge about the functions of affected genes is required. At present, however, the function is not yet known for a relatively large proportion of the genes affected by a treatment. These genes will be disregarded for biological interpretation while they will be included in the biomarker approach.

In addition, many processes will have an overlap in genes. The biological interpretation of pathway analysis should thus be done with caution and be confirmed by biochemical or cytological experiments.

In many cases, omics techniques will detect effects, for example on mRNA expression, of exposure levels lower than the NOAEL (e.g. Heneweer *et al*., 2007). This implied advantage of being very sensitive, also incurs a risk for being too sensitive. Phenotypic anchoring will maintain important to link induction or repression levels of genes to clinical manifestation, and thus for its application in risk assessment.

Omics investigations that follow a traditional dose-response design appeared to be quite rare so that the translation of omics information into the regulatory risk assessment framework, or into other decision making, is particularly difficult.

Earlier UK and US reviews identify significant challenges associated with quantification of risks based on omics datasets (e.g. Ein-Dor *et al*. 2006).

Most of the omics studies performed so far are not designed for the purpose of food safety risk assessment. So, a proper framework design for an omics study (e.g. the use of a range of doses to support dose-response modelling) is crucial to be able to perform (quantitative) risk assessment.

Statistically significant correlations between omics and physiology have not yet been established.

Fundamental aspects (e.g. study design, analysis of results and statistical approaches) of the omics investigations are often not sufficiently enough addressed in research papers.

It is often difficult to use data from previously-published omics studies in risk assessment since these are often generated for a different purpose and, therefore, processed in a different way.

It would be informative to reanalyse original omics data were it is available. Important in that respect is to define rules on data-generation to make datasets more accessible.

Most importantly, it must be noted that however promising all developments may be, they have not been realized in everyday food risk assessment (chemical or microbiological) to this date.

Finally, the following issues were identified which could support the further investigation of this topic:

- Should omics experiments always provide knowledge about the MOA of compounds?
- When the omics results for a “new compound” are very similar to that of a known toxic compound: is it then sufficient to state that the “new compound” is toxic as well?
- Should risk assessment always be based on *in vivo* omics results or are *in vitro* results sufficient as well?
- Should omics studies always be confirmed by a focused classical toxicological study?

- Is there a risk that omics analysis is too sensitive? Can we discriminate between a "healthy defence response" vs a toxic response? For example, should always a comparison be made to food components/supplements that are considered to be healthy?
- What are the risks that omics studies provide false positive or false negative results?
- How many cell systems have to be used to state that a compound is not toxic?
- Are there cases of successful bioassays resulting from omics findings?
- Could the integration of omics provide an adding value for risk assessment?
- Is the genotypic information obtained by whole genome sequencing sufficient to predict the phenotype?
- Can transcriptomics on the pathogen be used to predict host-pathogen interactions?
- What type of omics information is needed to predict growth and survival of pathogens in a given matrix?
- International cooperation is crucial for the full utilization of omics data in food safety risk assessment. Databases of genomes need to be accessible to all researchers in the area, so that genomes can be compared and past experience of others can be used to predict the virulence and other characteristics of an agent under study. Considerable additional research is needed for the application of omics to predict host-agent interactions and the behaviour of agents in given matrices.

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EXTERNAL SCIENTIFIC REPORT

A foresight study on emerging technologies: State of the art of omics technologies and potential applications in food and feed safety[1]

REPORT 2

Application of omics to hazard and emerging risks identification and foresight on potential future applications of omics in risk assessment

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ABSTRACT

Representative case studies were selected to document omics technologies for the identification of emerging hazards in food and feed related to chemical hazards, GMOs, nutrition and plant health, and biological hazards. The main knowledge gap in hazard identification using omics data is in the difficulty to establish a one to one relationship between the exposure and a biomarker. Translating omics data generated in animals and *in vitro* studies to human and plant physiology remains uncertain. In general, statistical issues concern the many different approaches for omics data analysis, both in the visualization and interpretation of the data, which makes it difficult to compare results from different studies. Pathway level descriptions, based on omics studies, provide the best opportunities for high throughput chemical risk assessment. The most relevant application of omics data to early detection of emerging microbiological hazards lies in the combination of genomics and epidemiology. The lack of microbiological omics data on host-pathogen interaction and changes in expression of pathogens under different conditions makes it difficult to use omics data for risk assessment. Whole genome sequencing is the most promising relevant technique for its application in risk assessment, source attribution and in detecting (diffuse) outbreaks. Still, the availability of (easily accessible) databases with genomic information that can predict the behaviour of a strain is a challenge. A methodology for hazard identification based on 'intermediates' for exposure and effect needs an extension of the current data available concerning with the elements of risk assessment. In addition, methods for statistical (pathway) analysis should become comparable between research studies such that a relational database could be implemented for valuable use.

KEY WORDS

Omics, hazard identification, risk assessment, data gaps, omics, emerging risk, foresight.

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The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the European Food Safety Authority is subject. It may not be considered as an output adopted by EFSA. EFSA reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the authors.

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SUMMARY

This report deals with the application of omics to hazard and emerging risks identification and a foresight study on the potential future developments of omics technologies and possible implications for risk assessment in food and feed safety.

Hereto, representative case studies were selected from the review of the literature to document if and how omics technologies have been used for the identification of new and emerging hazards in food and feed related to chemical hazards, GMOs, nutrition and plant health, and biological hazards. Data gaps related to methodology, experimental set-up, data availability and statistical approaches were identified. Furthermore, suggestions on how to improve the data collection for its optimal use in future hazard identification studies are given. Finally, plausible developments in the application of omics techniques have been described for the areas of greatest impact, challenges for implementation have been discussed and steps to take to bring the integration to a next level in the next 5-10 years.

Results show that, for chemical hazards, the role of omics in hazard identification and emergence of risk is dominantly a search for biomarkers of exposure, or a process to discover mechanisms that connect biomarkers of exposure with intermediate biomarkers of effect, in situations where there is an established statistical association between exposure and disease.

The main knowledge gap in hazard identification using omics data is in the difficulty to establish a one to one relationship between the exposure and the marker. Experimental designs often show very high exposure levels and un-physiological routes of exposure. Furthermore, translating omics data generated in animals and *in vitro* studies to human and plant physiology remains uncertain. In general, statistical issues concern the many different approaches for omics data analysis, both in the visualization (clustering techniques) and interpretation (subjective) of the data, which makes it difficult to compare different study results. Investigation of case-studies shows that pathway level descriptions, based on omics studies, provide the best opportunities for high throughput chemical risk assessments.

The most relevant application of omics data to early detection of emerging microbiological hazards lies in the combination of genomics and epidemiology. Genetic analysis of both pathogens that are related to sporadic disease cases and to outbreak events can be used to reduce public health risks through the detection of virulence factors, resistance genes and genetic diversity. Still, the lack of microbiological omics data on host-pathogen interaction and changes in expression of pathogens under different conditions makes it difficult to use omics data for risk assessment. Whole genome sequencing is the most promising relevant technique for its application in risk assessment, source attribution and in detecting (diffuse) outbreaks. Still, the availability of (easily accessible) databases with genomic information that can predict the behaviour of a strain is a challenge.

In general, it became apparent that a methodology for hazard identification based on ‘intermediates’ for exposure and effect needs an extension of the current data available concerned with the elements of risk assessment: biomarker identification and mode of action. In addition, methods for statistical (pathway) analysis should become comparable between research studies such that a relational database could be implemented for valuable use.

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Background as provided by EFSA

A key objective of EFSA is the evaluation of new methodologies and technologies for risk assessment applied to food and feed safety. These may present complex methodological challenges for risk assessment as well as opportunities for emerging risk identification, impacting EFSA's mid- to long-term work.

Firstly, new and emerging technologies can raise questions relating to information gaps (*e.g.* reliability and interpretation of the results, data quality standards) and methodological uncertainties (*e.g.* integration of new knowledge in the existing risk assessment framework). Secondly, scientific and technical innovation is a well established driver of change linked to the food and feed chain, with potential implications, in the case of omics, for the identification of emerging risks. For example, new analytical methods can improve our capability of identifying new hazards, new or increased exposures, or groups of the population whom may be more susceptible to certain environmental contaminants.

In the post-genomic era, the scientific community is now witnessing major advances in omics technologies (*e.g.* genomics, proteomics, metabolomics, toxicogenomics, etc.). Omics technologies are firmly established as research tools, and are gaining credibility in risk assessment, particularly toxicology, as they may offer advantages over traditional approaches. Compared to traditional methods, omics technologies appear to combine the benefits of relative simplicity, sensitivity and speed of generating information, potentially reducing the need for animal testing.

Emerging risks have been defined by the scientific committee of EFSA as follows: "an emerging risk to human, animal and/or plant health is understood as a risk resulting from a newly identified hazard to which a significant exposure may occur or from an unexpected new or increased significant exposure and/or susceptibility to a known hazard". A growing body of evidence is now becoming available on the application of omics for the identification of new hazards and emerging risks, including toxicological screening and prediction of chemical substances, and the identification of new and reliable biomarkers. Omics technologies have already been applied to the food and feed safety area, for example in the identification of biomarkers, the elucidation of mechanisms of action of toxic chemicals, the identification and screening of new and emerging contaminants in food, the evaluation of nutritional health claims, the safety assessment and evaluation of substantial equivalence of GMOs, the detection and characterisation of foodborne pathogens, and the investigation of the association between diet and cancer risk.

Whilst omics may have major implications for EFSA's scientific activities, current methodological and analytical uncertainties do not yet allow the identification of how and to what extent omics technologies can be integrated within the current risk assessment framework, and to which extent they can be fully exploited for emerging risk identification.

At an international level, risk assessment bodies, including US-EPA, WHO, and OECD are currently starting to consider the integration of omics in their risk assessment frameworks, mainly in the field of mechanistic toxicology.

Thus, this procurement aims to critically review the state of the art of omics technologies applied to food and feed safety, in order to understand possible future implications for risk assessment and emerging risk identification in the areas under EFSA's remit.

Terms of reference

Application of omics to hazard and emerging risks identification. The objectives of this part of the assignment is to provide EFSA with a scientific report documenting how omics technologies can be applied to hazard and emerging risks identification, in the areas under the remit of EFSA and a foresight study on the potential future developments of omics technologies and possible implications for risk assessment in food and feed safety for the next 5-10 years.

This contract was awarded by EFSA to:

Contractor: The National Institute for Public Health and the Environment (RIVM)

Contract title: Foresight study on emerging technologies: State of the art of omics technologies and potential application in food and feed safety.

Contract: CT/EFSA/EMRISK/2011/02

1. Introduction and objectives

This procurement aims to critically review the state of the art of omics technologies applied to food and feed safety, in order to understand possible future implications for risk assessment and emerging risk identification in the areas under EFSA's remit. The main objectives of the contract resulting from the present procurement include a review of the state of the art of omics technologies applied to risk assessment, with a focus on food and feed safety, and a foresight study on the potential future developments of omics technologies and possible implications for risk assessment in food and feed safety. This report deals with a foresight study on the potential future developments of omics technologies and possible implications for risk assessment in food and feed safety.

As a first step, case studies were reviewed with respect to their relevance for hazard identification (the first step in risk assessment). Representative case studies were selected to document if and how omics technologies have been used for the identification of new and emerging hazards in food and feed. Subsections address chemical hazards related to human health (2.1) and GMOs (2.1.1.), hazards related to nutrition and plant health (2.2), and a final section is devoted to biological hazards (2.3).

Subsequently, the expertise of the authors in risk assessment was used in section 3 to identify the data gaps related to methodology, experimental set-up, data availability and statistical approaches in order to improve the use of omics for future hazard identification in food and feed safety risk assessment. Here, a separate section was devoted to case studies as assigned to the chemical (3.1) and the microbiological (3.2) part. Hazards related to GMOs, nutrition and plant health are incorporated in section 3.1, because the data gaps resulting from these studies show a big overlap with those in the chemical-human interaction case studies. Specific or additional issues were addressed where appropriate.

Suggestions on how to improve the data collection for its optimal use in future hazard identification studies are given in section 4. Finally an overview is given of the current European and international projects concerning omics data and risk assessment.

Section 6 describes the potential future developments of omics technology and its implications for food and feed safety risk assessment. This section describes plausible developments in the areas of greatest impact, challenges for implementation and steps to take to bring the integration to a next level in the next 5-10 years.

Although separate sections have been devoted to chemical hazards, GMOs, nutrition, plant health and biological hazards, a strong overlap exists between these areas in data gaps, suggestions for improvement in data collection, scientific developments and challenges for implementation. It is, therefore, recommended to read through the whole document to get an overall view on the current state and future prospects in the application of omics techniques to improve food and feed safety.

In this document the terms hazard assessment and hazard identification are used as defined by the US EPA http://www.epa.gov/risk_assessment/health-risk.htm and further explained in section 2.

2. Omics data for the identification of new and emerging hazards in food and feed

Emergence, in relation to the hazards and risks associated with food and feed, is a complex concept that is generally applied at a population level. In practice a hazard or risk previously believed to be insignificant is emergent when it assumes a higher priority (in the absence of prior information this step might be described as hazard identification). In the simplest case, when hazards are associated with exposure to harmful agents, emergence can be classified as (i) the identification of a new adverse health effect (or at least biological activity) that is associated with an existing exposure (agent) (ii) the identification of increasing exposure (i.e. due to consumption) to an agent that is known to be associated with adverse health effects (iii) the identification of a sub-population that has a high rate of adverse health effects (sensitivity) associated with a particular agent with an established exposure.

This representation of emergence is distinct from the emergent behaviour that is commonly associated with non-linear physics or other 'order from chaos' ideas including non-equilibrium phase transitions and self organization etc. (e.g. Kauffman, 1995).

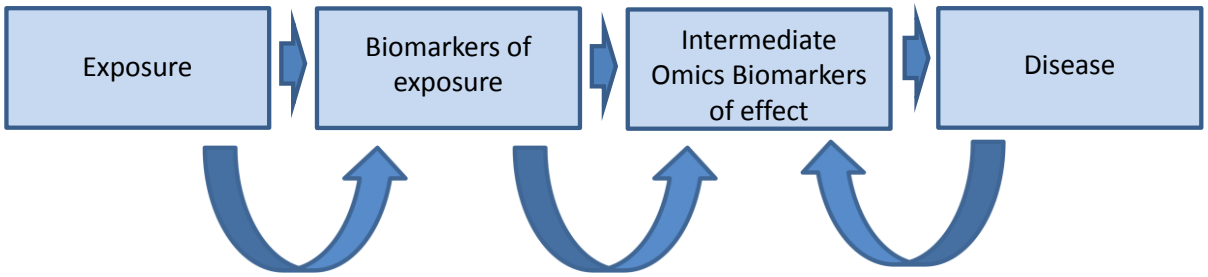
In relation to the hazards and risks from food and feed the concept of emergence centres on an identified association, i.e. statistically connected observations of an exposure and an effect (usually disease). In turn investigations that relate to emergence (i) can provide initial identification of the association (ii) can develop the statistical significance of an established association, (iii) can attempt to infer a causal relationship between the agent and the effect for an observed association (often by combining omics results with additional information).

2.1. Chemical hazards related to human health

In practice, for chemical hazards, the initial identification and the quantification of the associations, for exposure and disease, are dominated by classical methods and particularly by classical toxicology. The review conducted in the first part of this project on omics reports relating to chemical hazards confirms that omics methods are used predominantly in a search for causal relationships where a known association exists and particularly they are used for the identification of mode of action.

The complexity of human responses to chemical exposures ensures that complete details of a causal response are difficult to identify. Vineis *et al*. (2013) highlight the role of intermediaries, loosely described as biomarkers, that can be used to establish the structure of a causal link between exposure and disease in stages; a 'meet-in-the-middle' approach. Vineis *et al*. (2013) emphasize that omics methods are effective at the identification and quantification of the intermediaries and hence contribute to causal discovery in relation to emerging risks and hazard identification.

The figure below (adapted from a figure in Vineis *et al*. (2013)) illustrates the position of intermediaries in the causal route from exposure to disease. Small arrows illustrate the causal steps and curved arrows indicate the opportunities for experimental discoveries. Linking biomarkers of exposure with intermediate biomarkers of effects adds to observed association between exposure and disease. Investigations of these links are often categorized as the discovery of a mode of action. (According to the US EPA the mode of action is a sequence of key events and processes starting with the interaction of an agent with a cell, preceding through operational and anatomical changes, and resulting in an adverse effect. In addition, a key event is an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element; NRC, 2009). The development of a mode of action is an attempt to find mechanistic information that can explain or clarify an observed dose-response association but, within a database of omics reports relating to chemical hazards, omics data is usually too limited to explain fully (quantitatively) how a chemical exposure leads to an observed effect.



Exposure itself is a complex concept that has recently been expanded to include a life history of time varying chemical levels experienced by each individual – the exposome (e.g. Wild, 2012). Chemical hazards associated with food and feed contribute, dominantly, to the specific external component of the exposome and the corresponding real exposures are very difficult to quantify so that biomarkers of

exposure, such as serum levels of metabolites, accumulated alterations of DNA/proteins or changes in expression in transcriptomics, proteomics etc. often act as surrogates. Within a database of omics reports relating to chemical hazards, there are some efforts to establish markers of exposure using omics methods.

In turn, specific disease outcomes, particularly in relation to chronic effects, are difficult to ascertain in controlled conditions so that a prospective search for intermediaries is often a step in the hazard identification process. Case control studies, such as genome wide associations, are used to identify intermediate markers which predict disease states or bottom up approaches (systems biology) are used to unravel metabolic pathways that are essential for health.

Examples of omics reports illustrating components of hazard identification

Thus, for chemical hazards, the role of omics in hazard identification and emergence of risk is dominantly a search for biomarkers of exposure, or a process to discover mechanisms that connect biomarkers of exposure with intermediate biomarkers of effect, in situations where there is an established statistical association between exposure and disease (most often established from classical toxicology).

Examples have been selected from the review conducted in the first part of this project that represent the contribution of omics research and its (statistical) data analysis to unravel the link between exposure and effect for chemical agents.

Kellert *et al*. (2008) describe an explicit metabolomics search for biomarkers of furan exposure in the urine of treated rats. Principle components analysis is used to identify a small number of potential markers from an initial screening involving approximately 450 metabolites (including unknown species). However the report does not establish a strong statistical significance for the biomarkers of exposure so that additional results are required to initiate a mode of action assessment.

Feng and Lu (2011) used liquid chromatography and mass spectrometry to identify modification of human blood proteins caused by acrylamide (or glycidamide). The results clearly identify modifications (mainly N-terminal excess), but do not establish a quantitative relationship between the marker and the exposure. The authors indicate that the results could be the starting point for understanding metabolic pathways (i.e. a mode of action assessment) without providing details. In this case the identification of a marker of exposure is only a very preliminary step in the hazard identification process (i.e. it indicates a method for hazard identification rather than an identification itself).

Hispard *et al*. (2011) report a proteomic investigation aimed at the identification of biomarkers of exposure for cadmium in rats. Although comparative proteomic analysis clearly identifies biomarkers, including Cu/Zn superoxide dismutase, the report stresses that the method has some limitations for practical analysis of the effect of cadmium and hence the results cannot easily contribute to a search for a causal mechanism of action.

Bjork *et al*. (2008) report a transcriptomics investigation combined with a pathway analysis that identifies a causal route relating to the effects on the liver of PFOS (perfluorooctane sulfonate) exposure in rats. The investigation uses two intermediate markers of effect, peroxisome proliferation and decreases liver triglycerides, and identifies a corresponding significant signal in differential expression of approximately 450 genes in the rat liver. The report concludes that a mode of action for PFOS effects in neonate rats involves transcriptional control for the metabolism that is dominated by the PPARa pathway. Potentially this mode of action is significant in hazard identification for human exposures to PFOS.

Abdullah *et al*. (2012) report complex comparative proteomics experiments involving liver tissue of both wild type and Nrf2 knockout mice to explore defence mechanisms in response to BHA **(**butylated hydroxyanisole) exposure. The results confirm the role of the transcription factor in the induction of

the defence mechanism against xenobiotic toxicity. The investigation uses a set of established anti-oxidant response proteins as an intermediate marker of effect and establishes the relative significance of Nrf2 in coordinated control to identify causation within the pharmacodynamic mode of action of defence against BHA. The report indicates that the identification of the mode of action, for defence against BHA exposure, might have relevance for risk assessment in relation to other chemical exposures.

Duan *et al*. (2011) describe a case control metabolomics investigation for children exposed to melamine. This report identifies hypoxanthine as a biomarker of melamine induced nephrolithiasis (kidney stones) i.e. it identifies an intermediate marker of effect that contributes to a causal interpretation. The authors suggest that elevated levels of hypoxanthine are responsible for purine metabolism disorders and that maintaining normal levels of hypoxanthine can decrease risk. This report does not indicate a causal link between exposure and effect.

Mally *et al*. (2010) describe metabolomics initially aimed at full dose-response assessment for furan exposures but were unable to establish a statistically significant association with disease. However, the report identifies a significant increase in unconjugated bile acids associated with exposure and these are described as indicators of hepatic injury. In practice the report identifies intermediate biomarkers of effect which can contribute to the discovery of a mode of action and, ultimately, hazard identification.

Bakshi *et al*. (2008) explore the mode of action of low dose cadmium exposure in human prostate cells using transcriptomics. A set of genes involved in inflammation and immunomodulation act as an intermediate marker of carcinogenesis and network analysis of global transcriptomics reveals a network involving many of the genes where tumour necrosis factor (TNF) is a dominant element. The network adds a causative belief, transient over expression of TNF, to the mechanism for cancer development in prostate cells.

2.1.1. Chemical hazards related to GMOs

The hazard identification of genetically modified plants concerns three topics: 1) potential impact on the environment, 2) safety for humans and animals after consumption, and 3) nutritional quality. Two studies applied omics technology to assess the effects of consumption of genetically modified food. Sissener *et al.* (2010) fed zebrafish for 20 days with genetically modified soya (Roundup Ready®) or maize (YieldGard® Bt maize, MON810) or with their non-modified, maternal, near-isogenic lines. Omics (qPCR) revealed a decreased mRNA expression of SOD-1 in the liver of zebrafishes fed with GM maize. Although this approach is interesting, it is very difficult to link an effect on one gene in zebrafish to implications for risk assessment for humans. In another study (Cao *et al.*, 2012), rats were fed for 90 days to T1c-19 rice flour or its transgenic parent MH63 at 70% wt/wt. (1)H NMR analysis on urine sampled every month detected changes in levels of creatine, citric acid, a-ketoglutarate and hippurate. The effects were, however, very limited and mostly not consistent at the three time points. It is, therefore, questionable whether the observed differences are really due to the transgene.

In other studies, omics, mainly transcriptomics, was applied on the GM vs. the non-GM plant itself, for example GM maize vs. non-GM maize (Manetti *et al.*, 2006; Barros *et al.*, 2010) or GM vs. non-GM rice (Montero *et al.*, 2011). In general, differences between the GM and non-GM variants were detected. However, at present it is impossible to link these observed effects to an increased hazard upon consumption.

2.2. Hazards related to nutrition and plant health

Reports from the review conducted in the first part of this project in the area of nutrition concern vitamin A, lycopene, vitamin D, caffeine, zinc, fluoride, iodine and saponin that are, except for

caffeine, mainly related to health promotion. Omics studies are used to discover intermediate biomarkers of effect (gene expression) with molecular techniques as a new opportunity to unravel the mode of action (in all reports) or for direct hazard identification in particular (lycopene and vitamin D). It appears to be difficult to statistically correlate exposure to effect as of numerous differentially expressed genes involved in many pathways and the many effect modifiers at the level of biological processes (e.g. age and smoking).

Metabolomics, proteomics and transcriptomics studies are used to investigate molecular mechanisms underlying plant health exposed to pathogens, abiotic stress and pests or plant health in relation to biocontrol and xenobiotics response. The omics studies are generally set-up to identify biomarkers and investigate the mode of action to be used for cultivar selection and future crop protection. As large numbers of individual expression changes in response to test agents are identified results are difficult to interpret in terms of risk assessment.

2.3. Biological hazards

The main limitation in applying omics technologies to the identification of microbial new and emerging hazards is that in food microbiology omics data are focused on the microbes and less on the effect on the host. Novel approaches to overcoming this problem are being developed (Carrico *et al*., 2013; Ursell *et al*., 2012), but are not yet applicable at the moment of writing, spring 2013. The most significant application of omics data to early detection of new microbiological hazards lies in the combination of genomics and epidemiology (Carrico *et al*., 2013). The EU-sponsored SAFE FOODS project examined the role of horizontal gene transfer in creating new microbial food safety risks (Kelly *et al*., 2009). Since most of the scientific literature in the area of emerging microbiological risks to food and feed safety concentrates on the genetics of the pathogens, this aspect will be highlighted.

A literature search of past examples of identification of an emerging pathogen by means of genomics or another omics approach yielded no records. On the other hand, several reviews explore the potential of genomics for this aim (Gupta *et al*., 2009; Koser *et al*., 2012; Sabat *et al*., 2013). The strongly reduced costs of whole genome sequencing (WGS) make it now feasible to use WGS a as standard typing methodology. The Achilles heel of WGS was up till now the interpretation of the enormous amount of data generated. This situation should soon improve, as some major initiatives to facilitate data analysis are being developed. One example is the joint project of University of California at Davis and the Food and Drug Administration (http://100kgenome.vetmed.ucdavis.edu/) to collect at least 100,000 complete genomes of food pathogens. Another is the Pathogen-annotated tracking Resource Network (PATRN) system (Gopinath *et al*., 2013). The Food Microbe Tracker (http://www.foodmicrobetracker.com/login/intro.aspx) is a web-based tool that specializes in *Listeria monocytogenes*.

Bioinformatics at the genome level has the most potential to predict new and emerging microbiological risks, as these have very recently been used to explain events after they happened (Grad *et al*., 2012; Nilsson *et al*., 2012; Scheutz *et al*., 2011). The presence of virulence genes can be used to predict the properties and the associated risks of strains under investigation (Abee *et al*., 2004; Bhagwat and Bhagwat, 2008; Withee and Dearfield, 2007). The mere presence of virulence genes by itself may not be always diagnostic. The expression and the resulting virulence can still be influenced by a large variety of factors, but the potential to be expressed given certain conditions is of greater importance for risk assessment than whether they are actually expressed in the laboratory.

Microbial food safety has to deal with two situations: 1) pathogens that are often present on certain products, such as *Campylobacter* spp. on poultry meat and cause sporadic disease cases and 2) outbreak organisms that are usually found in very low numbers. New and emerging risks can also be the consequence of two types of events: 1) genetic changes in microorganisms and viruses, a good example is the STEC O104:H4 that caused the outbreak in spring 2011, and 2) environmental, economic and other factors that drive changes in the microbiota, resulting in new pathogen/product

combinations. In neither case are the possibilities mutually exclusive, but more they represent the extremes of a continuum. The combination of these two sets of parameters yields a matrix that can be used to describe each new hazard that is encountered. This should be kept in mind when reviewing new and emerging microbiological food safety risks.

For the disease load caused by sporadic cases, genetic analysis of pathogens such as *Campylobacter* or *Salmonella* spp. that are often present on products of animal origin, can be used to improve production on the one hand and food safety and public health on the other (Diaz-Sanchez *et al*., 2013). Changes in the composition of the gut microbiota can be monitored and intervention is possible before these alterations cause food safety risks. Genetics of the individual pathogens can be used to predict changes in the pathogenicity. The formation of biofilms, a major virulence factor in the case of STEC and *Listeria monocytogenes*, can be predicted based on genetic information (Sofos and Geornaras, 2010).

The case of zoonotic viruses deserves special attention, as the jump from animal to human may be accompanied by increased virulence (Haagmans *et al*., 2009). Genomic analysis on a monitoring basis can detect newly emerging pathogenic strains by comparing strains under investigation with closely related known pathogens. The adaptation of new variants can be documented and analyzed to understand the biological consequences of transmission. The cross-over of rotavirus between animal reservoirs and humans has been followed by genomic analyses and the outcome provided important insights in the manner of transmission and the consequences of the interspecies jumps (Midgley *et al*., 2012).

The first concern during an outbreak is the identification of the source. In fact, quite a lot of outbreaks go undetected, as the necessary data are not connected and the outbreak is not recognized (Boxrud *et al*., 2010). Now that WGS can be used as a tool for rapid typing, it will have the major advantage that the sequence data also provide information about characteristics that are relevant for food safety, as virulence factors and resistance genes can be identified (Zhang *et al*., 2010). If the outbreak concerns a modified strain, that combines genes from different origins, this will be recognized. Once more experience is gained in predicting the properties of new strains that combine known genes in combinations that have previously not yet been encountered, an important tool to counteract new and food pathogens has been obtained (Hoorfar, 2011). It will be imperative for the detection of outbreaks to connect information on isolates from human patients to strains isolated from foodstuffs.

Outbreak detection and rapid hazard characterization can be combined for *Campylobacter* spp, as virulence in this genus is well investigated. Genomic analysis can reveal the nature of the core lipo-oligosaccharide (LOS), known to affect human immune response (Matsumoto *et al*., 2010). Comparison of human and chicken isolates yielded epidemiological correlation between LOS and the invasion potential (Habib *et al*., 2009). In the past a variety of methods was used, but all can be replaced by WGS, so that the complete genomic information can be applied to both typing and risk assessment.

As a rule the genus *Salmonella* is considered genetically stable and new variants are supposed to not occur very often. More recent data do suggest more genetic diversity that previously assumed (Eisenstark, 2010). An example may be the Salmonella Thompson that caused an outbreak by contaminating salmon cuts (Friesema *et al*., 2012). The investigation into this strain is still ongoing, but there are indications that some genetic changes compared to older strains may have occurred. The distribution of serovars and their prevalence varies widely within the EU (Huehn *et al*., 2010). The consequent utilization of WGS on foodborne *Salmonella* is likely to yield a wealth of new information, once the data collection has reached a certain completeness.

The recent EFSA opinion (Biohaz panel, 2013) on STEC and EHEC bases the risk classification largely on genetic properties such as the presence of the different *stx* genes and the genes coding for adherence factors. In combination with the WGS tools that are being developed, both the genomics of an epidemiology can be analyzed (Grad *et al*., 2012) and an instant risk assessment performed (Sabat *et al*., 2013). More complete information on the various virulence factors will be needed to use this

tool with a high level of confidence, but even now strong indications as to the potential hazard can be obtained.

Application of risk management tools, such as microbiological criteria and the concept of "qualified presumption of safety" (QPS) can be based on genomics as well. As in most other examples studied, the genomic information is more relevant than the other types of omics. For QPS taxonomic status is of foremost importance. Taxonomic classification can be genome based (Klenk and Goker, 2010; Pearson *et al*., 2009). Microbiological criteria can be more specifically applied when not the taxon is subjected to a target value, but the organism can be further specified on the basis of genetic properties.

3. Data gaps to improve the use of omics for hazard identification in food and feed safety

3.1. Gaps in hazard identification exposed by a review of chemical omics reports

Most studies compiled in the review conducted in the first part of this project generated omics data that can be used for obtaining insight in the mode of action (MOA), by identifying pathways affected by the compounds, and as such can assist hazard identification, the first step in risk assessment. However, a number of gaps can be identified and should be tackled in order to improve the use of omics data for hazard identification in food and feed safety risk assessment. These gaps are related to methodology, experimental set-up, data availability, and statistical approaches used for analysis of the omics data.

Gaps in hazard identification methodology

A structure that includes intermediate markers for exposure and for effect provides a framework for organization and evaluation of data from omics reports in relation to the identification of chemical hazards. However, the structure also assists identification of gaps and weaknesses in an appreciation of omics investigations of the chemical hazards that are associated with food and feed:

- Primarily it is clear that hazard identification, based on omics methodology, is largely complementary to classical techniques that discover association between chemical exposure and disease. The dominant role of omics information concerns identification of the mode of action corresponding with particular chemical exposures. Some omics investigations identify biomarkers of exposure but, since omics responses are complex, it is difficult to establish the essential elements of a one to one relationship between the exposure and the marker.

- Several records in a database of omics reports relating to chemical hazards describe exposures that are mixtures; these include an investigation of mixtures of food additives by Radonjic *et al*. (2007), an investigation of the combined effects of perfluorinated compounds by Wei *et al*. (2009) and an investigation of the effects of melamine and cyanuric acid mixtures by Xie *et al*. (2010). Current data structures are usually organised according to pure agent types so that it is difficult to capture multi-component exposures. Potentially the responses to multi-agent exposures, measured by omics methods, are complex and cannot be analysed with respect to pure components. It is clear that multi-component exposures can be considered as emerging hazards of the second kind (increasing exposures) and that characterization of the exposure is a significant challenge.

- A macroscopic appreciation of chemical hazard identification concerns direct association of exposure with disease. However it is clear that, at a genetic or molecular level, many other processes such as control, defence or repair are involved and that these should be considered as part of a hazard identification step. A report by Abdullah *et al*. (2012), concerning anti-oxidant proteins in rat liver tissue, concerns the mode of action for BHA but also concentrates on cellular processes that protect against toxicity. The identification of defence mechanisms corresponds with emergence of the first kind (novel biological effect) and these do not fit easily into the current hazard identification (exposure-effect) framework.

- Complex metabolism ensures that chemical exposures, viewed at a molecular level, may trigger multiple responses and may contribute to more than one end point of concern (particularly in relation to different levels of exposure). It is difficult to represent the multiple modes of action within single hazard identifications so that currently each is identified independently. Current hazard identification often fails to integrate the distinct end points associated with different hazards associated with a single exposure.

- Despite of the common identification of morphological effects, the analysis of omics responses often suggest limited overlap at the gene level and at the level of biological processes. This can be due to the presence of confounding factors in case-control studies. Also, targeted investigations that do not assess both risks and benefits (as present in many of the 'nutrition' studies where hormesis plays an important role) are very difficult to interpret.

Experimental design and data availability

Many of the omics studies identified in the review have limited value for additional aspects of hazard identification, other than MOA of the compounds. Common limitations are related to the experimental design of the studies, i.e.:

- The use of very high exposure levels, way above the expected human (or plant) exposures. This is remarkable since one of the roles generally attributed for toxicogenomics in risk assessment is that it allows to analyse low-dose effects (with omics changes as possible biomarkers of overt effects that would occur at higher doses). Some of the papers identified potential biomarkers of risk (genes, metabolites, proteins), but exposures used were unrealistically high (e.g. vitamin A). The usefulness of these biomarkers can only be judged after testing at realistic exposure levels. Examples were shown for arsenic and lead, where exposure to levels which may occur in Europe, increased gene expression and protein levels of immune mediators (Kozul *et al*., 2009), and proteins involved in cardiovascular disease (Birdsall *et al*., 2010). Other examples are rodent studies that used low doses of estrogens (Heneweer *et al*. 2007; Kato *et al*. 2004; Newbold *et al*. 2007). Interestingly, these low doses affected the mRNA expression levels of many genes in either the uterus or liver while effects on classical toxicological parameters could not be detected.

- The lack of toxicokinetic (i.e. absorption, distribution, metabolism, and excretion, ADME) data. This is particularly the case for *in vitro* experiments but also *in vivo* studies often apply unphysiological routes of exposure (intravenous, intraperitoneal) meaning that the toxicokinetics of the compounds is not properly addressed.

- The uncertainty of translation of omics data generated in animals and *in vitro* studies to human and plant physiology. Although, for most of the compounds, experiments have been performed in different species, the information available on inter-species variability is too fragmentary to be useful. As for plant physiology, it is clear that plant cell cultures (as often used in omics experiments) will not express the full physiological interactions which are prevalent in a whole plant.

Statistical issues and data availability

Quite generally omics methods transform the appreciation of complex, multi-component, biological behaviour into the appreciation of complex, multivariate, data sets. Simple representations of the complex information from omics experiments correspond with large arrays of real numbers where rows are labelled by object identities (i.e. gene names) and columns are labelled by distinct realizations (i.e. experimental conditions, times etc.). Values correspond with the measured signals, such as fluorescent intensity, that represent activity; usually the number of rows is much greater than the number of columns. Visualization and analysis of these kind of large data sets revealed the following statistical issues.

- One observation from the data collected in the review is that the overlap in pathways affected by the same compound in different studies is rather low. In part of these studies that might be due to differences in species, gender, exposure route etc. However, in most studies identified, the overlap in pathways between studies and species generally is quite low. Even overlap in pathways in studies using the same species, gender, exposure route etc. the overlap in pathways may be remarkably low as was shown for dioxins (Nault *et al*., 2013, Kopec *et al*., 2010 and 2011). One of the reasons might be that the (raw) data of the omics studies are generated and processed in different ways, e.g. by applying different statistical and bioinformatics methods. This is a general gap that should be bridged in order to increase the usefulness of omics data for hazard identification.

- The combination of pre-processing, feature selection and classification ensures that there are many alternative predictive modelling approaches associated with omics data sources. Several reviews deal with the principles and established methods for analyses of microarray data in relation to safety evaluation, e.g. Irwin *et al* 2004, but the general approaches apply equally to data analysis for proteomics, metabolomics etc. The US Food and Drug Administration, within their Microarray Quality Control Study, have summarized this variety of approaches and have made recommendations for best practice for gene expression analysis (MAQC, 2010). Although the MAQC study is largely aimed at clinical decisions it is clear that the majority of modeling guidelines are equally appropriate for chemical safety evaluation. Furthermore, statistical analysis of omics data sets in relation to safety assessments has recently been reviewed by the UK Committee on Toxicity (COT, 2010).

- Raw data sets associated with omics technologies often require substantial manipulations, such as standardization, logarithmic transformation and normalization, prior to analysis. These processes reduce non biological variability associated with the data collection and are often integrated into the particular experimental platforms. Although this pre-processing step is essential for effective data analyses it is usually considered as an inert precursor for higher level approaches aimed at the discovery of biologically relevant information.

- Higher level analyses of omics generated data sets, which are aimed at biological pattern discovery, can usefully be partitioned into two complementary activities:

- Identification, classification and visualization of the biological response patterns (i.e. of mRNAs, proteins or metabolites) associated with a particular effect and
- Evaluation and classification of an identified response in terms of a biologically relevant frame of reference

Identification of the response is most often realized as the construction of a list of differentially expressed mRNAs, proteins or metabolites while evaluation of the response is often manifested as the interpretation of the identified expression changes in terms of known pathways or other schemes of organized biological activity. Each of these activities can be performed using a variety of data analysis strategies or statistical techniques. The majority of reports, concerning the use of omics generated data sets in relation to chemical safety, do not include detailed description of the statistical analyses.

- A list of differentially expressed genes (or proteins, or metabolites etc.) is most easily generated, one gene at a time, by direct comparison of measured expression values in distinct realizations of the complete system; most often a simple threshold for the relative expression selects the list members. The actual thresholds are, largely, subjective and it is very difficult to give a statistical interpretation (significance) of the generated list. Alternatively if the measured expression values for a particular gene, for distinct realizations of the system, can be assumed to arise from a single population of values, with known properties, the population statistics can be used to identify differential expression e.g. based on a measure of statistical significance and, again list members can be identified one gene at a time.

One by one identification of the list members can be replaced by statistical schemes that score the change in expression associated with groups of connected genes; in principle the collective

identification of differential expression is more sensitive but it also relies on a priori knowledge of functionally related groups of genes. Several computational schemes, which incorporate appropriate knowledge bases of known gene sets, are established e.g. gene set enrichment analysis (GSEA) (Subramanian *et al.*, 2005).

- The essential omics data structure (large number of genes and a relatively small number of treatments) ensures that the potential for false discovery, i.e. assigning a gene to the differential list as the result of observing a random fluctuation rather than as a result of causal behaviour, is a major statistical concern (e.g. Reiner *et al*., 2003). Replicating experiments, to address the statistics (dependency) of false discovery, is often considered impractical or uneconomic. Routines for controlling the false discovery rate of omics data analyses are incorporated into pattern identification methods but are rarely quantified in chemical safety investigations. The statistics of false discovery is often associated with an argument that restricts omics approaches to initial screening in relation to safety evaluation.

- A list of differentially expressed genes is usually the starting point for exploration of relevant biological effects. Hereto, a range of statistical approaches are applied. Clustering approaches (unsupervised learning) are employed to discover groups of genes that have similar expression profiles or to discover groups of treatments that initiate similar molecular responses. In turn, by systematic comparison with established knowledge bases, the groups of genes can be identified with coordinated biological responses (often 'pathways') and the grouped treatments can be classified as coherent conditions. Hierarchical cluster analysis is most intuitive (Eisen *et al*., 1998) but K-means clustering is also widely employed. Clustering methods satisfy many of the requirements for complexity reduction but resist probabilistic interpretations suitable for quantitative safety assessments.

Principal component analysis (singular value decomposition) is an alternative method for complexity (dimensionality) reduction that is often applied to omics data sets (e.g. Alter *et al*., 2000). Matrix operations reveal special combinations of genes (normalized linear combinations - eigenvectors) that are mutually orthogonal and are ranked in their ability to account for the variation in the data. Principal components (eigenvectors) are assumed to represent independent biological behaviours (pathways or regulatory systems) associated with particular realizations. The corresponding combinations of expression values (eigenvalues) quantify the ability of each eigenvector to represent the variation observed in the data; and hence quantify the power of low dimension representation of the complex system.

- Data generation and processing have probably been performed differently between the various case studies from Deliverable 1. Most studies do not have their raw or pre-processed data in public repositories (i.e. publicly accessible databases). Accessibility of omics data to other research groups allows to reanalyse data from a group of studies using a processing tool of interest. Important in that respect is to define rules on data generation to make datasets more suitable.

In summary, fundamental aspects related to methodology, experimental set-up (study design), generation and analysis of data and statistical approaches can often not sufficiently enough be taken into account in a data base study to fully exploit the omics data for hazard identification. A number of the aspects have systematically been addressed in the EPA dibutylphatalate case study (Euling *et al.*, 2011; 2013).

These issues, particularly the use of a range of doses, are equally crucial to move the use of omics data towards quantitative risk assessment (hazard characterization in particular). Recently, the first papers dealing with the use of omics data for quantitative risk assessment appeared (Thomas *et al.*, 2011; 2012). This topic will be discussed in more detail under Objective 4.

3.2. Gaps in hazard identification exposed by a review of microbiological omics reports

The lack of data in microbiological omics with regard to risk assessment is encyclopaedial. Most striking is the almost complete lack of omics data on host-pathogen interaction, but also very little is known on the changes in expression of pathogens under different conditions. Only between genomic information and pathogenicity some correlations have been established, the best example being the various virulence factors in (S)VTEC pathogens (EFSA, 2013). The correlation in this case is between the presence of a gene and the risk of disease. More complex correlations, such as between transcriptomics and pathogenicity have not yet been established. Metabolomics could in principle be used to predict efficacy of probiotics, but the knowledge to substantiate this is lacking. For the application of the QPS principle the various omics techniques might be able to contribute, but also in that case the existing knowledge is not sufficient.

Host-pathogen interactions have two aspects that could be studied by omics techniques without the need for technical developments. Transcriptomics on mRNA of the pathogen would reveal the internal adjustments the microbe makes in order to successfully attack the host. Similarly transcriptomics on the immune system seem to be the most relevant technique to investigate the reaction of the target cells within the host. Technical developments, or at least the development of a new microarray, would be needed to study the host-pathogen interaction at the transcriptomics level in a single integrative manner. If enough mRNA of both can be collected from a single experiment, than it would in theory be possible to perform an analysis without separating the two kinds of mRNA. In reality, considerable difficulties can be expected concerning both the techniques and the interpretation.

By far the most advanced insight on the basis of omics has been accomplished on the action of probiotics on the host (Bron *et al*., 2012). Specifically the prediction of individual differences in the reaction of hosts to probiotics is very promising. The complete knowledge on the human immune system underpins the application of the various omics. The characterization of the bacterial effect or molecules is less complete. While a lot of research still needs to be done in this area as well, it at least provides a good template for the application of omics in the other fields of microbial food safety.

Transcriptomics, possibly combined with metabolomics, might be applied to predict the behaviour of known or suspected pathogens under given conditions and those data could be utilized for risk assessment. The advantage would be that risk assessments could be fine-tuned to the extent that one could predict that for example a certain microorganism on tomatoes is harmless, while the same strain on meat can become invasive. This would prevent these tomatoes being removed from the market on suspicion of being a threat to the consumer, while in reality they posed no risk. It remains to be seen, however, how much confidence the risk manager will have in this type of data, given the natural tendency of authorities to err on the safe side.

The ability to predict the growth and persistence of bacteria on certain foodstuffs under specified conditions underlies the application of microbiological criteria, performance objectives, food safety objectives, etc. Data on the microbiological condition at certain stages in the production process, should allow to predict the quality and safety of the food on the plate of the consumer. There are very few, if any, food/pathogen or food/spoilage organism combinations for which this situation has actually been achieved. The thorough understanding of the physiology of the organism needed for this holy grail could be accomplished by means of an integrated omics approach. At present there are insufficient data to accomplish this goal.

Safety of a complete class of microorganisms is judged under the qualified presumption of safety (QPS) approach. Taxonomy has up till now been the sole foundation for decisions on inclusion on the QPS list. It can very well be imagined that not only genomics, but also the other omics approaches can be useful for the judgement on QPS status.

In general, omics on foodborne pathogens results have to be sufficiently reliable to function as a base for risk assessments. The statistics underlying the distinction between signal and noise for a marker and the relationship between the signal and the exposure for the consumer need to be very carefully

documented for omics to be dependable enough for food safety decisions. At the risk of making an overly sweeping statement, it seems that within microbiological food safety we have barely scratched the surface when it comes to relating the outcome of omics studies to risks for the consumer.

4. Data base for future hazard identification

One main goal of the review completed in the first part of this project was to enable an inventory on the added value of omics studies for risk assessment, in particular hazard identification. Chemical compounds, microbiological agents and GMOs of interest for EFSA were taken as case studies. For each study, the main results of the biological interpretation of the omics data as indicated by the authors of the article were summarized. In general, these concerned biological pathways or processes that were either up- or down-regulated which are informative for the mode of action of the compounds. Related to the main aim, to identify the added value of omics studies for hazard identification and risk assessment, the data collected for the review performs very well. This section describes some considerations for improving the data collection after its implementation for case study analysis.

4.1. Suggested improvements for data collection in relation to hazard identification for the 'chemical' records

It is clear that a structure for hazard identification based on intermediates for exposure and effect could be included within a database of omics reports by including fields for intermediates (although these fields may require free form). In addition, these changes would point to changes in the values associated with the field describing the element of risk assessment; biomarker identification could be partitioned to relate specifically to exposures or to effects and mode of action could be expanded to identify a relation with a statistical association between exposure and disease.

The present data collection contains some weak points when one would like to use it for new knowledge discovery, thus as a tool for hazard identification itself. A database yields an added value when it allows to compare the results of the records to each other. For this, it is important that the database allows to assess which compounds affect similar genes or similar pathways. For example, it would be very informative when the database reveals that a "compound A" with an unknown mode of action affects the same genes as a "compound B" with a known mode of action. This would be a strong indication that these two compounds have the same mode of action, providing important new insights about the mode of action of compound A. The same accounts for compounds that affect the same pathways and/or processes.

One weakness of the data collection performed is that the number of affected genes is limited to maximal five up- and five down-regulated genes while often, hundreds of genes are being affected. This very much limits its application. For example, compound A affects 150 genes in study 1 and compound B affects 250 genes in study 2 from which 80 genes are affected by both compound A and B. This overlap is highly significant indicating a similarity in mode of action of compounds A and B. However, it is very unlikely that this will be reflected in the five up- and five down-regulated genes reported in the data collected.

There are also drawbacks in comparing compounds to each other on pathways. Different researchers use different collections of pathways or processes. As a consequence, a pathway detected as significantly affected by one researcher might not be tested at all by another researcher. In addition, the type of statistics differ between researchers.

A database for new knowledge discovery would require other specifications. Such a database needs to contain the complete omics data results, thus for example the expression of 1) all mRNAs in 1,000 transcriptomics experiments, 2) all measured proteins (peptides) in 500 proteomics experiments, and 3) all measured metabolites in 300 metabolomics experiments. Such a database requires tools by

which similarities in expressions among studies can be identified. For example, it should enable to reveal which of the compounds within the database have a highly significant overlap in effects on mRNAs to that of compound A. Equally important, the database should allow comparing the effects of the compounds on pathways. For the latter, it is an advantage that such a database uses the same pathways and the same statistics for all analyses. Examples of approaches for those aims are the Connectivity Map (Lamb *et al.*, 2006), SRS (Veldhoven *et al.*, 2005), Oncomine (Rhodes *et al.*, 2004), Genevestigator (Laule *et al.*, 2006), GenomewidePDB (Jeong *et al.*, 2013), IPAD (Zhang and Drabier, 2012) and ArrayTrack (Xu *et al.*, 2010).

4.2. Suggested improvements in the data collection in relation to hazard identification for the 'microbiological' records

The data collected for the review on microbiological studies differs to quite some extent from those for the chemical hazards, again because the genomics in the case of microbiology almost invariably apply to the organism under study and not to the host. In addition, animals models are rarely relevant. Suggestions on how to improve the data collection reflect therefore the type of information one would like to see in future reports, rather than incompleteness of the database for the presently available studies. In short: what type of information is mostly missing these days and would be very useful for risk assessment and identification of emerging risks?

The information most sorely lacking is omics data on host-pathogen interactions. In contrast, in the field of probiotics, microorganisms that are supposed to provide a health benefit to the host, some data on the host reaction at the genetic level do exist (Bron *et al*., 2012; O'Sullivan, 2008). Conceptually the kind of data to be gathered is straightforward. The response of the genetic machinery of the host at the various levels needs to be documented. In practice this may be very hard to achieve. The omics data of the intestines of healthy and infected individuals needs to be compared and interpreted. The holy grail for food microbiology is to become predictive, in this case to predict which individuals will be affected by a certain pathogen and who will not suffer any consequences. It is stating the obvious that such predictions can be made in only very rare cases, even though the fact that large individual differences in infectivity exist is well established. However, when the area of omics of host response will be further developed, a few fields will have to be considered.

Right now a few fields have been included for the review on microbiological hazards because of their potential usefulness, even though the information that can be provided at this state of the art is not very helpful yet. Examples of these fields in the present data collection are:

- Omics applied for taxonomy
- Control analysis
- Number of genes/proteins/metabolites identified with the omics approach
- Genes/proteins/metabolites identified with the omics response
- Pathways identified with the omics response
- Target in host
- Host-pathogen interactions
- Virulence factors
- Epidemiology
- Ecological analysis

With the general introduction of WGS as typing technique, it is to be expected that genomics will be routinely used for taxonomic classification. The field "omics applied for taxonomy" now has a yes/no entry, but it can be imagined that specification will be useful. The "control analysis" field is in principle open, but "no" is by far the most used answer. Control analysis is a powerful tool to understand the interaction between a microbe and its environment. Several of the omics techniques, especially those that focus on metabolism, could be very well used to analyse metabolic control and

thus supply information that can be used to predict the behaviour of the organism under study. While not very practical yet, it can be expected to be of better use in the future.

The fields asking for the number and identity of the genes, proteins or metabolites identified are closely related, because when entering a study in the database with very many such items, listing all of them is impractical, while not very relevant if it concerns only a few. In fact the main issue may be which items to enter in the database, because when a search is performed, the results should not be contaminated with irrelevant hits. When expression of large numbers of genes is quantified using microarrays or similar techniques, it will be difficult to select those that are most useful for future reference. Pathways are more simple to name and indicate, but have in most studies available now rarely been identified.

Identification of the target in the host is closely related to host pathogen interactions and virulence factors are in turn specific genes involved in both. The target tissue of food pathogens will almost always be the intestines, but in the future more knowledge about specific cells that are targeted within the intestinal tissue may become available. New research on the host pathogen interaction may shed light on the specificity of the host and thus on the individual variability of that interaction. The effects of the presence of known and yet to be discovered virulence factors can also be more completely interpreted in that framework. Once future directions of this type of research become clear, these fields can be adapted accordingly.

Epidemiology and ecological analysis are closely interconnected, the ecology often being the explanation for the epidemiology. The epidemiology is restricted to outbreak analysis, but connecting the outbreak data to an understanding of the ecology of the organism can provide insight in the driving factors of the outbreak, or of the spread throughout the chain. These are therefore fields that can be expected to be further explored in future research on foodborne outbreaks and the spread of foodborne pathogens by other mechanisms. When new insights and reporting methodologies have been developed, adaptation or expansion of these fields may be warranted.

5. European and international projects concerning omics data and risk assessment

Several national reviews, and international projects, have addressed issues concerning the use of omics data (particularly toxicogenomics) to support risk assessment for food and environmental hazards. The UK Committee on Toxicity of chemicals in food, consumer products and the environment (COT, 2012a, b) have continually reviewed progress on the use of toxicogenomics data in risk assessment following an initial statement in 2004 (COT, 2004). The COT have identified roles for omics in risk assessments as well as important considerations for interpreting information but currently (February 2012) they conclude that “deciphering meaningful and useful biological information from toxicogenomics data remains a challenge”.

The United States Environmental Protection Agency reported on developments leading towards a systematic approach to using toxicogenomics data in health risk assessment and supported the general framework with an extensive case study for dibutyl phthalate (EPA, 2009). The systematic approach is summarized in seven steps;

- STEP 1: Compile the available epidemiologic, animal toxicology, toxicogenomic, and other studies
- STEP 2: Consider the quantitative and qualitative aspects of the risk assessment that these data may address
- STEP 3: Formulate questions to direct the toxicogenomic data set evaluation
- STEPS 4 and 5: Evaluate the toxicity and/or human outcome and genomic data sets
- STEP 6: Describe results of evaluations and analyses to answer the questions posed in Step 3

- STEP 7: Summarize the conclusions of the evaluation in the assessment

Crucially steps 4, 5 include considerations of phenotypic anchoring and mode of action (e.g. Wilson *et al*., 2011). The EPA report encourages the use of genomic data into risk assessment on a case by case basis in a weight-of-evidence approach, but only identifies qualitative evaluations.

The EPA report identifies other initiatives, which support using toxicogenomics data in risk assessment, from the US Food and Drug Administration, from the National Academy of Sciences and the National Research Council, e.g. NRC, 2007.

Currently the EPA is driving a large collaborative project, which involves several US agencies, to explore the new science and methods that can contribute to emerging and future risk assessments - NexGen: Advancing the Next Generation of Risk Assessment (http://www.epa.gov/risk/nexgen/). The NexGen project hosted a public dialogue conference in February 2011 and a workshop, "Systems biology: informed risk assessment", in Washington DC during June 2012 (http://nas-sites.org/emergingscience/workshops/omics-informed-risk-assessment/).

A European Union project called Safe Foods examined all aspects of the risk analysis framework and highlighted a role for omics technologies in safety assessment (Davies, 2010). In a series of workshops the European Centre for Ecotoxicology and Toxicology of Chemicals has considered the application of omic technologies to risk assessment (ECETOC, 2010). An EU project carcinogenomics, and others, have explored the role of omics information sources in the evaluation of human cancer risks (Paules *et al*. 2011).

In May 2011 the International Life Sciences Institute organized a workshop concerning the impact of omics technologies on microbial risk assessments; "Microbial Risk Assessment – Application of omics technology" (Brul *et al*., 2012). ILSI has identified advances for microbial risk assessment as a priority for its Risk Analysis in Food Microbiology task force.

In 2010 the Health and Environmental Sciences Institute conducted an online survey, among subject experts, to address the current and future applications of toxicogenomics (Pettit *et al*., 2010). The survey identified several key areas for which toxicogenomics can have major impact, but also concluded "the broad implementation and full impact of toxicogenomics for chemical and drug evaluation to improve decision making within organizations and by policy makers has not been achieved".

6. Potential future developments of omics technology and its implications for food and feed safety risk assessment.

6.1. Chemical and biological hazards related to human health, nutrition and plant health

It is immediately clear from the omics review that there is an enormous variation in omics studies that are aimed at the assessment of hazards in relation to food and feed. Other published sources indicate equivalent variety in omics studies that address, e.g., chemical safety in relation to environmental substances or pharmaceuticals. The variability is associated with many factors including resources, research objectives, distinct methods and protocols for data collection and analyses as well as inherent biological variability. The omics studies range from simple support for classical observational studies of adverse effects, e.g. El-Sayyed *et al*. (2011), to very large integrated projects that combine multiple data sources, develop novel analyses, identify research needs and contribute to decision making (EPA, 2009).

In addition to specific investigations there are an increasing number of reviews and reports that comment on the role of omics studies in relation to risk assessment of chemical hazards and which point to issues that should be addressed; these include COT (2011, 2012), Thomas *et al*. (2012), Wilson *et al*. (2011), Ioannidis and Khoury (2011), Crump *et al*. (2010), Hartung *et al*. (2012) and Paules *et al*. (2011). Together these sources can be used to identify several areas for further

consideration including complexity, methodology (reproducibility), interpretations (pathways) and translation (anchoring).

6.1.1. Plausible developments in the areas of greatest impact

Classical toxicology studies often begin with a dose-response structure in which a small number of groups (of animals) are exposed to an agent at different doses to establish a point of departure (i.e. a dose at which adverse effects, corresponding with a predefined endpoint, are significant). Benchmark doses and (no or lowest) observed adverse effect levels provide comparable measures for point of departure (e.g. Izadi *et al*., 2012) and are starting points for building regulations in relation to human exposures. Although transcription, or other omics information, can also be used to identify a point of departure the dose response methodology is not often employed in omics studies of toxicity. In most cases the integration of omics data into risk assessments involves informing a mode of action, i.e. identifying a mechanistic step in the transition from exposure to adverse effect, but increasingly omics signatures are interpreted at a pathway level. In this case an omics signature is used to map the interaction of toxic chemicals with biologically relevant pathways and, in turn, the pathways are associated with phenotype. Pathway level descriptions, based on omics studies, provide opportunities for high throughput chemical risk assessments (Judson *et al*., 2011). Potentially pathway level interpretations are more stable than e.g. gene level signatures (i.e. omics data sets are more similar at a pathway level than at a gene or protein level) and they provide opportunities for systematic (probabilistic) assessment of uncertainties (e.g. Chen *et al*., 2013). Reports in the omics review illustrate a wide range of schemes for the identification of pathway level descriptions ranging from methods based largely on expert opinions, e.g. Wei *et al*., 2008, to methods that use systematic searches of database information sources combined with causal discovery techniques e.g. Benton *et al*., 2011. The development of reliable pathway analysis tools such as Ingenuity Pathway Analysis, http://www.ingenuity.com/, is a step forwards in the systematic integration of omics data into risk assessment.

Establishing a 'traditional' dose-response relationship is not the initial goal in plant health research. Here, advances in omics techniques have facilitated investigations into the molecular basis of beneficial plant-microbe interactions. Understanding of the molecular processes underlying biocontrol, for example, are essential to overcome existing limitations (e.g. translating trial outcomes to diverse field situations) in the development of this technology for wide-spread use in agriculture (Mark *et al*., 2006). An additional area of impact for the development of omics technology related to plant research is in its efficiency compared to traditional technologies. For example, reducing crop yield losses due to abiotic stresses is highly labour and time consuming when selection through conventional breeding is applied. Alternatively, quantitative trait loci (QTL) for stress tolerance have been identified for a variety of traits in different crops (Ashraf, 2010). However, the accuracy and preciseness in QTL identification are problematic. A transgenic approach to the problem seems more convincing and practicable to improve abiotic stresses in different crops. Furthermore, dual metabolomics is a promising research area to study plant-pathogen interaction specific responses in both host and pathogen at the same time (Allwood *et al*., 2010).

The issues surrounding complexity, reproducibility and interpretation of complex global information obtained from omics studies relating to chemical risk assessments, and other issues concerning public engagement with regulatory science, may be viewed in terms of their impact on a current risk assessment framework and as indicators for future developments.

- Statistical issues, and particularly the integration of datasets from multiple sources into single assessments, ensure that proper management of data relating to omics studies will be a major element in the next generation of risk assessments (e.g. Baggerly, 2010). In particular open (shared) data, common data standards and validation may become requirements for the progression of risk assessment

- Complexity issues, such as pattern discovery in network models, combined with increasing concerns over reliance on animal models to indicate that a strongly computational component may become a major part of screening for toxicological properties of new chemicals as part of the next generation of risk assessments
- Issues surrounding the interpretation of complex observations, combined with a for communicating uncertainty with hazard assessment, indicate that pathway analysis may play a major role in a next generation of risk assessment methodologies and that the construction of well documented and harmonized libraries of pathway information will be a part of the next generation of risk assessments
- Emphasis on pathways, including those associated with adverse effects and other adaptive responses, combined with a movement away from traditional dose-response methodology ensures that the next generation of risk assessments will not fit neatly into an established framework and they may necessitate additional structures, and fora, for the management and communication of risks.

6.1.2. Challenges for implementation and steps to take

Thomas *et al*. (2012) indicate that approximately 30,000 chemicals are in commercial use in the United States but only a small fraction, ~5%, have a risk assessment that is recognised by the US EPA (in the Integrated Risk Information System). Similar complexity is recognised by the European Commission, and European Chemicals Agency, within the REACH initiative (EC, 2007). However, it is apparent from the omics review that the majority of research publications, each often representing several man years of effort, address single agents and single targets, and develop information at a single organizational level, in support of very targeted risk evaluations. Relatively few reports address toxic effects based on groups of chemicals or on mixtures and only small numbers of reports address multiple endpoints or multiple scales. This highlights an important disaggregation issue for which the number of identified hazards, based on new mixtures of chemicals or on increasingly specific definitions of targets and effects, grows much more rapidly than the number of assessments. Most recently several high throughput and high content screening programs, for chemicals, have been designed to address this situation i.e. an experimental design that maximizes throughput and minimizes false negative findings. The NexGen program prioritizes high throughput screening, based on quantitative structure activity relationships, as the first tier in a three step risk assessment process but even this advance is subject to criticism (e.g. Tannenbaum, 2012). Increasingly computationally intensive approaches e.g. Judsen *et al*. (2012), which combine high-throughput data from many sources, address complexity issues involved in predicting the toxicity of new chemicals or products.

The reports included in the omics review indicate that ranked lists of differentially expressed entities, genes or proteins or metabolites, are the dominant form of outputs used to represent a toxic effect observed in an omics study. Thomas *et al*. (2012) indicate that toxicity is not expected to occur without differential expression, particularly at the transcriptional level, although this is clearly distinct from an assertion that a pattern of differential expression is an indicator of a toxic effect. Some lists include only a few examples of expression that differs from a control while others identify several hundred (quantitative) expression changes, corresponding to different conditions, in extensive tables or in supplementary material. Lists of differentially expressed entities are relatively easy to visualize and comprehend, and can be used to support higher level understanding in terms of pathways or toxic endpoints, but the statistical properties of the results (sometimes obtained from a single realization) are often very difficult to appreciate. The reproducibility of ranked lists of differentially expressed genes obtained from high throughput omics experiments, particularly in relation to cancer outcomes, have caused significant concerns (e.g. Ein-Dor *et al*., 2006). Several reports, e.g. Venet *et al*. (2011), have

questioned whether an observed signature (expression pattern) from an omics study can be statistically associated with a biological effect. Currently stability measures (e.g. Boulesteix and Slawski, 2009) are rarely included to support the information presented in reports of omics studies and this leads to an expanding debate concerning the validation and translation of omics results into health assessments (e.g. Ioannidis and Khoury, 2011).

It might be clear that natural genetic variation in expression patterns and in those produced constitutively should be investigated more thoroughly to identify statistical significant associations between exposure and effects. That is, the expression of a cellular product in response to exposure to an agent does not imply its mandatory involvement in, e.g., plant defensive mechanisms. Moreover, experiments performed with a single plant variety cannot be generalized as of the many different biological factors outside the experimental design might influence its response. For example, genetic-environmental interaction, inconsistent repeatability and large number of genes regulating biological processes have hampered the generalisation of experimental results (Ashraf, 2010).

Pathway level organization may have significant value for interpretation of global expression information; leading to improved reproducibility and quantification of uncertainty. However, translation of pathway information into useable knowledge also relies on established associations between the perturbations of known pathways and particular adverse effects; sometimes called phenotypic anchoring (Paules, 2003). A searchable library of associations, mapping pathway perturbations onto phenotypic markers may under pin the next generation of high throughput chemical risk assessments but developing this library, based on ideas from systems biology and network science, is a major challenge (e.g. Vidal *et al.*, 2011).

In conclusion, improving effective food and feed safety risk assessment lies in combining knowledge derived from different information sources, e.g. disease phenotyping, cytological analysis, omics studies and targeted transgenics to identify the network components of cause - effect mechanisms.

6.1.3. Practical implications for chemical hazards or genetically modified food

Classical toxicity testing in animals has a number of drawbacks. The harmful effects on animal welfare is one main concern (Hartung, 2011). Another big problem is that the accuracy of animal tests for toxicity of chemicals or pharmaceuticals is rather low (Archibald *et al.*, 2011). In addition, classical toxicity testing is very time consuming and expensive which led to the so-called disaggregation issue. The number of identified hazards of chemicals grows much more rapidly than the number of risk assessments. For example, (Thomas *et al.*, 2012) indicate that approximately 30,000 chemicals are in commercial use in the United States but only a small fraction, ~5%, have a risk assessment that is recognised by the US EPA in the Integrated Risk Information System. Similar complexity is recognised by the European Commission, and European Chemicals Agency, within the REACH initiative (EC, 2007).

Chemical risk assessment is on the eve of undergoing two important changes: 1) the use of high-throughput cellular assays capable of testing large numbers of samples in a short time and in an accurate and cost-efficient manner (Schmidt, 2009; Hodgson, 2012), and 2) the application of omics techniques (Cote *et al.*, 2012; Zhou *et al.*, 2013).

It is our expectation for the future that the potential toxicity of a compound will be tested using a range of *in vitro* human cell systems (and not animal cell systems) that are models for relevant target tissues and organ systems such as the immune system. These cell systems will be exposed to a range of doses, including actual exposure doses, and effects will be followed by application of omics technology, e.g. metabolomics, proteomics and transcriptomics. In addition (or prior to this), the compound will be tested by a range of high throughput screening assays that comprehensively assess known toxic modes of action.

The omics results will be analysed by a systems biology approach that aims to relate the alterations of expression of metabolites, proteins and mRNAs to effects on organelles, transcription factors, enzymes and other cellular processes. This is enabled by the availability of extensive information about interactions of ten-thousands of proteins with metabolites, cellular processes and organelles. A network approach will be used that visualizes connections between proteins, metabolites and processes and the consequences of perturbations. This analysis will also discriminate whether effects on genes are due to toxicity or due to adaptive responses of the cells.

A second approach comprises a comparison of the omics results obtained to omics results of previous findings in the same cellular systems with other toxicants of known or unknown modes of action. This will be enabled via a database linkage system. A high overlap in effects on metabolites, proteins and mRNAs points to a resemblance of mode of action. An opposite effect (e.g. down-regulation vs. up-regulation) points to an antagonistic effect, for example inhibition of a nuclear receptor. Such a database will also contain sets of metabolites, mRNAs and proteins for which expression repeatedly alters in response to certain toxic modes of action. An example of such an approach is the Connectivity Map in which MCF7 cells are exposed to 1309 compounds (Lamb *et al.*, 2006).

This approach will mainly give information on the mode of action. Exposure to a range of doses will allow quantitative risk assessment and tell about the potency of the compound. Comparison of actual human exposure doses to the potency of the compound will be informative for risk assessment.

The use of human cell systems

As mentioned above, the use of human cell systems is key in the new approaches. The rationale behind this is the above mentioned poor predictive value of animal experiments and the effects on animal welfare (Archibald *et al.*, 2011).

Animal experiments acquired a very strong basis in human toxicology from the 1940s onwards. The rule that animal-based research should be conducted before human experiments was anchored in the Declaration of Helsinki on "Ethical principles for Medical Research involving Human Subjects", and has become the leading principle in regulations for testing of drugs and chemicals throughout the world (Greek *et al.*, 2012). This was based on the assumption that animals (mammals) and humans would react more or less the same to drugs, chemicals and diseases. However, this is often not the case. There are many examples showing this lack of correspondence between animals and humans. Many drugs that were "successfully" (safe and effective) tested in animals turned out to give serious adverse effects in humans. Because of this, there is much concern on adverse drug reactions which have reaches epidemic proportions. The European Commission estimated in 2008 that adverse drug reactions kill 197,000 EU citizens annually (Archibald *et al.*, 2011). The ToxCast project showed that not only humans and rats but also rabbits and rats differ often in their response to toxicants, in this case developmental toxicants (Knudsen *et al.*, 2013).

It is clear that animal-based research has led to wrong assumptions of safety of drugs and chemicals for humans causing serious adverse effects, and conversely has led to the withheld of promising drugs based on adverse effects or lack of affects in animals. Thus the predictive value of animal experiments is at least questionable.

On the other hand, the human cell systems presently being used for *in vitro* testing often comprises cancer cell lines that are immortal or blood cells taken from human volunteers. The cancer cells differ largely from normal cells, lack interaction with other cell types in the *in vitro* system and therefore very poorly represent the *in vivo* situation. Nevertheless, these cell systems can often be used to detect many types of toxic actions either by omics techniques or by biological/biochemical analysis. Pathways affected *in vitro* are often also being affected *in vivo* (e.g. (Katika *et al.*, 2012). More sophisticated systems that better mimic the *in vivo* situation and allow interaction between different types of cells certainly will improve the relevance of the omics results, and there is an urgent need to develop these *in vitro* systems. One new approach is the use of mechanical forces that are known to

play a very important role in regulation of transcription and differentiation (Mammoto *et al.*, 2012). With this technique in combination with microfluidic devices it is possible to reconstitute tissue arrangements observed in living organs (Huh *et al.*, 2012). Each cell culture chamber contains cells from a different organ. The micro-fluidic devices continuously re-circulate medium through the chambers enabling metabolites or proteins produced by one tissue to reach other tissues (Huh *et al.*, 2012).

It is important to be aware that *in vitro* testing of compounds by direct exposure to cell systems might generate false negative or false positive results. A false negative result will be obtained when the intact compound is not toxic but only the metabolites formed during digestion in the gastro-intestinal tract. A false positive result will be obtained when the compound exerts a harmful effect on the cell line, while *in vivo* the compound does not pass the intestines or is converted to a non-toxic metabolite. Therefore, *in vitro* models should also include 1) an oral gut digestion model, and 2) a model for translocation through and metabolism by intestinal cells. In addition, a liver cell system should be included to enable liver mediated metabolic conversions of the compound.

The use of systems biology

It is an enormous challenge to relate alterations in expression of mRNAs, miRNAs, proteins and metabolites to biological effects in organisms. In fact, in order to unravel which processes are being affected one needs to understand the interference of toxicants with the interplay between the thousands of proteins and metabolites. Systems biology is being considered as a tool to improve this understanding. Systems biology is the scientific discipline that encompasses and describes relationships between different types of omics findings and physiology (Geenen *et al.*, 2012). Preferably, knowledge is used that is based on confirmed findings.

A simplified theoretical example of information included in such a knowledge database: transcription factor X is known to induce upon activation an increased expression of 200 target genes. Five of these target genes are known to convert metabolites in secondary metabolites. An exposure experiment to a toxicant is done after which results of alterations of mRNAs and metabolites are loaded into the network. This shows that many of the 200 target genes of transcription factor X have a decreased expression. In addition, the cells have higher levels of the primary metabolites and lower levels of the secondary metabolites. This leads to the conclusion that both the results of the transcriptomics and metabolomics experiment point to an inhibiting effect of the toxicant on transcription factor X.

Multiple efforts are undertaken to provide information on interactions between toxicants, proteins, metabolites, organelles and pathways. The Toxin and Toxin Target Database (T3DB, http://www.t3db.org) provides information about the targets for more than 3,000 toxins (Lim *et al.*, 2010). The Human Metabolome Database ((www.hmdb.ca) provides quantitative chemical, physical, clinical and biological data about more than 40,000 experimentally 'detected' and biologically 'expected' human metabolites. The Comparative Toxicogenomics Database (CTD, http://ctdbase.org/) provides information about interactions between environmental chemicals and gene products and their relationships to diseases that are manually curated from literature (Davis *et al.*, 2013). Other databases provide networks of interactions between proteins, e.g. http://dip.doe-mbi.ucla.edu/dip/ (Xenarios *et al.*, 2002) or http://cic.scu.edu.cn/bioinformatics/Ensemble_PPIs/index.html) (Zhang *et al.*, 2012). Within this view, toxic effects are considered as perturbations of highly interlinked cellular networks (Vidal *et al.*, 2011).

It is important to realize that not all changes in metabolites, proteins or mRNAs reflect adverse effects. These alterations might also reflect adaptive effects of the system that do not lead to malfunction. Therefore, the system biology approach should enable to discriminate between adverse and adaptive effects. Interpretation of omics results will often be done on the level of pathways or processes. Multiscale interpretations such as pathways are superior to single scale interpretations of effects such as differential expression of individual genes. Pathway level descriptions, based on omics studies,

provide opportunities for high throughput chemical risk assessments (Judson *et al.*, 2012). Another advantage is that pathway level interpretations are more stable than gene level signatures. omics data sets are more similar at a pathway level than at a gene or protein level) and they provide opportunities for systematic (probabilistic) assessment of uncertainties (e.g. (Chen *et al.*, 2013)).

Statistical issues

This new type of risk assessment will also raise statistical issues particularly the integration of datasets from multiple sources into a single assessment (e.g. (Baggerly, 2010)). Another relevant issue is how to conclude whether a pathway is affected. Which proportion of the genes within a pathway has to be affected to consider the pathway as a whole to be affected?

When are the mRNAs or metabolites of a pathway being considered to be affected? Should these be up- or down-regulated at least 1.3, 1.5 or 1.7-fold? Which statistical test has to be used to determine the significance? Should all data be analysed in a similar way? How to detect very subtle effects when applying exposure levels close to real-life exposure levels? In addition, the next generation of risk assessment methodologies requires construction of well documented and harmonized libraries of pathway information.

The reproducibility of omics experiments, for example of ranked lists of differentially expressed genes particularly in relation to cancer outcomes, have caused significant concerns (Ein-Dor *et al.*, 2006). Criteria have to be defined to determine whether a pathway is affected or not. Several reports, e.g. (Venet *et al.*, 2011) have questioned whether an observed signature (expression pattern) of an omics study can be statistically associated with a biological effect. Currently stability measures (e.g. (Boulesteix and Slawski, 2009)) are rarely included to support the information presented in reports of omics studies and this leads to an expanding debate concerning the validation and translation of omics results into health assessments (e.g.(Ioannidis and Khoury, 2011)). In addition, it is very important that molecular changes like altered pathways can be linked to phenotypic changes, a process called phenotypic anchoring (Paules, 2003; Currie, 2012).

Exposure to a range of doses, including actual exposure doses

An important issue for risk assessment is to assess the lowest dose that exert an adverse effect. For this aim, multiple doses have to be tested. Examples of such an approach have been published by (Thomas *et al.*, 2011; Thomas *et al.*, 2013). In these studies, rats were treated with carcinogenic compounds and target organs were analyzed for traditional histological and organ weight changes and transcriptional changes using microarrays. Benchmark dose methods were used to identify non-cancer and cancer points of departure both for the traditional and the transcriptional changes. A main finding was that the benchmark dose for transcriptional changes of the most sensitive pathways did not occur at significantly lower doses than that of traditional parameters. However, the transcriptional changes could be detected much earlier (from day 5 onwards) than the traditional parameters (the rodent carcinogenesis bioassay takes in general two years)(Thomas *et al.*, 2013). In most cell systems, changes in metabolites, proteins and mRNAs are expected to occur not only earlier but also at lower exposure doses than changes of traditional parameters. For example, activation of nuclear receptors will quickly induce expression of many target genes while effects on viability or other parameters are not observed (e.g. (Heneweer *et al.*, 2007)). Therefore, omics techniques are expected to detect effects at lower exposure levels than traditional methods. This is an important issue since, as is also concluded on basis of the EFSA omics review, most toxicological studies applied doses that are often 100- to 1000-fold higher than actual exposure doses. Based on this observation, the National Research Council of the USA recommends in their vision document "Toxicity Testing in the 21st Century: A Vision and a Strategy" (National_Research_Council_(NRC), 2013), (http://www.nap.edu/catalog.php?record_id=11970) to perform exposures to doses that are more close to actual exposure levels or to doses measured in blood of human individuals. On the other hand, it is

reasonable to take in addition also doses that are for example 10-fold higher than actual exposure doses to compensate for the exposure to many other chemicals of which some will share modes of action (Schwarzenbach *et al.*, 2006). Methods such as a combination of physiologically based pharmacokinetic (PBPK) modelling and quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) have been developed to translate *in vitro* exposures to corresponding *in vivo* exposures (Yoon *et al.*, 2012).

Clearly, development of sensitive and very accurate methods will be required to enable detection of relatively subtle effects. One example of such a new method is next generation sequencing of mRNAs that displayed a higher sensitivity to differential expression than microarrays (Sirbu *et al.*, 2012).

Individual variations in susceptibility between humans

Human variability in response to chemical exposure is due to multiple intrinsic and extrinsic susceptibility factors, including age, nutrition, lifestyle, and genetic make-up. Integrative approaches in this field are also denoted as phenomics (Houle *et al.*, 2010). Also mice have been shown to differ widely in susceptibility to adverse effects of chemicals due to genetic diversity (Rusyn *et al.*, 2010). Information on human susceptibility variation should be used in risk assessment to characterize differences in response of individuals to chemicals. This could be used to determine an uncertainty factor of an estimated risk level in a population.

To generate data on human genetic susceptibility, both mechanistic data on gene targets of chemicals, and human population polymorphism data on these genes are needed. There are many open source databases on chemical toxicity, gene expression and pathways involved (summarised in (Mortensen and Euling, 2011)). However, these data will have to be integrated to fill major gaps in our knowledge on molecular mechanisms of chemicals in humans, including multiple modes of action and interacting pathways under different conditions. Large-scale sequencing projects to characterise human genetic variation are still running or completed, and this multitude of data is also available in open source databases (summarised in (Mortensen and Euling, 2011)). A targeted project on population-based toxicity phenotyping in human cells from 9 populations in 5 continents, the 1000 Genomes Toxicity Screening Project, was recently started by Rusyn at Texas A&M University (http://www.genomezoo.net/). Taken cytotoxicity as end point, cells from different individuals showed a 100-fold range in sensitivity for some compounds (Lock *et al.*, 2012). Again, it is still challenging to integrate these data and to link the biological genetic variants to a toxic response phenotype (Mortensen and Euling, 2011). A consequence of these findings is that toxicity studies have to be performed on a range of cells reflecting many different genotypes.

The use of omics technologies for risk assessment of GMOs in the future

Related to the question whether consumption of a GMO food component can impair the health of consumers, the same strategy can be followed as provided above for chemical compounds. Thus, the human *in vitro* models described above can also be used for risk assessment of GMO food components. One somewhat complicating factor is that extracts need to be made (for example with methanol) from the GMO food component to enable the exposures (De Vos *et al.*, 2007) which always induce a risk that metabolites responsible for the toxicity will not be included in the extract. Also for GMO food components, application of the oral gut digestion model in combination with metabolism by liver cells will improve the trustworthiness of the data.

Can once all animal experiments be replaced by *in vitro* human cell systems?

The vision given above recommends human *in vitro* methods in combination with omics technology, systems biology and high throughput screening. Since recent years, our understanding of the interactions of proteins, mRNAs and metabolites is rapidly increasing. On the other hand, we are still far away from understanding these networks of interactions of ten thousands of proteins, mRNAs and

metabolites completely. Importantly, as a result of genetic imprinting, different cell types express different mRNAs and proteins and therefore will have other networks. Thus distinct networks need to be identified for different cell types. Likely, we will never be able to build these networks in all details. On the other hand, the complexity can be reduced by focussing on networks related to toxic effects. Nevertheless, a very complex task remains.

In addition, much input and time have to be invested in the development of *in vitro* cell/tissue systems that very well mimic the organisation of cells and communication between these cells in tissues *in vivo*. Moreover, these *in vitro* produced tissues should be capable to communicate to each other. For example, metabolites formed from a toxicant in the liver should reach kidney cells. Certainly, it will take much effort and time before these systems mimic the processes in the human body sufficiently to assess a wide range of modes of action.

Another challenge is the phenotypic anchoring: the observation of subtle changes in expression of mRNAs or levels of metabolites has to be translated into potential consequences for malfunction of processes in humans. Omics results need to be strongly connected to disease phenotypes otherwise they will not enter the decision making process.

To our view, it is a realistic expectation that in the coming 10 years omics technology applied on human *in vitro* systems in combination with high throughput assays will gradually replace most animal experiments. This optimism is based on the rapid progression in technology, including omics techniques, bioinformatics and *in vitro* cell systems. Acceptance of these assays by regulators generally is slow. Important is that newly developed methods will be included in testing approaches as soon as these methods are validated.

The approach outlined above will likely not be perfect and will result in some rate of false positive or false negative findings but the outcome will certainly be much better than the predictability of animal tests and will be further improved in the years thereafter.

6.2. Biological hazards

6.2.1. Plausible developments in the areas of greatest impact

Within the general field of food microbiology omics are most relevant for risk assessment, source attribution, outbreak detection and exposure assessment. In the immediate future the contribution of genomics is likely to by far outweigh the impact of the other omics techniques, except possibly for risk assessment. For predictive microbiology, when attempts are made to foresee the behaviour of food microbes under conditions specific for certain foodstuffs, a large variety of omics techniques may be relevant (Brul *et al.*, 2008). Being primarily a risk management tool, predictive microbiology will only marginally be discussed below. Given the rapid developments within WGS and the immediate relevance for risk assessment, WGS will receive special attention in this foresight study on biological hazards.

Risk assessment aims to identify a hazard, to characterize it and to quantitatively predict the impact on public health. Within the general area of microbial food safety and foodborne microbiological hazards, omics have potential applications to provide insight in the response of the microbes to treatments and control measures and to the conditions they encounter within the food chain (Brul *et al.,* 2012). The variation induced by environmental conditions in the genetic properties of strains derived from common origins can be considerable (Eisenstark, 2010). As a consequence the interpretation of genomics becomes very complex, as not only the information obtained at a certain point in time is relevant, but also the induced modifications in later stages, under different circumstances. Therefore, while genomics may give perfectly valid information on an organism at a particular moment, conclusions may no longer be entirely reliable after prolonged growth under different conditions.

An important role for genomics in general and WGS in particular in microbial food safety has been predicted for some time (Abee *et al.*, 2004; Bhagwat and Bhagwat, 2008; Dieterich *et al.*, 2006;

Hyytia-Trees *et al.*, 2007). Given that WGS still has to live up to these great expectations, it may seem overly optimistic to predict that this will happen in the very near future. Still, there are convincing grounds for this optimism. The price per genome has gone down so much that by now WGS is a very cheap manner to type an organism, cheaper than most "classical" methodologies, such as phagetyping, PFGE and PCR involving multiple genes. Tools to analyse the flood of information that becomes available when a complete genome is sequenced are being developed (Jolley and Maiden, 2013; Koser *et al.*, 2012; Segerman *et al.*, 2011). One of the essential tools to interpret genomic data will be the availability of other genomes of foodborne pathogen for comparison. Several initiatives are being developed, such as the 100K genome project of UC at Davis and the FDA (http://100kgenome.vetmed.ucdavis.edu/). In an ideal situation, the genome of a strain under study can be largely matched with a genome in the database. The characteristics can then be assumed to be very similar. If a well-documented history of the strain that the genome is obtained from is included in the database, then a prediction can be made on the virulence and other relevant properties of the newly sequenced strain. This would enable the investigator to make an initial evaluation of the risk associated with this strain.

Matching patient strains with foodborne strains can be a powerful tool for source attribution when studying disease loads caused by foodborne pathogens. Obviously similarity alone is not a sufficient basis for firm conclusions, but epidemiological correlations can provide a strong indication on which further investigations can be based. Genome based testing of foodborne pathogens has been implemented by the USDA Food Safety and Inspection Service to provide data for foodborne illness source attribution (Withee and Dearfield, 2007). In New Zealand a very significant reduction of the incidence of campylobacteriosis was achieved by implementing control measures that were based on genomic analysis supported epidemiology (Muellner *et al.*, 2013). These examples show the great potential for omics techniques in surveillance and epidemiological source attribution.

Simplified outbreaks can be described as either of two extremes: point outbreaks and diffuse outbreaks. Point outbreaks are caused by a mishap late in the chain and can best be illustrated by an example statement such as "all participants in the church lunch who ate tuna salad got sick within 24 hours." Diffuse outbreaks are due to contamination far earlier in the chain and are therefore much more difficult to detect as the related cases are usually not connected by any information system. Several examples have been described of fruit pickers who contaminated food that was subsequently exported to many countries and caused considerable disease before this was noticed. Genomics can provide essential information linking individual cases with the causative pathogens and the patient strains with each other. This approach has been successful in detecting diffuse outbreaks, as shown by many examples (Cheung and Kwan, 2012; Friesema *et al.*, 2012; Sabat *et al.*, 2013; Verhoef *et al.*, 2012).

Developments in the other omics fields with regard to microbiological risk assessment have not yet reached the level that can they be called plausible for the near future. Hence, they are discussed below as applications that face challenges for implementation.

6.2.2. Challenges for implementation and steps to take

Challenges for implementation

The Achilles heel for the application of genomics and in particular WGS within food safety and microbiological risk assessment is the enormous overload of information that each sequenced genome supplies. It will be a considerable challenge to discern the useful information from the irrelevant noise. What is lacking at the moment of writing is a consistent system for annotating genomes and interpreting genomic information in a way that it can predict the actual properties of the strain. This problem is likely to be overcome when more foodborne pathogens are sequenced, databases that contain these sequences can be consulted freely and a consensus emerges on annotation. By then it should be possible to find the closest match for any newly sequenced organism and by comparing it with the strain identified as the best equivalent an initial risk assessment can be made. One must take proper precautions however, not to over-interpret the genomics data. Differences in environmental conditions can cause immensely different reactions in genetically almost identical strains, therefore the predictive value of the DNA sequence alone is limited.

An immediate challenge for the application of WGS and genomics in general for microbiological food safety is the identification of the primary virulence factors, the secondarily contributing factors and other undesirable properties, such as resistance genes. The mere presence or absence of the genes coding for such factors is not enough to reliably predict the behaviour of the microorganism concerned. The interaction between the different factors must be understood in the framework of the physiology of the cell as well. In the case of the STEC and EHEC pathogens, for example, the confusion over how the interpret the presence of the stx1, stx2, and other genes is considerable and these issues are not likely to be resolved soon. This problem can be overcome to a practical extent by comparing isolates from foodstuffs with patient strains using an epidemiological approach. However, epidemiologically established correlations do not by necessity provide proof of a causal relationship. Still, if a multitude of sequences of covering both patient and foodborne strains are available in databases, conclusions can be drawn about strains matching closely a well-described organism in the database. Obviously, the more isolates have been sequenced, the greater the chance that a sufficiently close match will be found. Once these challenges have been overcome, the ideal of an instant assessment based on automated genome analysis of the risks associated to a particular strain could be approached. However, except for very standard situations of well-known strains, it is to be expected that expert judgement of the data will always be required.

Following an outbreak by typing the strain involved using genomics seems a very logical approach. The often PCR based typing methods for viruses, are in fact genomics based and PFGE is a genome derived technique. Again, the initial challenge will not be the impossibility to distinguish between isolates, but the restraint needed not to over-interpret the invariably occurring small changes in the genome. It is likely, however that comparing whole genomes as a typing method will allow rapid correlations between isolates, which will be especially useful when patient and foodborne strains can be matched. Once many complete genomes have been entered into databases, it may be possible to foretell the origin of patient strains, based on matches with isolates from different types of food. If for example, *Salmonella*, from meat, fish or vegetables differs enough to be distinguished, then the first patient isolates from an outbreak could give investigators already an indication about the particular food involved. Reaching this level of knowledge and understanding will still require a considerable effort.

The many other omics techniques that could be relevant for food microbiology require far more research to fulfil their potential than genomics. Transcriptomics has the potential to contribute to our understanding of the interaction between food microbes and their environment, but to realize this promise the understanding of transcriptomics needs to progress considerably (Brul *et al.*, 2012). A thorough perception of transcriptomics may add to the power of prediction by genomics, but also in that case on the condition that not just each of these fields separately is well developed, the connection must be as well. Metabolomics can provide a much better insight in the effect of probiotics than can be

accomplished at the moment (Bron *et al.*, 2012), once a significantly deeper understanding of the role of metabolites in the interaction with the host has been reached. Proteomics provide more understanding of the inner workings of the organism than insight in host-pathogen interaction, except when attachment factors and other outer oriented proteins are involved. However, the surface for this type of explorations has barely been scratched.

Steps to be taken

In addition to scientific advances, considerable auxiliary efforts are required to exploit the potential of omics for microbiological risk assessment to the full. Many of these are of an organisational nature.

Ownership of genomics data is likely to become a sensitive topic. Ideally, from a scientific point of view, all sequenced genomes would be made available to researchers worldwide by depositing them in easily accessible databases, or even better a single database. However, by depositing the sequenced genome and the accompanying information in such a database, the owner gives up his rights, including the ability to control the way his data will be used. This may be a step that many of the institutes that are likely to generate this type of data, such as food safety authorities, are likely to have substantial reservations about. Some of these concerns might superficially be judged as irrational, others are the consequence of very real legal restrictions. For instance, information that can be linked to a single company may as a rule not be made public in most EU member states. A complete description of all bureaucratic hurdles to be overcome is outside of the scope of this prospective. Nonetheless, this issue must be addressed comprehensively to enable unhindered data sharing on an international level.

In parallel to addressing the issues of data ownership and other formal issues, the design of the database, the information that should accompany the sequences and the way they can be searched are to be given considerable thought as well. Any individual who has to make a decision about measures to be taken within the framework of food safety would want to be able to compare the genome of a suspect microorganism isolated from a foodstuff with the relevant known genomes and be able to review the results immediately. If several databases for genomes of foodborne pathogens are set-up, then a single common search should be possible. In fact it should seem as if a single database was searched. This will require strict coordination between the parties administrating the databases. From a scientific perspective a single database would be optimal, but if this cannot be achieved for practical reasons, then it should be approached as much as possible.

The information that is to be registered in addition to the genome sequence deserves ample consideration. A few elements can easily be named, such as the location and origin of the isolate, the species name, the contact information of the submitter. Additional information may be crucial for optimal use of the database. This is not a trivial point and will require thorough deliberations and consultations between all stakeholders.

For the other omics, other than genomics, to be useful for microbiological risk assessment, the old *cliché* applies: “More research is needed”.

CONCLUSIONS

This report deals with the application of omics to hazard and emerging risks identification and a foresight study on the potential future developments of omics technologies and possible implications for risk assessment in food and feed safety.

Case study investigations show that, for chemical hazards, the role of omics in hazard identification and emergence of risk is dominantly a search for biomarkers of exposure, or a process to discover mechanisms that connect biomarkers of exposure with intermediate biomarkers of effect, in situations where there is an established statistical association between exposure and disease (most often established from classical toxicology).

The main knowledge gap in hazard identification using omics data here is in the difficulty to establish a one to one relationship between the exposure and the marker. This concerns data analysis from multiple-component exposures, the many defence mechanisms on a molecular level underlying a single (or multiple) response(s) and the limited overlap between processes at the gene level and biological response level.

Experimental designs often show very high exposure levels and unphysiological routes of exposure. Furthermore, translating omics data generated in animals and *in vitro* studies to human and plant physiology remains uncertain. In general, statistical issues concern the many different approaches for omics data analysis, both in the visualization and interpretation of the data, which makes it difficult to compare different study results.

A list of differentially expressed genes is most often the starting point for further analysis. However, thresholds for a result being "differentially" are, largely, subjective. In addition, the often applied, different clustering methods for further data analysis satisfy many of the requirements for complexity reduction, but resist probabilistic interpretations suitable for quantitative safety assessments. Finally, replicating experiments, to address the potential for false discovery, is often considered impractical or uneconomic.

Potential future developments in the integration of omics data into chemical risk assessments involves pathway level analysis. Pathway level interpretations seem more stable than e.g. gene level signatures (i.e. omics data sets are more similar at a pathway level than at a gene or protein level) and they provide opportunities for systematic (probabilistic) assessment of uncertainties.

The main limitation in applying omics technologies to the identification of microbial emerging hazards is that in food microbiology omics data are focused on the microbes and less on the effect on the host. The most significant application of omics data to early detection of new microbiological hazards lies in the combination of genomics and epidemiology. Genetic analysis (using WGS) of both pathogens that are related to sporadic disease cases and to outbreak events can be used to reduce public health risks through the detection of virulence factors, resistance genes and genetic diversity. Still, the lack of microbiological omics data on host-pathogen interaction and changes in expression of pathogens under different conditions makes it difficult to use omics data for risk assessment. For example, correlations, such as between transcriptomics and pathogenicity have not yet been established. Technical developments would be needed to study the host-pathogen interaction at the transcriptomics level. Combined with metabolomics, these data might be applied to predict the behaviour of known or suspected pathogens under given conditions. This would underlie its application in setting microbiological criteria, performance objectives, food safety objectives, judgement on QPS status etc. It remains to be seen, however, how much confidence the risk manager will have in this type of data, given the natural tendency of authorities to err on the safe side.

WGS sequencing is the most promising relevant technique for its application in 1. risk assessment (improving insight in the response of microbes in the food chain, to treatments and control measures), 2. source attribution (combining surveillance and epidemiological data with strain typing) and 3. detecting (diffuse) outbreaks (linking individual cases with the causative pathogen). A drawback is that genomics may give perfectly valid information on an organism at a particular moment, conclusions may no longer be reliable under different conditions. In addition, one of the essential tools to interpret genomic data will be the availability of other genomes of foodborne pathogens for comparison. The availability of (easily accessible) databases with genomic information that can predict the behaviour of a strain is a challenge.

A commonality between chemical and biological agents in predicting their emerging risk from omics data lies in the fact that the presence of a biomarker (e.g. gene, metabolome, protein) may by itself not always be a good predictor, since the expression is influenced by a large variety of (biological) factors.

In general, our study is based on a methodology for hazard identification using omics data based on 'intermediates' for exposure and effect. A consistent application of this methodology in case study

analysis throughout the whole review revealed the need of an extension of the data collection on fields concerned with the 'elements of risk assessment: biomarker identification and mode of action'. In addition, methods for statistical (pathway) analysis should become comparable between research studies such that a relational database could be implemented for valuable use. An additional suggestion concerns the review on 'microbial' hazard identification in particular. Here, data from omics studies could contribute to establish the link between epidemiological and ecological information if appropriate information was to be collected.

Moreover, the management of large data sets, including its (statistically) complex structures, will ensure that the next generation of risk assessments will not fit neatly into an established framework for food and feed safety risk management and communication.

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