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Effect-based Analytics for Toxicological Screening — Concepts for Future Developments

ABSTRACT

The concept of effect-based analytics is based not on the detection of individual substances present in food or feed by analytic chemistry, but rather on determining effects these substances cause in biological systems. In this way, effect-based analytics allow for the detection of unknown toxins. In-depth knowledge of molecular principles of toxicity is required to develop suitable biological test systems for effect-driven analysis. An example of an established representative of a test system of this kind is the CALUX (Chemical-Activated Luciferase Gene Expression) test for dioxin-like effects, which measures xenobiotic activation of the aryl hydrocarbon receptor by means of a luciferase reporter system. Such test systems permit the reliable detection of specific molecular mechanisms of toxicity. Possibilities are sketched out here for further development of methods in effect-based analytics, with the help of which the parallel detection of various molecular modes of action mediating toxicity to biological systems could be possible in the future. A special focus is placed on novel developments in the field of transcript signature-based effect detection and their possible application in food analysis. A vision of the future of effect-based techniques in food and feed safety is mapped out, expanding far beyond current applications of effect-based systems.

INTRODUCTION

Unlike classic chemical-analytical methods that serve to identify individual substances, effect-based analytics fundamentally comprises the measuring of an effect in the form of a simple, easily accessible, reproducible and sensitive endpoint in a biological system, to the extent that this is possible. If specific effects on cellular regulatory systems are to be examined rather than general, comparatively nonspecific endpoints, such as *in vitro* cell death, mechanistic knowledge of the molecular principles of a specific biological effect is required to enable the identification of suitable endpoints and/or biomarkers. At present, the identified endpoints or effects are often recorded in cultures of mammalian cells *in vitro* via luciferase-based reporter gene analyses. In addition, in

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particular in the field of environmental toxicity, effect-based approaches on entire organisms, such as the zebrafish *(8)* or certain algae *(20)*, are pursued. An example of a cell culture-based biological test system, outlined in detail in previous overview articles on the status of effect-based analytics *(5, 6)*, is the CALUX (Chemical-Activated Luciferase Gene Expression) assay. This test is based on the specific binding of dibenzodioxins and similar substances to the aryl hydrocarbon receptor which, as a ligand-activated transcription factor, subsequently initiates the transcription of a firefly-derived luciferase reporter gene that is controlled by an aryl hydrocarbon receptor-responsive promoter region. Comparable reporter gene-based test systems already exist for various hormonal effects mediated via androgen or estrogen receptors *(20)*. The reporter genes used here can be integrated in a stable manner into the genome of a permanent mammalian cell line *(Fig. 1A)*.

There is currently a legal requirement in food control to conduct an analytical identification of the substance responsible for a positive finding in an effect-driven test system. Accordingly, effect-based approaches are currently used primarily as screening methods to identify in a large number of samples those that are to be subsequently examined by means of classic analytics for the presence of specific substances known to be toxicologically relevant. A "positive" finding in an effect-based sytem therefore involves the identification of substances by use of classic analytical methods, which can be complex and time-consuming. A tiered procedure of this kind involving effect-based screening followed by chemical analytics proved its value in the last dioxin crisis, where it was necessary to test a large number of samples within a short time (see also *(6)*). Repeated cycles of sample fractioning and the use of effect-based test systems prior to chemical-analytical substance identification can help to limit the chemical complexity of a sample *(3)*.

For this reason, a decisive boost for the further distribution and application of effect-based methods in the field of food and feed control could in the future lie in departing, at least partially, from the principle used now. For example, assuming the availability of appropriately validated effect-based methods, which enable not only the qualitative detection but also the quantifiability of a certain biological effect with sufficient precision, the mere detection of a biological effect above a certain potency, to be defined individually for each biological test system and which would assume the function of a limit value in classic chemical analytics, could be recognized as sufficient for any decisions to be made or consequences to be drawn. This would be desirable, especially in view of the fact that some toxicologically relevant xenobiotic-binding nuclear receptors bind substances that are hardly related from a chemical point of view (an aspect to be dealt with in more detail later in the text). This lack of chemical similarity could lead to difficulties in the subsequent search for a specific individual substance causing the effect at such a promiscuous receptor.

The current state of using luciferase reporter systems for effect-centered analysis in food control is limited to individual endpoints such as that addressed by the CALUX assay. This present state of effect-based analytics has been the focus of a previous paper in this journal *(5)*. The aim of this paper, however, is to sketch potential future development of existing techniques applied in effect-based analytics and to present novel approaches focused on the detection of transcriptional alterations of endogenous cellular gene transcription that appear promising for future application in effect-based analytics. This will be done in the following paragraphs, starting with possible further developments of luciferase-based methodologies and their possible limitations, and then continuing with the use of single transcript markers and transcriptomic signatures. This manuscript thereby reaches far beyond previously published work in the field, including our own previous paper *(5)*, thus making this work interesting for experimental researchers and method developers, as well as for readers who are concerned with food surveillance and regulatory issues.

Further development through establishment of a reporter gene test battery

In principle, a large number of different cellular effects or mechanisms of action can be recorded through effect-based test systems based on reporter assays of this kind. Reporter gene systems allow the precise quantification of the examined biological effect and enable the easy establishment of dose-response relationships, which does not automatically apply to the same extent to every other effect-based system. Established and well-characterized reporter systems are available in the meantime, in particular for a panel of nuclear receptors, which function as ligand-activated transcription factors and are known to constitute important molecular switchpoints with many toxicologically-relevant processes, as well as for numerous transcription factors acting downstream of important cellular signaling pathways. These assays could be prepared for use in routine analysis in validation studies. At the end of a development of this kind, a battery of reporter gene test systems that would be suitable for recording a large number of different, toxicologically relevant signals could be available *(Fig. 1B)*. Various reporter gene systems are available (firefly luciferase, Gaussia luciferase, Renilla luciferase, fluorescent proteins such as GFP), which differ in their substrates, reaction conditions and detection and which can, therefore, be used simultaneously for detection of several endpoints in a single biological system, for example in the classic "dual luciferase test" in which two luciferases with different substrate specificities and reaction

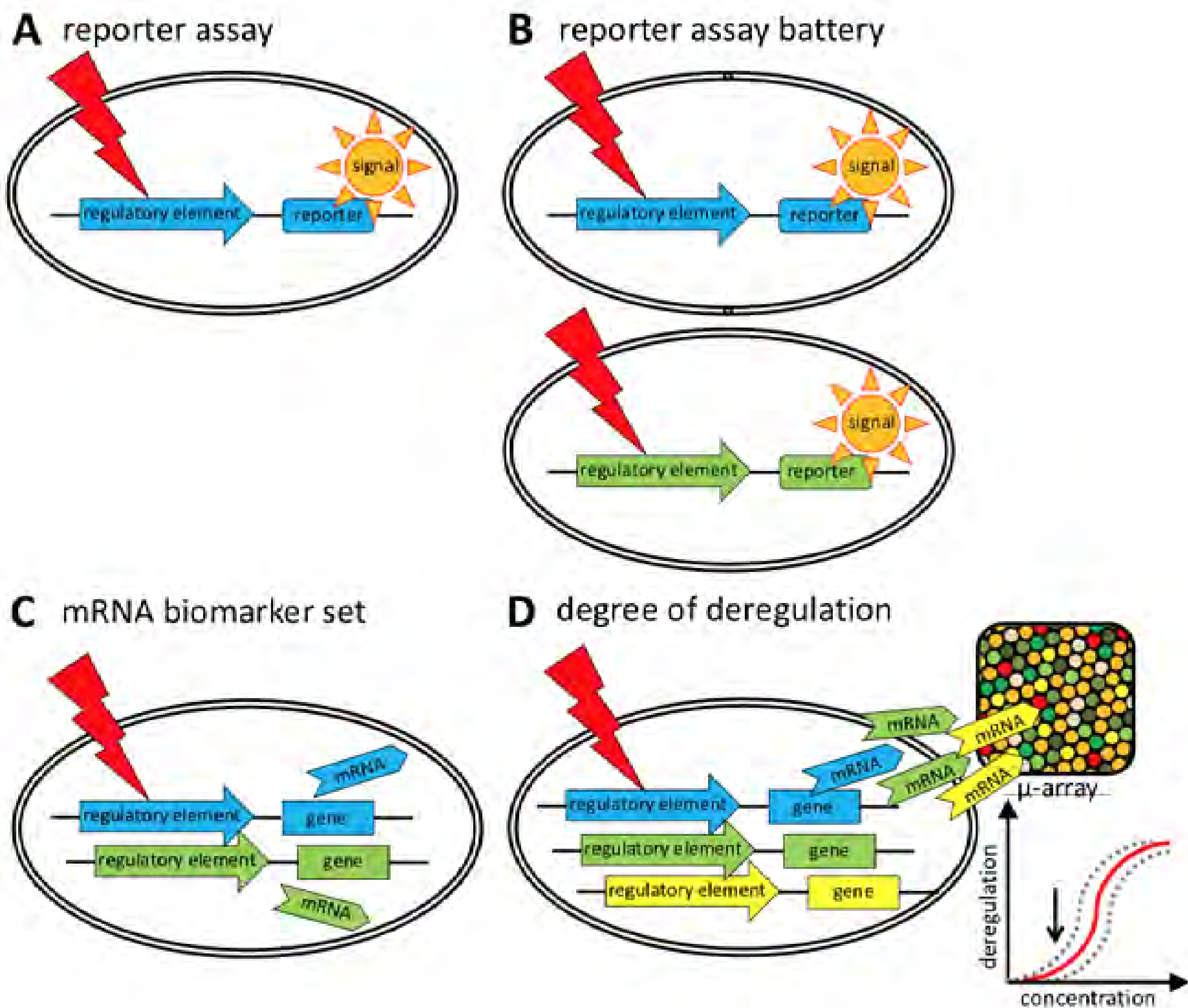

Figure 1. Graphic illustration of various approaches to effect-based analytics.

Footnote:

(A) Measurement of a specific signaling pathway/specific MoA (mode of action) in a biological system as the endpoint by means of a reporter gene test. This test principle is used on dioxin-like effects in the CALUX test, for example.

(B) A test battery comprising a combination of several biological test systems with different reporter systems permits the detection of several different types of effects.

(C) The parallel detection of several MoAs could be achieved by establishing specific transcript signatures in a single biological system.

(D) Turning away from mechanism-specific observation, a degree of biological deregulation could be determined by measuring the global gene transcription, which might then be used as an indicator of the effect of a sample once a certain, yet to be determined, value has been exceeded (cf. the arrow in the illustration).

conditions are determined in one sample. Nonetheless, only a small number of endpoints can be recorded in parallel for practical experimental reasons, so that the establishment of a reporter battery would be linked to the necessity of using numerous different reporter lines together, each one directed towards one or only a few individual endpoints (for instance, a cell line with reporter system A and B, a second cell line with reporter system C and D, etc.). Please also refer to the deliberations made further on in the text in this regard.

It must be pointed out here that classic luciferase reporter gene analysis and comparable approaches are not the only way of recording biological effects related to nuclear receptor and transcription factor activation. Modern imaging techniques could play a future role within the scope of the automated recording of the translocation of a fluorescence-marked toxin-activated receptor. For practical reasons, however, an infinite number of endpoints cannot be determined in parallel using methods of this kind either.

Simplification through the use of procaryotic systems

Many laboratories responsible for routine analysis of food and feed do not currently have the equipment or the expertise to conduct cell-based assays of effect-based analytics, in particular those that involve the germ-free cultivation of mammalian cells. This problem could no doubt be solved by acquiring the necessary equipment, and training staff accordingly, but — even though automated systems are becoming more and more suitable to take over sub-stages of the experimental workflow — cultivation of mammalian cell lines is still a more complex technique than the use of procaryotic systems. In the field of ecotoxicology, luciferase-expressing strains of *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus*, for example, are used to detect various heavy metals, with each bacteria strain being equipped with a luciferase reporter construct that is responsive to a particular heavy metal *(9, 10)*. It must be added at this point, however, that procaryotic cells differ greatly from eucaryotic cells in their biochemistry and signal transduction. Because of a lack of specific interaction partners, such as the coactivator proteins needed for the proper function of a certain transcription factor, the functionality of a mammalian protein introduced into procaryotes cannot simply be assumed. For this reason, procaryotic systems are not automatically suited for predicting effects on the more complex intercellular signaling pathways of a mammalian organism. Conversely, they could constitute a time-saving and low-cost alternative to mammalian cell-based test systems for endpoints that are easier to address, or in cases where corresponding regulation systems of sufficient specificity exist in procaryotes or can be implemented in biological systems of this kind.

Limitations of reporter gene-based methods

Only a few nuclear receptors exhibit high substrate specificity. Instead, a large number of substances whose chemical structures are only slightly related, if at all, are accepted by many receptors as ligands. Even the aryl hydrocarbon receptor examined in the CALUX assay, which is commonly regarded as a comparatively specific receptor for dibenzodioxins, also binds some naturally occurring substances as well as some amino acid derivatives formed under physiological conditions, in addition to other contaminants, such as dibenzofurans and several polychlorinated biphenyls *(2)*. Hormone receptors like the estrogen receptor show a comparatively broad substrate specificity, which is additionally accompanied by pronounced species differences in the affinity to various ligands *(13)*. The problem of a broad ligand specificity of a receptor and/or the resultant low specificity for a single, specific ligand is made even more difficult by the pronounced crosstalk of various cellular signaling pathways, i.e., by their mutual influencing. In particular, signaling cascades with fundamental significance for cell physiology and development appear to have a high degree of networking with other signaling pathways. For example, a reporter system that detects the activity of the so-called canonical Wnt/β-catenin signaling pathway, which is important for embryonic development, proved itself to be responsive in murine embryonic stem cells to a large number of structurally different chemical substances whose molecular target structures – at least as far as is currently known – are not components of the Wnt/β-catenin signaling pathway *(17, 18)*. All in all, this adds up to immense difficulty for unequivocal substance identification by means of classic methods of analytical chemistry, which is currently required by law following a "positive" result from an effect-based screening test.

In addition, the possible interference of a sample with the enzymatic reaction of the reporter system has to be considered. The routinely used firefly luciferase has proven to be comparatively susceptible to inhibition by many different chemicals *(4)*, while nonspecific interferences of the test substance and the transcription factors used in the reporter gene system can also occur *(11)*. The latter problems are controllable, however, through the inclusion of appropriate checks.

A further potential problem of a large battery of independent reporter gene-based test systems could lie in the occurrence of isolated "positive" test results in individual sub-test systems of the battery, which could ultimately lead to a large number of samples being categorized as suspicious. The validation of the test battery as a whole with a clearly prepared strategy for dealing with "positive" results in individual tests would be required here. How is the increased likelihood of a coincidentally wrong positive test result in a single sub-test system, as it is caused by a larger number of endpoints recorded in parallel, to be dealt with? Should different types of biological effects on different signaling cascades with different tasks in the organism, for example, be given different weightings in the interpretation of the test results? Or should every finding be given the same weighting as a matter of principle? Can an addition of certain values for different test systems in line with the concept of a hazard index *(21)* be applied, and if so, under which preconditions? In addition to these difficulties, economic aspects would have to be taken into

account, as conducting a large number of individual tests (see also the remarks made previously) within the scope of a test battery of this kind would involve significant time and resources.

Endogenous transcript biomarkers as an alternative to reporter genes

Multiplex approaches with various reporter gene systems, such as several biochemically different luciferases, whose signals can be read out separately, are possible in principle but are restricted to a very few parallel endpoints because of practical problems with the introduction of a larger number of constructs into the DNA and because of limitations in availability of different reporter genes that can be measured in parallel or in sequence. To solve the previously mentioned problem of a large number of reporter gene-bearing test systems to be operated in parallel, as was the case in the reporter gene battery already discussed, the use of methods that permit the simultaneous recording of a large number of endpoints and/or toxicity mechanisms in a single biological system would have to be considered. Ideally, the simultaneous analysis of various toxicity mechanisms in a single biological system would be achieved on the basis of the physiological reaction of a cell without having to introduce any foreign material into the system, such as the genetic elements of a reporter gene construct.

In a comparison of various technologies that could be considered here, the search for suitable predictive biomarkers (effect markers) at the mRNA level, as opposed to analyses at the protein or metabolite levels, should be categorized as the easiest to conduct; modern PCR and/or transcriptomics techniques are comparatively cheap and permit the measurement of specific target gene expression and the preparation of global expression profiles on well-standardized experimental platforms *(12)*.

As a rule, a toxicologically relevant effect on the cell will be reflected at the transcriptional level by changes to the cell's gene expression profile, which is why use of signatures of mRNA biomarkers appear to be suitable for the previously mentioned purpose. Either a single mRNA or a certain set of RNAs jointly regulated by a specific toxicological mode of action can be used in principle for each individual endpoint or mechansim. This is fundamentally not essentially different from the reporter gene battery approach already outlined, which is also based on altered transcription, except that a simultaneous determination of different endpoints or transcripts is made from a single biological sample and endogenous target genes, instead of a foreign reporter gene integrated into the genome of the test cells, serve as endpoints. Although this leads to a greater complexity of endpoints (several endogenous target RNAs have to be measured in parallel in a system instead of one reporter gene) and a methodology that has to be seen as more elaborate than reporter gene tests, it could prove to be less cost- and resource-intensive than the analysis of an entire battery of reporter gene systems. The suitable transcripts can be identified here via a non-targeted approach, with its main focus being on a transcriptome-wide search for suitable deregulated genes. Alternatively, based on mechanistic knowledge of toxicity-relevant signaling and metabolic pathways (adverse outcome pathway concept), the targeted addressing of important molecular interim stages of a toxic action, in this case the induction of characteristic target genes of a transcription factor activated by a toxin, for example, which in turn causally influences subsequent steps in the toxicity cascade, can be undertaken.

Application of single transcript biomarkers and transcript signatures

It is likely that hardly any single endogenous transcript will specifically signal the presence of a single substance (group) with sufficient sensitivity and predictivity by means of expression changes. A candidate for being such a rare entity could be the *CYP1A1* gene, which is regulated after the exposure of cells to agonists of the aryl hydrocarbon receptor, thereby making it suitable as a target transcript for the analysis of dioxin-like effects. Even in the event that a specifically regulated individual transcript is missing, however, a skillful combination of several transcripts into a specific signature of a certain toxicity effect is possible; this has been shown in several recently published studies. For example, a signature of eight transcripts to distinguish developmental toxins that work in different mechanistic ways was identified in an *in vitro* system with pluripotent stem cells *(15)*. Another recent publication describes the establishment of a transcript signature that can be used to identify agonists of the peroxisome proliferator-activated receptor PPARα *(14)*. The identification of estrogen receptor-influenced substances by means of a transcript signature is similarly possible *(7)*, while the use of transcript signatures in differentiating human pluripotent stem cells for the characterization of the developmental toxicity potential of chemical substances has also been proposed recently *(16)*. As an alternative to marker transcripts for distinct mechanisms, it may be possible to find biomarker batteries for more complex endpoints, such as organ toxicity or a specific aspect thereof (such as the induction of steatosis in liver cells) and not the effects produced via a single specific mechanism (such as aryl hydrocarbon receptor activation). A strategy offers itself here that focuses on already available adverse outcome pathways and in which important sub-stages of toxicity are condensed schematically, from the initial molecular occurrence all the way through to damage to a particular organ or the entire organism *(1)*. A distinction between non-hepatotoxins and cholestasis-inducing substances based on examination of a selection of marker

transcripts was published recently *(19)*. The establishment of biomarker signatures of hepatotoxicity *in vitro* is the goal of a current BMBF (German Federal Ministry for Education and Research)-funded research project that is being conducted with the involvement of the German Federal Institute for Risk Assessment (http://www.bfr.bund.de/de/modellierung_des_toxoms_ kultivierter_ menschlicher_hepatozyten__livsys_-193087.html).

On the basis of possibilities that already exist for identifying certain effects via transcriptional signatures, it is therefore conceivable in principle that test systems could be developed that either enable the measurement of a single biomarker set as an indicator of a certain effect, or that are suitable at the same time for determining several biomarker sets for the parallel identification of more than one molecular mechanism of action. From a methodological point of view, depending on the numbers of transcripts to be analyzed, standard RT-PCR methods, so-called low-density arrays that combine a number of target transcripts, or whole-transcriptome microarrays, might come into use. Provided that appropriate specific mRNA signatures independent of one another could be found for various mechanisms of action, a system of this kind would in principle be at least comparable to a reporter gene battery, but could be conducted in only a single experiment in a solitary biological system *(Fig. 1C)*. An analysis of this kind is without doubt comparatively complex, as it involves sample processing and extraction, cell incubation, RNA isolation, PCR and data evaluation. The possibility of covering in parallel and at a reasonable cost a large number of endpoints that would otherwise have to be determined separately could nevertheless make the execution of such a method appear feasible from a practical point of view.

Issues and challenges to be solved for routine application of transcript-based methods

There can be no doubt that a great deal of research still has to be done before transcript-based approaches can be put to use in routine operations in food and feed control. The challenges to be faced include the following:

- As the basis for development and application of effect-based analytical test systems, knowledge of molecular toxicity mechanisms has to be further widened. This includes the mechanistic analysis of the interactions of various mechanisms of action that could be expected in the event of co-exposure of a cell to mixtures of two or more chemicals with different effects.
- In order to establish multi-endpoint systems, i.e., biological test systems that permit simultaneous detection of several effects, the functionality of the identified molecular toxicity mechanisms has to be verified experimentally in each test system. Many cells are known to alter their properties as a consequence of transition into *in vitro* culture.
- The identification of specific biomarker signatures for individual molecular mechanisms of toxicity is a complex task requiring the interdisciplinary cooperation of toxicologists and bioinformaticians.
- The establishment of dose-response curves for signatures of several transcripts that may under certain circumstances be of different strengths or be regulated with different sensitivities also requires the inclusion of bioinformatic expertise.
- The majority of the laboratories that routinely monitor foods and feeds do not have the necessary prerequisites, with regard to technical equipment and specialized knowledge, to use biological test systems, including subsequent analysis at the transcript level. Efforts are required here to create the necessary infrastructure and expertise.
- Efforts are also required in the field of sample processing. Extraction and other methods are needed to ensure that the resultant samples do not have any toxic effects on the biological systems used, due to solvents, for example, and also to ensure that if a large number of effects that can be caused by structurally different chemicals are to be examined, all of the different types of chemicals are in the extract and do not get lost in the course of processing.
- In general, comprehensive validation has to be conducted before a new method in food and feed monitoring can be used, and one would also have to pay due consideration to the variability of biological systems.

NON-TARGETED EFFECT-BASED ANALYTICS ON A TRANSCRIPTOME BASIS

The approaches outlined previously aim at the detection of specific effects and signal modulations, and at the possible widening of methods of this kind through the parallel determination of several different endpoints or mechanisms of action, each of which should be as specific as possible. All of these would lie within the range of targeted analytics. If it is assumed that biological effects are reflected sensitively in the transcript pattern of a cell and that a large number of mechanistically different effects exist, this means ultimately that in principle, all mechanisms of action could be recorded in parallel by recording the expression change of all transcripts of a cell, at least to the extent that the selected biological model system was capable of reproducing them. Transferred to the concept of an "effect-orientated" as opposed to a mechanism-based approach, in the sense of non-targeted analytics without regard to specific signaling pathways influenced by a substance, a "degree of biological deregulation" could be determined as a measure of the disturbance of cellular equilibrium by exogenous substances, i.e., a parameter determined by the number of deregulated transcripts as well as the amplitude of their deregulation. Compared with the relatively insensitive effect-based endpoint of cytotoxicity *in vitro*, a para-

meter of the degree of deregulation of this kind would probably be capable of detecting an effect in concentrations well below the cytotoxicity threshold.

The transcriptome data would then be observed in principle as a mass of data points independent of causative mechanisms of action, biomarker transcripts or other specific deliberations. By means of the degree of deregulation, dose-response curves could then be used in a benchmark approach (the exceeding of a certain degree of deregulation beyond the biological fluctuation margins of untreated cultures) to determine the concentration of a test substance that disturbs cellular equilibrium in an *in vitro* experiment *(Fig.1D)*. The data could then be examined specifically as an indicator for possible mechanisms with a view toward the deregulation of certain transcripts.

SUMMARY

Effect-based analytical methods are still used only to a small extent in food and feed monitoring. Nonetheless, the novel methodological developments in the field of effect-based analytics might be suited to substantially increasing the application of effect-based assays in the future. This would make the topic relevant for research concerned with the elucidation of toxic mechanisms that form the basis of all effect-based approaches, for developers of biological test methods, for potential future users in industry or in public control laboratories, and for people who set the legal regulatory framework for the use of effect-based methods. One obstacle to the introduction of new methods of effect-base analytics in routine monitoring is the legal necessity to identify and quantify by classic chemical analysis a causative substance as a result of a "positive" finding from an effect-based test system. Therefore, it is currently possible to use effect-based test systems only as parts of step-by-step testing or as a screening system, and not as a separate, single test method. While in principle potentially harmful samples might be detected by both approaches, effect-based assays might be able to detect effects of yet unknown toxic substances that would yield negative results in analytical-chemical assays aimed at identifying distinct, known toxins. In the field of reporter gene analysis, which is already used to record the effects on specific nuclear receptor-dependent signal cascades, concrete further possibilities for development exist all the way through to a battery of different test systems that could jointly cover a selection of toxicologically highly important mechanisms of action. More recent publications show substantial progress in the identification of transcript signatures as indicators of certain mechanisms of action. Because of their potential use in recording many mechanistically different effects in parallel, transcript-orientated approaches could point out a new direction in future development of effect-based methods. Whereas perfected experimental platforms (e.g., microarrays) are already available, extensive experimental preliminary work on the mechanistic principles of gene transcription through various cellular signal cascades, and in particular in the field of bioinformatics on methods of data and evaluation and interpretation, is still required before the formal validation of such methods and their subsequent use in routine analytics is conceivable. Furthermore, changes on a legal level that distinguish between cases with and without the necessity for classical substance identification, or that include the significance and relevance of effect-based analytics as an alternative and/or supplement to classical chemical analysis within the scope of food and feed control, could enhance the appeal of effect-based test systems.

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REFERENCES

1. Anonymous. 8 December 2016. Adverse Outcome Pathway Wiki. Available at: www.aopwiki.org. Acccessed 26 June 2017.
2. Bock, K. W. 2013. The human Ah receptor: hints from dioxin toxicities to deregulated target genes and physiological functions. *Biol. Chem.* 394:729–739.
3. Brack, W., S. Ait-Aissa, R. M. Burgess, W. Busch, N. Creusot, C. Di Paolo, B. I. Escher, L. M. Hewitt, K. Hilscherova, J. Hollender, H. Hollert, W. Jonker, J. Kool, M. Lamoree, M. Muschket, S. Neumann, P. Rostkowski, C. Ruttkies, J. Schollee, E. L. Schymanski, T. Schulze, T. B. Seiler, A. J. Tindall, G. De Aragao Umbuzeiro, B. Vrana, and M. Krauss. 2016. Effect-directed analysis supporting monitoring of aquatic environments — an in-depth overview. *Sci. Total Environ.* 544:1073–1118.
4. Braeuning, A. 2015. Firefly luciferase inhibition: a widely neglected problem. *Arch. Toxicol.* 89:141–142.
5. Braeuning, A., H. Broll, and A. Lampen. 2016a. Effect-based analytics: new concepts for food control and safety. *Food Prot. Trends.* 36:412–419.
6. Braeuning, A., H. Broll, and A. Lampen. 2016b. Wirkungsbezogene Analytik: Neue Konzepte für die Überwachung und Lebensmittelsicherheit. *J. Verbrauchersch. Lebensm.* 11:91–96.
7. Corton, C. 2015. Identification of potential endocrine disrupting chemicals using gene expression biomarkers. *Toxicol. Letters* 238S:S36.
8. Di Paolo, C., T. B. Seiler, S. Keiter, H. Meng, M. Muz, W. Brack, and H. Hollert. 2015. The value of zebrafish as an integrative model in effect-directed analysis — a review. *Environ. Sci. Eur.* 27:8.
9. Hakkila, K., T. Green, P. Leskinen, A. Ivask, R. Marks, and M. Virta. 2004. Detection of bioavailable heavy metals in EILATox-Oregon samples using whole-cell luminescent bacterial sensors in suspension or immobilized onto fibre-optic tips. *J. Appl. Toxicol.* 24:333–342.
10. Lappalainen, J. O., M. T. Karp, J. R. J. Nurmi, and M. P. J. Virta. 2000. Comparison of the total mercury content in sediment samples with a mercury sensor bacteria test and *Vibrio* Fischeri Toxicity Test. *Environ. Toxicol.* 15:443–448.
11. Luckert, C., S. Hessel, A. Lampen, and A. Braeuning. 2015. Utility of an appropriate reporter assay: Heliotrine interferes with GAL4/upstream activation sequence-driven reporter gene systems. *Anal. Biochem.* 487:45–48.
12. Marx-Stoelting, P., A. Braeuning, T. Buhrke, A. Lampen, L. Niemann, M. Oelgeschlaeger, S. Rieke, F. Schmidt, T. Heise, R. Pfeil, and R. Solecki. 2015. Application of omics data in regulatory toxicology: report of an international BfR expert workshop. *Arch. Toxicol.* 89:2177–2184.

13. Matthews, J., T. Celius, R. Halgren, and T. Zacharewski. 2000. Differential estrogen receptor binding of estrogenic substances: a species comparison. *J. Steroid Biochem. Mol. Biol.* 74:223–234.

14. Oshida, K., N. Vasani, R. S. Thomas, D. Applegate, M. Rosen, B. Abbott, C. Lau, G. Guo, L. M. Aleksunes, C. Klaassen, and J. C. Corton. 2015. Identification of modulators of the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARalpha) in a mouse liver gene expression compendium. *PloS ONE* 10:e0112655.

15. Rempel, E., L. Hoelting, T. Waldmann, N. V. Balmer, S. Schildtknecht, M. Grinberg, J. A. Das Gaspar, V. Shinde, R. Stöber, R. Marchan, C. van Thriel, J. Liebing, J. Meising, N. Blüthgen, A. Sachinidis, J. Rahnenführer, J. G. Hengstler, and M. Leist. 2015. A transcriptome-based classifier to identify developmental toxicants by stem cell testing: design, validation and optimization for histone deacetylase inhibitors. *Arch. Toxicol.* 89:1599–1618.

16. Shinde, V., L. Hoelting, S. P. Srinivasan, J. Meisig, K. Meganathan, S. Jagtap, M. Grinberg, J. Liebing, N. Blüthgen, J. Rahnenführer, E. Rempel, R. Stoeber, S. Schildtknecht, S. Förster, P. Godoy, C. van Thriel, J. A. Gaspar, J. Hescheler, T. Waldmann, J. G. Hengstler, M. Leist, and A. Sachinides. 2017. Definition of transcriptome-based indices for quantitative characterization of chemically disturbed stem cell development: introduction of the STOP-Toxukn and STOP-Toxukk tests. *Arch. Toxicol.* 91:839–864.

17. Uibel, F., A. Muhleisen, C. Kohle, M. Weimer, T. C. Stummann, S. Bremer, and M. Schwarz. 2010. ReProGlo: a new stem cell-based reporter assay aimed to predict embryotoxic potential of drugs and chemicals. *Reprod. Toxicol.* 30:103–112.

18. Uibel, F., and M. Schwarz. 2015. Prediction of embryotoxic potential using the ReProGlo stem cell-based Wnt reporter assay. *Reprod. Toxicol.* 55:30–49.

19. Van den Hof, W. F., M. L. Coonen, M. van Herwijnen, K. Brauers, W. K. Wodzig, J. J. van Delft, and J. C. Kleinjans. 2014. Classification of hepatotoxicants using HepG2 cells: A proof of principle study. *Chem. Res. Toxicol.* 27:433–442.

20. Wernersson, A. S., M. Carere, C. Maggi, P. Tusil, P. Soldan, A. James, W. Sanchez, V. Dulio, K. Broeg, G. Reifferscheid, S. Buchinger, H. Maas, E. van der Grinten, S. O'Toole, A. Ausili, L. Manfra, L. Marziali, S. Polesello, I. Lacchetti, L. Mancini, K. Lilja, M. Linderoth, T. Lundeberg, B. Fjällborg, T. Porsbring, D. G. J. Larsson, J. Bengtsson-Palme, L. Förlin, C. Kienle, P. Kunz, E. Vermeirssen, I. Werner, C. D. Robinson, B. Lyons, I. Katsiadaki, C. Whalley, K. den Haan, M. Messiaen, H. Clayton, T. Lettieri, R. Negrao Carvalho, B. M. Gawlik, H. Hollert, C. Di Paolo, W. Brack, U. Kammann, and R. Kase. 2015. The European technical report on aquatic effect-based monitoring tools under the water framework directive. *Environ. Sci. Eur.* 27:7.

21. Wilkinson, C. F., G. R. Christoph, E. Julien, J. M. Kelley, J. Kronenberg, J. McCarthy, and R. Reiss. 2000. Assessing the risks of exposures to multiple chemicals with a common mechanism of toxicity: how to cumulate? *Regul. Toxicol. Pharmacol.* 31:30–43.

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