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Solutions for present and future emerging pollutants in land and water resources management

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Improved Bioassay Solutions for Environmental Monitoring Based on Adverse Outcome Pathways

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1.1 Summary

A central motivation of the SOLUTIONS project is to establish and provide advanced methods for environmental monitoring of freshwaters that are useful to identify contaminants of emerging concern in the context of the European Water Framework Directive. Recently it has become evident that contamination of European river basins with chemicals is not limited to a small number of priority compounds but rather contamination patterns are diverse and complex. This situation is not easily amenable by additional chemical analytical efforts. It has thus been suggested to complement chemical monitoring with effect-based tools which offers the scope required to capture groups of compounds as well as their transformation products and combined effects.

In this deliverable we report on the efforts within the SOLUTIONS project to improve the utility of bioassays for environmental monitoring. Improvements were sought with regard to provision of

(i) Mode-of-action collation for compounds that are currently being detected in surface water monitoring studies in order to compare this with the capability of suggested effect-based tools to detect these contaminants;

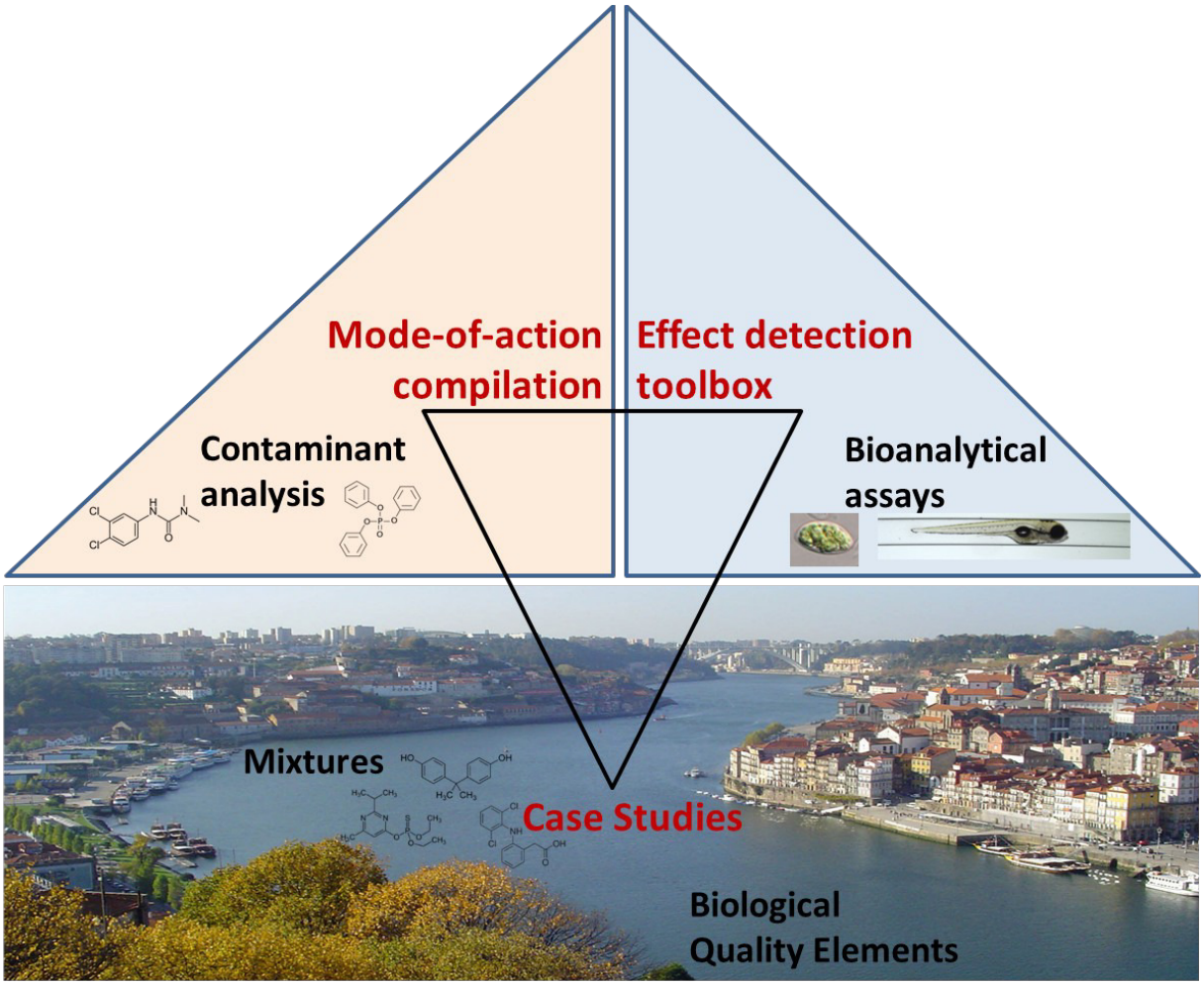
(ii) Uniform and transparent experimental procedures for the selected effect-based tools, that facilitate understanding of principle elements of their use and the data that they generate;

(iii) Case studies from the Danube and Rhine river basins employing chemical and effect-based tools in concert to study the coherence and complementarity between chemical

and bioanalytical information.

The next steps in the development of effect-based tools for water monitoring will include consideration and validation of mixture effects for the individual bioassays, verifying the relevance of combined effects in environmentally relevant mixtures, reflecting on the relationships between different bioanalytical tools and ecological effects, and developing guidelines for the use of effect-based tools for different monitoring purposes.

1.2 Graph



2. List of Contents

3. List of Abbreviations

AhR:	Arylhydrocarbon receptor
ANOVA:	Analysis of variance
AOP:	Adverse Outcome Pathway
AR:	Androgen receptor
ARE:	Antioxidant response element
ATP:	Adenosine 5'-Triphosphate
BEQ:	Bioanalytical equivalent concentration
BQE:	Biological Quality Elements
CAFLUX:	Chemically Activated FLUorescence eXpression
CALUX:	Chemically Activated LUminescence eXpression
DOC:	Dissolved organic carbon
DPP-4:	Dipeptidyl Peptidase-4
EBT:	Effect-based tools
EC:	Effect Concentration
EDA:	Effect Directed Analysis
ER:	Estrogen Receptor
FET:	Fish Embryo Toxicity assay
GR:	Glucocorticoid receptor
IC:	Inhibitory concentration
JDS:	Joint Danube Survey
KE:	Key Event
LOD:	Limit of detection
LOEC:	Lowest Observable Effect Level
LOQ:	Limit of quantification
LPS:	Lipopolysaccharide
LVSPE:	Large volume solid-phase extraction
MoA :	Mode of Action
MIE:	Molecular Initiating Event
PXR:	Pregnane X receptor
qPCR:	quantitative Polymerase Chain Reaction
QSAR:	Quantitative Structure-Activity Relationship
REP_i:	Relative effect potency
SOP:	Standard Operating Procedure
Tox21:	Toxicity Testing in the 21st Century
ToxCast:	Toxicity ForeCaster
US EPA:	United States Environmental Protection Agency
WWTP:	wastewater treatment plant

4. Project results

4.1 Bioassays – State of the art and needs for improvement

Chemical monitoring studies on surface waters demonstrate that contamination is not only expected to occur for the 45 priority pollutants currently considered under the WFD but many more chemicals can be detected in aquatic ecosystems using instrumental analytics (Kolpin et al. 2002, Loos et al. 2010). For example, the NORMAN network in February 2016 published a list of 1036 chemical entities they consider as emerging contaminants in surface waters (http://www.norman-network.net/?q=node/19). Taking into consideration that up to date the detection of chemical contamination in surface water relies on target analysis the exact number and identity of chemicals of emerging concern is unknown.

Chemical contamination also does not exclusively occur as individual compounds but occurrence of mixtures of contaminants appears to be the more realistic assumption (Ginebreda et al 2014, Altenburger et al. 2015). Furthermore, mixture exposure may lead to combined effects that can only be anticipated if all components are known and their toxicity is well characterised (Posthuma et al. 2008, Kortenkamp and Altenburger 2011). In realisation of such scenarios, it has been suggested that effect-based tools may complement chemical monitoring (Conon, Geist Werner, 2012, Ekman et al. 2013) by offering specific advantages: Using biological effects to detect contamination allows aggregating several chemical structures that produce the same effect irrespective of whether or not their identity and concentrations are known. This means one would always accommodate for the totality of mixture components producing a certain effect irrespective of whether or not we know the exact composition. Moreover, steps required in the assessment of a contamination subsequent to a chemical identification such as consideration of the bioavailability of the contaminants and its relevance for elucidation of subsequent adverse biological effect may also become informed through bioassay data.

For environmental risk assessment defined panels of standardised biological assays are performed under controlled exposure conditions. These bioassays are used to observe apical biological responses in intact organisms such as growth development or behaviour and have become widely accepted. Freshwater bioassays to protect pelagic organisms, include for example protocols for fish, daphnia, algae and macrophytes (EC, 2011). Biological response studies have also been key in elucidating the mechanisms and modes of action (MoA) of chemical effects on biological systems. For such efforts biochemical or physiological assays have been conducted capturing endpoints

ranging from ligand binding and modification of receptor activation to alteration of downstream events. With the paradigm shift in toxicity testing in the US (NRC 2007) a new era of high-throughput gene expression, enzyme, and cell-based bioassays were introduced for the testing of chemicals to support environmental and health assessment (see the NIH Tox 21 program https://ncats.nih.gov/tox21/ and the US-EPA ToxCast program https://www.epa.gov/chemical-research/toxicity-forecasting). By now several hundred enzymatic and receptor signaling assays mainly developed for pharmaceutical screening have been employed to characterise concentration-response patterns for many compounds (Sipes et al. 2013). In the first phase of the joint program some 2800 compounds were characterized in total while in the second phase more than 10000 compounds were tested focusing on assays covering nuclear receptors responses (AhR, AR, ERα, FXR, GR, PPARδ, PPARγ, TR and VDR) and stress response pathways (p53, NF-κB, pH2AX, endoplasmic reticulum stress, mitochondrial membrane potential, ARE/Nrf-2, heat shock response and DNA damage). Currently, planning for a third phase has begun which will amend the previous steps by 'using more physiologically relevant cells (i.e., primary or stem cells) in screening assays, increasing the number of molecular pathways tested by measuring gene expression, and expanding the compound library to include among others chemical mixtures' (https://ncats.nih.gov/tox21/about/operations). Despite the large numbers of assays established and used for chemical bioactivity screening major questions remain regarding their utility. They include questions as to the coverage of potential targets of chemicals in organisms or cells (Overington, Al-Lazikani, Hopkins 2006), how the translation of molecular interaction between chemicals and biomolecules into adverse effects can be made operational to be used for prediction (Patlewicz et al. 2013). Furthermore, most of the bioassays suggested for chemical MoA-profiling have not been investigated for their applicability and utility to situations other than individual pure compound testing.

So while there are substantial numbers of bioassays available, the need to provide and improve systematic links between contaminant exposure with biological adverse effects calls for mechanistic principles (Hendriks 2013) as it is neither technical not logistically feasible to investigate every exposure situation for all potentially relevant endpoints. The literature offers conceptual frameworks to address the relation between observation of specific biological effects and adverse outcomes (Ankley et al. 2010) and for addressing combined effects from mixtures (Altenburger et al. 2015). Operationalisation for water monitoring purposes is, however, rarely found. The variable matrix, low compound concentrations and unresolved mixtures are expected to pose specific challenges.

Meanwhile several practical efforts have been undertaken to prove the utility of bioassays to serve as effect-based tools for water monitoring. Early bioassay application to characterization of water contamination focused on the detection and quantification of dioxin-like compounds (Behnisch Hosoe, Skai 2001) which was followed by efforts to detect endocrine-active compounds or mutagenicity in water samples (e.g. Van der Linden et al. 2008, Reifferscheid et al. 2012) using bioassays. For other effect categories such as general stress response signals, inhibition of photosynthesis, geno- or immunotoxicity, proposed bioassays have not yet reached a similar degree of development. A benchmarking study characterising ten different samples representing different steps in a full water cycle using 103 different bioassays demonstrated water type characteristic bioanalytical profiles (Escher et al. 2014) and the complementarity of different effect detection principles. Furthermore, an EU-coordinated round robin study on an artificial mixture of priority pollutants provided evidence that effects for the mixture may occur even at concentration levels that EU legislation safety regulation would consider as safe (Carvalho et al. 2014). Also, efforts have been made allowing comparative protocol evaluation in simplified round robin like studies with spiked water samples to demonstrate recovery of compound specific effects (Di Paolo et al. 2016). So, in summary, we find that due to the ill-defined chemical contamination of surface waters, aquatic exposure and effect assessment might benefit from complementary effect-based characterisations. Despite many bioassays being potentially available for such efforts, there is as yet no systematic approach developed to define which panel of assays could be of greatest use.

What is needed for a more systematic approach in developing effect-based tools (EBTs) for water monitoring? First and foremost when considering the diversity of bioassay protocols suggested for effect-detection of compounds exposure, based on the knowledge laid out above it may help to know which effects are likely to be induced by water contaminants. We would then need to consider existing protocols in terms of their exposure regime, effect detection principles and quality assurance measures to define applicability criteria. Subsequently, proof-of-principle case studies evaluate their anticipated utility, demonstrating that matrix and mixture issues can be accounted for.

In this deliverable we report on the efforts within the SOLUTIONS project to investigate and improve the utility of bioassays for environmental monitoring purposes. In a stepwise approach we, first of all, collated MoA information for organic chemicals that have been detected in surface water monitoring studies (see section 4.2). This synopsis should help to cross-reference expected MoA with suggested bioassays and their capabilities to detect relevant water contamination. At a later

stage the compiled knowledge may also be used to benchmark the different assays. Secondly, for a set of 36 different bioassays that may eventually serve as effect-based tools for water monitoring we produced a compilation of experimental procedures, so-called standard operating procedures (SOP) using a uniform format (see section 4.3). A uniform layout of SOPs provides consistent access to existing methods, allows comparative exposure and effect detection consideration and provides options to derive more stringent and coherent data evaluation and quality control procedures. We strive to develop criteria that help for an improved understanding of principles of detected effect qualities. Finally, we report on SOLUTIONS case studies from the Danube and Rhine river basins (see section 4.4), where chemical and effect-based tools were employed in concert to study the coherence and complementarity between chemical and bioanalytical information.

4.2 Which modes-of-action can we expect from prevalent water contaminants?

To propose bioassays for effect-based monitoring of water one should consider up front what type of effects may be expected from present contamination of freshwaters. >One approach would be to consider all chemicals that potentially occur in freshwaters due to anthropogenic activities, which would include all compounds which undergo environmental risk assessments for their aquatic exposure potential, i.e. industrial chemicals, pesticides, biocides, pharmaceuticals, washing agents, personal care products and the like. Alternatively, perhaps it concerns only those compounds that have actually been identified and quantified in analytical monitoring studies. We decided to start from the latter perspective, i.e. listing compounds that have actually been found in monitoring studies using target screening methods on European freshwater samples. This approach will capture mostly past contamination which renders the aim of addressing compounds of emerging concern difficult. Therefore, to assess current and future contaminants requires an additional perspective that considers the dynamics of use, emission and contamination. The dynamics of water contamination are explicitly addressed in other activities of the SOLUTIONS consortium using emission-driven exposure modelling and future scenario building approaches, where results will become available later in the project. Here, we linited ourselves to comparing the findings for the expected biological effects between chemicals that have actually been detected in freshwaters with a larger list of compounds that could have been identified based on the analytical methods used, but were not found above detection limits. Most of the findings reported in the following have been published in Busch et al. (2016). The paper provides the methodological approaches and documentation of data as well as further details, e.g. on relative biological importance of the considered compounds.

Seven recent monitoring studies that employed multi-compound target screening methods for organic compounds on water samples from three major European water catchments, namely the Danube, Rhine and Elbe were taken to retrieve a list of 970 distinct organic chemicals that could be structurally annotated and quantified. They mainly represent compounds that are used in pesticides, pharmaceuticals and industrial products. This number and composition compares well with the NORMAN list of emerging substances (update February 2016, http://www.norman-network.net/?q=node/19) which comprises of 1036 entries for substances that have been detected in monitoring studies but which additionally include compounds of natural origin such as microcystins. One has to acknowledge that compounds detectable through targeted analysis comprise of not only intentionally produced and emitted substances but also transformation products, isomers and other compounds (around 30% of the entities). The detectable compound spectrum is to some degree driven by the analytical method used. At the same time none of the individual methods used detects all of the compounds compiled here, but they complement each other. At best some 400 different compounds were captured using a single method in the studies considered here for references, please refer to Busch et al. 2016). In turn this means one has to take additional efforts to compare the findings between different monitoring studies as findings or lack thereof may be due to the specific method used. Moreover, it has to be understood that the compound list we consider in the following, despite being 970 entries long, does not comprehensively cover all known water contaminants. For example, due to the studies selected no metals or organometallic compounds are covered. Furthermore, compounds that cannot be captured with current screening techniques such as glyphosate are also ignored here.

Of the 970 distinct organic chemicals that could be assigned some 420 compounds occurred above detection limits. Thirteen of these compounds were detected across all seven studies considered, while 219 compounds were unique for an individual study because of the method differences highlighting again the current difficulties regarding strong statements on typical contamination patterns or relevant mixtures. The concentrations quantified for the 13 compounds that were detected across all studies varied by about 4-5 orders of magnitude between 1 and 10000 ng/L. This is in line with the existence of sites with higher contaminant burden (e.g. for waste water emissions) versus almost pristine sites.

To derive an expectation for biological effects to be detected in a bioassay we retrieved effect information provided in publically accessible databases such as drugbank (http://www.drugbank.ca/) for the contaminants on the compiled list. The information available

relates to the interaction of chemicals with biological structures and functions. The type of information varies considerably in level of detail and precision and may cover well-characterized target molecules (such as hydroxymethylglutaryl-coenzyme A reductase) the naming of an affected pathway (e.g. cholesterin biosynthesis) or a notion on a functional disturbance, such as lipid metabolism or endocrine signaling. A molecular target which might seem the most specific type of information in this context could be attributed for 459 (i.e. 47%) of all compounds considered. We collated these different categories of knowledge, sorted them into different classes and aggregated them first into effect types and subsequently into broader MoA categories based on expert judgement. We ended up with 31 different MoA categories listed in table 1. These categories range from adenosine receptor to insects-specific modes of action. It emerges that some categories comprise a diversity of different targets and possibly different processes that are potentially affected. For example, for the MoA group neuroactivity we counted 18 different known receptors while others comprise only one specific receptor like the adenosine receptor or refer to processes e.g. DNA alkylation in the category of nucleic acid damage. These examples illustrate that grouping compounds by biological MoA on the one hand helps to reduce the large number of hundreds of compounds into an order of magnitude lower number of MoA categories, which might be captured through effect-based tools. On the other hand, the existing effect information is not structured to build simple effect categories readily translatable into detection principles. Another question is whether adverse outcome pathways as elaborated in the scientific and regulatory communities (see OECD efforts) for individual chemical risk assessment can be of further help here. This will be taken up when reflecting on the coverage of potential effects with the bioassays proposed for use as effect-based tools in water monitoring.

Table 1: Mode of action categories assigned for the following molecular targets or mechanism identified for the 970 organic chemicals detectable in water analytics according to Busch et al. (2016)

MoA category	**Molecular targets / molecular processes**
Adenosine receptor	Adenosine receptor
Analgetic	Opioid receptor
	unknown
Angiotensin Receptor or Enzyme	Angiotensin receptor
	Angiotensin-converting-enzyme (ACE)
Antibiotic	(Ionophoric process)
	30 S subunit of bacterial ribosome
	50 S subunit of bacterial ribosome
	Bacterial dihydrofolate reductase
	Bacterial dihydropteroate synthase
	Bacterial penicillin binding protein (PBP)
	Inosine monophosphate dehydrogenase (IMPDH)
	Mycobacterial arabinosyl transferase
	unknown
Antihistamine	Histamine H1 receptor
	Histamine H2 receptor
Antiinflammatory	Cyclooxygenases 1 and 2
	Prostaglandin G/H synthase
	unknown
ATP inhibition	ATPase
	ATP synthase
	Na+/K+ ATPase
Beta blocker	Beta adreneric receptors 1 and 2
Cancerogenic	multiple targets or unknown
Carotenoid biosynthesis inhibition	4-Hydroxyphenylpyruvate dioxygenase (4-HPPD)
	Phytoene desaturase
	unknown
Cell membrane disruption	(Actin disruption)
	(Bacterial dehydrogenases)
	3 keto reductase, C4 demethylase
	unknown
Cell wall biosynthethis	Cellulose synthase
	unknown
Chitin biosynthesis inhibition	unknown
DPP-4 inhibition	Dipeptidyl peptidase-4 (DPP-4)
Endocrine	Androgen receptors
	Aromatase
	Estrogen receptors
	Glucocorticoid receptors
	Multidrug transporters
	Progesterone receptors
	Retinoid X receptors

	Testosterone 5-alpha-reductase
	Thyroid receptors
	unknown
Ion channel modulation	Calcium channels
	Potassium channels
	Ryanodine receptors
	SLC12A1
	SLC12A3
	Sodium channels
	unknown
Iron chelator	Fe3+ ions
Lipid metabolism	Acetyl CoA carboxylase (ACCase)
	Bacterial ENR (enoyl-acyl carrier protein reductase enzyme)
	Phospholipid biosynthesis methyltransferase
	PPAR receptors
	unknown
Mitosis, Cell cycle	GGGP cyclase (Gibberillin synthese)
	Protein tyrosine kinase and topoisomerase II
	Tubulin
	unknown
Neuroactive	5-HT1 and 5-HT2 receptors
	Acetylcholine esterase
	Adrenergic receptors (alpha and beta)
	Benzodiazepine receptor (GABAA)
	Brain adenosine receptors
	Cannabinoid receptors
	Dopamine receptors (D3/D2)
	Ecdysone receptors
	GABA receptors
	Monoamine oxidase (MAO)
	Muscarinic acetylcholine receptors (mAChRs)
	Nicotinic acetylcholine receptors (nAChRs)
	N-methyl-D-aspartate receptor (NMDAR)
	Serotonin and noradrenalin transporters
	Serotonin receptors
	Serotonin transporters
	Sigma receptors
	Trace amine associated receptor 1
	μ-,δ- and κ-Opioidreceptors
Nucleic acid biosynthesis	50 S subunit of Bacterial ribosome
	Adenosine deaminase
	DNA gyrase
	DNA gyrase and topoisomerase IV
	RNA polymerase I
	Thymidylate synthetase
Nucleic acid damage	DNA (alkylation or binding)
	(AhR pathway)
Photosynthesis inhibition	Lycopene cyclase

	Protoporphyrinogen oxidase ferredoxin (PS I) D1 protein (PS II)
Protein biosynthesis inhibition	Acetolactate synthase (ALS) EPSP synthase (methionine biosynthesis) unknown
Respiration inhibition	Complex III cytochrome bc1 (ubiquinol oxidase) at Qo site Ubiquinol oxidase Ubiquinone reductase Complex II succinate-dehydrogenase Complex III electron transport protein Fumerate reductase (uncoupled oxidative phosphorylation) (unspecific membran disruption)
Signal transduction	Activated protein kinase (AMPK) Erythrocyte phosphodiesterase G-proteins in early cell signaling (proposed) MAP/Histidine-Kinase in osmotic signal transduction
Sterol biosynthesis inhibition	C14-demethylase in sterol biosynthesis Cholesterol-7-alpha-hydroxylase Delta14 reductase and delta8-delta7 isomerase Fungal squalene monooxygenase (squalene 2,3-epoxidase) Hydroxymethylglutaryl-CoA Reductase (HMG-CoA)
Synthetic Auxin	(plant hormone signalling)
Viral enzyme inhibition	Virus specific enzymes (e.g. influenza virus neuraminidase, HIV viral proteinase)
Vitamin K pathway	(Prothrombin synthesis) (Rodenticide via 4-hydroxycoumarin vitamin K antagonism) Vitamin K reductase
Insects-specific MoAs	Anopheles gambiae odorant binding protein 1 (AgamOBP1) Insect-specific cytochrome P450 system

As a next step we analyzed the distribution of chemicals from our compilation into the constructed MoA categories. The results are shown in figure 1. For 65% of the compounds detected in the monitoring studies a MoA could be assigned. The remaining fraction comprised mainly of transformation products or industrial chemicals where information required to assign a MoA was scarce. For two thirds of the compounds with an assigned MoA 28 out of the 31 MoA categories were indeed represented for the occurring water contaminants. Certain groups, such as neuroactive, photosynthesis inhibition, mitosis and cell cycle or sterol biosynthesis inhibition comprised of 19 and more chemicals. This picture did not substantially change when analysing the longer list of 970 compounds.

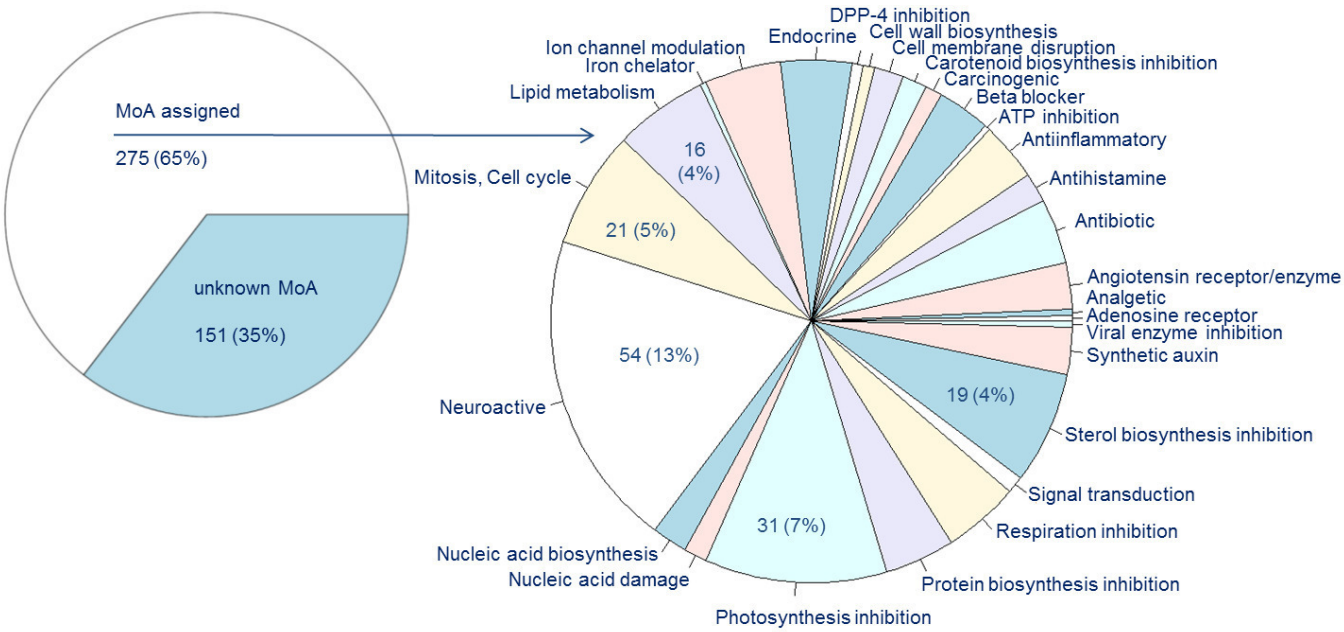


Figure 1: Distribution of 426 water contaminants detected in freshwater monitoring studies into mode of action categories according to Busch et al. (2016).
ATP = adenosine 5'-triphosphate; DPP-4 = dipeptidyl peptidase-4; MoA = mode of action. Figure reprinted from Busch, W., Schmidt, S., Kühne, R., Schulze, T., Krauss, M. and Altenburger, R. (2016). Micropollutants in European rivers: A mode of action survey to support the development of effect-based tools for water monitoring. Environmental Toxicology and Chemistry, 35(8): 1887-1899. Copyright 2016 with permission from Wiley.

Now the starting point for considering what bioassays are useful to detect the environmentally occurring compounds is derived from chemical specific MoA information. In a next step we need to compare the biological effects that can be measured by bioassays to identify which of the assays might be relevant to be developed into effect-based monitoring tools. It has to be stated though that we have as yet not considered the temporal and concentration scales that may also be relevant to attribute the identified MoA. A generic perspective in this respect may not be helpful to this end as too many potential variables (e.g. interspecies extrapolation or different receptor status of different cell types) may overcomplicate the picture. Instead in the following we deal with a set of bioassays that are available among the SOLUTIONS research groups and that cover a larger set of assays that have been suggested for use as effect-based tools in water monitoring.

4.3 What knowledge on bioassays do we have that could detect water contaminants?

Prior to application of the various bioassays to major contaminants and water samples we took on the task of generating a harmonized set of standard operating procedures (SOPs) for these assays. The aims of this work were: firstly, to provide transparency on the principles of effect detection of the available assays in order to reflect on the coverage for different modes of action that could be expected for different water contaminants (see 4.2). Secondly, we intended to ensure sufficient documentation for the methodology used; and thirdly, we strived to facilitate comparison of different experimental parameters between the different bioassays e.g. exposure length or sample volume requirement.

Although original descriptions of many of the bioassays have previously been published, it was considered prudent to collect current SOPs as several of the assays had been modified following their first publication, in several cases in order to reduce sample volume requirements and to increase throughput. Also, with a few exceptions most of the bioassay methods had been published in a rather condensed format within scientific articles rather than as elaborated or interlaboratory tested ISO methods or OECD test guidelines. It was therefore important to gather information in an extended and harmonised format elaborating on standard quality control practices. This will support future direct comparison between assays, greater transparency and allow areas of improvement to be identified as well as estimate experimental efforts.

Despite these clear objectives, it was challenging to identify a common structure for these SOPs which could accommodate the wide variety of bioassays available and the different status of development with regard to standardisation. The bioassays considered here (see Appendix 2 for documentation of the short names for the assays and the SOPs) range in complexity from *in vitro* nuclear- and cell-reporter assays providing highly mechanistic data on specific receptor-activation (e.g. ERE luciferase reporter gene in bioassay ER_MELN) or adaptive stress responses (e.g. antioxidant response element driven NRF-2 reporter in bioassay AREc32) to more classical organism-based bioassays capable of detecting apical responses (e.g. morphological observations on fish embryo development in bioassay FET). Readout methodology for the assays ranges from analysis of alterations in gene transcription by qPCR or the use of luciferase or fluorescent proteins to monitor transgene activation *in vivo*, to less mechanistic but more widely encompassing measures such as mortality, abnormalities in tail formation or abnormal behaviour.

Finally, in order to facilitate the inclusion of these SOPs into the upcoming NORMAN - ECOTOX Database, relevant fields required by this database were included in the SOP template to allow efficient retrieval of this information. This work resulted in the current SOP format, which, in order to accommodate the various bioassays, contains a number of fields which are relevant for any given assay. The retrieved SOP comprises of the bioassays main features, a brief description of the assay, a document history, regulatory aspects, experimental conditions, test media description, the test organism and provisions during the test conductance, statistical parameters and quality control measures, an experimental outline, a data analysis procedure, technical process control and references. The full documentation of collected SOPs can be found in Annex 2 of this document.

From the principles of effect detection for the various bioassays considered here we subsequently undertook to reflect which of the compounds identified in chapter 4.2 as water contaminants demonstrated to occur in freshwater could possibly be detected using an effect-based tool.

Adverse Outcome Pathways (AOPs) have been proposed as a means to organize the assembly, evaluation and portrayal of the causal relationships between the interaction of a stressor with its biological target (the molecular initiating event) and the adverse effect (adverse outcome) of regulatory concern (e.g. changes to growth, development or reproduction). Anchoring of effect data from the existing SOLUTIONS bioassay panel, ranging from *in vitro* cell based assays to *in vivo* toxicological assays with aquatic organisms, into an AOP framework may thus assist in bridging the gap between the different laboratory based and field based approaches. SOLUTIONS internal deliverable ID T4.1 aimed, in part, to propose approaches for using SOLUTIONS bioassays within an AOP framework by anchoring bioassay effect data to the different parts of relevant AOPs, and thereby to identify a number of AOPs that may be of relevance for the SOLUTIONS bioassay panel. Attempts were made to anchor the entire list of 52 available bioassays to key components of existing AOPs. This resulted in the identification of 4 AOPs which were highly relevant for SOLUTIONS bioassays, 18 AOPs which were partially relevant and 3 AOPs which were marginally relevant. Three example AOPs (photosystem disruption leading to growth inhibition in algae; acetylcholine esterase inhibition leading to acute mortality in crustaceans; and estrogen receptor 1 activation leading to reproductive dysfunction in fish) were populated with data from available effect databases (ECOTOX and TOXCAST) to study whether specific allocations between compounds and assays could be derived. Although the proposed strategy is appealing, a lack of ecologically relevant and mature AOPs, and a general lack of sufficient effect data were considered to be major limitations to practical use in SOLUTIONS. Ongoing efforts outside of

SOUTIONS to develop and evaluate novel and existing AOPs are expected to facilitate this line of thinking in the future.

Figure 2 illustrates the attempt to roughly categorise the bioassay panel within the AOP framework and it highlights the broad coverage of the different levels of the framework by the bioassays used within SOLUTIONS.

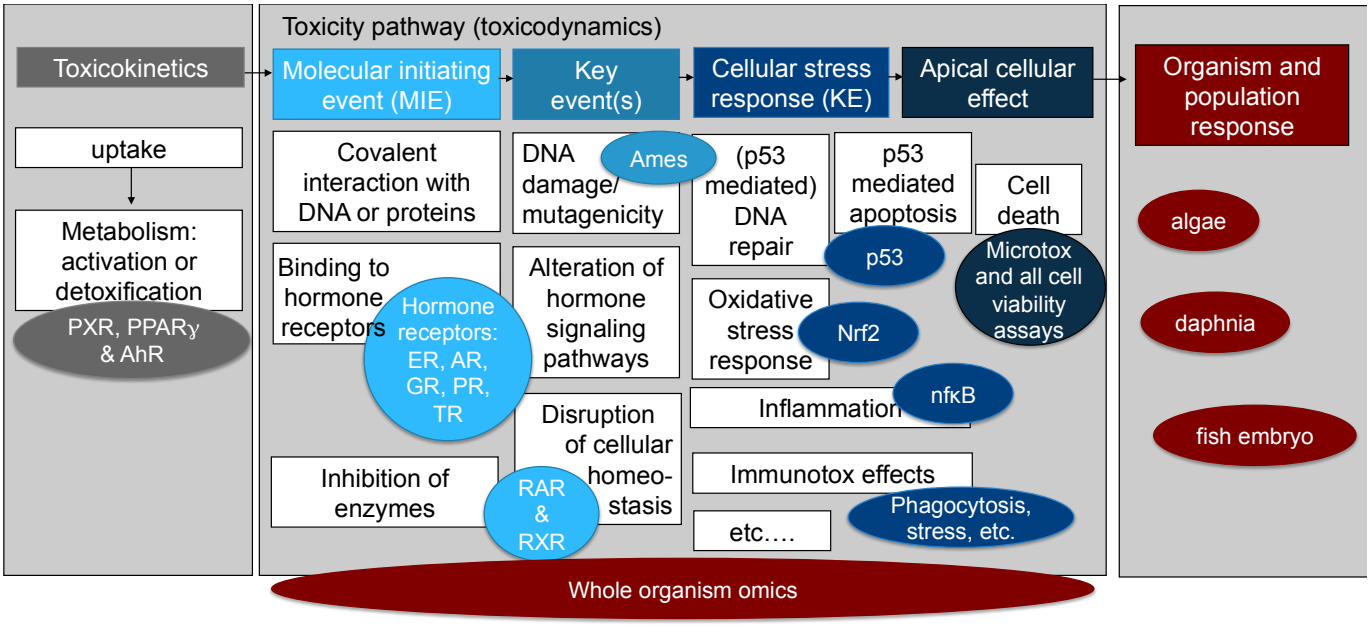


Figure 2: Relationship between the AOP conceptual framework and the bioassays used within SOLUTIONS.

So, the question which bioassay can we expect to detect which water contaminant is still unresolved. In section 4.2 we analysed the MoAs expected for detected water contaminants. In this section we compiled available bioassays and their effect detection principles. The subsequent step to match both using the AOP concept has not been successful. As an alternative approach to address the above question we compared the MoA categories as retrieved in chapter 4.2 with the effects laid out for the various bioassays in Annex II.

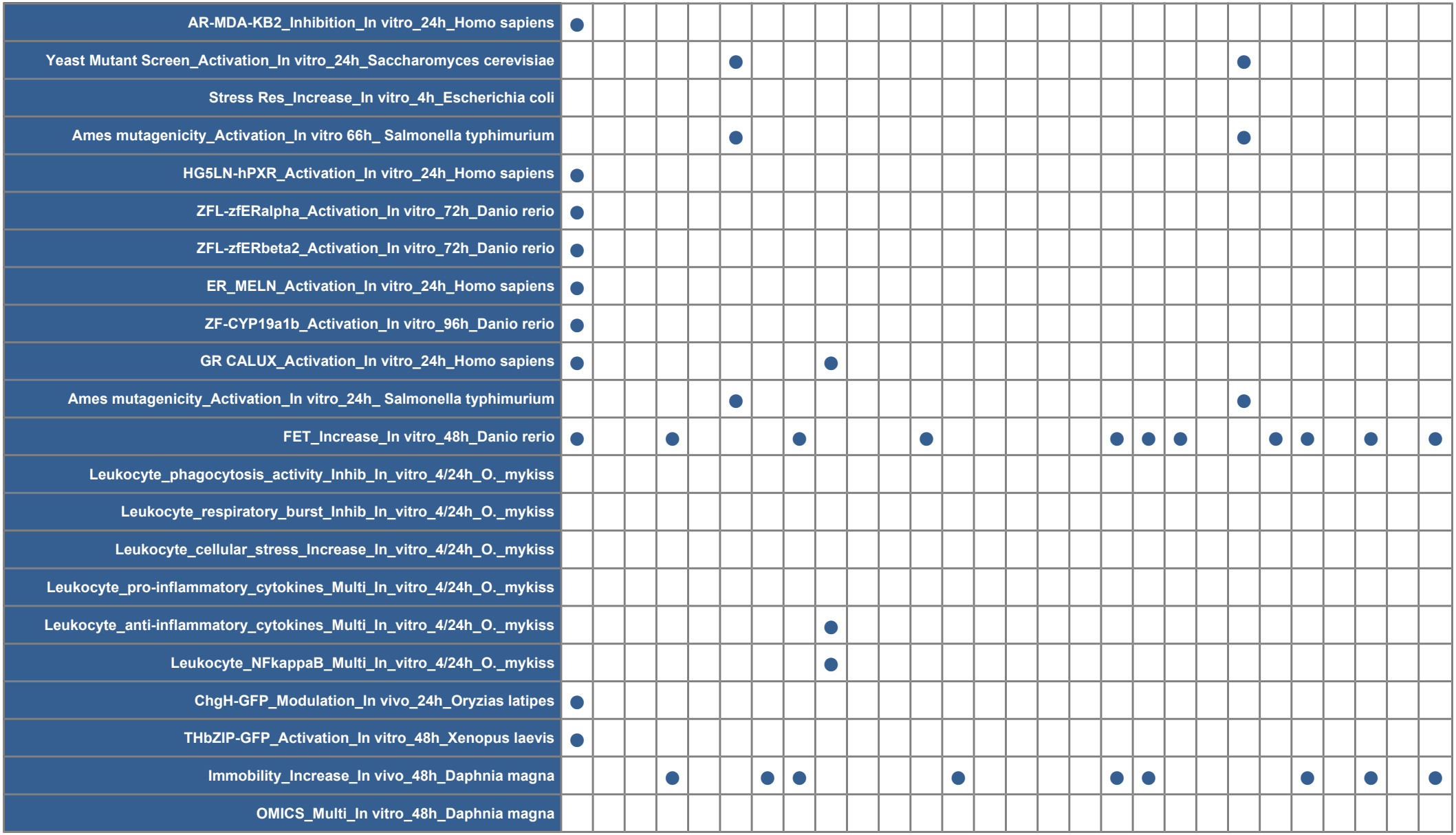
Table 2 depicts the coverage of the 28 MoA categories identified for the 426 organic contaminants found in freshwater monitoring studies (see 4.2) by the bioassays regarded here. A match was assumed if, based on expert knowledge, we expect that a MoA category should be relevant enough to elucidate measurable effects in a given bioassay. The expected MoA coverage for some assays is fairly arbitrary since many of the MoAs covered are theoretical and highly aggregated in terms of

mechanisms that they could comprise. This aggregation of mechanisms e.g. an endocrine MoA category would cover receptor and non-receptor based estrogen, androgen, glucocorticoid, thyroid agonistic as well as antagonistic effects. Thus, this MoA is covered by several assays of varying specificity, when really only a subset of mechanisms within the MoA category would be picked up in most assays. Furthermore, MoA-thinking is specific for taxonomic groups and it remains to be validated for specific assays whether they actually capture a specific MoA. And finally a qualitative match between a MoA and a bioassay does not indicate the exposure conditions (compound availability, dose, and time) required to produce measurable effects, which depend on kinetic and dynamic parameters.

Despite these principal limitations the displayed matches in table 2 clearly help to highlight several points. Certain MoAs, such as endocrine disruption, respiratory inhibition, or anti-inflammatory mechanisms, are covered by several assays, while there is clearly no available bioassay to cover for a number of MoAs such as adenosine receptor interactions, sterol biosynthesis inhibition, or synthetic auxins effects. Also, it is evident that some assays cover several MoAs, while others are matching with one specific MoA only. While the former are apical endpoint assays on whole organisms such as immobility of a daphnid after 48 h of exposure, the latter are typically receptor-transfected cells assaying a fluorescent of luminescent reporter product. Stress response assays in this approach are difficult to match with specific modes of actions but may be regarded as integrators of several key events of an AOP.

Table 2: Matching the mode of action categories for the 423 organic chemicals detected in water analytics (according to Busch et al. 2016) with the 36 bioassays described in Annex II

	Endocrine	DPP-4 inhibition	Cell wall biosynthesis	Cell membrane disruption	Carotenoid biosynthesis inhibition	Carcinogenic	Beta blocker	ATP inhibition	Anti-inflammatory	Antihistamine	Antibiotic	Angiotensin receptor/ enzyme	Analgesic	Andenosine receptor	Viral enzyme inhibition	Synthetic auxin	Sterol biosynthesis inhibition	Signal transduction	Respiratory inhibition	Protein biosynthesis inhibition	Photosynthesis inhibition	Nucleic acid damage	Nucleic acid biosynthesis	Neuroactive	Mitosis, cell cycle	Lipid metabolism	Iron chelator	Ion channel modulation
PSII_Inhibition_In vitro_48h_Chlamydomonas reinhardtii																					●							
Growth_Inhibition_In vitro_72h_Chlamydomonas reinhardtii			●	●	●																●				●			
AR-UAS-bla Griptite_Activation_In vitro_24h_Homo sapiens	●																											
ERα-UAS-bla Griptite_Activation_In vitro_24h_Homo sapiens	●																											
GR-UAS-bla HEK-293T_Activation_In vitro_24h_Homo sapiens	●								●																			
PR-UAS-bla HEK-293T_Activation_In vitro_24h_Homo sapiens	●																											
PPARγ-UAS-bla HEK-293T_Activation_In vitro_24h_Homo sapiens	●																											
AREc32																												
P53RE-bla HCT-116_Activation_In vitro_48h_Homo sapiens						●																●						
NFκB-bla THP-1_Activation_In vitro_24h_Homo sapiens									●	●																		
Microtox_Increase_In vitro_30min_Vibrio fischeri				●				●			●								●									
Extended_FET_Multi_In vitro_Danio rerio	●			●				●				●						●	●	●			●	●		●		●
AhR_CAFLUX_Activation_In vitro_24h_Rattus rattus	●					●																						
AR-MDA-KB2_Activation_In vitro_24h_Homo sapiens	●																											


AR-MDA-KB2_Inhibition_In vitro_24h_Homo sapiens
Yeast Mutant Screen_Activation_In vitro_24h_Saccharomyces cerevisiae
Stress Res_Increase_In vitro_4h_Escherichia coli
Ames mutagenicity_Activation_In vitro 66h_ Salmonella typhimurium
HG5LN-hPXR_Activation_In vitro_24h_Homo sapiens
ZFL-zfERalpha_Activation_In vitro_72h_Danio rerio
ZFL-zfERbeta2_Activation_In vitro_72h_Danio rerio
ER_MELN_Activation_In vitro_24h_Homo sapiens
ZF-CYP19a1b_Activation_In vitro_96h_Danio rerio
GR CALUX_Activation_In vitro_24h_Homo sapiens
Ames mutagenicity_Activation_In vitro_24h_ Salmonella typhimurium
FET_Increase_In vitro_48h_Danio rerio
Leukocyte_phagocytosis_activity_Inhib_In_vitro_4/24h_O._mykiss
Leukocyte_respiratory_burst_Inhib_In_vitro_4/24h_O._mykiss
Leukocyte_cellular_stress_Increase_In_vitro_4/24h_O._mykiss
Leukocyte_pro-inflammatory_cytokines_Multi_In_vitro_4/24h_O._mykiss
Leukocyte_anti-inflammatory_cytokines_Multi_In_vitro_4/24h_O._mykiss
Leukocyte_NFkappaB_Multi_In_vitro_4/24h_O._mykiss
ChgH-GFP_Modulation_In vivo_24h_Oryzias latipes
THbZIP-GFP_Activation_In vitro_48h_Xenopus laevis
Immobility_Increase_In vivo_48h_Daphnia magna
OMICS_Multi_In vitro_48h_Daphnia magna

4.4 Case study demonstration of bioassays as valuable tools in environmental monitoring

While the previous chapters described MoAs of potential relevance and improvements for providing standardised and reliable bioassay tools the following chapter will demonstrate the added value of these assays as monitoring tools when complementing traditional chemical analysis. It shall be stressed here that bioassays in the context of this deliverable are regarded as bioanalytical detectors for compound groups exhibiting a selected measurable effect. Thus, although addressing effect-based sum parameters rather than individual compounds, bioassays are regarded here in comparison with chemical analytical tools rather than with ecological tools used when assessing the impact of stressors on ecosystems. Thus, similar to chemical analysis bioanalytical detection of toxicants present in a water body relies on extraction and pre-concentration steps and attempts to quantify the degree of contamination rather than to conclude on possible impacts on ecosystems or human health.

In order to consider bioassays as tools for effect-based monitoring, we conducted several field case studies in the SOLUTIONS project. We therein addressed a number of distinct questions, of which the following two will be discussed in the following chapters:

1. Are the bioassays (including the pre-concentration steps) sensitive enough to detect and quantify contamination in typical surface waters in Europe and were they able to discriminate between more and less contaminated sites? Do the bioassays allow the detection of traces of toxicants with specific effects in complex mixtures with bulk compounds present in much higher concentrations?
2. Are the bioassays able to detect joint effects of chemicals in water samples and are bioassay responses consistent with chemical analytical results?

To answer these questions the field demonstration studies will be evaluated. The studies themselves will not be reported in detail here but are listed and referenced in the appendix as peer-reviewed publications. The studies considered include

- A study (case study 1) on the application of seven different bioassays on surface water samples from the River Danube, sampled during the Joint Danube Survey 3 (http://www.danubesurvey.org/) attempting to explain measured toxicity with analysed chemicals (Neale et al 2015).
- A field study (case study 2) applying fifteen *in vitro* bioassays for xenobiotic metabolism, nuclear receptor-mediated specific MoA and the induction of adaptive stress responses

together with extensive chemical analysis on three sites in the River Danube upstream and downstream of the discharge of untreated wastewater from the city of Novi Sad, Serbia (König et al. in press).

- An evaluation of the contribution of wastewater effluents on the micropollutant burden in small streams in the Rhine catchment (case study 3) integrating bioanalysis using eight different bioassays and chemical analysis (Neale et al. 2016).

Question 1 - Are the bioassays (including the pre-concentration steps) sensitive enough to detect and quantify contamination in typical surface waters in Europe and were they able to discriminate between more and less contaminated sites? Do the bioassays allow the detection of traces of toxicants with specific effects in complex mixtures with bulk compounds present in much higher concentrations?

Case Study 1. In total 22 sites along the River Danube have been sampled using large volume solid phase extraction and subjected to bioanalysis using seven different toxicological endpoints including the endpoints listed in Table 3.

Table 3: Overview of bioassays used in the current study

Endpoint	Assay	Method reference	Positive reference compound	EC or LC value
Activation of AhR	CAFLUX	Nagy, et al.	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	EC_{10}
Activation of PXR	HG5LN-hPXR	Lemaire, et al. ; Creusot, et al.	SR 12813*	EC_{10}
Activation of ER	MELN	Balaguer, et al., Kinani, et al.	17β-Estradiol	EC_{10}
Oxidative stress response	ARE-*bla*	Invitrogen	tert-Butylhydroquinone (tBHQ)	$EC_{IR1.5}$
p53 response	p53RE-*bla*	Neale, et al.	Mitomycin	$EC_{IR1.5}$
NF-κB response	NF-κB-*bla*	Jin, et al.	Tumor necrosis factor alpha (TNFα)	$EC_{IR1.5}$
Mortality	Fish embryo toxicity (FET)	OECD	3,4-Dichloroaniline	LC_{50}

*Tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate

In parallel, all extracts were analysed chemically for 264 organic chemicals among which 94 were detected at least at one site. Molar concentrations of all detected chemicals per site were added resulting in a factor of about 6 between the most and the least contaminated sites (Neale et al. 2015).

Despite the strong dilution of pollution in the River Danube and the comparable low concentrations of target compounds (Neale et al. 2015), the applied bioassays were successful in detection and quantification of the effects of enriched water samples on all seven toxicological endpoints.

In order to assess the power of the bioassays to discriminate between more and less toxic samples all bioassay responses (expressed as bioassay equivalent concentrations, BEQs) were normalised to the response to the least toxic sample in the same bioassay (Figure 3). For all endpoints mean values and standard deviations over all sites were calculated for every endpoint (Neale et al. 2015). Standard deviations normalised by the mean values are depicted in Figure 4.

While the variability of AhR, FET, oxidative stress (ARE), p53 and NF-kB responses across samples from various sites is within a factor of 6 and thus very much in line with overall contamination described by chemical analytical results, the variability of ER and PXR mediated responses was much higher with a factor of 270 for ER and 36 for PXR. Coefficient of variations (in figure 4 reported as relative standard deviations) for AhR, FET, ARE p53 and NF-kB assays were in the range of 50% of the mean values, while PXR (120%) and ER (180%) assays gave a stronger discrimination of the sites along the Danube. Thus, it may be summarised that PXR and ER are particularly powerful discriminators between sites along the River Danube. For PXR the compounds causing these effects are widely unknown. However, ER mediated effects have been shown to strongly depend on trace amounts of steroids in the water, which were below the analytical detection limits in this study. It may be hypothesized that the ER assay was able to identify peak concentrations of these compounds along the river, while chemical analysis failed to detect them. Interestingly, most endpoints including ER and PXR detect lowest responses at site 64 that was also indicated as least contaminated based on chemical analysis. At the same time maximum chemical contamination was also related to the greatest activation of ER.

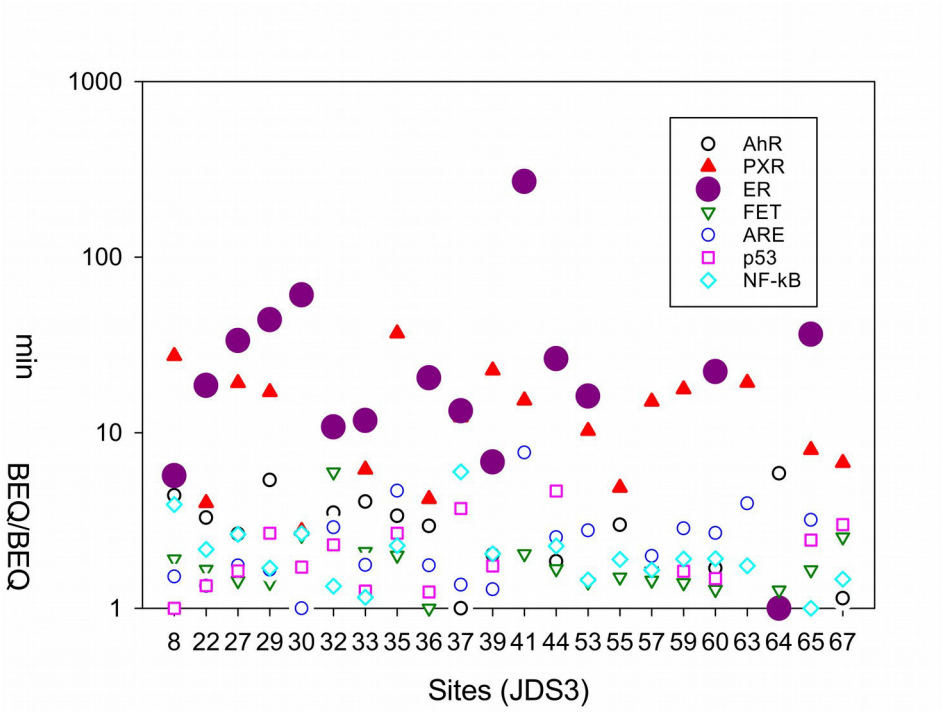


Figure 3: Case study 1 -BEQs for arylhydrocarbon receptor binding (AhR), activation of PXR, activation of ER, fish embryo toxicity (FET), oxidative stress response (ARE), p53 response and NF-kB response provide as a ratio to the minimum BEQ for each endpoint. The original data published by Neale et al. 2015 listed in Appendix I to this deliverable.

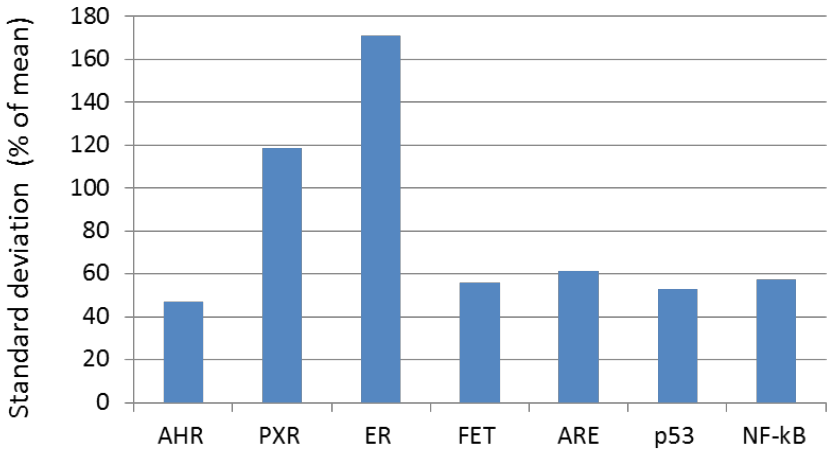


Figure 4: Relative standard deviation of the responses of the different bioassays over all sites normalised to the mean for each endpoint. The original data published by Neale et al. 2015 listed in Appendix I to this deliverable.

Case Study 2. An extended set of bioassays was applied to three sites at the River Danube around the city of Novi Sad (König et al. in press). One site (NS1) was situated upstream of the discharge of untreated wastewater of the city, one (NS2) was located 200 m downstream of this discharge, while another sample was taken about 7 km downstream of the discharge (NS3) all along the same

shoreline. Typical chemical concentrations at NS2 were about a factor of 20 above the concentrations at sites NS1 and NS3 with chemical concentrations in the same order of magnitude.

The applied bioassays together with the BEQs at the three sampling sites are presented in Table 3, indicating that the higher chemical concentrations at NS2 were well reflected by the biological responses. To estimate the discriminative power of the assays, the quotient of BEQs after the discharge of wastewater BEQ_{NS2} and upstream of this contamination source (BEQ_{NS1}) was calculated. Eight of the assays exhibited quotients in the range of 10 to 22 and thus were well in agreement with the chemical analysis. The endpoints AhR, RXR and ARE were less discriminative with quotients below 10 while NF-kB showed an even greater response to the upstream sample. The latter might indicate masking effects of the pollution from Novi Sad rather than an induction of the expected effect.

The discrimination of the directly impacted site NS2 from the two less impacted sites is visualized in figure 5. This figure also illustrates the great similarity between NS1 and NS3 that is in good agreement with chemical data and highlights the robustness of the bioassay results.

Table 4: Summary of bioassay results of the water extracts as BEQ_{bio} expressed in $ng_{reference\ compound}/L$ (from König et al., in press).

		BEQ_{bio} ($ng_{reference\ compound}/L$)			
Bioassay	Reference compound	NS1	NS2	NS3	BEQ_{NS2}/BEQ_{NS1}
Xenobiotic Metabolism					
AhR CAFLUX H4.G1.1c2	2,3,7,8-Tetrachloro-dibenzodioxin (TCDD)	$(1.2 \pm 0.1)*10^{-2}$	cytotoxic	$(2.2 \pm 0.2)*10^{-2}$	
AhR CAFLUX H1.G1.1c3	2,3,7,8-Tetrachloro-dibenzodioxin (TCDD)	$(4.9 \pm 0.5)*10^{-2}$	$(1.6 \pm 0.2)*10^{-1}$	$(8.2 \pm 0.9)*10^{-2}$	3.26
PPARγ GeneBLAzer	Rosiglitazone (Rosi)	$(6.1 \pm 0.9)*10^{-1}$	(8.9 ± 1.5)	$(5.9 \pm 1.2)*10^{-1}$	14.59
anti PPARγ GeneBLAzer	2-Chloro-5-nitro-*N*-4-pyridinylbenzamide (T0070907)	$(5.9 \pm 2.5)*10^{2}$	$(1.0 \pm 0.4)*10^{4}$	$(6.0 \pm 2.6)*10^{2}$	16.95
Endocrine disruption (agonistic effect)					
AR GeneBLAzer	Metribolone (R1881)	$(5.2 \pm 1.8)*10^{-1}$	(5.8 ± 2.0)	$(9.0 \pm 2.4)*10^{-1}$	11.15
AR-MDA-KB2	Dihydrotestos-terone	$< 2.1*10^{-2}$	(2.3 ± 0.3)	$(1.0 \pm 0.2)*10^{-1}$	110.00
Erα GeneBLAzer	17ß-Estradiol (E2)	$(5.3 \pm 0.2)*10^{-3}$	$(2.6 \pm 0.1)*10^{-1}$	$(1.2 \pm 0.1)*10^{-2}$	21.60
BG1Luc4E(2)	E2	$(1.4 \pm 0.4)*10^{-2}$	$(6.7 \pm 0.2)*10^{-1}$	$(3.1 \pm 0.9)*10^{-2}$	21.61
E-SCREEN	E2	$(1.9 \pm 0.7)*10^{-1}$	(2.4 ± 0.6)	$(3.4 \pm 1.2)*10^{-2}$	12.63
GR GeneBLAzer	Dexametha-sone	< 1.1	cytotoxic	cytotoxic	
PR GeneBLAzer	Promegestone	cytotoxic	cytotoxic	cytotoxic	
RXR GeneBLAzer	9-cis-Retinoic acid	$< 2.0*10^{-2}$	$(1.5 \pm 1.0)*10^{-1}$	$(3.5 \pm 6.1)*10^{-2}$	7.50
RAR GeneBLAzer	All-trans-Retinoic acid	(7.2 ± 5.8)	cytotoxic	(7.0 ± 5.7)	
Endocrine disruption (antagonistic effect)					
anti AR GeneBLAzer	Cyproterone Acetate	$< 7.1*10^{-1}$	$(1.2 \pm 0.9)*10^{1}$	(1.0 ± 0.8)	17.14
anti AR-MDA-KB2	Flutamide	(2.2 ± 1.9)	agonistic effect	(1.1 ± 0.2)	
anti Erα GeneBLAzer		$< 4.8*10^{2}$	cytotoxic	$< 4.81*10^{2}$	
anti GR GeneBLAzer	Mifepristone	$< 1.8*10^{-4}$	$(2.5 \pm 0.7)*10^{-3}$	$< 1.8*10^{-4}$	13.90
anti PR GeneBLAzer	Mifepristone	cytotoxic	cytotoxic	cytotoxic	
Adaptive Stress Response					
ARE GeneBLAzer	tert-Butyl-hydroquinone	$(1.3 \pm 0.1)*10^{4}$	$(5.6 \pm 0.3)*10^{4}$	$(1.6 \pm 0.1)*10^{4}$	3.50
NFκB GeneBLAzer	Tumor necro-sis factor alpha	(2.9 ± 0.4)	(2.4 ± 0.2)	$(1.3 \pm 0.4)*10^{-1}$	0.83
p53 GeneBLAzer	Mitomycin	$<6.2*10^{1}$	cytotoxic	cytotoxic	

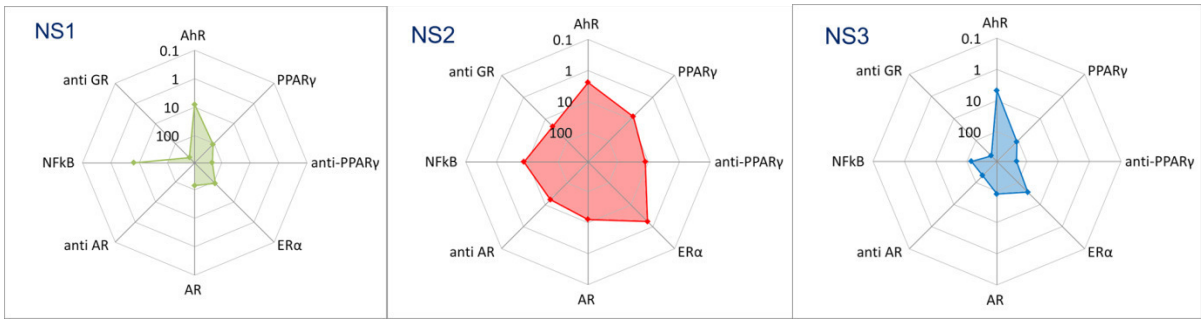


Figure 5: Case study 2 - Biological responses of selected endpoints to River Danube water samples at the three sites NS1, NS2 and NS3. The effect concentrations are expressed in units of REF. Figure reprinted from König, M. et al. Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environ Pollut (in press). DOI:10.1016/j,envpol.2016.11.011. Copyright 2016 with permission from Elsevier.

Case Study 3. The Rhine case study was used to assess whether a similar panel of bioassays is able to discriminate the impact of treated wastewater in small Swiss streams at three different sites at different Rhine tributaries (Birmensdorf, Muri and Reinach) (Neale et al. 2016). The results clearly indicate a good discrimination between upstream sites, effluents and downstream sites for all endpoints with the effluents giving the greatest response followed by the downstream site and the upstream site (Table 5). In agreement with the results in the Danube, the discriminative power of AhR activation is rather low (factor of 2 to 3 between effluent and upstream sample), for ER *in vitro* (MELN) it is much higher with a factor of 7 to 27. Activation of ER in *Danio rerio* and of AR *in vitro* could not be assessed for its discriminative power due to mortality caused by the effluent or non-detectability of the response upstream and downstream. The inhibition of PSII after 2h and 24 h exhibited effects in the effluents which were by a factor of 10 to 80 greater than those exhibited by upstream samples, while the discriminative power of algal growth was lower with a maximum ratio of 20. Again in agreement with the results in the Danube, oxidative stress (ARE) exhibited only ratios of 3 to 6 between effluent and upstream samples.

Compared to the bioassay responses, the differences in concentrations of target chemicals upstream, downstream and in the effluents (Figure 6) were much more pronounced with many compounds below the detection limit upstream the discharge of the treated effluent. Thus, only parts of the chemical contamination measured in the effluents seems to contribute to measurable effects, while at the same time upstream toxicity was detectable that is not reflected by measured chemical concentrations. Possible seems an increasing relative impact of unknown natural and background compounds on bioassay responses at lower levels of anthropogenic contamination.

Table 5: Summary of bioassay results of the water extracts from three different sites including an upstream, a downstream and the effluent sample for the activation of AhR and ER in vitro, ER in Danio rerio (ERD) and AR (in vitro), the inhibition of PSII after 2h (PSII2h) and 24 h (PSII24h), inhibition of algal growth and oxidative stress (ARE) (Neale et al. 2016)

				BEQ 10^{-13} M					
	Birmensdorf			Muri			Reinach		
	upstr.	effluent	downstr.	upstr.	effluent	downstr.	upstr.	effluent	downstr.
AhR	1	2	1.2	0.9	3.1	2.0	0.8	2.4	2.2
ER	9.5	72.8	18.8	16.5	155	34.9	5.59	153	31.3
ER_D	<98.4	Mortality	<97.4	<97.4	789	<97.7	<76.0	Mortality	<98.2
AR	<14.3	88.9	<14.2	<14.2	107	29.7	<12.5	422	<14.3
$PSII_{2h}$	438	5,530	1,580	261	11,100	2870	335	2,840	983
$PSII_{24h}$	492	7,970	2,130	306	24,000	7970	584	4,720	1,790
Algae	<1800	13,300	4,540	3,190	67,300	15,300	<1,800	12,400	3,460
ARE	406,000	1,430,000	743,000	589,000	2,080,000	1,090,000	423,000	2,730,000	1,180,000

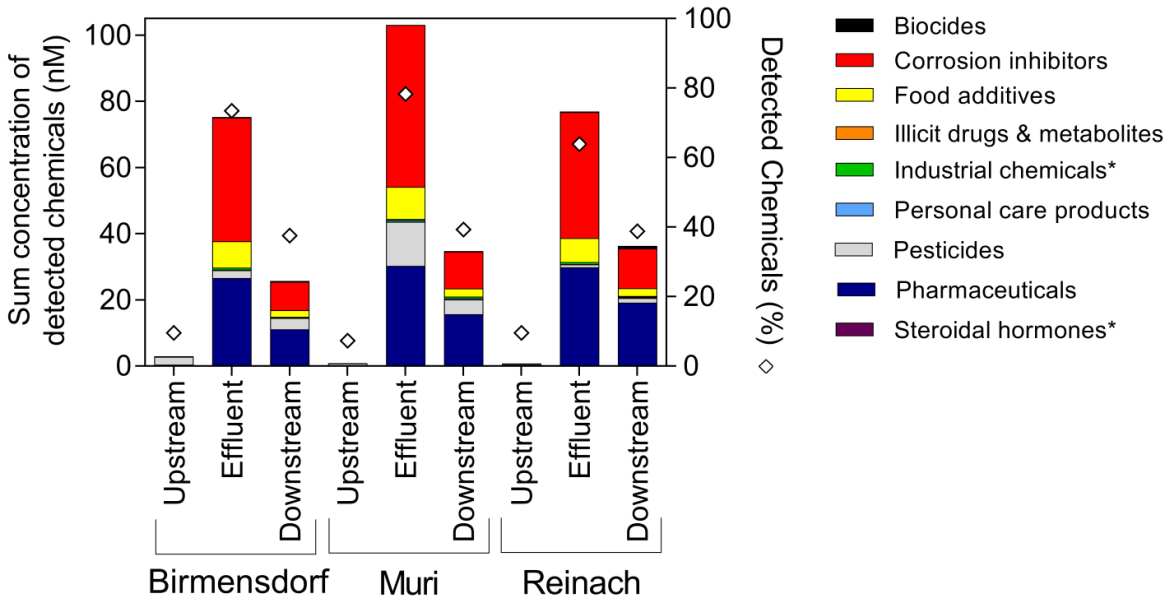


Figure 6: Case study 3 - Chemical concentrations upstream, downstream and in the effluent of three wastewater treatment plants in the Rhine case study.

Figure reprinted from Neale, P.A., et al. (2016). Integrating Chemical Analysis and Bioanalysis to Evaluate the Contribution of Wastewater Effluent on the Micropollutant Burden in Small Streams. Science of the Total Environment: 576: 785-795. Copyright 2016 with permission from Elsevier.

Summary. The case studies showed that almost all bioassays are well suited to detect and quantify the exposure to toxicants, to discriminate contaminated from less contaminated sites and to indicate the impact of treated and untreated wastewater on water quality when applied on enriched samples using (LV)SPE for enrichment. Despite the enormous complexity of the contaminant mixtures present in waste- and surface waters most of the assays addressing specific effects were also well suited to detect these effects in complex mixtures. This holds true even for the sample downstream of the discharge of untreated wastewater in Novi Sad, which can be considered as a kind of worst case scenario, with large amounts of unspecific organic contamination that might have the potential to mask specific effects. Even under these circumstances the *in vitro* assays addressing ER and AR mediated effects (agonistic and antagonistic) were demonstrated to be well suited to detect specific (endocrine disrupting) potencies of wastewater components. Effects on PR and GR could not be detected in wastewater-impacted surface waters due to overlaying toxicity. However, preliminary results show that responses can be demonstrated after fractionation and thus separation of cytotoxic compounds from specific receptor-binding chemicals. The inhibition of PSII and algal growth inhibition could be shown to respond most sensitively to phytotoxic chemicals in treated wastewater, with specific PSII inhibition exhibiting slightly greater discriminative power than unspecific algal growth inhibition. Tests for adaptive stress responses (ARE, NF-kB and p53) as well as for AhR activation exhibited relatively low discriminative power in all case studies. These effects are obviously driven only to a minor extent by anthropogenic pollutants but to a major extent by natural factors.

Question 2 - Are the bioassays able to detect joint effects of chemicals in water samples and are bioassay responses consistent with chemical analytical results?

In all three case studies bioassays have been used jointly with extensive chemical analysis. To compare findings from chemical analysis and bioassays we used a component-based mixture effect approach to estimate biologically equivalent concentrations (BEQ) and compare these with bioassay-detected effect estimates. For the detected chemicals, effect concentrations were retrieved from the literature and the ToxCast database. The subsequently employed mixture effect prediction/observation comparison can be considered an effect concentration or mass balance approach. It compares chemically derived BEQs (based on the chemical analysis) with the biologically derived BEQs (based on the biotesting of dilution series of enriched water samples). It was used in order to quantify the fraction of the activity of the samples which can be explained by

the compound concentrations detected.

Case Study 1. In samples from JDS3 that were tested for seven different effect endpoints 264 target chemicals were analysed with detection of 94 of them at least once in the 22 JDS3 samples (Neale et al. 2015). For all bioassays except NF-kB mass balance calculations were performed (Figure 7). For NF-kB no effect data for individual chemicals are available that would allow for estimation of contribution of individual chemicals.

In this case study, only a few percent of the observed AhR activation could be explained by terbutylazine, while in 7 out of 22 sites the phytohormone daidzein was found to be responsible for 10 to 70% of the effect. ER activation could be explained in several cases by the phytohormone genistein and the steroid estrone. Other steroid hormones such as estradiol, estriol and ethinyl estradiol were below the detection limit at all sites. It may be hypothesised that a more sensitive chemical analysis of these steroid hormones could have significantly enhanced the fraction of explained effect. The response in p53, activation of PXR, ARE and FET could only be explained by target chemicals to less than 0.25%. This may result from three different hypotheses: 1) the assays are detecting effects of individual unknown chemicals that are not targeted by the chemical analysis, or 2) the detected effect is caused by the complex mixture with many chemicals contributing, 3) there are interactive combination effects. First results of ongoing EDA studies indicate that at least for oxidative stress and FET the second hypothesis is more probable, while the experience with p53 and PXR is too limited to draw conclusions.

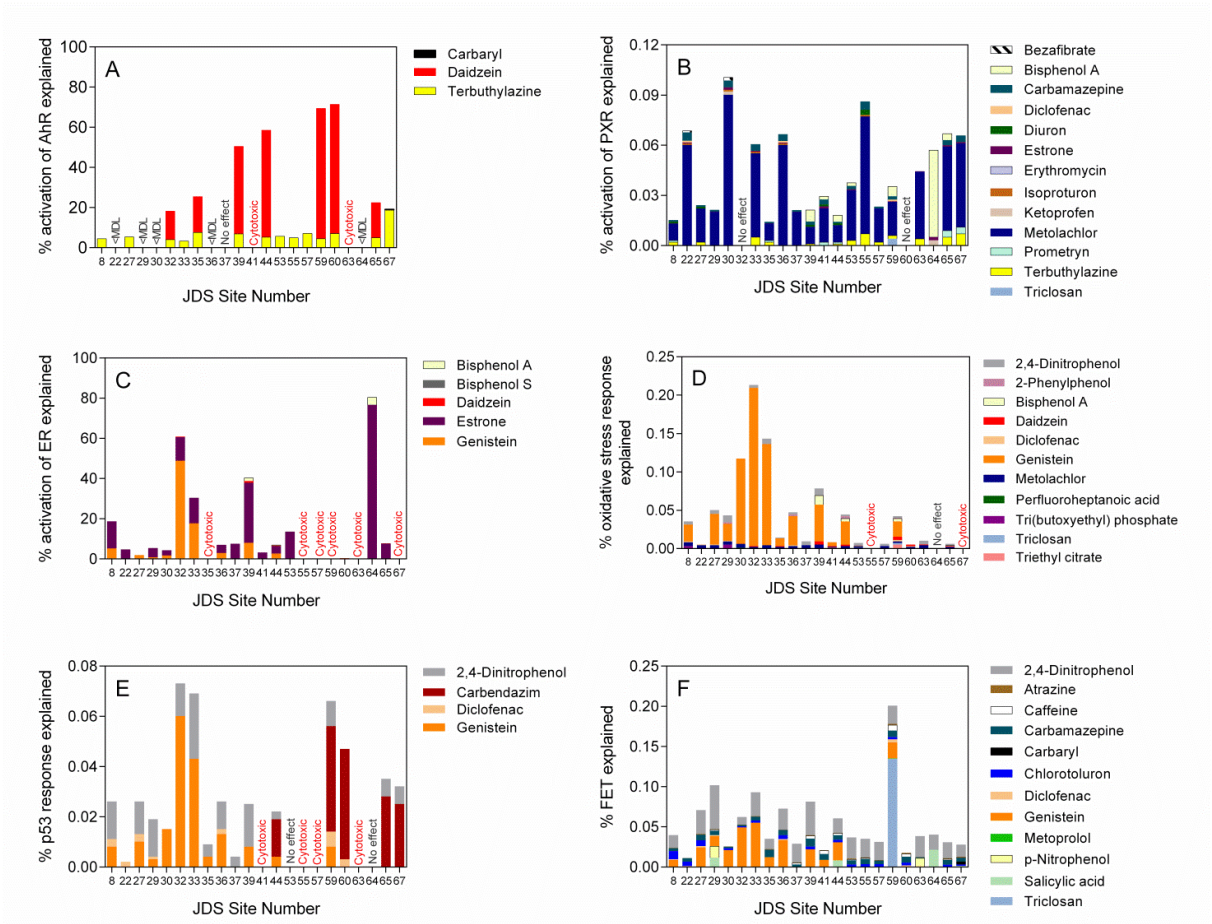


Figure 7: Case study 1 - Percent of the biological effect explained by individual detected chemicals for A) activation of AhR, B) activation of PXR, C) activation of ER, D) oxidative stress response, E) p53 response and F) fish embryo toxicity (FET)

Figure reprinted from Neale, P.A., et al. (2015). Linking in vitro effects and detected organic micropollutants in surface water using mixture-toxicity modeling. Environmental Science & Technology, 49: 14614-14624. Copyright 2016 with permission from ACS.

Case Study 2. Danube water samples upstream and downstream of Novi Sad have been analysed for 276 organic micropollutants detecting 125 compounds in at least one sample. Particular efforts were being made to detect endocrine disrupting steroids at low concentrations. While maximum concentrations upstream of Novi Sad of individual compounds were in the range between 100 and 200 ng/L, downstream of the wastewater effluent discharge (NS2) five chemicals were detected in concentrations from 1 to 4 µg/L including the stimulant caffeine, the anti-diabetes drug metformin, the surfactants lauryl diethanolamide and N,N-dimethyldodecylamine-N-oxide and the primary bile acid cholic acid. For the mass balances (Figure 8) assessing the percentage of detected bioassay responses that can be explained with measured chemicals, compounds with high concentrations are

of minor importance only except for caffeine which explained about 10 % of the observed oxidative stress (ARE) at NS2.

In total, 20 to 200 % of the responses in bioassays on ER, AR and GR activation or inhibition could be explained with targeted chemicals at the most contaminated site NS2. The natural estrogens estrone, estriol and 17-beta estradiol were considered to be the major ER agonists, consistently confirmed in both bioassays (activation of ERα-GeneBlazer and of BG1Luc4E(2)).

The inhibition of the anti-AR GeneBlazer response was explained mainly by the phytohormones genistein and daidzein which are present in soy products and are probably related to food production and consumption. The xenobiotics bisphenol A and 2,4-dinitrophenol were minor but also significant contributors to the observed anti-androgenicity in Danube waters. Inhibition of anti-GR GeneBlazer response was predominated by progesterone with several other hormones and the xenobiotic 1,2-benziso-thiazolinone contributing.

While this approach clearly indicated relevant pollutants contributing to the measured effects the quantitative uncertainty was relatively high. Individual effect potencies of the target chemicals were derived from ToxCast data which resulted in substantial uncertainty. Thus, the explanation of in total 20 to 200 % of the effects should be regarded in relation to this uncertainty and do neither proves nor excludes that there are contributions from other non-measured compounds.

The very high concentrations of caffeine downstream of the wastewater effluent discharge could explain about 10 % of the oxidative stress response (activation of ARE GeneBlazer) exhibited by the Danube water sample. This indicates that also responses in relatively unspecific biotests can explain large contributions of individual pollutants if their concentrations are high enough.

At the less contaminated sites upstream and further downstream of the effluent discharge much smaller fractions of the responses could be explained. This is probably caused by the fact that major contributors are present at concentrations below the detection limit of the chemical analytics.

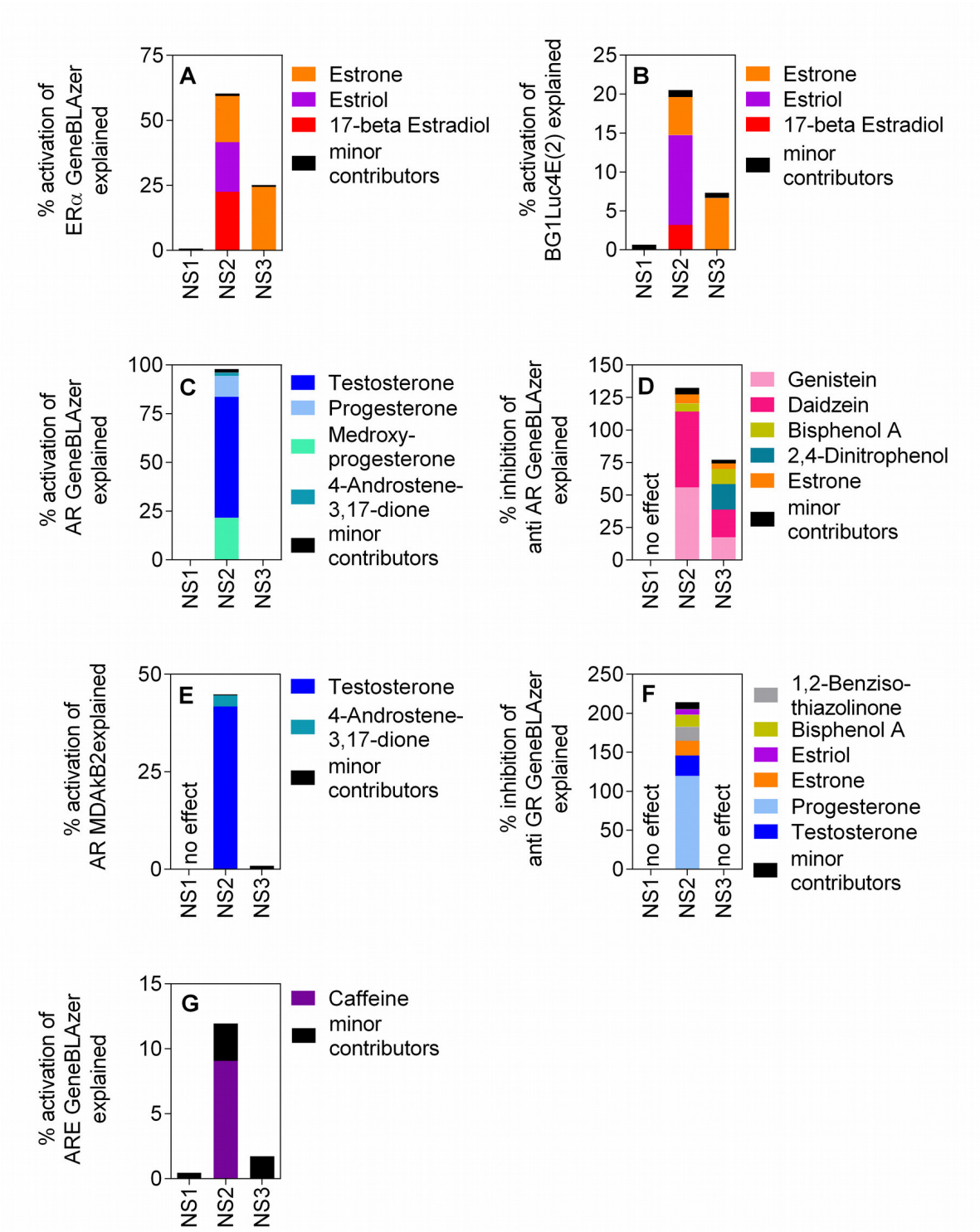


Figure 8: Case study 2 - Percentage effect explained by individual detected chemicals for A) activation of ERα (GeneBLAzer), B) activation of ERα (BG1Luc4E2)), C) activation of AR (GeneBLAzer), D) inhibition of AR (GeneBLAzer), E) activation of AR (MDAkB2), F) inhibition of GR (GeneBLAzer) and G) oxidative stress response.

Figure reprinted from König, M. et al. Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environ Pollut (in press). DOI:10.1016/j,envpol.2016.11.011. Copyright 2016 with permission from Elsevier.

Case Study 3. In water samples upstream and downstream of treated wastewater effluent discharges as well as in the effluents themselves 400 different chemicals were analysed and about half of them detected in at least one of the samples. A specific focus of chemical analysis was set on the detection and quantification of pesticides (including herbicides) together with bioassays for PSII inhibition in green algae and algal growth inhibition. The total fractions of effects explained by diuron, terbutylazine, terbutryn, isoproturon, metribuzin and others where in the range of 20 to 100 % with typically lower fractions for the less specific endpoint growth inhibition compared to PSII inhibition (Figure 9).

The fraction of ER and AR activation explained by target chemicals was below 0.4 % and thus very low. This is probably related to the insufficient detection limits of chemical analysis of natural hormones and other steroids. These may act as contributors to endocrine disruption at very low concentrations (see case study 2). Estrone was the only steroid that could be detected contributing the majority of BEQs to the in total very low fraction of explained effect.

Interestingly, fractions of up to 30% of AhR activation could be explained by the individual pesticides propiconazole and terbutylazine. Only a minor fraction of oxidative stress (< 2% of ARE GeneBlazer activation) could be explained by the analysed chemicals with many different and site-specific chemicals contributing to the measured effect.

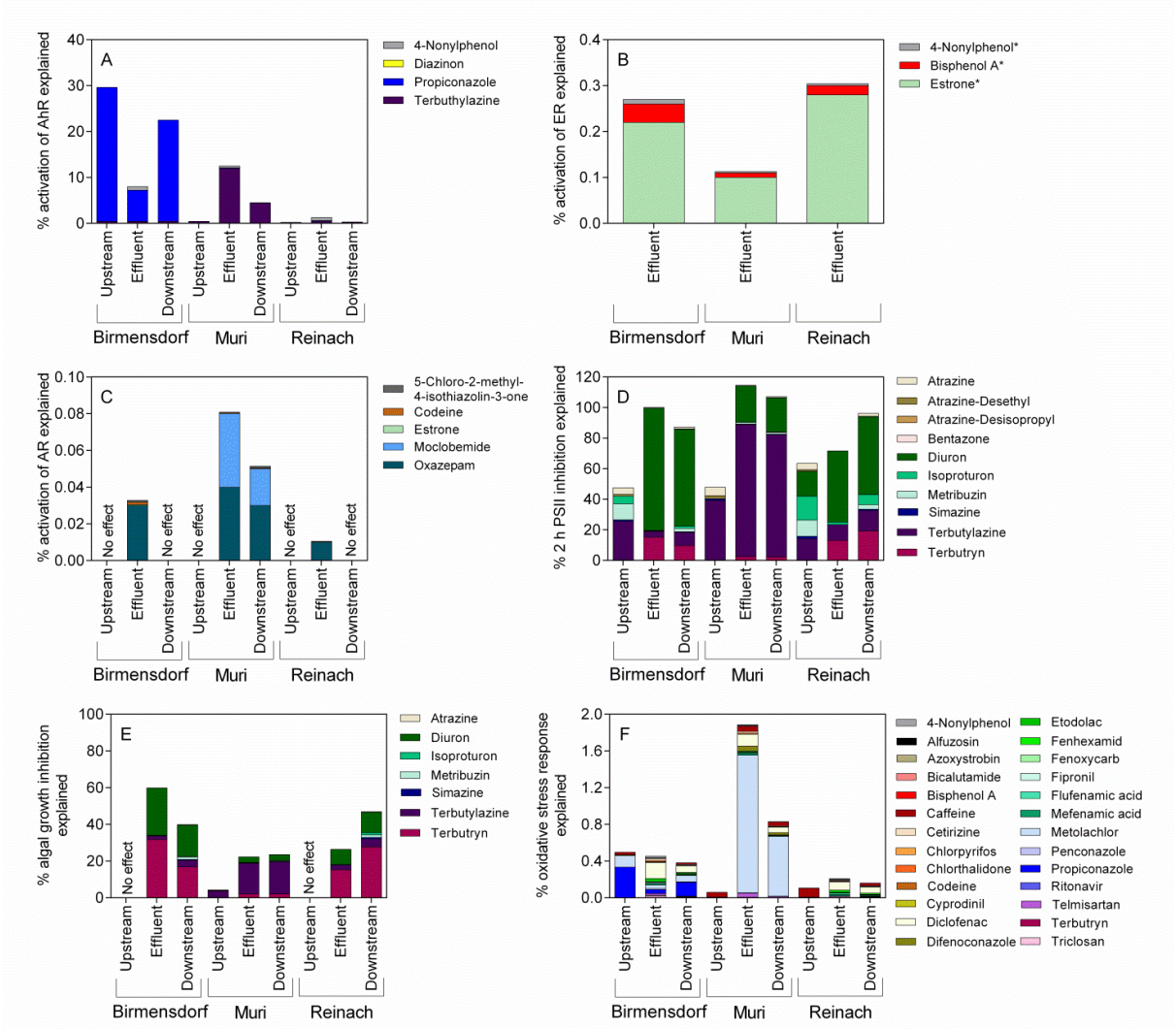


Figure 9: Case study 3 - Percentage effect explained by individual detected chemicals for A) activation of AhR, B) activation of ER (MELN), C) activation of AR, D) 2 h PSII inhibition, E) algal growth inhibition and F) oxidative stress response. *Estrogenic compounds were only measured in the effluent samples.

Figure reprinted from Neale, P.A., et al. (2016). Integrating Chemical Analysis and Bioanalysis to Evaluate the Contribution of Wastewater Effluent on the Micropollutant Burden in Small Streams. Science of the Total Environment: 576: 785-795. Copyright 2016 with permission from Elsevier.

Summary. The case studies yielded conclusive results for the application of bioassays for the detection of four groups of toxicological endpoints:

(1) Endocrine disruption. ER, AR and GR activation and inhibition responses were typically dominated by natural and synthetic steroids with some contribution of phytohormones such as genistein and daidzein and xenobiotics such as bisphenol A. Thus, a high degree of effects can be explained if highly specific chemical analysis for steroids (with very low detection limits) is applied

rather than screening methods. Such sophisticated target analysis is available only in few specialised laboratories and is rather expensive. The bioassays addressing ER, AR and GR activation and inhibition are sensitive enough to detect these chemicals as a group and provide a cost-efficient and feasible monitoring alternative to chemical analysis and may be seen as a good measure for the contamination with these compounds. Linking effects to individual compounds, different test systems addressing the same type of responses (ER or AR activation) may lead to a different ranking of chemicals due to their compound-specific sensitivity. However, since the sources and abatement options for these chemicals are similar, the practical relevance of this drawback in water management seems to be limited.

(2) Bioassays for PSII inhibition and algal growth reflected very well the contamination with PSII inhibiting herbicides and are well suited for effect-based detection and quantification of this group of chemicals independent from the exact composition of the herbicide mixture. Assuming that the environmental risk depends on the overall load of herbicidal chemicals rather than on the exact composition of the applied cocktail and thus abatement requires a reduction of this overall load rather than the replacement of one herbicide by another with the same effect, the applied bioassays provide an excellent measure for the contamination with the whole use group of chemicals.

(3) Non-specific effects such as oxidative stress could be explained by target analysis only to a minor degree. However, they may indicate the overall contamination and are thus a good complementary tool to specific assays reflecting the contamination beyond what is accessible by current chemical analysis.

(4) Uncertainties in explaining detected effects based on chemical quantifications and component based mixture effect prediction relate to chemical concentration estimates for components that are bioactive at concentrations below detection limits, knowledge on the components bioactivities in the specific assays and validity of the mixture effect predictions. The quantification of low concentrations specifically of steroids may be improved by employing markers of waste water contaminant dilutions that would offer estimations of how far below detection limits steroid concentration due occur. Such proxies would allow a refined handling of left censored values, i.e. samples with concentrations below the method detection limit. Uncertainties from extrapolation of individual components bioactivities from ToxCast data are currently addressed in an effort to benchmark a subset of identified major water contaminants (see 4.2) representing different modes of action for the bioassays provided here. The reliability of component-based mixture effect

predictions relying on the assumption of non-interactive combined effect for situations where few bioactive compounds are present in a complex mixture of contaminants will be investigated in a designated round robin mixture study across different bioassays.

5. Conclusions

5.1. Towards improved bioassay solutions for environmental monitoring based on adverse outcome pathways

From the SOLUTIONS project efforts in this area we learned that the several hundreds of distinct organic water contaminants that are currently detected in European freshwater systems may be aggregated into less than 30 MoA groups from a bioanalytical effect perspective. This offers a hypothesised scope to capture mixtures of diverse contaminants via effect detection using effect-based tools. There is a substantial battery of bioassays available for potential use in water monitoring both in the literature and in the SOLUTIONS consortium where 36 bioassays standard operating procedures were documented. A straightforward match between a compound MoA and a bioassays detection principle is, however, as yet for selective assays but not generally possible. The comparison between the knowledge on MoA prevalent for water contaminants and the effects detectable with the available bioassays show that few prominent modes of action are covered by several assays e.g., endocrine effects, while we have assays that capture effects for which no clear link to contaminants occurring could be demonstrated and several known MoA were not captured by any of the mechanism specific cell-based assays. Apical assays such as short-term studies with fish eggs, daphnids and algae may help out if we are not dealing with delayed effects or system-specific mechanisms such as carcinogenicity or viral enzyme inhibition. Case studies applications of bioassays for water monitoring showed that if 10-1000 fold pre-concentration of water samples was performed, bioassays were sensitive enough to detect effects of contaminants and discriminate between samples from different sites. The pre-concentration step requires additional quality control measures that need to be further elaborated to render it a consented approach. In conjunction with advanced chemical analytical efforts it was furthermore demonstrated that bioassays captured exposure to mixtures rather than to individual components. From the differences between chemical analytical derived effect expectations and observed effects one might draw conclusions on compounds not captured in chemical analysis or on non-additive mixture effects. Differentiating between the two hypotheses is not straightforward as variance of the assay responses and precision of reported relative effect potencies is severely understudied. The way towards establishing bioassays as effect-based tools useful for routine water monitoring is thus clearly moving forward but several issues remain to be solved.

5.2. Identified knowledge gaps and next steps

To improve our ability of linking specific contamination with specific bioassays for monitoring and thus to improve sensitive and biologically meaningful effect-based monitoring we need a better

understanding of how MoA knowledge for compounds and experimental effect detection can be linked. The AOP concept provides conceptual guidance but proved to be currently of little help for the cases at hand. A next step therefore will be a literature study to reflect in further detail on the matching of MoA information available for contaminants with the knowledge available on bioassays. For the latter it would help to study how far ToxCast high throughput bioscreening information for defined single chemicals can be used for effect predictions for the bioassays suggested for effect-based monitoring. In continuation of the quest to improve the coherence of covered relevant MoA in monitoring we will make efforts to define a strategy for deriving bioassay panels for monitoring and reflect on the value of untargeted assays such as transcriptome studies to amend for non-captured MoA.

For the central issues of mixture toxicity assessment in improved water monitoring efforts, the crucial knowledge gap is to provide evidence for the hypothesis that non-interactive combined effects prevail even for environmental mixtures of multiple components of highly differing bioactivities. The assumption that combined effects derive from additive contributions of the mixture components would allow conclusion about whether or not chemicals under consideration are sufficient to explain relevant effects detected in the bioassays. To evaluate this hypothesis we want to improve the quality of knowledge for a component-based validation of mixture prediction along different lines: (i) We will study the evidence collated in ToxCast and likewise databases or modelling tools by comparing expectable individual compound bioactivities with observable compounds effects in an wide array of bioassays and water contaminants. (ii) We presently carry out a cross-bioassay round robin mixture study. Here, again, we use a selection of water contaminants identified as relevant, derive a multi-component mixture of components showing various MoA and run these in different mixture ratios across an array of different bioassays. By comparing component-based mixture effect predictions with observations we want to learn about the precision of effect prediction and possible confounding factors. (iii) Finally, we intend to address technical quality issues relevant for employing bioanalytics routinely for water monitoring. In particular this regards derivation of quality controls for treating enriched water samples.

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8. Appendices

Appendix I – Relevant publications from the SOLUTIONS consortium

Altenburger, R., Ait-Aissa, S., Antczak, P., Backhaus, T., Barceló, D., Seiler, T.-B., Brion, F., Busch, W., Chipman, K., López de Alda, M., Umbuzeiro, G.d.A., Escher, B.I., Falciani, F., Faust, M., Focks, A., Hilscherova, K., Hollender, J., Hollert, H., Jäger, F., Jahnke, A., Kortenkamp, A., Krauss, M., Lemkine, G.F., Munthe, J., Neumann, S., Schymanski, E.L., Scrimshaw, M., Segner, H., Slobodnik, J., Smedes, F., Kughathas, S., Teodorovic, I., Tindall, A.J., Tollefsen, K.E., Walz, K.-H., Williams, T.D., Van den Brink, P.J., van Gils, J., Vrana, B., Zhang, X. and Brack, W. (2015). Future water quality monitoring--adapting tools to deal with mixtures of pollutants in water resource management. The Science of the Total Environment, 512-513: 540-551.

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Wastewater Effluent on the Micropollutant Burden in Small Streams. Science of the Total Environment: 576: 785-795

Smetanová, S., Riedl, J., Zitzkat, D., Altenburger, R., and Busch, W. (2015): High-throughput concentration–response analysis for omics datasets. Environmental Toxicology and Chemistry, Volume 34, Issue 9, pages 2167–2180

The full publications can be accessed through the SOLUTIONS website under:
http://www.solutions-project.eu/results-products/

Appendix II – Standard operating procedures for WP12 bioassays

Appendix II – Standard operating procedures for WP12 bioassays

Bioassay main features

Bioassay ID	WP12 bioassay_ER_MELN
Bioassay type	*in vitro*
Adverse Outcome Pathway	Estrogen receptor activation leading to reproductive adverse effects
Test species	Homo sapiens
Cell line/ cell strain	MELN (MCF-7 cells stably transfected with ERE-luciferase reporter gene driven by endogenous hERalpha)
Transgene	luciferase reporter gene under control of an estrogen response element

Measured endpoints/ molecular targets	**Measured via**
Luciferase/ ER	Luminescence measured with reader

Brief description of test

Document history

Version	Modification	Author	Date
A	creation	Hélène SERRA	17/05/16

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	16-24h
Main determinant	EC_{50} from log-logistic concentration effect model
Effect equivalent EQ	17β-Estradiol
Unit of effect equivalent	ng/L
Concentration range in standard curve	0.0001- 10nM (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	Balaguer et al. 1999
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:

n/a

Assay format	96-well plate
Volume per well/ vessel	Final volume 150µl (100µl cell seeding, 50µl dosing of compounds)

Test media

Solvent	DMSO
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
Phenol red free DMEM – Gibco 11880-028 2% dextran charcoal coated and decomplemented FBS – Fisher Invitrogen F7524 1% nonessential amino acids - Gibco 11140-050 1% Penicillin/streptomycin (50 U/mL each) – Gibco 15070-063 1% L-Glutamine - Gibco 25030-032

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	80 000 cells per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	DMEM – Gibco 21885-025 5% foetal bovine serum (FBS) - Fisher Invitrogen F7524 1% nonessential amino acids - Gibco 11140-050 Penicillin/streptomycin (50 U/mL each) – Gibco 15070-063 1mg/mL G418 – Gibco 11811-064

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	7
Positive control tested?	Yes
Positive control substance	17β-Estradiol (DRC with 8 concentrations, concentration range 0.0001- 10nM)
Solvent control tested?	Assay medium and solvent control used as negative controls
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	Not measured
pH adjusted	-
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
1000-times concentrated (1000X) stock solutions of reference (estradiol) and test chemicals are prepared in DMSO.
Both reference (estradiol) and test chemicals are serially diluted in DMSO and each dilution is diluted in cultrue medium so that each conditions contains the same final DMSO concentration, i.e. 0.1 % v/v

Cell seeding:
80 000 cells per well, 100µL per well in 96well white opaque plates (Greiner cellStar ; D. Dutscher, Brumath, France). Plates are placed in incubator (humidified 37°C/5% CO_2) for 24h.

Dosing of cells:
Addition of 50 µl/well of dosing media containing the test chemical or sample (3X concentrated), and incubated for 16h in incubator (humidified 37°C/5% CO_2)

Detection:
- Remove of the media out of the plate and addition of 50µL/well of DCC medium D-luciferin at 37.5µg/mL (Sigma)
- Read of luminescence in living cells using a microtiter luminometer (KC-4, BioTek Instruments, France)

Data analysis

Software used: Excel and Regtox 7.0.7 Microsoft Excel™ macro (Vindimian et al., 1983). This macro uses the Hill equation model and allows calculation of EC50.

Cytotoxicity data analysis: MTT test
After luciferase assay, culture medium is replaced by 100 µl of DCC with 0.5 mg/mL of MTT. Cells are incubated for 3 h. In metabolically active cells, MTT is reduced by the mitochondria into a blue formazan precipitate, which is solubilized by adding 80 µl of DMSO and agitation for 10 min. Plates are then read at 570 nm against a 640 nm reference wavelength on a microplate reader (KC-4, BioTek Instruments, France). Cell viability was expressed as a percentage of the solvent control value.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
Each plate includes negative (both medium and solvent) and positive (estradiol 10 nM) controls, as well as usually 6-7 dilution points of test chemical dosed in triplicates. Luminescence units data may vary from plate to plate. Therefore data are analyzed plate by plate and expressed as the % of maximum Estradiol effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-runs (inter-assay) replication;

3 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

References:

Balaguer P, Francois F, Comunale F, Fenet H, Boussioux AM, Pons M, Nicolas JC, Casellas C (1999) Reporter cell lines to study the estrogenic effects of xenoestrogens. Sci Tot Environ 233:47–56

Vindimian E. Robaut C. & Fillion G. (1983) A method for cooperative and non cooperative binding studies using non linear regression analysis on a microcomputer. J. Appl. Biochem. 5, 261-268.

Bioassay main features

Bioassay ID	WP12 bioassay_hPXR_HG5LN
Bioassay type	*in vitro*
Adverse Outcome Pathway	hPXR activation leading to transcription of detoxification enzymes
Test species	Homo sapiens
Cell line/ cell strain	HG5LN cells (HeLa cells stably transfected with GAL4-luciferase reporter gene)
Transgene	luciferase reporter gene under control of a truncated hPXR having a GAL4 and the LBD of hPXR

Measured endpoints/ molecular targets	**Measured via**
Luciferase/hPXR	Luminescence measured with reader

Brief description of test

The HG5LN-hPXR cell line results from a two-step stable transfection (Lemaire et al., 2006). As a first step, HeLa cells were stably transfected with a GAL4RE5-BGlob-Luc-SVNeo plasmid, leading to the HG5LN cell line which expresses constitutively luciferase activity. Then, HG5LN cells were stably transfected, with the pSG5-GAL4(DBD)-hPXR(LBD)-puro plasmid to obtain the HG5LN-hPXR cell line. The HG5LN cell line was used to assess toxic or unspecific effects on luciferase in the bioassay, hence providing information on the specificity of hPXR activation in HG5LN-hPXR cells.

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Hélène SERRA	17/05/16

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	16h
Main determinant	EC_{50} from log-logistic concentration effect model
Effect equivalent EQ	SR 12813
Unit of effect equivalent	µg/L
Concentration range in standard curve	0.001- 10µM (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	Lemaire et al., 2006
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	96-well plate
Volume per well/ vessel	Final volume 150µl (100µl cell seeding, 50µl dosing of compounds)

Test media

Solvent	DMSO
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
Phenol red free DMEM – Gibco 11880-028 5% dextran charcoal coated FBS – Fisher Invitrogen F7524 1% Penicillin/streptomycin (50 U/mL each) – Gibco 15070-063

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	60 000 cells per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	DMEM – Gibco 21885-025 5% foetal bovine serum (FBS) - Fisher Invitrogen F7524 1% nonessential amino acids - Gibco 11140-050 Penicillin/streptomycin (50 U/mL each) – Gibco 15070-063 0.3 mg/mL G418 – Gibco 11811-064 0.5 µg/mL puromycin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	7
Positive control tested?	Yes
Positive control substance	SR 12813 (DRC with 8 concentrations, concentration range 0.001- 3µM)
Solvent control tested?	Assay medium and solvent control used as negative controls
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	Not measured
pH adjusted	-
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Stock solutions are prepared in DMSO and diluted 1000-times in medium before dosing
SR 12813 stock solution (3mM) is prepared in DMSO and diluted 1000-times in medium before dosing

Cell seeding:
60 000 cells per well, 100uL per well in 96well white opaque plates (Greiner cellStar ; D. Dutscher, Brumath, France). Plates are placed in incubator (humidified 37°C/5% CO_2) for 24h.

Dosing of cells:
50 µl/well of dosing media containing the test chemical or sample, and incubated for 16h in incubator (humidified 37°C/5% CO_2)

Detection:

- Remove the media out of the plate and add 50µL/well of DCC medium containing D-luciferin at 0,15mg/mL (Sigma)
- Read of luminescence in living cells using a microtiter luminometer (KC-4, BioTek Instruments, France)

Data analysis

Software used: Excel and Regtox 7.0.7 Microsoft Excel™ macro (Vindimian et al., 1983). This macro uses the Hill equation model and allows calculation of EC50

Cytotoxicity data analysis:
After luciferase assay, culture medium containing the luciferin is removed and replaced by 100 µl of DCC with 0.5 mg/mL of MTT. Cells were incubated for 3 h. In metabolically active cells, MTT is reduced by the mitochondria onto a blue formazan precipitate, which was solubilized by adding 80 µl of DMSO and agitation for 10 min. Plates were then read at 570 nm against a 640-nm reference wavelength on a microplate reader (KC-4, BioTek Instruments, France). Cell viability was expressed as a percentage of the control value.

Quality control
Pipettors: Ensure that all pipettors are calibrated at least once a year. Plate setup : On every plate include a negative (medium) control and usually 6-7 dilution points dosed in triplicates. Relative luminescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum Estradiol effect. Determine : 1) Within-plate (internal) replication indicative of the operational variability; 2) Between-runs (inter-assay) replication; 3 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

References:

Lemaire G, Mnif W, Pascussi JM, Pillon A, Rabenoelina F, Fenet H, Gomez E, Casellas C, Nicolas JC, Cavailles V, Duchesne MJ, Balaguer P (2006) Identification of new human pregnane X receptor ligands among pesticides using a stable reporter cell system. Toxicol Sci 91:501–509

Vindimian E. Robaut C. & Fillion G. (1983) A method for cooperative and non cooperative binding studies using non linear regression analysis on a microcomputer. J. Appl. Biochem. 5, 261-268.

Bioassay main features

Bioassay ID	WP12 bioassay_zfCYP19a1b_GFP
Bioassay type	*in vivo*
Adverse Outcome Pathway	ER activation leading to development and reproductive effects
Test species	*Danio rerio*
Cell line/ cell strain/transgenic line	Transgenic Cyp19a1b-GFP zebrafish
Transgene	GFP reporter gene under control of the zebrafish *cyp19a1b* promoter

Measured endpoints/ molecular targets	**Measured via**
GFP fluorescence / *cyp19a1b* gene expression	GFP fluorescence measured with a fluorescent microscope

Brief description of test

EASZY: Detection of **E**ndocrine **A**ctive **S**ubstance, acting through estrogen receptors, using transgenic cyp19a1b-GFP **Z**ebrafish embryos. EASZY assay is a rapid (96hours of exposure) and cost-effective *in vivo* embryo fish screening assay for estrogenic activity of chemicals. The test provides mechanistic information regarding the capacity of chemicals to activate the ER-signaling pathway *in vivo* in radial glial cells (RGC), while considering the biodisponibility and pharmacodynamics of test chemicals. It allows the quantification of the estrogenic activity of chemicals through the measurement of GFP that faithfully mimics the expression of the ER-regulated *cyp19a1b* gene. Because the skull of early developmental stages of zebrafish is transparent, GFP is observed, imaged and quantified *in vivo* without sacrificing the fish.

Document history

Version	**Modification**	**Author**	**Date**
A	creation	François BRION/ Hélène SERRA	03/05/16

Regulatory aspects

According to the EU Directive 2010/63/EU, the EASZY assay does not fall into the regulatory frameworks dealing with animal experimentation

Experimental conditions

Exposure duration	96h
Main determinant	EC_{50} from log-logistic concentration effect model
Effect equivalent EQ	17β-Estradiol
Unit of effect equivalent	ng/L
Concentration range in standard curve	-
Bioassay performed to standard guideline?	No (EASZY is under validation process at VMG-ECO, OECD)
Guideline no. or reference for published method	Brion et al., (2012) Plos One 7(5): e36069
Deviation from standard guideline?	

Describe deviation from standard guideline:

Assay format	Glass crystalizers
Volume per well/ vessel	25mL

Test media

Solvent	DMSO
Max solvent concentration in test media	0.01%
Reference media	Water

Composition of reference media
For water control, we use reconstituted water produced by mixing osmosis water (conductivity < 10 µS/cm) with mains water previously subjected to mechanical and charcoal filtrations and UV disinfections. The produced water is of constant pH (pH=7 ± 0.5), conductivity (350 µS.cm-1) and temperature (27°C ± 1) and aerated to oxygen saturation.

Renewal type	100% - semi static
Renewal frequency	daily renewal

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	20 fertilized embryos per vessel
Age of organisms	< 4hpf
Developmental stage of organisms	Embryo-larval
Feeding?	no
Frequency of feeding	no
Culturing conditions	Incubator with controlled temperature of 27°C ± 1°C and light/dark photoperiod (14hrs light :10hrs dark)
Growth Medium	Same as reference media

Statistical parameters and quality control

Number of experimental repetitions	1 to 2
Number of replicates per treatment	1 replicate per treatment with n=20 fertilized embryos
Number of concentrations tested per sample	3 to 5
Positive control tested?	Yes
Positive control substance	17α-ethinylestradiol 0.05nM or 17β-estradiol 10nM
Solvent control tested?	Assay medium and solvent control used as negative control
Other controls?	-
pH of sample in test medium tested?	Yes
pH adjusted	no
DO of sample in test medium measured?	no
Conductivity of sample in test medium measured?	Yes
Ammonium/ nitrite content measured?	no

Experimental outline

Stock solution preparation:

Stock solutions are prepared in DMSO and diluted 10 000-times in medium before dosing
Ethinylestradiol stock solution (0,5µM) or 17β-Estradiol(100 µM) is prepared in DMSO and diluted 10000-times in water before dosing

Embryos:

20 fertilized (<4 hpf) are randomely put in each treatment. Every day, dead eggs are removed from media

Dosing of embryos:

25ml/vessel of dosing media containing the test chemical, and incubated for 96h.

In vivo imaging:

Fluorescence imaging is realized using a fluorescence microscope equipped with a 10X objective, a GFP filter, an external light source (e.g., HBO lamp) and a fluorescence camera**.** For each exposure condition, zebrafish larvae are carefully transferred from the exposure crystallizer to a multi-well fluorescence hydrophobic glass slide using a Pasteur pipette. Each transgenic zebrafish is then photographed dorsally. A good positioning of the fish is mandatory to ensure comparison of GFP expression from a fish to another.
For GFP quantification, photomicrographs are imported in the image analysis software (ImageJ software) and GFP analyzed in the region of interest (ROI) corresponding to brain regions where expression of GFP is normally observed in transgenic cyp19a1b-GFP zebrafish. GFP intensity is quantified as integrated density.

Data analysis

Data are expressed as mean fold ± standard error of the mean (S.E.M) above water control or solvent control (if solvent control is used).

Software used: Excel and Regtox 7.0.7 Microsoft Excel™ macro (Vindimian et al., 1983). This macro uses the Hill equation model and allows calculation of EC50

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Experiment setup :
Each experiment must include two negative (medium and solvent) controls, a positive control (17α-ethinylestradiol 0.05nM or 17β-estradiol 10nM) and from 3 to 5 concentrations of each substance.
Medium and solvent control should induce a similar fluorescence level and mortality in controls should not be above 20%. In the positive control, the mean GFP intensity should be 10-fold the mean GFP intensity meaured in medium and solvent controls.

References:

Brion, F., Le Page, Y., Piccini, B., Cardoso, O., Tong, S.K., Chung, B.C., et al., 2012. Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1bgfp) zebrafish embryos. PLoS One 7, e36069.

Vindimian E. Robaut C. & Fillion G. (1983) A method for cooperative and non cooperative binding studies using non linear regression analysis on a microcomputer. J. Appl. Biochem. 5, 261-268.

Bioassay main features

Bioassay ID	WP12 bioassay_ZFL_ZELHalpha
Bioassay type	*in vitro*
Adverse Outcome Pathway	Estrogen receptor alpha activation leading to reproductive
Test species	Danio rerio
Cell line/ cell strain	ZELH (Zebrafish liver (ZFL) cells stably transfected with ERE-luciferase reporter gene driven by zfERalpha)
Transgene	luciferase reporter gene under control of a estrogen response element

Measured endpoints/ molecular targets	**Measured via**
Luciferase/zfERalpha	Luminescence measured with reader

Brief description of test

The zebrafish *in vitro* assay was derived from the zebrafish liver cell (ZFL) line that was stably transfected by first an ERE-driven luciferase gene, yielding the ZELH cell line. Then, the ZELH cell line was transfected with zfERalpha, yielding the ZELH-zfERalpha cell lines (Cosnefroy et al., 2012). Establishment of the cell model and its response to different classes of well-known xenoestrogens have been previously described (Cosnefroy et al., 2012).

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Hélène SERRA	17/05/2016

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	72h
Main determinant	EC_{50} from log-logistic concentration effect model
Effect equivalent EQ	17β-Estradiol
Unit of effect equivalent	ng/L
Concentration range in standard curve	0.0001- 10nM (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published	Cosnefroy et al. 2012

method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	96-well plate
Volume per well/ vessel	Final volume 150µl (100µl cell seeding, 50µl dosing of compounds)

Test media

Solvent	DMSO
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
5 % Charcoal-Dextran treated Fetal Bovine Serum – Fisher Invitrogen F7524 50% of L-15 – Gibco 31415-29 35% of DMEM HG – Gibco 52100-039 15%of Ham's F12 – Gibco 21700-026 15mM of HEPES – Gibco 15630-056 0.15 g/L of sodium bicarbonate - PAN biotech 0.01 mg/ml of insulin – PAN biotech 50 ng/ml of EGF – Sigma Aldrich SRP3027

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	25 000 cells per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 28°C
Growth Medium	5 % Charcoal-Dextran treated Fetal Bovine Serum – Fisher Invitrogen F7524 50% of L-15 – Gibco 31415-29 35% of DMEM HG – Gibco 52100-039 15%of Ham's F12 – Gibco 21700-026 15mM of HEPES – Gibco 15630-056 0.15 g/L of sodium bicarbonate - PAN biotech 0.01 mg/ml of insulin – PAN biotech 50 ng/ml of EGF – Sigma Aldrich SRP3027 50U/mL Penicillin/streptomycin (50 U/mL each) – Gibco 15070-063 1 mg/mL G418 - Gibco 11811-064

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	7
Positive control tested?	Yes
Positive control substance	17β-Estradiol (DRC with 8 concentrations, concentration range 0.0001- 10nM)
Solvent control tested?	Assay medium and solvent control used as negative controls
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	Not measured
pH adjusted	-
DO of sample in test medium measured?	Not tested
Conductivity of sample in test medium	Not measured

measured?	
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Stock solutions are prepared in DMSO and diluted 1000-times in medium before dosing
Estradiol stock solution (10 µM) is prepared in DMSO and diluted 1000-times in medium before dosing

Cell seeding:
25 000 cells per well, 100uL per well in 96well white opaque plates (Greiner cellStar ; D. Dutscher, Brumath, France). Place plates in incubator (humidified 28°C) for 24h

Dosing of cells:
50 µl/well of dosing media containing the test chemical or sample, and incubated for 72h in incubator (humidified 28°C)

Detection:
- Remove the media out of the plate and add 50µL/well of of DCC medium containing D-luciferin at 37.5µg/mL (Sigma)
- Read of luminescence in living cells using a microtiter luminometer (KC-4, BioTek Instruments, France)

Data analysis

Software used: Excel and Regtox 7.0.7 Microsoft Excel™ macro (Vindimian et al., 1983). This macro uses the Hill equation model and allows calculation of EC50

Cytotoxicity data analysis:
After luciferase assay, culture medium containing the luciferin is removed and replaced by 100 µl of DCC with 0.5 mg/mL of MTT. Cells were incubated for 3 h. In metabolically active cells, MTT is reduced by the mitochondria onto a blue formazan precipitate, which was solubilized by adding 80 µl of DMSO and agitation for 10 min. Plates were then read at 570 nm against a 640-nm reference wavelength on a microplate reader (KC-4, BioTek Instruments, France). Cell viability was expressed as a percentage of the control value.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and usually 6-7 dilution points dosed in triplicates. Relative luminescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum Estradiol effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-runs (inter-assay) replication;

3 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

References:

Cosnefroy, A., Brion, F., Maillot-Marechal, E., Porcher, J.M., Pakdel, F., Balaguer, P., et al., (2012). Selective activation of zebrafish estrogen receptor subtypes by chemicals by using stable reporter gene assay developed in a zebrafish liver cell line. Toxicol. Sci. 125, 439–449.

Vindimian E. Robaut C. & Fillion G. (1983) A method for cooperative and non cooperative binding studies using non linear regression analysis on a microcomputer. J. Appl. Biochem. 5, 261-268.

Bioassay main features

Bioassay ID	WP12 bioassay_ZFL_ZELHbeta2
Bioassay type	*in vitro*
Adverse Outcome Pathway	Estrogen receptor beta2 activation leading to reproductive
Test species	Danio rerio
Cell line/ cell strain	ZELH (Zebrafish liver (ZFL) cells stably transfected with ERE-luciferase reporter gene driven by zfERbeta2)
Transgene	luciferase reporter gene under control of a estrogen response element

Measured endpoints/ molecular targets	**Measured via**
Luciferase/zfERbeta2	Luminescence measured with reader

Brief description of test

The zebrafish *in vitro* assay was derived from the zebrafish liver cell (ZFL) line that was stably transfected by first an ERE-driven luciferase gene, yielding the ZELH cell line. Then, the ZELH cell line was transfected with zfERbeta2, yielding the ZELH-zfERbeta2 cell lines (Cosnefroy et al., 2012). Establishment of the cell model and its response to different classes of well-known xenoestrogens have been previously described (Cosnefroy et al., 2012).

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Hélène SERRA	17/05/2016

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	72h
Main determinant	EC_{50} from log-logistic concentration effect model
Effect equivalent EQ	17β-Estradiol
Unit of effect equivalent	ng/L
Concentration range in standard curve	0.0001- 10nM (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published	Cosnefroy et al. 2012

method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	96-well plate
Volume per well/ vessel	Final volume 150µl (100µl cell seeding, 50µl dosing of compounds)

Test media

Solvent	DMSO
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
5 % Charcoal-Dextran treated Fetal Bovine Serum – Fisher Invitrogen F7524 50% of L-15 – Gibco 31415-29 35% of DMEM HG – Gibco 52100-039 15%of Ham's F12 – Gibco 21700-026 15mM of HEPES – Gibco 15630-056 0.15 g/L of sodium bicarbonate - PAN biotech 0.01 mg/ml of insulin – PAN biotech 50 ng/ml of EGF – Sigma Aldrich SRP3027

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	25 000 cells per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 28°C
Growth Medium	5 % Charcoal-Dextran treated Fetal Bovine Serum – Fisher Invitrogen F7524 50% of L-15 – Gibco 31415-29 35% of DMEM HG – Gibco 52100-039 15%of Ham's F12 – Gibco 21700-026 15mM of HEPES – Gibco 15630-056 0.15 g/L of sodium bicarbonate - PAN biotech 0.01 mg/ml of insulin – PAN biotech 50 ng/ml of EGF – Sigma Aldrich SRP3027 50U/mL Penicillin/streptomycin (50 U/mL each) – Gibco 15070-063 1 mg/mL G418 - Gibco 11811-064

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	7
Positive control tested?	Yes
Positive control substance	17β-Estradiol (DRC with 8 concentrations, concentration range 0.0001- 10nM)
Solvent control tested?	Assay medium and solvent control used as negative controls
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	Not measured
pH adjusted	-
DO of sample in test medium measured?	Not tested
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Stock solutions are prepared in DMSO and diluted 1000-times in medium before dosing
Estradiol stock solution (10 µM) is prepared in DMSO and diluted 1000-times in medium before dosing

Cell seeding:
25 000 cells per well, 100uL per well in 96well white opaque plates (Greiner cellStar ; D. Dutscher, Brumath, France). Place plates in incubator (humidified 28°C) for 24h

Dosing of cells:
50 µl/well of dosing media containing the test chemical or sample, and incubated for 72h in incubator (humidified 28°C)

Detection:

- Remove the media out of the plate and add 50µL/well of of DCC medium containing D-luciferin at 37.5µg/mL (Sigma)
- Read of luminescence in living cells using a microtiter luminometer (KC-4, BioTek Instruments, France)

Data analysis

Software used: Excel and Regtox 7.0.7 Microsoft Excel™ macro (Vindimian et al., 1983). This macro uses the Hill equation model and allows calculation of EC50

Cytotoxicity data analysis:
After luciferase assay, culture medium containing the luciferin is removed and replaced by 100 µl of DCC with 0.5 mg/mL of MTT. Cells were incubated for 3 h. In metabolically active cells, MTT is reduced by the mitochondria onto a blue formazan precipitate, which was solubilized by adding 80 µl of DMSO and agitation for 10 min. Plates were then read at 570 nm against a 640-nm reference wavelength on a microplate reader (KC-4, BioTek Instruments, France). Cell viability was expressed as a percentage of the control value.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and usually 6-7 dilution points dosed in

triplicates. Relative luminescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum Estradiol effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-runs (inter-assay) replication;

3 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

References:

Cosnefroy, A., Brion, F., Maillot-Marechal, E., Porcher, J.M., Pakdel, F., Balaguer, P., et al., (2012). Selective activation of zebrafish estrogen receptor subtypes by chemicals by using stable reporter gene assay developed in a zebrafish liver cell line. Toxicol. Sci. 125, 439–449.

Vindimian E. Robaut C. & Fillion G. (1983) A method for cooperative and non cooperative binding studies using non linear regression analysis on a microcomputer. J. Appl. Biochem. 5, 261-268.

Bioassay main features

Bioassay ID	Growth_Inhibition_In vivo_72h_Chlamydomonas reinhardtii
Bioassay type	*In vivo*
Adverse Outcome Pathway	Inhibition of PSII leading to growth Inhibition; Multiple MIE leading to growth inhibition
Test species	Algae *Chlamydomonas reinhardtii*
Cell line/ cell strain	Strain = NIVA-CHL 153 (Norwegian Institute for Water Research, Oslo, Norway)
Transgene	NR

Measured endpoints/ molecular targets	**Measured via**
Average growth rate	Fluorescence

Brief description of test

The purpose of this test is to determine the effects of a compound on the growth of freshwater microalgae *Chlamydomonas reinhardtii*. Exponentially growing algae are exposed to the test compound in batch cultures over a period of normally 72 hours. The system response is the reduction of growth in response to various concentrations of the test compound. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. Test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. The results are analysed in order to calculate the NOECs and EC_{50} at 24, 48 and 72h.

Document history

Version	Modification	Author	Date
B	Revision of version A	Tânia Gomes and Ana Catarina Almeida	09/06/16

Regulatory aspects
Standardised test used for regulatory testing (OECD TG 201)

Experimental conditions

Exposure duration	72h
Main determinant	NOEC, EC_{50}, EC_{10} etc.
Effect equivalent EQ	3,5-Dichlorophenol (DCP)
Unit of effect equivalent	M
Concentration range in standard curve	10^{-6}–10^{-3}
Bioassay performed to standard guideline?	Yes
Guideline no. or reference for published method	OECD Guideline 201 (OECD, 2011)
Deviation from standard guideline?	No

Describe deviation from standard guideline

Assay format	96-well plate
Volume per well/ vessel	200 µL

Test media

Solvent	DMSO
Max solvent concentration in test media	1%
Reference media	1) Talaquil media, 2) High Salt media (HSM) media

Composition of reference media
1) The composition of Talaquil media is the following: $2x10^{-5}$ Na_2EDTA; $5x10^{-4}$ M $CaCl_2.2H_2O$; $1.5x10^{-4}$ M $MgSO_4.7H_2O$; $1.2x10^{-3}$ M $NaHCO_3$; $5x10^{-5}$ M $K_2HPO_4.3H_2O$; $1x10^{-3}$ M NH_4Cl; $5x10^{-8}$ M $CoCl_2.6H_2O$; $5x10^{-5}$ MH_3BO_3; $8x10^{-8}$ M $Na_2MoO_4.2H_2O$; $1.63x10^{-7}$ M $CuSO_4.5H_2O$; $1.22x10^{-6}$ M $MnCl_2.4H_2O$; $1.58x10^{-7}$ M $ZnSO_4.7H_2O$ and

$9x10^{-7}$ M $FeCl_3.6H_2O$ in $1x10^{-2}$ M MOPS buffer (pH 7.5).

2) The composition of HSM media is the following: $9.34x10^{-3}$ NH_4Cl M, $8x10^{-5}$ $MgSO_4.7H_2O$ M, $7\ x10^{-5}$ $CaCl_2.2H_2O$ M, $0.83x10^{-3}$ K_2HPO_4 M, $0.53x10^{-3}$ KH_2PO_4 M, $0.13x10^{-3}$ EDTA disodium salt M, $8x10^{-5}$ $ZnSO_4.7H_2O$ M, $0.18x10^{-3}$ H_3BO_3 M, $0.25x10^{-3}$ $MnCl_2.4H_2O$ M, $7x10^{-6}$ $CoCl_2.6H2O$ M, $6x10^{-6}$ $CuSO_4.5H_2O$ M, $9x10^{-7}$ $(NH_4)6Mo_7O_{24}.4H_2O$ M, $2x10^{-5}$ $FeSO_4.7H_2O$ M (pH 7).

Renewal type	None (72h assay)
Renewal frequency	None (72h assay)

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	$10x10^3$ cells/mL (at start of study)
Age of organisms	NA
Developmental stage of organisms	NA
Feeding?	NA
Frequency of feeding	NA
Temperature controlled?	Yes
Temperature	20±2°C
Photoperiod	Continuous light
Light intensity	83±6 µmol/m^2/s^1

Statistical parameters and quality control

Culturing conditions	Incubator at 20±2°C
Growth Medium	Talaquil or HSM media
Number of experimental repetitions	2-3
Number of replicates per treatment	4
Number of concentrations tested per sample	6-10
Positive control tested?	Yes
Positive control substance	3,5-Dichlorophenol (DCP)
Solvent control tested?	Yes (DMSO)
Other controls tested?	Yes, algae with medium + medium with only test compound (blanks)
pH of sample in test medium	Yes
pH adjusted	Talaquil media buffered with MOPS at pH 7.5
DO of sample in test medium measured?	No

Conductivity of sample in test medium measured?	No
Ammonium/ nitrite content measured?	No

Experimental outline

Stock solution preparation:

- Sample from extract or compound and controls are dissolved in DMSO.
- Final DMSO concentration is constant across all experimental treatments (1%).

Sample and control preparation:

- Stock solutions for each sample extract or compound at highest concentration are serially diluted in assay medium into required test concentrations until a concentration of 2% DMSO.
- Solvent control is equally diluted as required for extracts/compound stock solutions.

Algae preparation:

- The algae used to provide the inoculum are cultured for 72h and under the conditions described for the test.
- Algae concentrations are measured with a Beckman-Coulter Multisizer 3 Coulter Counter in triplicate and adjust to an initial cell density of 20 $x10^3$ $cell.mL^{-1}$.

Plate preparation:

- 100 µL of algae are added in each well to reach $10x10^3$ cells/mL, along with 100 µL extract/compound/control per well in a 96-well plates (FalconTM,Oslo, Norway), final 1% DMSO.
- Microplates are placed in an incubator (continous light, 20°C) for 24, 48 and 72h with orbital shaking at 90 rpm.

Experimental read out:

- At 0, 24, 48 and 72 hours, density of algal cells in each well is determined by fluorescence at 485 nm excitation and 685 nm emission.

Data analysis

Software used: Microsoft Office Excel 2010 and GraphPad Prism 6 software (GraphPad Software Inc., San Diego California USA)

GIT data analysis:

- Fluorescence values for each sample/control are normalised with the mean fluorescence of corresponding blank.
- The average growth rate (µ) for each test concentration is calculated from the initial cell

concentration and cell concentration at the time of the last cell count using the formula:

$$\mu_{n-0} = \frac{\ln(N_n) - \ln(N_0)}{t_n - t_0} \times 24\ (day^{-1})$$

- μ_{n-0} is the average specific growth rate from time 0 to *n*, N_n is the cell density at time n and N_0 is the cell density at time 0.
- Growth inhibition is calculated as a percentage of control (%CT).
- Results are modelled using a sigmoidal concentration-response curve (CRC) with variable slope:

$$\gamma = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC_{50} - logX) \times Slope}}$$

- Y is the effect, X is the concentration, Bottom is the baseline effect (control), top is the maximal effect plateau (full growth inhibition), and log EC_{50} is the concentration causing 50% effect.

Quality control

Pipettes:
Ensure that all pipettors are calibrated at least once a year.

Algae cultures:
All flasks and glassware used for media preparation and experiments are autoclaved before use to avoid any microbial contamination. Culture samples are checked microscopically to detect the presence of any microbial contamination.

Assay replicates:
At least two to three independent repeats of the assay (intra-assay) with tetraplicates (internal replication) is made for each sample for final tests. Replicate variability is compared between repeats to make sure it doesn't exceed pre-determined levels. Screening performed only with one test.

Exposure verification:
Exposure concentrations measured whevener required for the test design.

Bioassay main features

Bioassay ID	PSII_Inhibition_*In vivo*_72h_Chlamydomonas reinhardtii
Bioassay type	*In vivo*
Adverse Outcome Pathway	Inhibition of PSII leading to growth Inhibition; Multiple MIE leading to growth inhibition
Test species	Algae *Chlamydomonas reinhardtii*
Cell line/ cell strain	Strain = NIVA-CHL 153 (Norwegian Institute for Water Research, Oslo, Norway)
Transgene	NR

Measured endpoints/ molecular targets	**Measured via**
Photochemical efficiency of PSII	Fluorescence

Brief description of test

The purpose of this test is to determine the effects of a compound on the Photosystem II (PSII) efficiency of the freshwater microalgae *Chlamydomonas reinhardtii*. Exponentially growing algae are exposed to the test compound in batch cultures over a period of normally 72 hours. The system response is the increase in chlorophyll a fluorescence in response to various concentrations of the test compound. The response is evaluated as a function of the exposure concentration in comparison with the average of replicate, unexposed control cultures. Test endpoint is inhibition of PSII primary photochemical efficiency and results are analysed in order to calculate the NOEC and EC_{50} at 24, 48 and 72h.

Document history

Version	**Modification**	**Author**	**Date**
B	Revison of version A	Tânia Gomes and Ana Catarina Almeida	06/06/16

Regulatory aspects
Not applicable.

Experimental conditions

Exposure duration	72h
Main determinant	NOEC, EC_{50}, EC_{10} etc.
Effect equivalent EQ	Atrazine
Unit of effect equivalent	M
Concentration range in standard curve	10^{-8}–10^{-3}
Bioassay performed to standard guideline?	No
Guideline no. or reference for published method	Kitajima and Butler (1975), adapted to a 96-well microplate (Almeida et al., 2015)
Deviation from standard guideline?	NA

Describe deviation from standard guideline

Assay format	96-well plate
Volume per well/ vessel	200 µL

Test media

Solvent	DMSO
Max solvent concentration in test media	1%
Reference media	Talaquil media, 2) High Salt media (HSM) media

Composition of reference media

1) The composition of Talaquil media is the following: $2x10^{-5}$ Na_2EDTA; $5x10^{-4}$ M $CaCl_2.2H_2O$; $1.5x10^{-4}$ M $MgSO_4.7H_2O$; $1.2x10^{-3}$ M $NaHCO_3$; $5x10^{-5}$ M $K_2HPO_4.3H_2O$; $1x10^{-3}$ M NH_4Cl; $5x10^{-8}$ M $CoCl_2.6H_2O$; $5x10^{-5}$ MH_3BO_3; $8x10^{-8}$ M $Na_2MoO_4.2H_2O$; $1.63x10^{-7}$ M $CuSO_4.5H_2O$; $1.22x10^{-6}$ M $MnCl_2.4H_2O$; $1.58x10^{-7}$ M $ZnSO_4.7H_2O$ and $9x10^{-7}$ M $FeCl_3.6H_2O$ in $1x10^{-2}$ M MOPS buffer (pH 7.5).

The composition of HSM media is the following: $9.34x10^{-3}$ NH_4Cl M, $8x10^{-5}$ $MgSO_4.7H_2O$ M, $7 x10^{-5}$ $CaCl_2.2H_2O$ M, $0.83x10^{-3}$ K_2HPO_4 M, $0.53x10^{-3}$ KH_2PO_4 M, $0.13x10^{-3}$ EDTA disodium salt M, $8x10^{-5}$ $ZnSO_4.7H_2O$ M, $0.18x10^{-3}$ H_3BO_3 M, $0.25x10^{-3}$ $MnCl_2.4H_2O$ M, $7x10^{-6}$ $CoCl_2.6H2O$ M, $6x10^{-6}$ $CuSO_4.5H_2O$ M, $9x10^{-7}$ $(NH_4)6Mo_7O_{24}.4H_2O$ M, $2x10^{-5}$ $FeSO_4.7H_2O$ M (pH 7).

Renewal type	None (72h assay)
Renewal frequency	None (72h assay)

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	$10x10^3$ cells/mL
Age of organisms	NA
Developmental stage of organisms	NA
Feeding?	NA
Frequency of feeding	NA
Temperature controlled?	Yes
Temperature	20±2°C
Photoperiod	Continuous light
Light intensity	83±6 µmol/m^2/s^1

Statistical parameters and quality control

Culturing conditions	Incubator at 20±2°C
Growth Medium	Talaquil or HSM media
Number of experimental repetitions	2-3
Number of replicates per treatment	4
Number of concentrations tested per sample	6-10
Positive control tested?	Yes
Positive control substance	Atrazine
Solvent control tested?	Yes
Other controls tested?	Yes, only medium (blank)
pH of sample in test medium	Yes
pH adjusted	No
DO of sample in test medium measured?	No
Conductivity of sample in test medium measured?	No
Ammonium/ nitrite content measured?	No

Experimental outline

Stock solution preparation:

- Sample from extract or compound and controls are suspended in DMSO.
- Final DMSO concentration is constant across all experimental treatments (1%).

Sample and control preparation:

- Stock solutions for each sample extract or compound at highest concentration are serially dilluted in assay medium into required test concentrations until a concentration of 2% DMSO.
- Solvent control are equally dilluted as required for extracts stock solutions.

Algae preparation:

- The algae used to provide the inoculum are cultured for 72h and under the conditions described for the test.
- Algae concentrations are measured with a Beckman-Coulter Multisizer 3 Coulter Counter in triplicate and adjust to an initial cell density of 20 $x10^3$ $cell.mL^{-1}$.

Plate preparation:

- 100 µL of algae are added in each well to reach $10x10^3$ cells/mL, along with 100 µL extract/compound/control per well in a 96-well plates (FalconTM,Oslo, Norway), final concentration of DMSO of 1%.
- Microplates are placed in an incubator (continous light, 20°C) for 24, 48 and 72h with orbital shaking at 90 rpm.

Experimental read out:

1) Fluorescence method – microplate reader

- At 0, 24, 48 and 72 h, chlorophyll a in each well is determined by fluorescence at 485 nm excitation and 685 nm emission.
- At each time point, chlorophyll a fluorescence measurement is made after 20 min adaption to dark to determine the fluorescence yield of PSII in a dark adapted state (F_o).
- 5 µl of diuron (final concentration 10 µM) is added to block the electron transport in the PSII.
- A second fluorescence measurement is performed to determine the maximal fluorescence yield in a light adapted state (F_m).

2) Fluorescence method – FluoroPen

- At 24, 48 and 72 h, chlorophyll a in each well is determined by fluorescence using a FluoroPen FP100 PAM (Photo System Instruments, Czech Republic).
- At each time point, chlorophyll a fluorescence measurement is made after 30 min dark adaption to determine the fluorescence yield of PSII in a dark adapted state (F_o) by measuring modulated light, which was sufficiently low (<0.1 µmol m^{-2} s^{-1}) to not induce any significant variation in fluorescence.
- A second fluorescence measurement is performed to determine the maximal fluorescence (Fm) using a 0.8 s saturating pulse at 8000 µmol m^{-2} s^{-1}.

Data analysis

Software used: Microsoft Office Excel 2010 and GraphPad Prism 6 software (GraphPad Software Inc., San Diego California USA)

PSII data analysis:

- Fluorescence values for each sample is normalised with the mean fluorescence of corresponding blank.
- The fluorescence of variable yield (F_v) is calculated as Fm-Fo, and F_v/F_m is used to express PSII primary photochemical efficiency and calculated using the formula: $F_v/F_m = (F_m-F_0)/F_m$.
- F_v is the fluorescence of variable yield, F_m the maximal fluorescence yield in a light adapted state and F_0 the fluorescence yield of PSII in a dark adapted state.
- PSII inhibition is calculated as a percentage of control (%CT).
- Results are modelled using a sigmoidal concentration-response curve (CRC) with variable slope:

$$\gamma = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC_{50} - logX) \times Slope}}$$

- Y is the effect, X is the concentration, Bottom is the baseline effect (control), top is the maximal effect plateau (full PSII inhibition), and log EC_{50} is the concentration causing 50% effect.

Quality control

Pipettes:
Ensure that all pipettors are calibrated at least once a year.

Algae cultures:
All flasks and glassware used for media preparation and experiments are autoclaved before use to avoid any microbial contamination. Culture samples are checked microscopically to detect the presence of any microbial contamination.

Assay replicates:
At least three independent repeats of the assay (intra-assay) with tetraplicates (internal replication) is made for each sample. Replicate variability is compared between repeats to make sure it doesn't exceed pre-determined levels.

References

A.C. Almeida, 2015. Toxicity of single biocides and their mixtures in the algae Chlamydomonas reinhardtii. Philosophiae Doctor (PhD) thesis. Department of Environmental Sciences, Faculty of Environmental Science and Technology, Norwegian University of Life Sciences, Ås, Norway.

M. Kitajima and W.L. Butler, 1975. Quenching of chlorophyll fluorescence and primary photochemistry in chloroplast by dibromothymoquinone. Biochim Biophys Acta 326, 105-115.

Bioassay main features

Bioassay ID	WP12 bioassay_E.coli_stress
Bioassay type	In vitro
Adverse Outcome Pathway	Various
Test species	*E.coli (Escherichia coli)*
Cell line/ cell strain	K12 MG1665
Transgene	GFP

Measured endpoints/ molecular targets	Measured via
Cytotoxicity	Optical density (OD600)
GFP expression	Fluroescence

Brief description of test

For each strain, fusion of stress promoters to GFP protein gene provides a mechanism for detection of modulation of cellular signaling, which makes analyses of differential expression of genes easier and more accurate. The short time required to complete a test makes use of live cell arrays rapid, economical, high-throughput biosensor systems for detecting toxicity and determining effects on specific signaling pathways.

Document history

Version	Modification	Author	Date
A	creation	Miao Guan, Xiaowei Zhang	20/09/2015

Regulatory aspects

Not regulated, in vitro test.

Experimental conditions

Exposure duration	4 h
Main determinant	IC10, GFP fluroescence
Effect equivalent EQ	-
Unit of effect equivalent	-
Concentration range in standard curve	-
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	Assessing the Toxicity of Naphthenic Acids Using a Microbial Genome Wide Live Cell Reporter Array System
Deviation from standard guideline?	No guideline available

Describe deviation from standard guideline
N/A

Assay format	384-well plate
Volume per well/ vessel	75 μ L

Test media

Solvent	DMSO
Max solvent concentration in test media	5%
Reference media	LB-Lennox plus 25 mg/L kanamycin

Composition of reference media
Every 1L LB-Lennox medium contains 5g NaCl (JKHD, Beijing), 5g Yeast Extract (OXOID, LP0021) and 10g Tryptone (OXOID, LP0042). The medium should be sterilized, and then cool down to room temperature. Add 1mL 25 mg/L kanamycin of every 1L medium. The medium should be stored at 4°C.

Renewal type	Static
Renewal frequency	None (4 h assay)

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	OD600=0.1 (~10^8cells)
Age of organisms	logarithmic phase
Developmental stage of organisms	logarithmic phase
Feeding?	none
Frequency of feeding	-
Temperature controlled?	Yes
Temperature	37°C
Photoperiod	No light
Light intensity	No light
Culturing conditions	37°C
Growth Medium	LB-Lennox plus 25 mg/L kanamycin

Statistical parameters and quality control

Number of experimental repetitions	2
Number of replicates per treatment	1
Number of concentrations tested per sample	3
Positive control tested?	No
Positive control substance	-
Solvent control tested?	Yes
Other controls tested?	Yes – promoterless strains
pH of sample in test medium tested?	Yes
pH adjusted	Yes
DO of sample in test medium measured?	No
Conductivity of sample in test medium measured?	No
Ammonium/ nitrite content measured?	No

Experimental outline

Stock solution preparation:

- Sample extracts and controls are suspended in DMSO.
- Final DMSO concentration is constant across all experimental treatments.

Sample and control preparation:

- For the highest concentrations for each sample extract/ control, stock solutions are added to assay medium to give a final concentration of ≤ 5% DMSO.
- They are then serial diluted as required in solvent control (DMSO).

Reporter live cell array:

- Strains of E. coli Stress Responsive Reporter genes were inoculated into a fresh 96-well plate from a stock plate by use of disposable replicators.
- Cells were incubated at 37 ℃ for 3 h in 96-well plate and then transferred into 384-well plate (Thermo, 142761, LOT 1073030) using multi-pipettes to make each well containing 58μL medium with bacteria. 2 μl of DMSO (solvent control) or sample solutions were added into individual wells on the 384-well plate.
- GFP intensity of each well was consecutively monitored every 10 min for 4 h by a Synergy H4 hybrid microplate reader (excitation/emission: 485 nm /528 nm) (BioTek Instruments Inc, Winooski, VT, USA).

Image analysis

- The gene expression was calculated by comparing the fold change between the sample and the control of the same reporter gene after some statistical analysis.
- Changes in gene expression with a correlation between response and concentrations as well as time with α=0.001 and a fold change >1.5 were considered as significant.

Data analysis

Software used: R and GraphPad Prism

Cytotoxicity test:

- Only concentrations below IC10 of the extract samples are considered for further reporter gene assay.

Reporter gene live cell array data analysis:

- Raw GFP readings are divided by the OD value, we get a preliminary value that reflect the activity of our target genes.
- The result matrix is smoothed by calculating the moving average of every neighboring three time points.
- To eliminate the type of background noise, the GFP expression produced by the eight promoterless plasmid values are averaged (two promoterless plasmids at four treatments) and subtracted from the values of each gene at the corresponding time point in both experimental and control tests.
- The promoter activity of each gene might be different at the onset of the experiment, so the values of the same gene at time point one in four treatments are averaged, and the differences between the averages and each of the 4 values are calculated. Then, the differences are subtracted from the values of each gene at all of the subsequent time points to eliminate the internal measurement noise. In order to filter the system noise, any value will be set to zero if it is less than twice the amount of the standard deviation of the aforementioned processed promoterless values.
- Evaluate the effects of gene expression fold change between the chemicals and the control of the same reporter gene.
- Hierarchical clustering analysis on concentration- and time-dependent gene expression patterns are performed for the selected genes by use of ToxClust, which is a method for evaluating multivariate responses programmed in R.
- The gene expression was calculated by comparing the fold change between the sample and the control of the same reporter gene after some statistical analysis.
- Changes in gene expression with a correlation between response and concentrations as well as time with α=0.001 and a fold change >1.5 were considered as significant.

Quality control

Pipettes:

Ensure that all pipettors are calibrated at least once a year.

Assay replicates:

Minimum 2 independent repeats of the assay. Responsive genes are considered when they are responsive at both of the 2 independent repeats.

Bioassay main features

Bioassay ID	WP12 bioassay_Yeast_mutant
Bioassay type	In vitro
Adverse Outcome Pathway	Genotoxicity
Test species	*Yeast (Saccharomyces cerevisiae)*
Cell line/ cell strain	BY4743 strains
Transgene	Single-gene knockout

Measured endpoints/ molecular targets	**Measured via**
Cytotoxicity	Optical density (OD600)
Reads of each strain	Sequencing

Brief description of test

Yeast genome-wide knockout mutant library pool, which contains 4000 strains of single gene mutant. We screen the whole library pool with the concentration of wild-type IC10, IC20, and IC50 of each chemical. Bioinformatics were used to analysis the enriched strains and sensitive strains of different concentrations and different chemicals. Knockout mutants were considered to be sensitive strains or resistant strains if the reads in the presence of the compound was significantly reduced or increased compared to the DMSO blank, respectively. Chemicals could be grouped due to hierarchical cluster analysis of hit mutants. We put chemicals into specific mode-of-action groups by finding the “chemical-genetic interaction” profiles, and then interpret toxicity mechanism of novel chemicals with similar activities.

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Miao Guan, Xiaowei Zhang	20/09/2015

Regulatory aspects

The described test is carried out on *Yeast* cellls with optical density at 600 nm(OD600) 0.1.

Experimental conditions

Exposure duration	24 h
Main determinant	Reads of each strain with the

	concentration of IC10, IC20 and IC50
Effect equivalent EQ	-
Unit of effect equivalent	-
Concentration range in standard curve	-
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	-
Deviation from standard guideline?	No guideline available

Describe deviation from standard guideline
N/A

Assay format	sequencing
Volume per well/ vessel	1.5mL

Test media

Solvent	DMSO
Max solvent concentration in test media	1%
Reference media	YPD medium with 200 μg/ml G418

Composition of reference media
All yeast mutant strains are grown in YPD medium plus 200 μg/ml G418 (Invitrogen, 11811) at 30°C with 200 rpm shaking. Every 1L YPD medium contains 5g yeast extract (OXOID, LP0021) and 5g tryptone (OXOID, LP0042) and 10g glucose (SIGMA, G7021). The medium with yeast extract and tryptone should be sterilized using sterilizer and glucose should be filtration sterilization with 22 μm filtration membrane, and then cool down to room temperature. Add 1mL 200 mg/mL G418 of every 1L medium. The medium should be stored at 4°C.

Renewal type	Static
Renewal frequency	None (24 h assay)

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	OD600=0.1 (~10^6cells)
Age of organisms	logarithmic phase
Developmental stage of organisms	logarithmic phase
Feeding?	none
Frequency of feeding	-
Temperature controlled?	Yes
Temperature	30°C
Photoperiod	No light
Light intensity	No light
Culturing conditions	30°C
Growth Medium	YPD medium with 200 μg/ml G418

Statistical parameters and quality control

Number of experimental repetitions	1
Number of replicates per treatment	3
Number of concentrations tested per sample	3
Positive control tested?	No
Positive control substance	-
Solvent control tested?	Yes
Other controls tested?	No
pH of sample in test medium tested?	Yes
pH adjusted	Yes
DO of sample in test medium measured?	No
Conductivity of sample in test medium measured?	No
Ammonium/ nitrite content measured?	No

Experimental outline

Stock solution preparation:

- Sample extracts and controls are suspended in DMSO.
- Final DMSO concentration is constant across all experimental treatments.

Sample and control preparation:

- For the highest concentrations for each sample extract/ control, stock solutions are added to assay medium to give a final concentration of ≤ 1% DMSO.

- They are then serial diluted as required in solvent control (DMSO).

Yeast mutant screen array:

- Strains of pooled mutant strains from the freezer are recovery and then add 10 μl stock pooled strains into 3ml fresh YPD medium using 15ml centrifuge tube. The stock pooled strains are returned to -80 °C fridge. Cells are incubated at 30 °C with 200rpm shaking overnight.
- 10 μL pooled strains from overnight are added into fresh medium to make the OD600 0.1±0.02 which cells are in logarithmic phase.
- 30 μl test sample or chemical with concentration of IC50 (IC20/ IC10) is added to reach 3ml culture system and shaking incubated at 30℃ for 24 hours.
- Centrifuge the 3ml cells at 4,000*g for 10 min at room temperature. Extract the yeast DNA using OMEGA yeast DNA Kit.
- PCR the target DNA sequence with designed primer. The PCR reaction system and thermal cycle environment is listed (Table 1 and Table 2).
- Specified length DNA fragment is obtained by agarose gel electrophoresis and gel purification. Purify the DNA fragment from agarose gels using Promega Wizard SV Gel and PCR Clean-Up System.
- DNA is diluted to 100 pM for Ion Proton Sequencer sequencing. The sequencing standard operation protocol can be seen in Ion PIT^{M} Sequencing 200 Kit V3.

Table 1. PCR reaction system.

PCR reaction system	Volume (μL)
Nuclease-Free Water	11.2
F518 5X Thermo phusion HF buffer	4
2.5 mM dNTPs	2
10 μM F primer	0.8
10 μM R primer	0.8
Thermo phusion hot start II DNA polymerase 2U/μl	0.2
DNA template	1
total	20

Table 2. PCR thermal cycle.

Temperature	Time

	94℃	3min	
	94℃	30s	28 cycles
	55℃	30s	
	72℃	30s	
	72℃	3min	
	4℃	∞	

Image analysis

- Individual unique barcode was used to distinguish different samples. The reads of each strain from each sample were normalized to balance the abundance of the total reads of each sample. Mutant strains were considered to be sensitive strains (log2folchange>0) or resistant strains (log2folchange<0) when padj<0.01 using DEseq2 package in the statistical environment R.

Data analysis

Software used: R and GraphPad Prism

Cytotoxicity test:

- Concentrations C10, IC20 and IC50 of the extract samples are considered for further yeast mutant screen assay.

Mutant screen data analysis:

- Cutoff length of the reads to obtain the target sequences.
- Individual unique barcode is used to distinguish different samples (or chemicals).
- The reads of each strain from each sample (or chemical) are normalized to balance the abundance of the total reads of each sample (or chemicals).
- Mutant strains are considered to be sensitive strains or resistant strains when padj<0.01 using DEseq2 package in the statistical environment R. Mutants with foldChange>0 are resistant mutants and mutants with foldchange<0 are sensitive mutants.
- Gene enrichment analysis is done using clusterProfiler package.

Quality control

Pipettes:
Ensure that all pipettors are calibrated at least once a year.

Assay replicates:
Minimum 3 independent repeats of the assay. DEseq2 package in the statistical environment R will determine the responsive mutants via the 3 independent repeats between the treatment and control.

Bioassay main features

Bioassay ID	WP12 bioassay MDAkb2_androgenicity
Bioassay type	*in vitro*
Adverse Outcome Pathway	Androgen receptor activation leading to reproductive disfunction in fish
Test species	-
Cell line/ cell strain	MDA-kb2
Transgene	Luciferase protein reporter gene under control of ARE response element

Measured endpoints/ molecular targets	Measured via
Activation of AR receptor	Luciferase activity measured with reader

Brief description of test

MDA-kb2 is human mammary carcinoma derived cell line transfected with luciferase gene under control of androgenic receptor (AR).
The bioassay is used in an attempt to identify a variety of compounds that could potentially elicit effects such as carcinogenicity, developmental toxicity and endocrine disruption since these effect have been connected with affecting activity of AR.

Document history

Version	Modification	Author	Date
A	creation	Jiří Novák	23/09/15

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{20} from log-logistic concentration effect model
Effect equivalent EQ	Dihydrotestosterone (DHT)
Unit of effect equivalent	ng/L
Concentration range in standard curve	10^{-11} – 10^{-8} M
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline

Assay format	96-well plate
Volume per well/ vessel	Final volume 150µl (100µl cell seeding, 50µl dosing of compounds)

Test media

Solvent	DMSO
Max solvent concentration in test media	0.5%
Reference media	Assay medium

Composition of reference media
L-15 (Sigma Aldrich, L1518) 10% dextran-charcoal-treated Fetal Bovine Serum (Sigma Aldrich, F6765)

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	40 000 cell per well, 100uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C incubator
Growth Medium	L-15 (Sigma Aldrich, L1518) 10% Fetal Bovine Serum superior (Biochrom, S 0615)

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	6
Positive control tested?	Yes
Positive control substance	dihydrotestosterone (DRC with 6 concentrations, concentration range 10^{-11} – 10^{-8} M)
Solvent control tested?	0.5% DMSO in assay medium
Other controls?	
pH of sample in test medium tested?	Yes- visually by change of colour of assay medium
pH adjusted	Buffered by medium to pH 7.3- adjusted with NaOH if needed
DO of sample in test medium measured?	Not tested
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured
Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in DMSO; DHT stock solution (200 x10^{-8} M) is prepared in DMSO and diluted 200-times in medium before dosing

Cell seeding:
40 000 cells per well, 100uL per well in 96-well (Bio Greiner One 655098)
Place plates in incubator (humidified 37°C) for 24h

Dosing of cells:
50 µl/well of assay media containing the test chemical or sample in DMSO (final cocnetration 0.5% v/v), and incubated for 24 h in incubator (humidified 37°C)

Detection:

- Assay medium removed, cell washed with PBS and 25 uL of lysis buffer added (Promega E153A)
- After 15 min of shaking, measurement of luminescence with luminometer

Data analysis

Software used: Excel and GraphPad

Luminescence data analysis:

- calculate the average and SD for the triplicates cells for the fluorescence data
- Normalize the data (average data for solvent control= 0, maximal response of 10^{-8} M DHT= 100

- Subtract the average background from all controls and samples emissions (= net signal)
  $L_{norm} = (L - L_{solventcontrol}) / (L_{max} - L_{solventcontrol})$
- The concentration-response model is a log- logistic model of signal which is normalised to the maximum effect induced by a reference compound and the minimum effect of the controls, from which an EC_{50} or EC_{10}, the effect concentration for 50% of activation of DHT is derived.
  $$effect = \frac{100}{1+10^{slope(\log EC_{50} - \log concentraion)}}$$

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and positive control (10^{-8} M DHT) in triplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum DHT effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication of calibration series of DHT (dose-response curve, EC50);
3) Between-runs (inter-assay) replication of calibration series of DHT (dose-response curve, EC50);

Bioassay main features

Bioassay ID	WP12 bioassay MDAkb2_anti-androgenicity
Bioassay type	*in vitro*
Adverse Outcome Pathway	Androgen receptor activation leading to reproductive disfunction in fish
Test species	-
Cell line/ cell strain	MDA-kb2
Transgene	Luciferase protein reporter gene under control of ARE response element

Measured endpoints/ molecular targets	Measured via
Activation of AR receptor	Luciferase activity measured with reader

Brief description of test

MDA-kb2 is human mammary carcinoma derived cell line transfected with luciferase gene under control of androgenic receptor (AR).
The bioassay is used in an attempt to identify a variety of compounds that could potentially elicit effects such as carcinogenicity, developmental toxicity and endocrine disruption since these effect have been connected with affecting activity of AR.

Document history

Version	Modification	Author	Date
A	creation	Jiří Novák	25/09/15

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	IC_{50} or IC_{20} from log-logistic concentration effect model
Effect equivalent EQ	Flutamide
Unit of effect equivalent	ug/L
Concentration range in standard curve	0,033-5 uM
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline

Assay format	96-well plate
Volume per well/ vessel	Final volume 150µl (100µl cell seeding, 50µl dosing of compounds)

Test media

Solvent	DMSO
Max solvent concentration in test media	1%
Reference media	Assay medium

Composition of reference media
L-15 (Sigma Aldrich, L1518) 10% dextran-charcoal-treated Fetal Bovine Serum (Sigma Aldrich, F6765)

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	40 000 cell per well, 100uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C incubator
Growth Medium	L-15 (Sigma Aldrich, L1518) 10% Fetal Bovine Serum superior (Biochrom, S 0615)

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	6
Positive control tested?	Yes
Positive control substance	flutamide (DRC with 6 concentrations, concentration range 0,033-5 uM) Fixed concentration of dihydrotestosterone (10^{-10} M) throughout the assay except solvent control
Solvent control tested?	1% DMSO in assay medium
Other controls?	negative control - dihydrotestosterone (10^{-10} M; 1% DMSO)
pH of sample in test medium tested?	Yes- visually by change of colour of assay medium
pH adjusted	Buffered by medium to pH 7.3- adjusted with NaOH if needed
DO of sample in test medium measured?	Not tested
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured
Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in DMSO; DHT stock solution ($200x10^{-8}$ M) is prepared in DMSO and diluted 200-times in medium before dosing

Cell seeding:
40 000 cells per well, 100uL per well in 96-well (Bio Greiner One 655098)
Place plates in incubator (humidified 37°C) for 24h

Dosing of cells:
50 µl/well of assay media containing the test chemical or sample in DMSO and dihydrotestosterone 10^{-10} M (final concentration od DMSO 1% v/v), and incubated for 24 h in incubator (humidified 37°C) together with negative control (10^{-10} M dihydrotestosterone) and solvent control.

Detection:

- Assay medium removed, cell washed with PBS and 25 uL of lysis buffer added (Promega E153A)
- After 15 min of shaking, measurement of luminescence with luminometer

Data analysis

Software used: Excel and GraphPad

Luminescence data analysis:

- calculate the average and SD for the triplicates cells for the fluorescence data
- Normalize the data (average data for solvent control= 0, maximal response of 10^{-10} M DHT= 100

- Subtract the average luminescence background from all controls and samples luminescence (= net signal)
  $L_{norm} = (L - L_{solventcontrol}) / (L_{max} - L_{solventcontrol})$
- The concentration-response model is a log- logistic model of signal which is normalised to the maximum effect induced by a DHT and the minimum effect of the controls, from which an EC_{50} or EC_{10}, the effect concentration for 50% of activation of DHT is derived.
  $$effect = \frac{100}{1+10^{slope(\log EC_{50} - \log concentraton)}}$$

Quality control
Pipettors: Ensure that all pipettors are calibrated at least once a year. Plate setup : On every plate include a negative (medium) control and positive control (10^{-8} M DHT) in triplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum DHT effect. Determine : 1) Within-plate (internal) replication indicative of the operational variability; 2) Between-plates (intra-assay) replication of calibration series of DHT (dose-response curve, EC50); 3) Between-runs (inter-assay) replication of calibration series of DHT (dose-response curve, EC50);

Bioassay main features

Bioassay ID	WP12 bioassay_CAFLUX
Bioassay type	*in vitro*
Adverse Outcome Pathway	Aryl hydrocarbon receptor activation leading to multiple toxic outcomes in fish (e.g. immunotoxicity, carcinogenesis, endocrine disruption)
Test species	-
Cell line/ cell strain	CAFLUX H4G1.1c2
Transgene	Green fluorescent protein reporter gene under control of DRE response element

Measured endpoints/ molecular targets	**Measured via**
Activation of AhR receptor	Fluorescence measured with reader

Brief description of test

CAFLUX H4G1.1c2 is based on rat hepatocarcinoma cell line and provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to compounds. Reporter plasmid vectors have been constructed that contain the enhanced green fluorescence protein (GFP) under control of aryl hydrocarbon receptor (AhR) activity. Great advantage of the bioassay is fact that the fluorescence signal can be read in living cells (without lysis) and the cells can be later used for cytotoxicity detection. The bioassay is used in an attempt to identify a variety of compounds that could potentially elicit effects such as immunotoxicity, carcinogenicity, developmental toxicity and endocrine disruption since these effect have been connected with activation of AhR.

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Jiří Novák	23/09/15

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{20} from log-logistic concentration effect model
Effect equivalent EQ	TCDD
Unit of effect equivalent	pg/L
Concentration range in standard curve	0.41-500 pM
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline

Assay format	96-well plate
Volume per well/ vessel	Final volume 150µl (100µl cell seeding, 50µl dosing of compounds)

Test media

Solvent	DMSO
Max solvent concentration in test media	0.5%
Reference media	DMEM

Composition of reference media
DMEM (Biosera LM-D1109/50) 10% Fetal Bovine Serum superior (Biochrom, S 0615)

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	15 000 cell per well, 100uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	DMEM (Biosera LM-D1109/50) 10% Fetal Bovine Serum superior (Biochrom, S 0615)

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	6
Positive control tested?	Yes
Positive control substance	Tetrachlorodibenzo-*p*-dioxin (DRC with 6 concentrations, concentration range 0.41- 500 pM)
Solvent control tested?	0.5% DMSO in assay medium
Other controls?	
pH of sample in test medium tested?	Yes- visually by change of color of assay medium
pH adjusted	Buffered by medium to pH 7.3- adjusted with NaOH if needed
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured
Number of experimental repetitions	
Number of replicates per treatment	Three intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in DMSO; TCDD stock solution (2 µM) is prepared in DMSO and diluted 200-times in medium before dosing

Cell seeding:
15 000 cells per well, 100uL per well in 96well (Bio Greiner One 655096)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
50 µl/well of assay media containing the test chemical or sample in DMSO, and incubated for 24 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Assay medium removed, cell washed with PBS and 50 uL of DMEM w/o phenol red (Biosera added
- Requirements reader are excitation filter 485nm, emission filter 520 nm

Data analysis

Software used: Excel and GraphPad

GFP data analysis:

- calculate the average and SD for the triplicates cells for the fluorescence data
- Normalize the data (average data for solvent control= 0, maximal response of 500 pM TCCD= 100

- Subtract the average green background from all controls and samples green emissions (= net green signal)

$$F_{norm} = (F - F_{solventcontrol}) / (F_{max} - F_{solventcontrol})$$

- The concentration-response model is a log- logistic model of the B/G ratios which are normalised to the maximum effect induced by a reference compound and the minimum effect of the controls, from which an EC_{50} or EC_{10}, the effect concentration for 50% of activation of TCDD is derived.

$$effect = \frac{100}{1 + 10^{slope(\log EC_{50} - \log concentration)}}$$

Quality control
Pipettors: Ensure that all pipettors are calibrated at least once a year. Plate setup : On every plate include a negative (medium) control and positive control (500 pM TCDD) in triplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum TCDD effect. Determine : 1) Within-plate (internal) replication indicative of the operational variability; 2) Between-plates (intra-assay) replication of calibration series of TCDD (dose-response curve, EC50); 3) Between-runs (inter-assay) replication of calibration series of TCDD (dose-response curve, EC50);

Bioassay main features

Bioassay ID	Ames mutagenicity_Activation_In vitro_48h_ Salmonella typhimurium
Bioassay type	in vivo
Adverse Outcome Pathway	
Test species	*Salmonella typhimurium* TA 98
Cell line/ cell strain	-
Transgene	-

Measured endpoints/ molecular targets	**Measured via**
His-revertants	pH indicator bromocresol purple

Brief description of test

The *Salmonella / microsome* fluctuation assay (Ames fluctuation test) was originally developed by Bruce Ames and co-workers using agar plates and later developed as a fluctuation assay incubated in liquid culture in microplates. It uses the bacteria *Salmonella typhimurium* to determine the mutagenic potential of samples. All bacteria used in the test are histidine-deficient mutants of *Salmonella typhimurium* that are unable to grow in histidine-free culture media. The TA98 strain contains a frameshift mutation (+2 type) hisD3052. Mutagenic agents may be able to induce mutations in the marker genes, and the bacteria can be reverted to synthesize the amino acid histidine again. The so called His-revertants can grow in histidine-deficient medium. During the incubation period, nutrients in the exposure medium are metabolized, leading to acidification. Since only reverted bacteria can survive in a histidin deficiency solution, the acidification is an indicator of the reverse mutation of bacteria. Such an acidification is indicated by a change in colour of the pH indicator bromocresol purple. The mutagenicity of the test sample is obtained by counting the number of wells that shifted from purple to yellow, compared to the negative control. In addition, as the bacteria has no metabolic capacity to activate promutagenic substances into DNA damaging metabolites, the S9 fraction (rat liver microsomal activation system) can be included in exposure.

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Ying Shao	23/09/15

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	48h
Main determinant	EC10 from log-logistic concentration effect model and NOEC value
Effect equivalent EQ	Number of revertant wells
Unit of effect equivalent	-
Concentration range in standard curve	-
Bioassay performed to standard guideline?	Yes
Guideline no. or reference for published method	ISO 11350:2012 Water quality -- Determination of the genotoxicity of water and waste water - Salmonella/microsome fluctuation test (Ames fluctuation test)
Deviation from standard guideline?	Yes

Describe deviation from standard guideline
In ISO GUILINE, sample were prepared in 24-well Plates directly. In this test, sample frist prepared in 96-well plates, then transfer to 24-well plates with tester strains.

Assay format	384-well plate
Volume per well/ vessel	50 µl

Test media

Solvent	DMSO
Max solvent concentration in test media	2%
Reference media	Reversion indicator medium

Composition of reference media

Substance	Molecular weight in [g/mol]	Molarity in [mM]	Concentration in [g/L]	Concentration in % [w/v]
Magnesiumsulfat-7-Hydrat	246,48	0,9	0,2	0,02
Citrat-Monohydrat	210,14	10,3	2,2	0,2
Di-Kaliumhydrogenphosphat	174,18	62,1	10,8	1,1
Natriumammoniumhydrogenphosphat -4-Hydrat	209,07	18,1	3,8	0,38
Bromkresolpurpur	563,21	0,05	0,03	0,003
D-Biotin	244,31	$10,6*10^{-3}$	$2,6*10^{-3}$	$2,6*10^{-4}$

D-Glukose	180,16	24	4,3	0,43

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	The cell density should be 180 FAU in the test cultures
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Temperature controlled?	Yes
Temperature	37°C ± 1°C
Photoperiod	-
Light intensity	-

Statistical parameters and quality control

Culturing conditions	37°C ± 1°C
Growth Medium	• 7.5 g meat extract 'Lab-Lemco' / L • 7.5 g Peptone / L • 5.0 g of Sodium chloride / L
Number of experimental repetitions	3
Number of replicates per treatment	48 wells per concentration
Number of concentrations tested per sample	6
Positive control tested?	Yes
Positive control substance	For assays with TA 98 without S9: 4-nitro-o-phenylenediamine(4-NOPD) For assay with TA 98 ,with S9: 2-aminoanthracene (2-AA)
Solvent control tested?	Yes
Other controls tested?	no
pH of sample in test medium	7.5 ± 0.1
pH adjusted	Yes
DO of sample in test medium measured?	Not test
Conductivity of sample in test medium measured?	10.4 mS / cm.
Ammonium/ nitrite content measured?	No

Experimental outline

Overnight culture

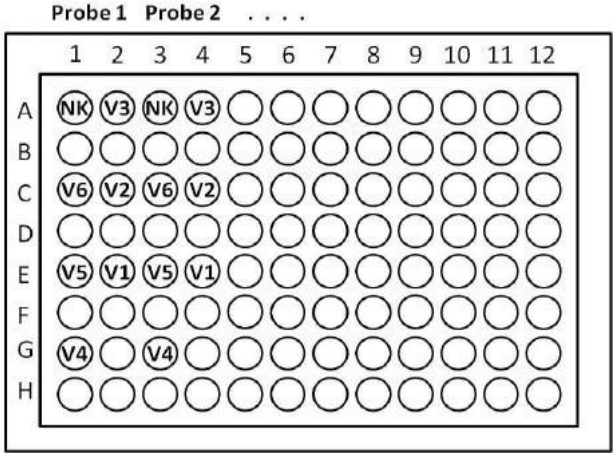
- Pipette 20 mL growth medium (autoclaved) into 100 mL Erlenmeyer flask for each strain.
- Add 20 µL ampicillin solution to each flask and mix gently.
- Immediately after thawing add 20 µL of the respective test strain (TA98 or TA100).
- Incubate the bacteria culture overnight in a shaking incubator at 37°C ± 1°C, 150 rpm for 8-10h (9h45m). After the overnight period, keep bacteria on ice before continuation of test procedure.

OD measurement

- Measure the optical density (OD) of the overnight cultures (ONC) immediately before exposure.
- Measure the OD with a 1:10 dilution of the ONC otherwise the FAU will be out of range. Therefore, pipette 900 µL of the medium into a cuvette, and add 100 µL of bacteria (after mixing well the Erlenmeyer flask with the ONC). For the blanks pipette 1 mL exposure medium into the cuvette.
- Measure cell densities (OD595) . Subtract the blank from the OD values of each strain.
- Calculate the required dilution factor and the exposure medium volume to be added to the ONC in order to adjust the cell density to 180 FAU for TA 98

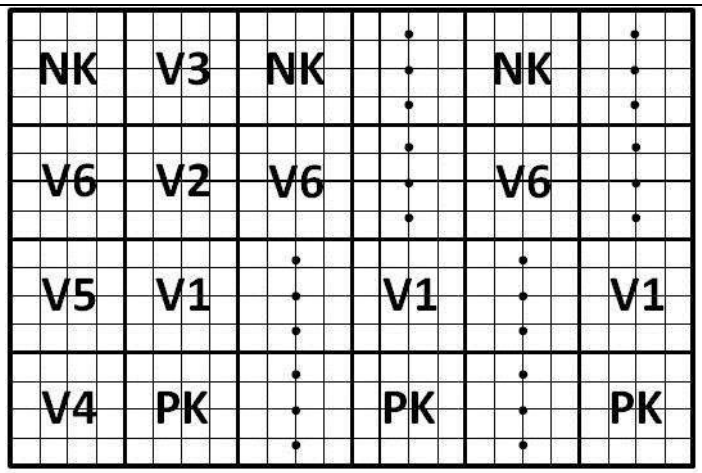
Sample dilution

- Homogenize samples before testing. Work under sterile conditions. Do serial dilution of samples in DMSO in the 96-well plates following 1:2 dilution steps. DMSO is used as negative control.
- Add 24 µL of DMSO to the negative control, and to the wells V2, V3, V4, V5 and V6
- Pipette 48 µL of the sample extract in V1 , then transfer 24 µL from V1 to V2 and mix well; repeat this procedure until V6.



Exposure:

- Sample exposure in 24 well plates, each well of the 96-well plate corresponds to one well in the 24-Well plate, so each sample is pipetted into two columns.
- Pipette 490 µL of the bacteria dilution , and then 10 µL of control or sample into each well .
- Add the S9 mix into each +s9 well.
- Put the plates in a shaking incubator (150 rpm, 37°C) for 100 min.
- Pipette 2.5mL of the reversion indicator medium in each well of the 24-well plate (60 mL per plate). Avoid contact between tip and solution.
- Transfer 50 µL from the 24-well plates into the 384-well plates for controls and samples according to Figure 3.
- Incubate the 384-well plates at 37°C for 48 h.

- 

Data acquisition

Measurement of revertant growth: Score each 384-well plate for the number of positive (yellow) and negative (purple) wells in each 48 well area.

Data analysis

- The test sample is regarded as mutagenic if there is a significant concentration-dependent increase of the number of wells with revertant growth over the tested concentration range; and/or a reproducible increase in the number of revertant wells per 48-well group at one or more concentrations in at least one strain with or without S9-mix.
- For pair-wise comparisons use ANOVA methods.The data is transformed (arcsin) and checked for normal-distribution and for homogeneity of variances. To determine NOEC/LOEC use Dunnett´s, Williams-test, or the Welch-t test (Bonferroni).

Quality control

The test is considered valid if:

- The mean value of negative controls is > 0 and ≤ 10 wells with revertant growth per 48-well area at all testing conditions (±S9-mix, tester strains TA98 and TA100).
- The mean value for positive controls is ≥25 wells with revertant growth per 48-well area at all testing conditions (±S9-mix, tester strains TA98 and TA100).

If one or both of these criteria are not met, a part of the test (e.g. only one testing condition) or the entire test is invalid.

Bioassay main features

Bioassay ID	WP12 bioassay_FET_RWTH
Bioassay type	*in vivo*
Adverse Outcome Pathway	-
Test species	*Danio rerio*
Cell line/ cell strain	-
Transgene	-

Measured endpoints/ molecular targets	Measured via
mortality	microscopic observation

Brief description of test

Zebrafish embryo is one of the most popular model organisms in developmental genetics and (eco)toxicity assessment. Owing to the very well correlation with acute toxicity in adults, easy to use, practical, rapid, and sensitive for assessing aquatic quality, it has gained growing interest as a refinement or even replacement for the acute fish test. From these advantages, the zebrafish embryo has been widely used to test the toxicities of environmental relevant contaminants, polluted water, waste water, suspended particulate matter, whole sediments, and sediment eluates or extracts. After exposure to the test chemical or environmental sample the lethal and sublethal effects such as rare or no pigments, edema, malformed spine, no somite primordium are recorded by inspection using an inverse microscope. Embryo toxicity and teratogenicty are then determined by these malformations and effects.

Document history

Version	Modification	Author	Date
A	creation	Ying Shao	23/09/15

Regulatory aspects

Embryos 120 hpf are not considered animals and thus the assay does not require any animal experiment permission

Experimental conditions

Exposure duration	48h
Main determinant	LC50
Effect equivalent EQ	Percent mortality
Unit of effect equivalent	-
Concentration range in standard curve	-
Bioassay performed to standard guideline?	Yes
Guideline no. or reference for published method	OECD Guidelines for the Testing of Chemicals, Section 2 Test No. 236: Fish Embryo Acute Toxicity (FET) Test
Deviation from standard guideline?	Yes

Describe deviation from standard guideline
In the OECD GUIDELINE zebrafish embryos are individually exposed in 24-well microtiter plates and 20 eggs for each test concentration. In this test, zebrafish embryos are individually exposed in 96-well microtiter plates and 10 eggs for each test concentration.

Assay format	96-well plate
Volume per well/ vessel	200 µl

Test media

Solvent	DMSO
Max solvent concentration in test media	0.5 %
Reference media	Artificial water

Composition of reference media

- 294.0 mg/l Calcium chloride, $CaCl_2 \cdot 2H_2O$ (10 ml stock solution)
- 123.3 mg/l Magnesium sulfate, $MgSO_4 \cdot 7H_2O$ (10 ml stock solution)
- 63.0 mg/l Sodium hydrogen carbonate, $NaHCO_3$ (10 ml stock solution)
- 5.5 mg/l Potassium chloride, KCl (10 ml stock solution)

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	50
Age of organisms	Not later than 60 minutes post fertilization
Developmental stage of organisms	4, 8, 16 and 32 blastomeres
Feeding?	-
Frequency of feeding	-
Temperature controlled?	Yes
Temperature	26 ± 1 °C
Photoperiod	-
Light intensity	-

Statistical parameters and quality control

Culturing conditions	26 ± 1 °C
Growth Medium	Artificial water
Number of experimental repetitions	10
Number of replicates per treatment	3
Number of concentrations tested per sample	5
Positive control tested?	Yes
Positive control substance	3,4-dichloroaniline (DCA)
Solvent control tested?	Yes
Other controls tested?	Yes
pH of sample in test medium	between pH 6.5 and 8.5
pH adjusted	Yes
DO of sample in test medium measured?	No
Conductivity of sample in test medium measured?	No
Ammonium/ nitrite content measured?	No

Experimental outline

Stock solution preparation:

- Sample extracts and controls are suspended in DMSO
- Final DMSO concentration is constant across all experimental treatments

Sample and control preparation:

- For the highest concentrations for each sample extract/control, stock solutions are added to assay medium to give a final concentration of 0.5% DMSO

- They are then serial diluted as required in DMSO

Plate preparation:

- 200 µl of sample/control per well in 96-well plates (polystyrene; TTP, Trasadingen, Switzerland; cat. Dominique Dutscher #009206)
- Place plates in incubator for 48h

Evaluation of the test is carried out with an inverse microscope at magnifications of 100x and 40x.

Data acquisition

Evaluation of the test is carried out with an inverse microscope at magnifications of 100x and 40x. The obtained information is based on the progress of development by comparing exposed and control groups :

- coagulation of eggs – eggs cloudy white and dark in microscopic light
- blastula – apparently granular, hemispheric structure on top of yolk
- epiboly – epibolic front of cells as distinct bulge
- somites – prime segments of the embryo (anlage after 16 h)
- eye anlage – check for existence of eye anlage
- tail detachment - check for tail detachment from the yolk sac
- heart beat – dinstict heart contractions
- blood circulation – best obeservable on the yolk and in the tail artery
- pigmentation – melanocytes as starlike dots
- teratogenic effects – malformation or underdevelopment of embryos
- edema – bubble-like tissue extension

An embryo is counted as dead, if any one or more of the four following applies after 48 h:

- coagulation
- no heartbeat
- no tail detachment
- lack of somite formation

Data analysis

Software used: Excel and GraphPad Prism

The results are expressed as mortality (%) in difference concentrations of each sample extract/ control.

$$Mortality\,(\%) = \frac{N_{dead-eggs}}{10} \times 100\%$$

The average mortality (%) and appropriate variability indices (S.D.) of respective treatments (usually triplicates) are calculated for each sample. Then the concentration–response curve is established, from which an LC50, the concentration of 50% of mortality of zebraish embryo is derived.

Quality control

Embryo selection :

- Only those eggs are chosen that exhibit normal development and are at least in the 8-cell stage.
- Prior to use, a final control of the selected eggs should be carried out to reduce background mortality in the test.

The test is considered valid if:

- Fertilization rate is above 50 %, with > 80 % being optimal and expectable under normal conditions.
- Mortalities below 10% in negative control
- Mortalities between 20 % and 90 % in positive control

Assay replicates:

Minimum 3 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined level. If replicates vary more than a predetermined value, the assay is performed a fourth time.

Bioassay main features

Bioassay ID	WP12 bioassay_GR-CALUX
Bioassay type	in vitro
Adverse Outcome Pathway	Glucocorticoid receptor activation leading to endocrine dysfunction
Test species	-
Cell line/ cell strain	Human osteoblastic cells (U2-OS)
Transgene	-

Measured endpoints/ molecular targets	Measured via
Binding of chemicals to GR receptor	Fluroescence measured with reader

Brief description of test

GR-CALUX bioassay is based on human osteoblastic osteosarcoma U2-OS cells transfected with a hGRα expression plasmid and a luciferase reporter construct. The U2-OS are genetically engineered to produce the enzyme luciferase in response to a defined pathway that is activated. When performing a bioassay, cells are seeded into 96 well plates. The next day, the medium is replaced by medium containing the compounds/samples to be tested. After a fixed time of exposure, the amount of luciferase is determined using a luminometer. The amount of luciferase produced by the samples is related to known concentrations of reference compound and the final results are therefore expressed as reference compound equivalents.

Document history

Version	Modification	Author	Date
A	creation	Ying Shao	23/09/15

Regulatory aspects

German S1 conformity for labs working with GMOs

Experimental conditions

Exposure duration	24h
Main determinant	EC50 or EC10 from log-logistic concentration effect model
Effect equivalent EQ	Dexamethasone
Unit of effect equivalent	ng/L
Concentration range in standard curve	0.03-100 nM (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline
n/a

Assay format	96-well plate
Volume per well/ vessel	200 µL

Test media

Solvent	DMSO
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
• DMEM without Phenol red, - Gibco 10569-010 • 5% Charcoal-Dextran treated Fetal Bovine Serum – Gibco 12676-011 0.2% Penicillin-Streptomycin – Gibco 15140-122 • 1% non-essential amino acids (MEM 100x)-Gibco 11140-035

Renewal type	No Renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	10*104 cells per ml, 6*104 cells per concentration 5.4*105 cells per replicate
Age of organisms	-

Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Temperature controlled?	Yes
Temperature	37°C
Photoperiod	-
Light intensity	-

Statistical parameters and quality control

Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	• DMEM (high-glucose), with GlutaMAX™ (Invitrogen 10569-010), • 7.5% FBS, dialyzed (Invitrogen 26400-036), • 0.2% Penicillin-Streptomycin – Gibco 15140-122 • 1% non-essential amino acids (MEM 100x)-Gibco 11140-035
Number of experimental repetitions	3 wells
Number of replicates per treatment	3
Number of concentrations tested per sample	9
Positive control tested?	Yes
Positive control substance	Dexamethasone
Solvent control tested?	Yes
Other controls tested?	Yes
pH of sample in test medium	No
pH adjusted	-
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	No
Ammonium/ nitrite content measured?	No

Experimental outline

Stock solution preparation:

- Sample extracts and controls are suspended in DMSO.
- Final DMSO concentration is constant across all experimental treatments.

Cell seeding:

10 000 cells per well, 100uL per well in 96well Poly-D-Lysine coated plates. Place plates in incubator (humidified 37°C/5% CO_2) for 24h.

Sample and control preparation:

- For the highest concentrations for each sample extract/ control, stock solutions are added to assay medium to give a final concentration of 0.1% DMSO.
- They are then serial diluted as required in solvent control.

Plate preparation:

- 200 µl per of sample/ control per well in 96 well plates TTP polystyrene (cat. Dominique Dutscher 009206).
- Place plates in incubator (humidified 37°C/5% CO_2)for 24h.

Data acquisition

Harvesting the cells

- Carefully remove all the medium from the cells.
- wash the cells with 100 µl PBS and carefully
- remove all the PBS.
- Add 30 = µl lysis reagent to the cells in each well.
- Shake the plate for at least five minutes (300 rpm) without heating the plate

Measuring the luciferase activity.

- Prime the appropriate tubes of the luminometer with glow-mix and 0.2 M NaOH.
- Measurement of the microtiter plates with the Luminometer

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:

- Calculate cell viability with the conventional MTT cytotoxicity assay. The MTT cytotoxicity results are expressed as percentase inhibition (%) of Absorbance in treated cells in comparison with control cells (i.e. blank or solvent-treated cells).

$$cell\,viability = \frac{A_{492nm}(sample) - A_{492nm}(cell - freecontrol)}{A_{492nm}((solvent)control) - A_{492nm}(cell - freecontrol)} \times 100\%$$

- Only concentrations that its cellviability is exceed 80% are used for quantification of the GR activity assay.

GR data analysis:

Results expressed in Dexamethasone equivalents(DEQ) were processed using a MS Excel template (provided by BDS) together with an add-in "Solver".

- Relative luminescent units (RLU) calculation

$$Y = \frac{A}{1 + (\frac{x}{B})^c}$$

y : Relative luminescent units (RLU);
x : The concentration in pM(Dexamethasone)/well;
A : maximum responses ;
B : the EC50 (median effective concentration) of the curve;
C : the slope of the curve, respectively ;

- Dex-EQ calculation

$$Dex - EQ = \frac{EC50_{sample}}{EC50_{dexamethasone}} (ngdex - equiv / L)$$

Quality control

The test is considered valid if:

- the results that reached the predefined parameters of r2 of calibration curve ≥0.99,
- a concentration between the LOQ and EC50 (median effective concentration) and a relative standard deviation ≤15%,

Determine:
Between-runs (inter-assay) replication.

Plate setup :
On every plate include a negative (medium) control and usually 9 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum GR activity assay.

Bioassay main features

Bioassay ID	WP12 bioassay_immune_ qPCR
Bioassay type	*in vitro*
Adverse Outcome Pathway	(immunotoxicity)
Test species	Trout
Cell line/ cell strain	primary head kidney leucocytes
Transgene	N/A

Measured endpoints/ molecular targets	Measured via
Cytokines & NFkB	qRT-PCR

Brief description of test

The cytokines are important signal proteins, mediating numerous functions, both in the innate and the adaptive immune system. In this study, a set of representative cytokines, which are related to the pro- or anti-inflammatory response, will be analyzed. The samples for the cytokine measurements are frozen (-80°C) and, once the testing is finished, they will be analyzed all together. For the analysis of the production of cytokines we are going to focus on a combination of pro- and anti-inflammatory cytokines (IL1β, TNFα, IFNγ, IL10, TGFβ) plus NFκB as a central mediator of the inflammatory response.

Document history

Version	Modification	Author	Date
A	creation	Kristina Rehberger	November 2015

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	3 and 19 h
Main determinant	mRNA level of immune-related genes
Effect equivalent EQ	
Unit of effect equivalent	
Concentration range in standard curve	
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	

Describe deviation from standard guideline

Assay format	96-well plate
Volume per well/ vessel	200 µl

Test media

Solvent	Depending on the test compound
Max solvent concentration in test media	
Reference media	Assay medium

Composition of reference media
RPMI Medium (Sigma-Aldrich, R8755) plus 50 mM HEPES, 7mM $NaHCO_3$ (and for cell isolation plus 10 U/ml Heparin)

Renewal type	No renewal of test compound
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	2*10^5 cells / well
Age of organisms	-
Developmental stage of organisms	Juvenile
Feeding?	-

Frequency of feeding	-
Temperature controlled?	-
Temperature	-
Photoperiod	-
Light intensity	-

Statistical parameters and quality control

Culturing conditions	Humidified 17 °C, incubator
Growth Medium	PRMI medium (see above)
Number of experimental repetitions	three technical replicate
Number of replicates per treatment	Min. 3
Number of concentrations tested per sample	3-4
Positive control tested?	Yes
Positive control substance	LPS (lipopolysaccarid)
Solvent control tested?	If used, yes
Other controls tested?	
pH of sample in test medium	
pH adjusted	Buffered by medium to pH 7.4
DO of sample in test medium measured?	no
Conductivity of sample in test medium measured?	no
Ammonium/ nitrite content measured?	no

Experimental outline

Cell isolation :

- Dissection of trout head kidney, mechanically disaggregated
- leukocytes are separated from cell debris and non-immune cells including erythrocytes by density centrifugation using a discontinuous percoll gradient
- After washing, the cell suspension is adjusted to 10^6 viable cells / ml (trypan blue staining).
- Leukocytes are seeded into 96-well plates, in which they incubate overnight to let the cells recover and attach to the bottom of the culture plates; FBS-containing media (0.5 % FBS)

Pre-stimulation:

- FBS-containing medium is removed, together with the non-attached cells
- The obtained adherent cell population is used for the *in vitro* exposure experiments
- For each assay and each time point (short- and long-term exposure; see below) a different 96-well plates is used.

- Halfe of the obtained adherent cells are stimulated with pathogen derived molecules (10 µg/ml lipopolysaccharides; LPS; 3 h) prior to the chemical exposure

Toxicant exposure:

- toxicant exposure is initiated (removing the media by discharging the plate) and the cells are exposed to different concentrations of the test chemicals in fresh, stimuli-free and FBS-free medium.
- The negative control (NC) cells receive toxicant-free medium.
- Three technical replicates are conducted for each test concentration and, at least, three independent experiments.
- The compound concentrations, which are applied in the immunotoxicity assays, correspond to non-cytotoxic concentrations

Data acquisition

Software used: qRT-PCR (Applied Biosystems; 7500 fast system) and Excel

The expression level will be analyzed by the Applied Biosystems software. Results are transferred to excel for further analysis.

Data analysis

Using Excel (preliminary):

- Housekeeping genes etc still need to be chosen

Quality control

The layout of the plates is kept the same for all assays in order to avoide pipetting mistakes

Viability of the cells is determined in range finding assays prior to the compound testing. Parallel to the compound testing, the viability is checked, too.

Bioassay main features

Bioassay ID	WP12 bioassay_NBT_assay
Bioassay type	*in vitro*
Adverse Outcome Pathway	(immunotoxicity)
Test species	Trout
Cell line/ cell strain	primary head kidney leucocytes
Transgene	N/A

Measured endpoints/ molecular targets	Measured via
ROS	Plate reader

Brief description of test

As soon as a microorganism gets ingested by phagocytes, the cells start to digest it. For this intracellular dissolution and destruction of particles, reactive oxygen species (ROS) are produced by the phagocytes. Consequently, this respiratory burst activity is also an essential part of the innate immune response. For the detection of those ROS, the NBT assay is performed. The yellowish, soluble substrate nitroblue tetrazolium (NBT; 1 mg/ml, 2 h incubation) will be reduced within the cells to a blue, insoluble di-formazan dye. This product can be detected at a wavelength of 630 nm (photometric measurement). An increased ROS-production results in increased di-formazan dye formation (if NBT is given in excess). The data is normalized against the blank (cells and media, but no NBT).

Document history

Version	Modification	Author	Date
A	creation	Kristina Rehberger	November 2015

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	3 and 19 h
Main determinant	ROS

Effect equivalent EQ	
Unit of effect equivalent	
Concentration range in standard curve	
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	

Describe deviation from standard guideline

Assay format	96-well plate
Volume per well/ vessel	200 µl

Test media

Solvent	Depending on the test compound
Max solvent concentration in test media	
Reference media	Assay medium

Composition of reference media
RPMI Medium (Sigma-Aldrich, R8755) plus 50 mM HEPES, 7mM $NaHCO_3$ (and for cell isolation plus 10 U/ml Heparin)

Renewal type	No renewal of test compound
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	2*10^5 cells / well
Age of organisms	-
Developmental stage of organisms	Juvenile
Feeding?	-
Frequency of feeding	-
Temperature controlled?	-
Temperature	-
Photoperiod	-

Light intensity	-

Statistical parameters and quality control

Culturing conditions	Humidified 17 °C, incubator
Growth Medium	PRMI medium (see above)
Number of experimental repetitions	three technical replicate
Number of replicates per treatment	Min. 3
Number of concentrations tested per sample	3-4
Positive control tested?	Yes
Positive control substance	LPS (lipopolysaccarid)
Solvent control tested?	If used, yes
Other controls tested?	Background control (no NBT)
pH of sample in test medium	
pH adjusted	Buffered by medium to pH 7.4
DO of sample in test medium measured?	no
Conductivity of sample in test medium measured?	no
Ammonium/ nitrite content measured?	no

Experimental outline

Cell isolation :

- Dissection of trout head kidney, mechanically disaggregated
- leukocytes are separated from cell debris and non-immune cells including erythrocytes by density centrifugation using a discontinuous percoll gradient
- After washing, the cell suspension is adjusted to 10^6 viable cells / ml (trypan blue staining).
- Leukocytes are seeded into 96-well plates, in which they incubate overnight to let the cells recover and attach to the bottom of the culture plates; FBS-containing media (0.5 % FBS)

Pre-stimulation:

- FBS-containing medium is removed, together with the non-attached cells
- The obtained adherent cell population is used for the *in vitro* exposure experiments
- For each assay and each time point (short- and long-term exposure; see below) a different 96-well plates is used.
- Halfe of the obtained adherent cells are stimulated with pathogen derived molecules (10 µg/ml lipopolysaccharides; LPS; 3 h) prior to the chemical exposure

Toxicant exposure:

- toxicant exposure is initiated (removing the media by discharging the plate) and the cells are exposed to different concentrations of the test chemicals in fresh, stimuli-free and FBS-free medium.
- The negative control (NC) cells receive toxicant-free medium.
- Three technical replicates are conducted for each test concentration and, at least, three independent experiments.
- The compound concentrations, which are applied in the immunotoxicity assays, correspond to non-cytotoxic concentrations

Data acquisition

Software used: plate reader (EnSpire) and Excel

The amount of produced ROS is measured with EnSpire. The results are transferred to excel for further analysis.

Data analysis

Using Excel (preliminary):

- Based on the three technical replicates for each test concentration (including the negative control, NC) the means were calculated for each run
- The values of the NC (or the solvent control, if needed) are set as 100 %.
- The mean of the individual tests (based on the technical replicates) were taken together for each concentration and the mean as well as the corresponding SEM were calculated

Quality control

Every plate includes a negative (medium) control.

The layout of the plates is kept the same for all assays in order to avoide pipetting mistakes

Viability of the cells is determined in range finding assays prior to the compound testing. Parallel to the compound testing, the viability is checked, too.

If needed, blanks are included for the assays

Bioassay main features

Bioassay ID	WP12 bioassay_phago_activity
Bioassay type	*in vitro*
Adverse Outcome Pathway	(immunotoxicity)
Test species	Trout
Cell line/ cell strain	primary head kidney leucocytes
Transgene	N/A

Measured endpoints/ molecular targets	**Measured via**
percentage of phagocytically active cells	Flow cytometry

Brief description of test

The phagocytic activity of the immune cells is an essential part of the innate immune response. Particles, like microorganism and pathogens, are ingested by phagocytes in order to degrade them. In this study, the phagocytic activity of trout leukocytes (mainly phagocytes, as they are enriched by the cell isolation procedure) is analyzed by means of fluorescent-labelled latex beads (1 µm in diameter; with applying a cell:bead ratio of 1:12) and flow cytometry (FACS; Fig. 3). The beads are incubated simultaneously with the test chemicals. The analysis of phagocytically active cells is based on gating of different populations, first the cells, afterwards single cells, live cells (by staining of dead cells with PI, propidium iodide), FITC+/- cells (and also a gating based on the amount of ingested beads). The data is normalized against the blank (= beads are added directly before processing the cells for the FACS measurement).

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Kristina Rehberger	November 2015

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	3 and 19 h

Main determinant	Percentage of phagocytically active cells
Effect equivalent EQ	
Unit of effect equivalent	
Concentration range in standard curve	
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	

Describe deviation from standard guideline

Assay format	96-well plate
Volume per well/ vessel	250 µl

Test media

Solvent	Depending on the test compound
Max solvent concentration in test media	
Reference media	Assay medium

Composition of reference media
RPMI Medium (Sigma-Aldrich, R8755) plus 50 mM HEPES, 7mM $NaHCO_3$ (and for cell isolation plus 10 U/ml Heparin)

Renewal type	No renewal of test compound
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	2.5*10^5 cells / well
Age of organisms	-
Developmental stage of organisms	Juvenile
Feeding?	-
Frequency of feeding	-
Temperature controlled?	-
Temperature	-

Photoperiod	-
Light intensity	-

Statistical parameters and quality control

Culturing conditions	Humidified 17 °C, incubator
Growth Medium	PRMI medium (see above)
Number of experimental repetitions	three technical replicate
Number of replicates per treatment	Min. 3
Number of concentrations tested per sample	3-4
Positive control tested?	Yes
Positive control substance	LPS (lipopolysaccarid)
Solvent control tested?	If used, yes
Other controls tested?	Background control (no phago)
pH of sample in test medium	
pH adjusted	Buffered by medium to pH 7.4
DO of sample in test medium measured?	no
Conductivity of sample in test medium measured?	no
Ammonium/ nitrite content measured?	no

Experimental outline

Cell isolation :

- Dissection of trout head kidney, mechanically disaggregated
- leukocytes are separated from cell debris and non-immune cells including erythrocytes by density centrifugation using a discontinuous percoll gradient
- After washing, the cell suspension is adjusted to 10^6 viable cells / ml (trypan blue staining).
- Leukocytes are seeded into 96-well plates, in which they incubate overnight to let the cells recover and attach to the bottom of the culture plates; FBS-containing media (0.5 % FBS)

Pre-stimulation:

- FBS-containing medium is removed, together with the non-attached cells
- The obtained adherent cell population is used for the *in vitro* exposure experiments
- For each assay and each time point (short- and long-term exposure; see below) a different 96-well plates is used.
- Halfe of the obtained adherent cells are stimulated with pathogen derived molecules (10 µg/ml lipopolysaccharides; LPS; 3 h) prior to the chemical exposure

Toxicant exposure:

- toxicant exposure is initiated (removing the media by discharging the plate) and the cells are exposed to different concentrations of the test chemicals in fresh, stimuli-free and FBS-free medium.
- The negative control (NC) cells receive toxicant-free medium.
- Three technical replicates are conducted for each test concentration and, at least, three independent experiments.
- The compound concentrations, which are applied in the immunotoxicity assays, correspond to non-cytotoxic concentrations

Data acquisition

Software used: FlowJo and Excel

Percentage of phagocytically active cells is calculated by specific gating of the cell population. Results are transferred to excel for further analysis.

Data analysis

Using Excel (preliminary):

- Based on the three technical replicates for each test concentration (including the negative control, NC) the means were calculated for each run
- The values of the NC (or the solvent control, if needed) are set as 100 %.
- The mean of the individual tests (based on the technical replicates) were taken together for each concentration and the mean as well as the corresponding SEM were calculated

Quality control

Every plate includes a negative (medium) control.

The layout of the plates is kept the same for all assays in order to avoide pipetting mistakes

Viability of the cells is determined in range finding assays prior to the compound testing. Parallel to the compound testing, the viability is checked, too.

If needed, blanks are included for the assays

Bioassay main features

Bioassay ID	WP12 bioassay_ FET
Bioassay type	*In vivo*
Adverse Outcome Pathway	Wide range of substances exhibiting diverse modes of action
Test species	Zebrafish (*Danio rerio*)
Cell line/ cell strain	–
Transgene	–

Measured endpoints/ molecular targets	Measured via
Mortality (coagulated egg/ fungi, no formation of somites, no detachment of the tail, no heartbeat)	Microscopic observation (Olympus IX70)
Sublethal effects (no formation of eyes, no spontaneous movements, no blood circulation, heart frequence (increase/ decrease), no/ low pigmentation, edema (yolk/ pericard), early hatching, hatching without movement)	Microscopic observation (Olympus IX70)
Teratogenic effects (retardation, malformation of head, malformation of sacculi/ otoliths, malformation of tail, malformation of tip of tail, malformation of heart, modified structure of chorda, scoliosis, rachischisis, yolk deformation, length of tail, behaviour (shivering/ tremor))	Microscopic observation (Olympus IX70)

Brief description of test

The test method intends to determine the acute or lethal toxicity of chemicals on embryonic and larval stages of fish (*Danio rerio*). Newly fertilised zebrafish eggs are exposed to the test chemical for a period of 120 h. The test includes maximum eight decreasing concentrations (serially diluted) of the chemical tested and a control. Every 24 h, four apical observations are recorded as indicators of lethality including coagulation of fertilised eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. Additionally observations of sublethal endpoints (no formation of eyes, no spontaneous movements, no blood circulation, heart frequence (increase/

decrease), no/ low pigmentation, edema (yolk/ pericard), early hatching, hatching without movement), as well as teratogenic endpoints (retardation, malformation of head, malformation of sacculi/ otoliths, malformation of tail, malformation of tip of tail, malformation of heart, modified structure of chorda, scoliosis, rachischisis, yolk deformation, length of tail, behaviour (shivering/ tremor)) are recorded. At the end of the exposure period, acute toxicity is determined based on a positive outcome in any of the observations recorded, and the LC50 and EC50 is calculated, respectively. The test report also includes information related to the conduct of the test, in particular: the oxygen saturation and pH in the beginning and at the end of the test.

Document history

Version	Modification	Author	Date
A	creation	Wibke Busch, Janet Krüger, David Leuthold	09/24/15

Regulatory aspects

The described test is carried out on embryo and larva stages of development (day post fertilisation 0 – 5) excluded from the definition of a laboratory animal according to EU Directive EC86/609.

Experimental conditions

Exposure duration	2 – 120 h
Main determinant	LC50, EC50
Effect equivalent EQ	Percent mortality, percent sublethal effects, percent teratogenic effects
Unit of effect equivalent	µmol/L
Concentration range in standard curve	5.71 – 30.72 µmol/L (3,4-dichloraniline)
Bioassay performed to standard guideline?	OECD test guideline 236
Guideline no. or reference for published method	DOI: 10.1787/9789264203709-en
Deviation from standard guideline?	Yes

Describe deviation from standard guideline
The number of test organisms is reduced to 9 per tested concentration instead of 20. Incubation of the assay is executed under shaking. The test is elongated from 96 h up to 120 h. Besides mortality, sublethal and teratogenic effects are recorded additionally. Furthermore, aerated standard dilution water (ISO 7346-3) is used as test medium.

Assay format	7.5 mL GC-vial
Volume per well/ vessel	6 mL

Test media

Solvent	MeOH/ DMSO
Max solvent concentration in test media	0.1% v/v
Reference media	Standard dilution water according to ISO 7346-3

Composition of reference media

Compound	**Concentration in seperate stock solution (g L^{-1})**	**Final concentration in reference medium (g L^{-1})**
$CaCl_2x2H_2O$	11.76	$294.00x10^{-3}$
$MgSO_4x7H_20$	4.93	$123.25x10^{-3}$
KCl	0.23	$5.75x10^{-3}$
$NaHCO_3$	2.59	$64.75x10^{-3}$

Renewal type	Static
Renewal frequency	–

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	3
Age of organisms	2 – 120 h
Developmental stage of organisms	4-cell – larva
Feeding?	No
Frequency of feeding	–
Temperature controlled?	Yes
Temperature	26 °C
Photoperiod	12:12 h (light:dark)
Light intensity	130 µmol s^{-1} m^{-2}

Statistical parameters and quality control

Culturing conditions	26 °C, 12:12 h (light:dark), light intensity: 130 µmol s^{-1} m^{-2}, pH 6.5 – 8.5
Growth Medium	Standard dilution water according to ISO 7346-3
Number of experimental repetitions	1 – 4
Number of replicates per treatment	3
Number of concentrations tested per sample	≤ 8
Positive control tested?	Yes (approximately five times a year in separate tests)
Positive control substance	3,4-dichloraniline
Solvent control tested?	Yes
Other controls tested?	No
pH of sample in test medium	6.5 – 8.5
pH adjusted	7.4 ± 0.1 (standard dilution water according to ISO 7346-3)
DO of sample in test medium measured?	Yes
Conductivity of sample in test medium measured?	No
Ammonium/ nitrite content measured?	No

Experimental outline

Preparation of standard dilution water according to ISO 7346-3:

- The following solutions are prepared seperately using bidistilled water: (i) 11.76 g L^{-1} calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), (ii) 4.93 g L^{-1} magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), (iii) 2.59 g L^{-1} sodium hydrogen carbonate ($NaHCO_3$), and 0.23 g L^{-1} potassium chloride (KCl)
- Solutions are stored at 4 °C
- 25 mL of each of the four solutions are mixed and are diluted to 1 L with bidistilled water
- The standard dilution water is aerated with oxygen at least for one day
- The pH of the aerated standard dilution water is adjusted to 7.4 ± 0.1 using sodium hydroxide solution or hydrochloric acid, respectively
- Storage of the standard dilution water for maximum one week

Exposure stock solution preparation:

- Depending on the logk_{ow} (*n*-octanol-water partition coefficient), the chemical tested is either

dissolved in standard dilution water ($\log k_{ow} \leq 3$) or in MeOH ($\log k_{ow} > 3$). If the solubility in MeOH is negligible, the substance is dissolved in DMSO. The final concentration of MeOH/ DMSO in the stock solution does not exceed 0.1% (v/v), i.e., the concentration of the tested chemical in MeOH/ DMSO should be 1000-fold higher compared to the most concentrated test medium

- Preparation of stock solutions is carried out maximum one day before exposure (by stirring and, if necessary, by heating to ≤ 50 °C for 4 – 8 h)
- If pH < 6.5 or pH > 8.5, the pH of the stock solution is adjusted using sodium hydroxide solution or hydrochloric acid, respectively
- pH and oxygen saturation of the most concentrated exposure medium and control (standard dilution water with maximum 0.1% (v/v) MeOH/ DMSO) are determined in the beginning and at the end of the test

Exposure conditions:

- 3 newly fertilised eggs (≤ 2 hours post fertilisation) per 7.5 mL GC vial are exposed to 6 mL exposure medium in triplicate for each test concentration
- Six 7.5 mL GC vials each containing 3 newly fertilised eggs in standard dilution water (with maximum 0.1% (v/v) MeOH/ DMSO) serve as negative control
- The sealed test vessels are incubated at 26 °C under shaking and with a photoperiod of 12:12 h (light:dark)

Microscopic observations:

- Every 24 h, observations are recorded as indicators of mortality, sublethal and teratogenic effects:

	Endpoint	t_{24}	t_{48}	$t_{72} – t_{120}$
Lethal	Coagulated egg/ fungi	X	X	X
	No formation of somites	X	X	X
	No detachment of tail	X	X	X
	No heartbeat		X	X
Sublethal	No formation of eyes	X	X	X
	No spontaneous movements	X		
	No blood circulation		X	X
	Heart frequence (increase/ decrease)		X	X
	No/ low pigmentation		X	X
	Oedemata (yolk/ pericard)		X	X
	Early hatching		X	
	Hatching without movement			
Teratogenic	Retardation	X	X	X
	Malformation of head	X	X	X
	Malformation of sacculi/ otoliths	X	X	X
	Malformation of tail	X	X	X
	Malformation of tip of tail	X	X	X
	Malformation of heart	X	X	X
	Modified structure of chorda	X	X	X

	Scoliosis	X	X	X
	Rachischisis	X	X	X
	Yolk deformation	X	X	X
	Length of tail	X	X	X
	Behaviour (shivering, tremor)	X	X	X

General remarks:

- A typical experiment consists of two steps. First, a range-finding experiment with a serial 2/ 3-fold dilution series is performed. Then, a denser serial 1.2 – 1.3-fold dilution series is tested in the range of the lowest exposure concentration causing maximum effect (100% mortality) and the highest exposure concentration causing minimum effect (0% mortality). In case still no data are received above 0% and below 100% mortality, the procedure is repeated with a denser dilution series until sufficient data points are generated.

Data analysis

Software used:

- Excel
- SigmaPlot

Mortality/ sublethal/ teratogenic data analysis:

- Calculate the percentage effect (lethal/ sublethal/ teratogenic) for each exposure concentration tested:

$$\%\ \text{effect} = \frac{N(\text{test organisms showing effect})}{N_{\text{total}}(\text{test organisms})} \cdot 100\%$$

- Create concentration-response curves using a logistic Hill 4 parameter model:

$$y = y_0 + \frac{ax^b}{c^b + x^b}$$

with y = % effect, x = exposure concentration, c = EC50/ LC50, b = Hill slope, a = maximum effect (100%), and y_0 = minimum effect (0%)

- The EC50/ LC50 is estimated from the logistic model

Quality control

Pipettes:

- Pipettes are calibrated at least once a year

FET test setup:

- Every test includes a negative (dilution-water) control and maximum eight dilution points dosed in triplicates

- pH and oxygen saturation of the most concentrated exposure medium and control (standard dilution water with maximum 0.1% (v/v) MeOH/ DMSO) are determined in the beginning and at the end of the test to preclude the interference of these parameters with chemical-induced effects observed in the FET test
- The incubation temperature (26 °C) within the climate chamber is checked frequently
- The test is repeated independently at least once if the examined chemical caused biological effects in the preliminary screening experiment

Validity of the test:

- Overall survival of control treatments should be ≥ 80% until the end of exposure
- pH should be in the range of 6.5 – 8.5 throughout the test period
- oxygen saturation should not fall below 60% until the end of the test

Bioassay main features

Bioassay ID	AhR_CALUX_Activation_In vitro_24h_Rattus rattus
Bioassay type	*in vitro*
Adverse Outcome Pathway	AhR activation leading to embryo toxicity in fish (https://aopwiki.org/wiki/index.php/Aop:21) AhR activation leading to hepatic steatosis (https://aopwiki.org/wiki/index.php/Aop:57) AhR activation leading to uroporphyria (https://aopwiki.org/wiki/index.php/Aop:131) Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicity (https://aopwiki.org/wiki/index.php/Aop:150)
Test species	-
Cell line/ cell strain	H4L1.1c4; genetically engineered from rat hepatoma H4IIe
Transgene	stable expression of AhR responsive luciferase reporter gene plasmid pGudLuc7.5, containing 5 concatenated dioxin responsive domain fragments

Measured endpoints/ molecular targets	Measured via
In response to AhR-active chemicals expression of luciferase is initiated	Luminescence measured with reader

Brief description of test

The AhR rat cell line H4L1.1c4 (Brennan et al., 2015), is a stable AhR responsive luciferase reporter cell line based on the H4IIe rat hepatoma cells. Based on the induction of the reporter gene by the reference compound TCDD (2,3,7,8-Tetrachlordibenzodioxin), AhR rat cells give substantially levels of induction of the reporter gene luciferase.

Brennan, J.C., He, G., Tsutsumi, T., Zhao, J., Wirth, E., Fulton, M.H. and Denison, M.S. (2015). Development of Species-Specific Ah Receptor-Responsive Third Generation CALUX Cell Lines with Enhanced Responsiveness and Improved Detection Limits. Environmental Science & Technology, 49(19): 11903-11912.

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting/Beate Escher	12/09/16

Regulatory aspects
This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} from log-logistic concentration effect model or or $_{EC10}$ from linear cocnentration-effect model up to 40% for weak agonists
Effect equivalent EQ	TCDD
Unit of effect equivalent	ng/L
Concentration range in standard curve	124 – 0.12 pM (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 40µl (30µl cell seeding, 10µl dosing of compounds)

Test media

Solvent	MeOH (DMSO for TCDD reference compound)
Max solvent concentration in test media	1%
Reference media	Assay medium

Composition of reference media

90% DMEM with Glutamax (Gibco 31966-021) 10% FBS (Gibco 10099-141) 100 U/ml penicillin 100 µg/ml streptomycin

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	3500 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	90% DMEM with Glutamax (Gibco 31966-021) 10% FBS (Gibco 10099-141) 100 U/ml penicillin 100 µg/ml streptomycin 0.4 mg/ml geneticin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	TCDD (DRC with 11 concentrations, concentration range 124 – 0.12 pM)
Solvent control tested?	Assay medium used as negative control
Other controls?	-
pH of sample in test medium tested?	No
pH adjusted	Buffered by medium to pH 7.4
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are diluted in assay medium with the final concentration of MeOH equal or less to 1%; a stock of TCDD in DMSO was used and as a last step diluted in medium with the highest concentration in well being 124 pM.

Cell seeding:
3500 cells per well, 30uL per well in 384well black, clear bottom, Poly-D-Lysine coated plates (Corning, cat. # 3845), Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 23 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Remove 20µl of medium and add PrestoBlue solution according to manufacture's protocol (Thermo Fisher Scientific, A13261) for detection of cytotoxicity
- Requirements reader for cytotoxicity detection are excitation filter 560 nm, emission filter 590 nm
- Read fluorescence immediately after adding the reagent (time 0h for potential autofluorescence)
- incubate for 1h at 37°C, read fluorescence again using the same gain for both measurements
- for the detection of the activity of the luciferase reporter gene wash cells twice with PBS, remove PBS, add 20µl lysis buffer (25mM Tris, 1% Triton-X 100, 2mM EDTA, 2mM DTT, 10% Glycerol) and incubate for 10 min at room temperature
- add 20µl of luciferase substrate buffer (20 mM Tricine, 2.67 mM MgSO4, 33.3 mM DTT, 0.1 mM EDTA, 0.261 mM Coenzym A, 0.53 mM ATP, 0.47 mM D-Luciferin), mix and transfer 40µl to white 384well plate (Corning, cat. # 3570)
- Read luminescence immediately after mixing

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 590 nm

$$\text{cell viability} = \frac{F_{665nm}(\text{sample})}{F_{665nm}((\text{solvent})\text{control})}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 \quad \frac{1}{1+10^{\text{slope}(\log EC_{50} \ \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s}\log\left(\frac{10}{90}\div\right)$$

Only concentrations below the EC10 for cell viability are used for quantification of the luciferase activity.

luciferase data analysis:

- calculate the average RLU for the unexposed cells (negativ control)
- The concentration-response model is a log- logistic model of the RLUs which are normalised to the maximum effect induced by a reference compound (typically TCDD) and the minimum effect of the controls

$$\text{effect} = \frac{\text{RLU} \quad \min(\text{RLU})}{\max(\text{RLU}) \quad \min(\text{RLU})}$$

A log-logistic concentration effect curve is then used to derive the EC_{50}

$$\text{effect}(\%) = \frac{100\%}{100\% + 10^{(\log EC50 \ \log C)\,\text{slope}}}$$

For compounds or samples that do not exceed 50% of maximum effect , a linear concentration response modell is used to derive EC_{10}:

$$\text{effect}(\%) = \text{slope} * \text{concentration}$$

$$EC10 = \frac{10\%}{\text{slope}}$$

General remarks:

A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where induction of AhR and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the induction signal and those concentrations cannot be used for the induction data evaluation.

Then concentrations are chosen that are not cytotoxic and a linear dose-response is measured for induction only.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.
Plate setup :
On every plate include a negative (medium) control and usually 11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum TCDD effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

Bioassay main features

Bioassay ID	WP12 bioassay_AREc32
Bioassay type	*in vitro*
Adverse Outcome Pathway	Not defined in the OECD-Wiki
Test species	-
Cell line/ cell strain	genetically engineered from MCF7
Transgene	stable antioxidant response element-driven NRF-2 reporter gene cell line coupled to a luciferase gene

Measured endpoints/ molecular targets	**Measured via**
In response to oxidative stress Nrf2 translocates to the nucleus and initiates transcription of ARE-containing genes	Luminescence measured with reader

Brief description of test

The AREc32 cell line (Wang et al., 2006), is a stable antioxidant response element-driven NRF-2 reporter gene cell line based on the MCF7 breast cancer cells. These cells are derived from the human breast cancer cell line MCF-7, with the addition of a luciferase gene construct attached to the ARE cis-element. The antioxidant response of the AREc32 cells can be measured by luciferase expression, similar to the HepG2 and Hepa 1c1c7 cells mentioned above. Based on the induction of reporter genes by the reference compound t-BHQ (tert-Butylhydroquinone), AREc32 cells give substantially greater levels of induction than HepG2 cells (Wang et al, 2006). The murine cell line Hepa-1c1c7 gives the same levels of induction when challenged with t-BHQ as AREc32 (Wang et al, 2006), yet it lacks the relevance of a human cell line in this context, making the AREc32 cell line a more appropriate bioanalytical tool for assess the effects of toxicants on human health.

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting	08/05/16

Regulatory aspects
This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	$EC_{IR1.5}$ from linear concentration effect model
Effect equivalent EQ	tBHQ
Unit of effect equivalent	ng/L
Concentration range in standard curve	0.05 - 50 µM (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 40µl (30µl cell seeding, 10µl dosing of compounds)

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
90% DMEM with Glutamax (Gibco 31966-021) 10% FBS (Gibco 10099-141) 100 U/ml penicillin 100 µg/ml streptomycin 5 mg/ml geniticin

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	2500 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	90% DMEM with Glutamax (Gibco 31966-021) 10% FBS (Gibco 10099-141) 100 U/ml penicillin 100 µg/ml streptomycin 5 mg/ml geneticin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	tBHQ (DRC with 11 concentrations, concentration range 0.05 - 50 µM)
Solvent control tested?	Assay medium used as negative control
Other controls?	-
pH of sample in test medium tested?	No
pH adjusted	Buffered by medium to pH 7.4
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in assay medium; aliquots of tBHQ are weighed in and stored until the day of the experiment. As a last step of the sample preparation tBHQ is disolved in Methanol (final concentration of MeOH stock 12.5mM) and diluted in medium which corresponds to a concentration of 50 µM in medium before dosing (equals highest concentration in dosing plate, with highest tested concentration in well being 12.5 µM)

Cell seeding:
2500 cells per well, 30uL per well in 384well black, clear bottom, TC treated (Corning, cat. # 3712)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 23 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Remove 20µl of medium and add PrestoBlue solution according to manufacture's protocol (Thermo Fisher Scientific, A13261) for detection of cytotoxicity
- Requirements reader for cytotoxicity detection are excitation filter 560 nm, emission filter 590 nm
- Read fluorescence immediately after adding the reagent (time 0h for potential autofluorescence)
- incubate for 1h at 37°C, read fluorescence again using the same gain for both measurements
- for the detection of the activity of the luciferase reporter gene wash cells twice with PBS, remove PBS, add 20µl lysis buffer (25mM Tris, 1% Triton-X 100, 2mM EDTA, 2mM DTT, 10% Glycerol) and incubate for 10 min at room temperature
- add 40µl of luciferase substrate buffer (20 mM Tricine, 2.67 mM MgSO4, 33.3 mM DTT, 0.1 mM EDTA, 0.261 mM Coenzym A, 0.53 mM ATP, 0.47 mM D-Luciferin), mix and transfer 40µl to white 384well plate (Corning, cat. # 3570)
- Read luminescence immediately after mixing

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 590 nm

$$cell\,viability = \frac{F_{665nm}(sample)}{F_{665nm}((solvent)control)}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 \quad \frac{1}{1+10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s}\log\frac{10}{90}\div$$

Only concentrations below the EC10 for cell viability are used for quantification of the luciferase activity.

luciferase data analysis:

- calculate the average RLU for the unexposed cells (negativ control)
- Calculate the induction ratio IR by dividing the RLU of the sample by the average RLU of the unexposed cells

$$IR = \frac{RLU(sample)}{\frac{\sum_{i=1}^{n} RLU(control)}{n}}$$

- The concentration-response model is a linear model of IR versus concentrations, from which an $EC_{IR1.5}$, the effect concentration that caused an IR of 1.5 is derived.

  $IR = 1 + \text{slope} \times \text{concentration}$

  $EC_{IR1.5}$ =0.5/slope

General remarks:
A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where induction of ARE and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the induction signal and those concentrations cannot be used for the induction data evaluation.
Then concentrations are chosen that are not cytotoxic and a linear dose-response is measured for induction only. Often the window between induction and cytotoxicity is small and no maximum induction can be reached, therefore concentrations are chosen for that step in a way that the maximum induction ratio is 5.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.
Plate setup :
On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum Estradiol effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_GeneBla_AR
Bioassay type	*in vitro*
Adverse Outcome Pathway	Androgen receptor activation leading to reproductive dysfunction in fish
Test species	-
Cell line/ cell strain	GeneBLAzer® AR -UAS-bla GripTite™ cells (genetically engineered from HEK293)
Transgene	beta-lactamase reporter gene under control of a UAS response element

Measured endpoints/ molecular targets	Measured via
Binding of chemicals to AR receptor	Fluroescence measured with reader

Brief description of test

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to compounds. Reporter plasmid vectors have been constructed that contain the Beta-lactamase Reporter under hormone-inducible control of an androgen receptor ligand-binding domain/Gal4 DNA binding domain chimera. The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, substrate concentration, excitation path length, fluorescence detectors, and volume changes. GeneBLAzer® Technology is used in an attempt to identify a variety of compounds that could potentially disrupt normal androgen receptor homeostasis and to examine the mechanism by which they may exert their actions.
(see also manual Invitrogen GeneBLAzer® AR alpha GripTite DA and AR alpha-UAS-bla GripTite Cell-based Assay)

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting	25/09/15

Regulatory aspects
This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{10} from log-logistic concentration effect model
Effect equivalent EQ	R1881
Unit of effect equivalent	ng/L
Concentration range in standard curve	3 pM- 100 nM
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 40µl (30µl cell seeding, 10µl dosing of compounds for exposure), for detection additional 8µl detection reagent

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
Opti-MEM without Phenol red, - Gibco 11058-021 2% Charcoal-Dextran treated Fetal Bovine Serum – Gibco 12676-011 1% Penicillin-Streptomycin – Gibco 15140-122 1mM Sodium Pyruvate – Gibco 11360-070 0.1 mM NEAA – Gibco 11140-050

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	4 000 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	DMEM (high-glucose), with GlutaMAX™ (Invitrogen 10569-010), 10% FBS, dialyzed (Invitrogen 26400-036), 0,1mM NEAA (Invitrogen 11140-050), 25mM HEPES (1 M, pH 7.3, Invitrogen 15630-080), 100 U/ml penicillin, 100 µg/ml streptomycin, 80 µg/ml hygromycin, 80 µg/ml zeocin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	R1881 (DRC with 11 concentrations, concentration range 3 pM- 100 nM)
Solvent control tested?	Assay medium used as negative control
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	Yes
pH adjusted	Buffered by medium to pH 7.3
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured

Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in assay medium; R1881 stock solution is prepared in DMSO and diluted in medium before dosing

Cell seeding:
4 000 cells per well, 30uL per well in 384well Poly-D-Lysine coated plates (Corning, cat. # 3845)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 22 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Add 8ul of FRET detection including ToxBlazer mixture per well
- Requirements reader for blue and green detection are excitation filter 409nm, emission filter 460 nm and 530 nm
- Requirements reader for cytotoxicity detection are excitation filter 600 nm, emission filter 665 nm
- Read fluorescence immediately after adding the substrate buffer (time 0h for potential autofluorescence)
- incubate for 2h at room temperature, read fluorescence again using the same gain for both measurements

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 665 nm

$$\text{cell viability} = \frac{F_{665nm}(\text{sample}) \quad F_{665nm}(\text{cell} \quad \text{free control})}{F_{665nm}((\text{solvent})\text{control}) \quad F_{665nm}(\text{cell} \quad \text{free control})}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 - \frac{1}{1+10^{\text{slope}(\log EC_{50} - \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s}\log\frac{10}{90}$$

Only concentrations below the EC10 for cell viability are used for quantification of the ß-Lactamase activity.

ß-Lactamase data analysis:

- calculate the average for the no cells background control for both the blue (460 nm) and green (530 nm) channels (=average blue background and average green background)
- Subtract the average blue background from all controls and sample blue emission (= net blue signal)

  $$signal_{460nm} = F_{460nm} - F_{460nm}(\text{cell free control})$$
- Subtract the average green background from all controls and samples green emissions (= net green signal)

  $$signal_{530nm} = F_{530nm} - F_{530nm}(\text{cell free control})$$
- Calculate the ratio of blue to green fluorescence (net blue/net green = ratio B/G)

  $$B/G = \frac{signal_{460nm}}{signal_{530nm}} = \frac{F_{460nm} - F_{460nm}(\text{cell free control})}{F_{530nm} - F_{530nm}(\text{cell free control})}$$
- Calculate the average blue to green ratio for your unexposed cells (negative control)
- The concentration-response model is a log- logistic model of the B/G ratios which are normalised to the maximum effect induced by a reference compound and the minimum effect of the controls, from which an EC_{50} or EC_{10}, the effect concentration for 50% of activation of ER is derived.

  $$\text{effect} = \frac{\max(B/G) - \min(B/G)}{\min(B/G) + 10^{\text{slope}(\log EC_{50} - \log\text{concentration})}}$$

General remarks:

A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where induction of ER and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the induction signal and those concentrations cannot be used for the induction data evaluation.

Then concentrations are chosen that are not cytotoxic and a linear dose-response is measured for induction only. Often the window between induction and cytotoxicity is small and no maximum induction can be reached, therefore concentrations are chosen for that step in a way that the maximum induction ratio is 5.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum R1881 effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_GeneBla_ARE
Bioassay type	*in vitro*
Adverse Outcome Pathway	Not defined in the OECD-Wiki
Test species	-
Cell line/ cell strain	ARE-bla Hep G2
Transgene	beta-lactamase reporter gene under control of a UAS response element

Measured endpoints/ molecular targets	Measured via
Binding of chemicals to ARE receptor	Fluroescence measured with reader

Brief description of test

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to compounds. Reporter plasmid vectors have been constructed that contain the Beta-lactamase Reporter under hormone-inducible control. CellSensor™ ARE-bla Hep G2 cells contain a beta-lactamase reporter gene under control of the Anti-oxidant Response Element (ARE) that has been stably integrated into Hep G2 cells. ARE-bla Hep G2 cells respond to tert-butylhydroquinone and Sulforaphane.

The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, substrate concentration, excitation path length, fluorescence detectors, and volume changes.

(see also manual Invitrogen CellSensor ARE-bla Hep G2 Cell-based Assay Protocol)

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting	25/09/15

Regulatory aspects
This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{10} from log-logistic concentration effect model
Effect equivalent EQ	tert-butylhydroquinone
Unit of effect equivalent	ng/L
Concentration range in standard curve	1.97 – 7.52 µM
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 48µl (30µl cell seeding, 10µl dosing of compounds, 8µl detection reagent)

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
Opti-MEM without Phenol red, - Gibco 11058-021 2% Charcoal-Dextran treated Fetal Bovine Serum – Gibco 12676-011 1% Penicillin-Streptomycin – Gibco 15140-122 1mM Sodium Pyruvate – Gibco 11360-070 0.1 mM NEAA – Gibco 11140-050

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	5 000 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	DMEM (high-glucose), with GlutaMAX™ (Invitrogen 10569-010), 10% FBS, dialyzed (Invitrogen 26400-036), 0,1mM NEAA (Invitrogen 11140-050), 25mM HEPES (1 M, pH 7.3, Invitrogen 15630-080), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml blasticidin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	tert-butylhydroquinone (DRC with 11 concentrations, concentration range 1.97 – 7.52 µM)
Solvent control tested?	Assay medium used as negative control
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	Yes
pH adjusted	Buffered by medium to pH 7.3
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in assay medium; aliquots of tBHQ are weighed in and stored until the day of the experiment. As a last step of the sample preparation tBHQ is disolved in Methanol and diluted in medium which corresponds to a concentration of 75 mM in medium before dosing (equals highest concentration in dosing plate, with highest tested concentration in well being 200 µM)

Cell seeding:
5 000 cells per well, 30uL per well in 384well Poly-D-Lysine coated plates (Corning, cat. # 3845)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 22 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Add 8ul of FRET detection including ToxBlazer mixture per well
- Requirements reader for blue and green detection are excitation filter 409nm, emission filter 460 nm and 530 nm
- Requirements reader for cytotoxicity detection are excitation filter 600 nm, emission filter 665 nm
- Read fluorescence immediately after adding the substrate buffer (time 0h for potential autofluorescence)
- incubate for 2h at room temperature, read fluorescence again using the same gain for both measurements

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 665 nm

$$\text{cell viability} = \frac{F_{665nm}(\text{sample}) \quad F_{665nm}(\text{cell} \quad \text{free control})}{F_{665nm}((\text{solvent})\text{control}) \quad F_{665nm}(\text{cell} \quad \text{free control})}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 \quad \frac{1}{1+10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s} \log \frac{10}{90} \div$$

Only concentrations below the EC10 for cell viability are used for quantification of the ß-Lactamase activity.

ß-Lactamase data analysis:

- calculate the average for the no cells background control for both the blue (460 nm) and green (530 nm) channels (=average blue background and average green background)
- Subtract the average blue background from all controls and sample blue emission (= net blue signal)

  $$signal_{460nm} = F_{460nm} \quad F_{460nm}(cell \quad free\ control)$$
- Subtract the average green background from all controls and samples green emissions (= net green signal)

  $$signal_{530nm} = F_{530nm} \quad F_{530nm}(cell \quad free\ control)$$
- Calculate the ratio of blue to green fluorescence (net blue/net green = ratio B/G)

  $$B/G = \frac{signal_{460nm}}{signal_{530nm}} = \frac{F_{460nm} \quad F_{460nm}(cell \quad free\ control)}{F_{530nm} \quad F_{530nm}(cell \quad free\ control)}$$
- Calculate the average blue to green ratio for your unexposed cells (negative control)
- The induction ratio IR is the ratio between B/G of a given sample well and the associated control (cell or cell with solvent control (up to 0.1% DMSO or blown down methanol) unexposed

  $$IR = \frac{B/G(sample)}{B/G((solvent)\ control)}$$
- The concentration-response model is a linear model of IR versus concentrations, from which an $EC_{IR1.5}$, the effect concentration that caused an IR of 1.5 is derived.

  $$IR = 1 + slope \times concentration$$

  $$EC_{IR1.5} = 0.5/slope$$

General remarks:

A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where activation of the transcription factor and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the induction signal and those concentrations cannot be used for the induction data evaluation.

Then concentrations are chosen that are not cytotoxic and a linear dose-response is measured for induction only. Often the window between induction and cytotoxicity is small and no maximum induction can be reached, therefore concentrations are chosen for that step in a way that the maximum induction ratio is 5.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum tert-butylhydroquinone effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_GeneBla_ER
Bioassay type	*in vitro*
Adverse Outcome Pathway	Estrogen receptor activation leading to reproductive dysfunction in fish (https://aopwiki.org/aops)
Test species	-
Cell line/ cell strain	GeneBLAzer® ER alpha -UAS-bla GripTite™ cells (genetically engineered from HEK293)
Transgene	beta-lactamase reporter gene under control of a UAS response element

Measured endpoints/ molecular targets	**Measured via**
Binding of chemicals to ER receptor	Fluroescence measured with reader

Brief description of test

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to compounds. Reporter plasmid vectors have been constructed that contain the Beta-lactamase Reporter under hormone-inducible control of estrogen -responsive DNA enhancer elements. The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, substrate concentration, excitation path length, fluorescence detectors, and volume changes. GeneBLAzer® Technology is used in an attempt to identify a variety of compounds that could potentially disrupt normal estrogen receptor homeostasis and to examine the mechanism by which they may exert their actions.
(see also manual Invitrogen GeneBLAzer® ER alpha GripTite DA and ER alpha-UAS-bla GripTite Cell-based Assay)

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting	11/09/15
	Corrections, cell number adapted	Rita Schlichting / Escher	05/09/2016

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{10} from log-logistic concentration effect model
Effect equivalent EQ	17β-Estradiol
Unit of effect equivalent	ng/L
Concentration range in standard curve	0.001- 2 nM (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 40µl (30µl cell seeding, 10µl dosing of compounds for exposure), for detection additional 8µl detection reagent

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
DMEM without Phenol red, - Gibco 10569-010 2% Charcoal-Dextran treated Fetal Bovine Serum – Gibco 12676-011 1% Penicillin-Streptomycin – Gibco 15140-122 1mM Sodium Pyruvate – Gibco 11360-070 0.1 mM NEAA – Gibco 11140-050

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	3 500 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	DMEM (high-glucose), with GlutaMAX™ (Invitrogen 10569-010), 10% FBS, dialyzed (Invitrogen 26400-036), 0,1mM NEAA (Invitrogen 11140-050), 25mM HEPES (1 M, pH 7.3, Invitrogen 15630-080)

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	17β-Estradiol (DRC with 11 concentrations, concentration range 0.001- 2 nM)
Solvent control tested?	Assay medium used as negative control
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	Yes
pH adjusted	Buffered by medium to pH 7.3
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in assay medium;
Estradiol stock solution (2 µM) is prepared in DMSO and diluted 200-times in medium before dosing

Cell seeding:
3 500 cells per well, 30uL per well in 384well Poly-D-Lysine coated plates (Corning, cat. # 3845)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 22 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Add 8ul of FRET detection including ToxBlazer mixture per well
- Requirements reader for blue and green detection are excitation filter 409nm, emission filter 460 nm and 530 nm
- Requirements reader for cytotoxicity detection are excitation filter 600 nm, emission filter 665 nm
- Read fluorescence immediately after adding the substrate buffer (time 0h for potential autofluorescence)
- incubate for 2h at room temperature, read fluorescence again using the same gain for both measurements

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 665 nm

$$\text{cell viability} = \frac{F_{665nm}(\text{sample}) \quad F_{665nm}(\text{cell free control})}{F_{665nm}((\text{solvent})\text{control}) \quad F_{665nm}(\text{cell free control})}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 \quad \frac{1}{1+10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s}\log \frac{10}{90}\div$$

Only concentrations below the EC10 for cell viability are used for quantification of the ß-Lactamase activity.

ß-Lactamase data analysis:

- calculate the average for the no cells background control for both the blue (460 nm) and green (530 nm) channels (=average blue background and average green background)
- Subtract the average blue background from all controls and sample blue emission (= net blue signal)

$$signal_{460nm} = F_{460nm} \quad F_{460nm}(cell \quad free\ control)$$

- Subtract the average green background from all controls and samples green emissions (= net green signal)

$$signal_{530nm} = F_{530nm} \quad F_{530nm}(cell \quad free\ control)$$

- Calculate the ratio of blue to green fluorescence (net blue/net green = ratio B/G)

$$B/G = \frac{signal_{460nm}}{signal_{530nm}} = \frac{F_{460nm} \quad F_{460nm}(cell \quad free\ control)}{F_{530nm} \quad F_{530nm}(cell \quad free\ control)}$$

- Calculate the average blue to green ratio for your unexposed cells (negative control)
- The concentration-response model is a log- logistic model of the B/G ratios which are normalised to the maximum effect induced by a reference compound and the minimum effect of the controls, from which an EC_{50} or EC_{10}, the effect concentration for 50% of activation of ER is derived.

$$effect = \frac{\max(B/G) \quad \min(B/G)}{\min(B/G) + 10^{slope(\log EC_{50} \quad \log concentration)}}$$

General remarks:

A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where induction of the nuclear receptor and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the B/G signal and those concentrations cannot be used for the data evaluation of the induction of the nuclear receptor.
Then concentrations are chosen that are not cytotoxic for dose response assessment of the activity. For those compounds that do not reach more than 30 % of maximim effect, a linear dose-response is measured for the induction of the nuclear receptor. Often the window between induction and cytotoxicity is small and no maximum induction can be reached.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum Estradiol effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;

2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_GeneBla_GR
Bioassay type	*in vitro*
Adverse Outcome Pathway	Glucocorticoid Receptor Activation Leading to Increased Disease Susceptibility (https://aopwiki.org/aops)
Test species	-
Cell line/ cell strain	GeneBLAzer® GR -UAS-bla GripTite™ cells (genetically engineered from HEK293)
Transgene	beta-lactamase reporter gene under control of a UAS response element

Measured endpoints/ molecular targets	Measured via
Binding of chemicals to GR receptor	Fluroescence measured with reader

Brief description of test

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to compounds. Reporter plasmid vectors have been constructed that contain the Beta-lactamase Reporter under hormone-inducible control of a glucocorticoid receptor ligand-binding domain/Gal4 DNA binding domain chimera. The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, substrate concentration, excitation path length, fluorescence detectors, and volume changes. GeneBLAzer® Technology is used in an attempt to identify a variety of compounds that could potentially disrupt normal receptor homeostasis and to examine the mechanism by which they may exert their actions.
(see also manual Invitrogen GeneBLAzer® GR alpha GripTite DA and GR alpha-UAS-bla GripTite Cell-based Assay)

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting	25/09/15

	Corrections, cell numbers adjusted	Beate Escher	09/05/2016

Regulatory aspects
This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{10} from log-logistic concentration effect model
Effect equivalent EQ	Dexamethasone
Unit of effect equivalent	ng/L
Concentration range in standard curve	10 pM- 100 nM
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 40µl (30µl cell seeding, 10µl dosing of compounds for exposure), for detection additional 8µl detection reagent

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
Opti-MEM without Phenol red, - Gibco 11058-021 2% Charcoal-Dextran treated Fetal Bovine Serum – Gibco 12676-011 1% Penicillin-Streptomycin – Gibco 15140-122 1mM Sodium Pyruvate – Gibco 11360-070 0.1 mM NEAA – Gibco 11140-050

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	4 000 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	DMEM (high-glucose), with GlutaMAX™ (Invitrogen 10569-010), 10% FBS, dialyzed (Invitrogen 26400-036), 0,1mM NEAA (Invitrogen 11140-050), 25mM HEPES (1 M, pH 7.3, Invitrogen 15630-080), 100 U/ml penicillin, 100 µg/ml streptomycin, 80 µg/ml hygromycin, 80 µg/ml zeocin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	Dexamethasone (DRC with 11 concentrations, concentration range 10 pM - 100 nM)
Solvent control tested?	Assay medium used as negative control
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	No
pH adjusted	Buffered by medium to pH 7.4
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium	Not measured

measured?	
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in assay medium; Dexamethasone stock solution (2 µM) is prepared in DMSO and diluted in medium before dosing

Cell seeding:
4 000 cells per well, 30uL per well in 384well Poly-D-Lysine coated plates (Corning, cat. # 3845)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 22 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Add 8ul of FRET detection including ToxBlazer mixture per well
- Requirements reader for blue and green detection are excitation filter 409nm, emission filter 460 nm and 530 nm
- Requirements reader for cytotoxicity detection are excitation filter 600 nm, emission filter 665 nm
- Read fluorescence immediately after adding the substrate buffer (time 0h for potential autofluorescence)
- incubate for 2h at room temperature, read fluorescence again using the same gain for both measurements

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 665 nm

$$\text{cell viability} = \frac{F_{665nm}(\text{sample}) \quad F_{665nm}(\text{cell} \quad \text{free control})}{F_{665nm}((\text{solvent})\text{control}) \quad F_{665nm}(\text{cell} \quad \text{free control})}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 \quad \frac{1}{1+10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s}\log\frac{10}{90}\div$$

Only concentrations below the EC10 for cell viability are used for quantification of the ß-Lactamase activity.

ß-Lactamase data analysis:

- calculate the average for the no cells background control for both the blue (460 nm) and green (530 nm) channels (=average blue background and average green background)
- Subtract the average blue background from all controls and sample blue emission (= net blue signal)

  $$\text{signal}_{460nm} = F_{460nm} \quad F_{460nm}(\text{cell} \quad \text{free control})$$
- Subtract the average green background from all controls and samples green emissions (= net green signal)

  $$\text{signal}_{530nm} = F_{530nm} \quad F_{530nm}(\text{cell} \quad \text{free control})$$
- Calculate the ratio of blue to green fluorescence (net blue/net green = ratio B/G)

  $$B/G = \frac{\text{signal}_{460nm}}{\text{signal}_{530nm}} = \frac{F_{460nm} \quad F_{460nm}(\text{cell} \quad \text{free control})}{F_{530nm} \quad F_{530nm}(\text{cell} \quad \text{free control})}$$
- Calculate the average blue to green ratio for your unexposed cells (negative control)
- The concentration-response model is a log- logistic model of the B/G ratios which are normalised to the maximum effect induced by a reference compound and the minimum effect of the controls, from which an EC_{50} or EC_{10}, the effect concentration for 50% of activation of ER is derived.

  $$\text{effect} = \frac{\max(B/G) \quad \min(B/G)}{\min(B/G)+10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

General remarks:

A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where induction of GR and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the induction signal and those concentrations cannot be used for the induction data evaluation.

Then concentrations are chosen that are not cytotoxic and a linear dose-response is measured for induction only. Often the window between induction and cytotoxicity is small and no maximum induction can be reached, therefore concentrations are chosen for that step in a way that the maximum induction ratio is 5.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum Dexamethasone effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_GeneBla_NFkappaB
Bioassay type	*in vitro*
Adverse Outcome Pathway	Not defined in the OECD AOP Wiki
Test species	-
Cell line/ cell strain	CellSensor® NFκB-bla THP-1 cells
Transgene	beta-lactamase reporter gene under control of a UAS response element

Measured endpoints/ molecular targets	**Measured via**
Binding of chemicals to Nuclear Factor kappa B (NFκB) response element	Fluroescence measured with reader

Brief description of test

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to compounds. Reporter plasmid vectors have been constructed that contain the Beta-lactamase Reporter under control of the Nuclear Factor kappa B (NFκB) response element. The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, substrate concentration, excitation path length, fluorescence detectors, and volume changes.
(see also manual Invitrogen CellSensor® NFκB-bla THP-1 Cell-based Assay)

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Rita Schlichting	25/09/15
	Corrections, cell number adapted	Rita Schlichting / Escher	05/09/2016

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{10} from log-logistic concentration effect model
Effect equivalent EQ	TNFalpha (Note: protein has to be dissolved in PBS)
Unit of effect equivalent	ng/L
Concentration range in standard curve	0.00027 – 0.55 mg/L
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 40µl (30µl cell seeding, 10µl dosing of compounds for exposure), for detection additional 8µl detection reagent

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
Opti-MEM without Phenol red, - Gibco 11058-021 2% Charcoal-Dextran treated Fetal Bovine Serum – Gibco 12676-011 1% Penicillin-Streptomycin – Gibco 15140-122 1mM Sodium Pyruvate – Gibco 11360-070 0.1 mM NEAA – Gibco 11140-050

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	20 000 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	RPMI 1640 (Invitrogen 11875-093), 10% FBS, dialyzed (Invitrogen 26400-036), 0,1mM NEAA (Invitrogen 11140-050), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml blasticidin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	TNFalpha (DRC with 11 concentrations, concentration range 0.00027 – 0.55 mg/L)
Solvent control tested?	Assay medium used as negative control
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	No
pH adjusted	Buffered by medium to pH 7.4
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in assay medium; TNFalpha stock solution is prepared in PBS and diluted in medium before dosing

Cell seeding:
20 000 cells per well, 30 uL per well in 384well Poly-D-Lysine coated plates (Corning, cat. # 3845)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 22 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Add 8ul of FRET detection including ToxBlazer mixture per well
- Requirements reader for blue and green detection are excitation filter 409nm, emission filter 460 nm and 530 nm
- Requirements reader for cytotoxicity detection are excitation filter 600 nm, emission filter 665 nm
- Read fluorescence immediately after adding the substrate buffer (time 0h for potential autofluorescence)
- incubate for 2h at room temperature, read fluorescence again using the same gain for both measurements

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 665 nm

$$\text{cell viability} = \frac{F_{665nm}(\text{sample}) \quad F_{665nm}(\text{cell} \quad \text{free control})}{F_{665nm}((\text{solvent})\text{control}) \quad F_{665nm}(\text{cell} \quad \text{free control})}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 \quad \frac{1}{1+10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s}\log \frac{10}{90} \div$$

Only concentrations below the EC10 for cell viability are used for quantification of the ß-Lactamase

activity.

ß-Lactamase data analysis:

- calculate the average for the no cells background control for both the blue (460 nm) and green (530 nm) channels (=average blue background and average green background)
- Subtract the average blue background from all controls and sample blue emission (= net blue signal)

$$signal_{460nm} = F_{460nm} \quad F_{460nm}(cell \quad free\ control)$$

- Subtract the average green background from all controls and samples green emissions (= net green signal)

$$signal_{530nm} = F_{530nm} \quad F_{530nm}(cell \quad free\ control)$$

- Calculate the ratio of blue to green fluorescence (net blue/net green = ratio B/G)

$$B/G = \frac{signal_{460nm}}{signal_{530nm}} = \frac{F_{460nm} \quad F_{460nm}(cell \quad free\ control)}{F_{530nm} \quad F_{530nm}(cell \quad free\ control)}$$

- Calculate the average blue to green ratio for your unexposed cells (negative control)
- The induction ratio IR is the ratio between B/G of a given sample well and the associated control (cell or cell with solvent control (up to 0.1% DMSO or blown down methanol) unexposed

$$IR = \frac{B/G(sample)}{B/G((solvent)\ control)}$$

- The concentration-response model is a linear model of IR versus concentrations, from which an $EC_{IR1.5}$, the effect concentration that caused an IR of 1.5 is derived.

$$IR = 1 + slope \times concentration$$

$$EC_{IR1.5} = 0.5/slope$$

General remarks:

A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where activation of the transcription factor and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the induction signal and those concentrations cannot be used for the induction data evaluation.

Then concentrations are chosen that are not cytotoxic and a linear dose-response is measured for induction only. Often the window between induction and cytotoxicity is small and no maximum induction can be reached, therefore concentrations are chosen for that step in a way that the maximum induction ratio is 5.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum TNFalpha effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_GeneBla_p53
Bioassay type	*in vitro*
Adverse Outcome Pathway	Estrogen receptor activation leading to reproductive dysfunction in fish
Test species	-
Cell line/ cell strain	CellSensor p53RE-bla HCT-116 cells
Transgene	beta-lactamase reporter gene under control of a UAS response element

Measured endpoints/ molecular targets	Measured via
Binding of chemicals to p53 response elements	Fluroescence measured with reader

Brief description of test

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to compounds. Reporter plasmid vectors have been constructed that contain the Beta-lactamase Reporter under hormone-inducible control of the p53 response elements. The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, substrate concentration, excitation path length, fluorescence detectors, and volume changes.
(see also manual Invitrogen CellSensor p53RE-bla HCT-116 Cell-based Assay)

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting	25/09/15
	Corrections, cell number adapted	Rita Schlichting / Escher	05/09/2016

Regulatory aspects

This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{10} from log-logistic concentration effect model
Effect equivalent EQ	Nutlin
Unit of effect equivalent	ng/L
Concentration range in standard curve	9 pM- 1.1 µM
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 48µl (30µl cell seeding, 10µl dosing of compounds, 8µl detection reagent)

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
Opti-MEM without Phenol red, - Gibco 11058-021 2% Charcoal-Dextran treated Fetal Bovine Serum – Gibco 12676-011 1% Penicillin-Streptomycin – Gibco 15140-122 1mM Sodium Pyruvate – Gibco 11360-070 0.1 mM NEAA – Gibco 11140-050

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	4 000 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	McCoy's 5A Medium (Invitrogen 16600-082), 10% FBS, dialyzed (Invitrogen 26400-036), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml blasticidin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	Nutlin (DRC with 11 concentrations, concentration range 9 pM- 1.1 µM)
Solvent control tested?	Assay medium used as negative control
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	Yes
pH adjusted	Buffered by medium to pH 7.3
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in assay medium; Nutlin stock solution is prepared in DMSO and diluted in medium before dosing

Cell seeding:
4 000 cells per well, 30uL per well in 384well Poly-D-Lysine coated plates (Corning, cat. # 3845)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 22 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Add 8ul of FRET detection including ToxBlazer mixture per well
- Requirements reader for blue and green detection are excitation filter 409nm, emission filter 460 nm and 530 nm
- Requirements reader for cytotoxicity detection are excitation filter 600 nm, emission filter 665 nm
- Read fluorescence immediately after adding the substrate buffer (time 0h for potential autofluorescence)
- incubate for 2h at room temperature, read fluorescence again using the same gain for both measurements

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 665 nm

$$\text{cell viability} = \frac{F_{665nm}(\text{sample}) \quad F_{665nm}(\text{cell} \quad \text{free control})}{F_{665nm}((\text{solvent})\text{control}) \quad F_{665nm}(\text{cell} \quad \text{free control})}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 \quad \frac{1}{1+10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s}\log \frac{10}{90} \div$$

Only concentrations below the EC10 for cell viability are used for quantification of the ß-Lactamase activity.

ß-Lactamase data analysis:

- calculate the average for the no cells background control for both the blue (460 nm) and

green (530 nm) channels (=average blue background and average green background)

- Subtract the average blue background from all controls and sample blue emission (= net blue signal)

$$signal_{460nm} = F_{460nm} \quad F_{460nm}(cell \quad free\ control)$$

- Subtract the average green background from all controls and samples green emissions (= net green signal)

$$signal_{530nm} = F_{530nm} \quad F_{530nm}(cell \quad free\ control)$$

- Calculate the ratio of blue to green fluorescence (net blue/net green = ratio B/G)

$$B/G = \frac{signal_{460nm}}{signal_{530nm}} = \frac{F_{460nm} \quad F_{460nm}(cell \quad free\ control)}{F_{530nm} \quad F_{530nm}(cell \quad free\ control)}$$

- Calculate the average blue to green ratio for your unexposed cells (negative control)
- The concentration-response model is a log- logistic model of the B/G ratios which are normalised to the maximum effect induced by a reference compound and the minimum effect of the controls, from which an EC_{50} or EC_{10}, the effect concentration for 50% of activation of ER is derived.

$$effect = \frac{\max(B/G) \quad \min(B/G)}{\min(B/G) + 10^{slope(\log EC_{50} \ \log concentration)}}$$

General remarks:

A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where induction of ER and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the induction signal and those concentrations cannot be used for the induction data evaluation.

Then concentrations are chosen that are not cytotoxic and a linear dose-response is measured for induction only. Often the window between induction and cytotoxicity is small and no maximum induction can be reached, therefore concentrations are chosen for that step in a way that the maximum induction ratio is 5.

Quality control

Pipettors:

Ensure that all pipettors are calibrated at least once a year.

Plate setup :

On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum Nutlin effect.

Determine :

1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_GeneBla_PPARgamma
Bioassay type	*in vitro*
Adverse Outcome Pathway	PPAR gamma activation leading to impaired fertility in males and PPAR gamma activation leading to impaired fertility in females (https://aopwiki.org/aops, accessed 9 May 2016)
Test species	-
Cell line/ cell strain	GeneBLAzer® PPAR gamma-UAS-bla 293H cells (genetically engineered from HEK293)
Transgene	beta-lactamase reporter gene under control of an UAS response element

Measured endpoints/ molecular targets	Measured via
Binding of chemicals to peroxisome proliferator-activated receptor gamma (PPARγ)	Fluroescence measured with reader

Brief description of test

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to compounds. Reporter plasmid vectors have been constructed that contain the Beta-lactamase Reporter under hormone-inducible control of estrogen - responsive DNA enhancer elements. The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, substrate concentration, excitation path length, fluorescence detectors, and volume changes. GeneBLAzer® Technology is used in an attempt to identify a variety of compounds that could potentially disrupt normal estrogen receptor homeostasis and to examine the mechanism by which they may exert their actions.
(see also manual Invitrogen GeneBLAzer® PPAR gamma 293H DA and PPAR gamma-UAS-bla 293H Cell-based Assay Protocol)

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting	09/05/16

Regulatory aspects

This is an *in vitro* assay, not regulated.

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{10} from log-logistic concentration effect model
Effect equivalent EQ	Rosiglitazone
Unit of effect equivalent	ng/L
Concentration range in standard curve	1- 1000 nM (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 40µl (30µl cell seeding, 10µl dosing of compounds for exposure), for detection additional 8µl detection reagent

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
Opti-MEM without Phenol red, - Gibco 11058-021 2% Charcoal-Dextran treated Fetal Bovine Serum – Gibco 12676-011 1% Penicillin-Streptomycin – Gibco 15140-122 1mM Sodium Pyruvate – Gibco 11360-070 0.1 mM NEAA – Gibco 11140-050

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	4 000 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	90% DMEM with Glutamax (Gibco, 31966-021) 10% FBS, dialyzed (Gibco 26400-036) 0,1mM NEAA (Gibco 11140-050), 25mM HEPES (1 M, pH 7.3, Gibco 15630-080) 1 mM Sodiumpyruvate (Gibco, 11360-039) 100 U/ml penicillin 100 µg/ml streptomycin 100 µg/ml hygromycin 500 µg/ml geniticin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	Rosiglitazone (DRC with 11 concentrations, concentration range 1- 1000 nM)
Solvent control tested?	Assay medium used as negative control
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	NO
pH adjusted	Buffered by medium to pH 7.4
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in assay medium; Rosiglitazone is weighed and aliquots are blown down that allow preparation of a stock solution (4 µM) in medium before dosing

Cell seeding:
4 000 cells per well, 30uL per well in 384well Poly-D-Lysine coated plates (Corning, cat. # 3845)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 22 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Add 8ul of FRET detection including ToxBlazer mixture per well
- Requirements reader for blue and green detection are excitation filter 409nm, emission filter 460 nm and 530 nm
- Requirements reader for cytotoxicity detection are excitation filter 600 nm, emission filter 665 nm
- Read fluorescence immediately after adding the substrate buffer (time 0h for potential autofluorescence)
- incubate for 2h at room temperature, read fluorescence again using the same gain for both measurements

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 665 nm

$$\text{cell viability} = \frac{F_{665nm}(\text{sample}) \quad F_{665nm}(\text{cell} \quad \text{free control})}{F_{665nm}((\text{solvent})\text{control}) \quad F_{665nm}(\text{cell} \quad \text{free control})}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 \quad \frac{1}{1+10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s} \log \frac{10}{90} \div$$

Only concentrations below the EC10 for cell viability are used for quantification of the ß-Lactamase activity.

ß-Lactamase data analysis:

- calculate the average for the no cells background control for both the blue (460 nm) and green (530 nm) channels (=average blue background and average green background)
- Subtract the average blue background from all controls and sample blue emission (= net blue signal)

  $$\text{signal}_{460nm} = F_{460nm} \quad F_{460nm}(\text{cell} \quad \text{free control})$$
- Subtract the average green background from all controls and samples green emissions (= net green signal)

  $$\text{signal}_{530nm} = F_{530nm} \quad F_{530nm}(\text{cell} \quad \text{free control})$$
- Calculate the ratio of blue to green fluorescence (net blue/net green = ratio B/G)

  $$B/G = \frac{\text{signal}_{460nm}}{\text{signal}_{530nm}} = \frac{F_{460nm} \quad F_{460nm}(\text{cell} \quad \text{free control})}{F_{530nm} \quad F_{530nm}(\text{cell} \quad \text{free control})}$$
- Calculate the average blue to green ratio for your unexposed cells (negative control)
- The concentration-response model is a log- logistic model of the B/G ratios which are normalised to the maximum effect induced by a reference compound and the minimum effect of the controls, from which an EC_{50} or EC_{10}, the effect concentration for 50% of activation of ER is derived.

  $$\text{effect} = \frac{\max(B/G) \quad \min(B/G)}{\min(B/G) + 10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

General remarks:

A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where induction of PPARgamma and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the induction signal and those concentrations cannot be used for the induction data evaluation. Then concentrations are chosen that are not cytotoxic and a linear dose-response is measured for induction only.

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum Estradiol effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_GeneBla_PR
Bioassay type	*in vitro*
Adverse Outcome Pathway	Not defined in the OECD-Wiki
Test species	-
Cell line/ cell strain	GeneBLAzer® PR -UAS-bla GripTite™ cells (genetically engineered from HEK293)
Transgene	beta-lactamase reporter gene under control of a UAS response element

Measured endpoints/ molecular targets	Measured via
Binding of chemicals to PR receptor	Fluroescence measured with reader

Brief description of test

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to compounds. Reporter plasmid vectors have been constructed that contain the Beta-lactamase Reporter under hormone-inducible control of progesterone receptor ligand binding domain/Gal4 DNA binding domain chimera. The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, substrate concentration, excitation path length, fluorescence detectors, and volume changes. GeneBLAzer® Technology is used in an attempt to identify a variety of compounds that could potentially disrupt normal PR receptor homeostasis and to examine the mechanism by which they may exert their actions.
(see also manual Invitrogen GeneBLAzer® PR alpha GripTite DA and PR alpha-UAS-bla GripTite Cell-based Assay)

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting	25/09/15
	Corrections, cell number adapted	Rita Schlichting / Escher	05/09/2016

Regulatory aspects
This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	24h
Main determinant	EC_{50} or EC_{10} from log-logistic concentration effect model
Effect equivalent EQ	Promegestone
Unit of effect equivalent	ng/L
Concentration range in standard curve	10 pM- 10 nM
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	
Deviation from standard guideline?	n/a

Describe deviation from standard guideline:
n/a

Assay format	384-well plate
Volume per well/ vessel	Final volume 40µl (30µl cell seeding, 10µl dosing of compounds for exposure), for detection additional 8µl detection reagent

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	Assay medium

Composition of reference media
Opti-MEM without Phenol red, - Gibco 11058-021 2% Charcoal-Dextran treated Fetal Bovine Serum – Gibco 12676-011 1% Penicillin-Streptomycin – Gibco 15140-122 1mM Sodium Pyruvate – Gibco 11360-070 0.1 mM NEAA – Gibco 11140-050

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	4 500 cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	humidified 37°C/5% CO_2 incubator
Growth Medium	DMEM (high-glucose), with GlutaMAX™ (Invitrogen 10569-010), 10% FBS, dialyzed (Invitrogen 26400-036), 0,1mM NEAA (Invitrogen 11140-050), 25mM HEPES (1 M, pH 7.3, Invitrogen 15630-080), 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml hygromycin, 80 µg/ml zeocin

Statistical parameters and quality control

Number of experimental repetitions	
Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	Promegestone (DRC with 11 concentrations, concentration range 10 pM- 10 nM)
Solvent control tested?	Assay medium used as negative control
Other controls?	No cells background control = cellfree wells with medium only
pH of sample in test medium tested?	No
pH adjusted	Buffered by medium to pH 7.4
DO of sample in test medium measured?	Not tested, CO_2 level 5% in incubator
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Methanol extracts of samples are blown down to dryness and resuspended in assay medium; Promegestone stock solution (2 µM) is prepared in DMSO and diluted in medium before dosing

Cell seeding:
4 500 cells per well, 30uL per well in 384well Poly-D-Lysine coated plates (Corning, cat. # 3845)
Place plates in incubator (humidified 37°C/5% CO_2) for 24h

Dosing of cells:
10 µl/well of dosing media containing the test chemical or sample, and incubated for 22 h in incubator (humidified 37°C/5% CO_2)

Detection:

- Add 8ul of FRET detection including ToxBlazer mixture per well
- Requirements reader for blue and green detection are excitation filter 409nm, emission filter 460 nm and 530 nm
- Requirements reader for cytotoxicity detection are excitation filter 600 nm, emission filter 665 nm
- Read fluorescence immediately after adding the substrate buffer (time 0h for potential autofluorescence)
- incubate for 2h at room temperature, read fluorescence again using the same gain for both measurements

Data analysis

Software used: Excel and GraphPad

Cytotoxicity data analysis:
Calculate cell viability from fluoresence measured at 665 nm

$$\text{cell viability} = \frac{F_{665nm}(\text{sample}) \quad F_{665nm}(\text{cell} \quad \text{free control})}{F_{665nm}((\text{solvent})\text{control}) \quad F_{665nm}(\text{cell} \quad \text{free control})}$$

The concentration-response model is a log- logistic model, from which an EC_{50} or EC_{10}, the effect concentration for 10% of inhibition of cell viability is derived.

$$\text{cell viability} = 1 - \frac{1}{1+10^{s\times(\log EC_{50} - \log(\text{concentration of reference compound or REF of sample}))}}$$

$$\text{cell viability} = 1 \quad \frac{1}{1+10^{\text{slope}(\log EC_{50} \quad \log\text{concentration})}}$$

$$\log EC_{10} = \log EC_{50} - \frac{1}{s}\log\frac{10}{90}\div$$

Only concentrations below the EC10 for cell viability are used for quantification of the ß-Lactamase activity.

ß-Lactamase data analysis:

- calculate the average for the no cells background control for both the blue (460 nm) and green (530 nm) channels (=average blue background and average green background)
- Subtract the average blue background from all controls and sample blue emission (= net blue signal)

$$signal_{460nm} = F_{460nm} \quad F_{460nm}(cell \quad free\ control)$$

- Subtract the average green background from all controls and samples green emissions (= net green signal)

$$signal_{530nm} = F_{530nm} \quad F_{530nm}(cell \quad free\ control)$$

- Calculate the ratio of blue to green fluorescence (net blue/net green = ratio B/G)

$$B/G = \frac{signal_{460nm}}{signal_{530nm}} = \frac{F_{460nm} \quad F_{460nm}(cell \quad free\ control)}{F_{530nm} \quad F_{530nm}(cell \quad free\ control)}$$

- Calculate the average blue to green ratio for your unexposed cells (negative control)
- The concentration-response model is a log- logistic model of the B/G ratios which are normalised to the maximum effect induced by a reference compound and the minimum effect of the controls, from which an EC_{50} or EC_{10}, the effect concentration for 50% of activation of ER is derived.

$$effect = \frac{\max(B/G) \quad \min(B/G)}{\min(B/G) + 10^{slope(\log EC_{50} \quad \log concentration)}}$$

General remarks:

A typical experiment consists of two steps (each performed in duplicate). First, a range finder with a 10-fold or serial (2-fold) dilution series, where induction of ER and cytotoxicity are evaluated. Interference by cytotoxicity causes a suppression of the induction signal and those concentrations cannot be used for the induction data evaluation.

Then concentrations are chosen that are not cytotoxic and a linear dose-response is measured for induction only. Often the window between induction and cytotoxicity is small and no maximum induction can be reached, therefore concentrations are chosen for that step in a way that the maximum induction ratio is 5.

Quality control

Pipettors:

Ensure that all pipettors are calibrated at least once a year.

Plate setup :

On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum promegestone effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_Microtox
Bioassay type	*in vitro*
Adverse Outcome Pathway	Not in https://aopwiki.org/aops
Test species	-
Cell line/ cell strain	*Aliivibrio fischeri*
Transgene	-

Measured endpoints/ molecular targets	Measured via
Inhibition of bioluminescence after 30 min incubation with sample	Luminescence measured with reader

Brief description of test

The luminescent bacteria test (MICROTOX) is a quick non-specific test, which is simple to handle and able to measure the total toxicity of a sample. The test organism *Aliivibrio fischeri* is a gram negative naturally luminescent marine bacterium. The measuring criterion is the bioluminescence of the organism. The luminescent process is an anaerobic oxidation process.

$$FMNH2 + O2 + R–CHO \longrightarrow FMN + H2O + R–COOH + hv\ (490nm)$$

The bacterial luminescence is coupled directly to the metabolic condition of the cell. Toxic substances, which cause changes in the status of the cell, cause thus an inhibition of the metabolism. This is apparent by the decreasing of the luminescence. Thus the bioluminescent inhibition can be used as measure for the toxicity of a sample. Due to the short exposure time of the test of 30 min, it mainly registers baseline toxicity (with exception of uncouplers and antibiotics).

Document history

Version	Modification	Author	Date
A	creation	Rita Schlichting	29/04/16

Regulatory aspects
This is an *in vitro* assay, not regulated

Experimental conditions

Exposure duration	30 min
Main determinant	EC_{50} from log-logistic concentration effect model
Effect equivalent EQ	Baseline TEQ with virtual baseline toxicant (Escher BI, Bramaz N, Mueller JF, Quayle P, Rutishauser S, Vermeirssen ELM. 2008. Toxic equivalent concentrations (TEQs) for baseline toxicity and specific modes of action as a tool to improve interpretation of ecotoxicity testing of environmental samples. J Env Monitor 10:612-621.
Unit of effect equivalent	mg/L
Concentration range in standard curve	Phenol 0.02 to 21.25mM (serially diluted)
Bioassay performed to standard guideline?	Yes
Guideline no. or reference for published method	DIN ISO 11348-1:2007
Deviation from standard guideline?	Yes

Describe deviation from standard guideline:
• Incubation at room temperature instead of 15°C • 384well format instead of cuvettes • Bacteria are grown as liquid culture in a cultivation automate (Regensburger Leuchtbakterientest – Kultivierungsautomat)

Assay format	384-well plate
Volume per well/ vessel	Final volume 60µl (30µl seeding of bacteria, 30µl dosing of compounds)

Test media

Solvent	MeOH
Max solvent concentration in test media	3%

Reference media	buffer

Composition of reference media
20.22 g Sodium chloride 2.035 g Magnesium chloirde - Hexahydrate 0.298 g Potassium chloride 4.185 g MOPS (Morpholin0-propanesulfonic acid) 1.0 g Sodium hydroxide (Plates) dissolve in 1l MQ-Water and adjust pH to 7 +/- 0,2, store at 4°C

Renewal type	No renewal
Renewal frequency	-

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	$6*10^5$ cells per well, 30uL per well
Age of organisms	-
Developmental stage of organisms	-
Feeding?	-
Frequency of feeding	-
Culturing conditions	in the dark at 20°C with mild stirring
Growth Medium	30 g Sodium chloride 0.204g Magnesium sulfate - Heptahydrate 2.75g Dipotassium hydrogenphosphate trihydrate 6.1g Sodium dihydrogenphosphate mono-hydrate 0.5g Diammonium sulfate 0.5 g Yeast-extract 5.0 g Tryptone 3ml Glycerol dissolve in 1L MQ-Water and adjust pH to 7.0 +/- 0.2, autoclave at 121°C, store at 4°C

Statistical parameters and quality control

Number of experimental repetitions	

Number of replicates per treatment	Two intra-plate replicates plus minimum 2 independent repeats of the assay (inter-assay replication)
Number of concentrations tested per sample	11
Positive control tested?	Yes
Positive control substance	phenol (DRC with 11 concentrations, concentration range 0.02 to 21.25mM)
Solvent control tested?	no
Other controls?	-
pH of sample in test medium tested?	Yes
pH adjusted	According to DIN ISO range of 6.5 to 8 acceptable
DO of sample in test medium measured?	Not tested
Conductivity of sample in test medium measured?	Not measured
Ammonium/ nitrite content measured?	Not measured

Experimental outline

Stock solution preparation:
Samples are dissolved in Methanol. Concentrations of samples are calculated to achieve maximum concentration of dose response curve by diluting 16µl of methanol stock solution in 500µl buffer.

Cell seeding:
6*10^5 cells per well, 30uL per well in 384well white plates (Corning, cat. # 3570) and incubate for 30 min at RT in the dark.

Dosing of cells:
30 µl/well of dosing media containing the test chemical or sample, and incubated for 30min at RT in the dark

Detection:
- Read bioluminescence 30 min after dosing of cells

Data analysis

Software used: Excel and GraphPad

Data analysis of bioluminescence:

- calculate correction factor

$$\text{for } t_0 \text{ corrected bioluminescence } IC = \frac{I_{30}}{I_0}$$

- calculate inhibition of bioluminescence

$$\text{Inhibition of bioluminescence} = \frac{(IC_{control} - IC)}{IC_{control}} \cdot 100\% = \left(1 - \frac{IC}{IC_{control}}\right) \cdot 100\% = \left(1 - \frac{I_{30}}{I_0} \cdot \frac{\text{average}(I_{control,0})}{\text{average}(I_{control,30})}\right) \cdot 100\%$$

- The concentration-response model is a log- logistic model of the inhibition of bioluminescence, from which an EC_{50}, the effect concentration for 50% of inhibition of bioluminescence is derived.
-
- $$\text{inhibitionofbioluminescence}(\%) = \frac{100\%}{10^{\text{slope}(\log EC_{50} \ \log\text{concentration})}}$$

General remarks:

Quality control

Pipettors:
Ensure that all pipettors are calibrated at least once a year.

Plate setup :
On every plate include a negative (medium) control and usually 6-11 dilution points dosed in duplicates. Relative fluorescence units data may vary from plate to plate. Therefore data are analyzed by plate and expressed as the % of maximum Estradiol effect.

Determine :
1) Within-plate (internal) replication indicative of the operational variability;
2) Between-plates (intra-assay) replication;
3) Between-runs (inter-assay) replication;

2 replicates per plate, minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels (ususally 10-15% for internal and intra-assay replication and 15-20% for inter-assay replication). If the coeficient of variance is more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	Ames mutagenicity – Activation - *In vitro* 66h - *Salmonella typhimurium*
Bioassay type	*in vitro*
Adverse Outcome Pathway	Mutations leading to cancer and/or germ cell alterations
Test species	*Salmonella enterica* subspecies I, serovar Typhimurium (*S. typhimurium*)
Cell line/ cell strain	TA1538; TA98; YG1041; YG5185; TA1535; YG7108
Transgene	n/a

Measured endpoints/ molecular targets	**Measured via**
frameshift and base-pair substitutions in DNA	Number of revertants colonies

Brief description of test

The Microplate Agar (MPA) is an adpatation of the Microsuspension Salmonella/microsome assay (Kado test) to reduce media, sample and material. It is based on the ability different strains of bacteria to revert an histidine mutation and grow in a culture media after exposure to single chemical, mixture or environmental sample. The test is performed with and without exogenous metabolic activation (S9). The number of revertants is proportional to the quantity/potency of the mutagens present in the analyzed sample.

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Gisela de Aragão Umbuzeiro	24/09/15

Regulatory aspects

This is an *in vitro* assay, not regulated.

Experimental conditions

Exposure duration	90 minutes
Main determinant	n/a
Effect equivalent EQ	n/a
Unit of effect equivalent	n/a
Concentration range in standard curve	n/a
Bioassay performed to standard guideline?	No
Guideline no. or reference for published method	Manuscript in preparation
Deviation from standard guideline?	Kado NY, Langley D, Eisenstatd E. 1983. A simple modification of the Salmonella liquid incubation assay. *Mutat Res* 121:25–32 modified by DeMarini DM, Dallas MM, Lewtas J. 1989. Cytotoxicity and effect on mutagenicity of buffers in a microsuspension assay. *Teratog Carcinog Mutagen* 9:287–295.

Describe deviation from standard guideline
Instead of using regular petri dishes plates with 2 ml of top agar, we use microplates of 12 wells with 0.2 ml of top agar.

Assay format	Microplate
Volume per well/ vessel	25.5 ul of incubation mixture (0.5 ul of sample, 12.5 ul of bacteria, 12.5 ul of PBS or S9 mix)

Test media: Minimum agar containing biotin and traces of histidine

Solvent	DMSO
Max solvent concentration in test media	2%
Reference media	See Mortelmans K, Zeiger E. 2000. The Ames Salmonella/microsome

	mutagenicity assay. *Mutat Res* 455:29–60.

Composition of reference media
See Mortelmans K, Zeiger E. 2000. The Ames Salmonella/microsome mutagenicity assay. *Mutat Res* 455:29–60.

Renewal type	static
Renewal frequency	n/a

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	1-2 10E10 cells/ml in the culture
Age of organisms	overnight
Developmental stage of organisms	n/a
Feeding?	n/a
Frequency of feeding	n/a
Temperature controlled?	Yes
Temperature	37 °C
Photoperiod	No
Light intensity	n/a

Statistical parameters and quality control

Culturing conditions	180 rpm, 37°C
Growth Medium	Nutrient broth
Number of experimental repetitions	1
Number of replicates per treatment	4
Number of concentrations tested per sample	6
Positive control tested?	Yes
Positive control substance	They vary according to the strain/condition (- / + S9)
Solvent control tested?	Yes
Other controls tested?	Extraction Blank
pH of sample in test medium	n/a
pH adjusted	No
DO of sample in test medium measured?	No

Conductivity of sample in test medium measured?	No
Ammonium/ nitrite content measured?	No

Experimental outline

Overnight cultures (around 10^9 cells/mL) were concentrated 5-fold by centrifugation (10,000g at 4°C for 10 min) and resuspended in 0.015 M sodium phosphate buffer. A volume of 12.5 µL of cell suspension, 12.5 µL of 0.015 M sodium phosphate buffer or S9 mix, and 0.5 µL of the sample were incubated at 37 °C for 90 min without shaking. To this mixture, 200 mL of molten agar was added and poured onto a microplate agar. Colonies were counted after 66 hr incubation at 37 °C by hand using of a stereomicroscope. Toxicity was also carefully evaluated by observing the background of the microplate agar. Metabolic activation was provided by Aroclor 1254-induced Sprague Dawley rat liver S9 mix (MolTox, Boone, NC) and prepared at 5% vol/vol and supplemented with the required co-factors. Data is analyzed by ANOVA followed by Tukey and regression analysis using Salanal Program.

Data acquisition

Excel file

Data analysis

Salanal software provided by Integrated Laboratory Systems, Research Triangle Park, NC.

Quality control

QA/QC procedures :

- Negative and positive controls control charts
- Sterility of the samples
- Blank extraction control
- S9 efficiency

Bioassay main features

Bioassay ID	WP12 bioassay _TW_Daphtox
Bioassay type	*in vivo*
Adverse Outcome Pathway	1,21,22 (Measures Adverse Outcome/ mortality)
Test species	*Daphnia magna*
Cell line/ cell strain	Bham2
Transgene	none

Measured endpoints/ molecular targets	Measured via
Intoxication/ immobilization	observation

Brief description of test

The OECD Guideline for Testing of Chemicals 202 (April 2004) is followed (http://www.oecd-ilibrary.org/environment/test-no-202-daphnia-sp-acute-immobilisation-test_9789264069947-en). Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilisation is recorded at 24 hours and 48 hours and compared with control values. The results are analysed in order to calculate the EC50 at 48h Determination of the EC50 at 24h is optional.

Document history

Version	Modification	Author	Date
B	1	Tim Williams	25/04/16

Regulatory aspects

The described test is carried out on an invertebrate species excluded from the definition of a laboratory animal according to EU Directive EC86/609.

Experimental conditions

Exposure duration	48 h
Main determinant	EC50 or EC10
Effect equivalent EQ	Not applicable
Unit of effect equivalent	Not applicable
Concentration range in standard curve	Not applicable
Bioassay performed to standard guideline?	Yes
Guideline no. or reference for published method	OECD Guideline for Testing of Chemicals 202 (April 2004) (http://www.oecd-ilibrary.org/environment/test-no-202-daphnia-sp-acute-immobilisation-test_9789264069947-en).
Deviation from standard guideline?	Yes

Describe deviation from standard guideline
For LV-SPE exposures the low quantities available necessitated reducing exposure volume to 5ml containing 5 neonate Daphnia. This procedure was also employed with chemical exposures to allow cros-comparison. Environmental samples and chemicals were dissolved in methanol.

Assay format	Single vial
Volume per well/ vessel	5ml

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	OECD medium

Composition of reference media
'OECD Media' Calcium chloride CaCl2, 2H2O 11.76 g/l Magnesium sulfate MgSO4, 7H2O 4.93 g/l Sodium bicarbonate NaHCO3 2.59 g/l

Potassium chloride KCl 0.23 g/l pH 7.4 to 7.6

Renewal type	static
Renewal frequency	n/a

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	5
Age of organisms	<24h
Developmental stage of organisms	neonates
Feeding?	no
Frequency of feeding	n/a
Temperature controlled?	Yes
Temperature	18°C to 22°C
Photoperiod	16-hour light and 8-hour dark cycle
Light intensity	Not measured

Statistical parameters and quality control

Culturing conditions	18°C to 22°C
Growth Medium	'OECD Media' Calcium chloride CaCl2, 2H2O 11.76 g/l Magnesium sulfate MgSO4, 7H2O 4.93 g/l Sodium bicarbonate NaHCO3 2.59 g/l Potassium chloride KCl 0.23 g/l pH 7.4 to 7.6
Number of experimental repetitions	To be decided
Number of replicates per treatment	3-4
Number of concentrations tested per sample	At least five test concentrations
Positive control tested?	Yes
Positive control substance	Potassium dichromate
Solvent control tested?	Yes
Other controls tested?	Yes: Untreated control
pH of sample in test medium	Buffered by medium to pH 7.4 to 7.6
pH adjusted	Not adjusted (as per OECD Guideline)

DO of sample in test medium measured?	no
Conductivity of sample in test medium measured?	no
Ammonium/ nitrite content measured?	no

Experimental outline

Daphnia acute toxicity testing

- Equipment: 7ml glass 'bijou' vials (labelled), 5ml Gilson pipette, Plastic transfer pipette
- At least 5 toxicant concentrations should be tested alongside a control. Ideally the lowest concentration will cause 0% mortality and the highest concentration will cause 100% mortality. A log or half-log scale is a good guideline for concentration spacing
- Three experimental replicates will be tested at each concentration typically with 5 neonates per replicate. Therefore:
- Transfer 4ml (Gilson) of clean, aerated OECD media to all vials
- Filter and pool required neonates (<24hrs) from culture beakers to a separate 250ml beaker
- Transfer 5 neonates from the pooled group to each vial (plastic transfer pipette) in 1ml volume of OECD media.
- Add appropriate volume of toxicant (pipettor). Noting the dose for each vial on the exposure record sheet
- Randomise the vials under the bulbs on the exposure shelf
- After 24 hours note the number of immobilised neonates. If a neonate cannot propel itself after gentle vial agitation it is denoted immobilised despite any limb movement
- Replace vials
- After an additional 24 hours again note the immobilised neonates.

Data acquisition

Daphnia toxicity test data acquisition

- After 24 and 48 hours note the number of immobilised neonates. If a neonate cannot propel itself after gentle beaker agitation it is denoted immobilised despite any limb movement.

Data analysis

- Data should be summarised in tabular form, showing for each treatment group and control, thenumber of daphnids used, and immobilisation at each observation.
- The percentages immobilised at 24 hours and 48 hours are plotted against test concentrations.
- Data are analysed by appropriate statistical methods (e.g. probit analysis, prism) to calculate the slopes of the curves and the EC50 with 95% confidence limits (p = 0.95).
- Where the standard methods of calculating the EC50 are not applicable to the data obtained, the highest concentration causing no immobility and the lowest concentration producing 100 per cent immobility should be used as an approximation for the EC50 (this being considered the geometric mean of these two concentrations).

Quality control

QA/QC procedures :

- For a test to be valid, the following performance criteria apply:- In the control, including the control containing the solubilising agent, not more that 10 percent of the daphnids should have been immobilised;
- Not more than 10 percent of the control daphnids should show immobilisation or other signs of disease or stress, for example, discoloration or unusual behaviour such as trapping at surface of water.

Bioassay main features

Bioassay ID	WP12 bioassay_Daphnia_arrays
Bioassay type	*ex vivo*
Adverse Outcome Pathway	Multiple
Test species	Daphnia magna
Cell line/ cell strain	Bham2
Transgene	none

Measured endpoints/ molecular targets	**Measured via**
60,000 probes representing 41153 distict Daphnia transcripts	Oligonucleotide microarray transcriptomics

Brief description of test

Daphnia magna gene expression is assessed using an Agilent Daphnia magna 8x60k format microarray

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Tim Williams	25/04/16

Regulatory aspects

The described test is carried out on an invertebrate species excluded from the definition of a laboratory animal according to EU Directive EC86/609.

Experimental conditions

Exposure duration	48 h
Main determinant	Gene expression
Effect equivalent EQ	Not applicable
Unit of effect equivalent	Not applicable
Concentration range in standard curve	Not applicable

Bioassay performed to standard guideline?	Yes
Guideline no. or reference for published method	OECD Guideline for Testing of Chemicals 202 (April 2004) (http://www.oecd-ilibrary.org/environment/test-no-202-daphnia-sp-acute-immobilisation-test_9789264069947-en).
Deviation from standard guideline?	Yes

Describe deviation from standard guideline
For LV-SPE exposures the low quantities available necessitated reducing exposure volume to 5ml containing 5 neonate Daphnia. This procedure was also employed with chemical exposures to allow cros-comparison. Environmental samples and chemicals were dissolved in methanol.

Assay format	Single vial
Volume per well/ vessel	5ml

Test media

Solvent	MeOH
Max solvent concentration in test media	0.1%
Reference media	OECD medium

Composition of reference media
'OECD Media' Calcium chloride CaCl2, 2H2O 11.76 g/l Magnesium sulfate MgSO4, 7H2O 4.93 g/l Sodium bicarbonate NaHCO3 2.59 g/l Potassium chloride KCl 0.23 g/l pH 7.4 to 7.6

Renewal type	static
Renewal frequency	n/a

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	5
Age of organisms	<24h
Developmental stage of organisms	Neonates
Feeding?	No
Frequency of feeding	n/a
Temperature controlled?	Yes
Temperature	18°C to 22°C
Photoperiod	16-hour light and 8-hour dark cycle
Light intensity	Not measured

Statistical parameters and quality control

Culturing conditions	18°C to 22°C
Growth Medium	'OECD Media' Calcium chloride CaCl2, 2H2O 11.76 g/l Magnesium sulfate MgSO4, 7H2O 4.93 g/l Sodium bicarbonate NaHCO3 2.59 g/l Potassium chloride KCl 0.23 g/l pH 7.4 to 7.6
Number of experimental repetitions	To be decided
Number of replicates per treatment	3-4
Number of concentrations tested per sample	At least five test concentrations
Positive control tested?	Yes
Positive control substance	Potassium dichromate
Solvent control tested?	Yes
Other controls tested?	Yes: Untreated control
pH of sample in test medium	Buffered by medium to pH 7.4 to 7.6
pH adjusted	Not adjusted (as per OECD Guideline)
DO of sample in test medium measured?	no
Conductivity of sample in test medium measured?	no
Ammonium/ nitrite content measured?	no

Experimental outline

Daphnia magna toxicant exposure and transcriptomics

***Daphnia magna* toxicant exposure**

- Equipment: 7ml glass 'bijou' vials (labelled), 5ml Gilson pipette, Plastic transfer pipette
- Toxicant concentrations are informed by the results of previous Daphnia acute toxicity tests
- One to six experimental replicates will be tested at each concentration typically with 15 neonates per replicate in 3 vials. Therefore:
- Transfer 4ml (Gilson) of clean, aerated OECD media to all vials
- Filter and pool required neonates (<24hrs) from culture beakers to a separate 250ml beaker
- Transfer 5 neonates from the pooled group to each vial (plastic transfer pipette) in 1ml volume of OECD media.
- Add appropriate volume of toxicant (pipettor). Noting the dose for each vial on the exposure record sheet
- Randomise the vials under the bulbs on the exposure shelf
- After 24 hours note any immobilised neonates. If a neonate cannot propel itself after gentle vial agitation it is denoted immobilised despite any limb movement
- Replace vials
- After an additional 24 hours again note any immobilised neonates.
- Filter neonates from each vial and transfer into Precellys tubes (Soft tissue homogenizing CK14 - 2 mL; Precellys, Bertin Technologies, Montigny le Bretonneux, France) using a fine paintbrush. Immediately freeze in liquid nitrogen then store at -80C.

Total RNA preparation

- Total RNA preparation employs Qiagen RNeasy microkit, see protocols here; https://www.qiagen.com/gb/resources/resourcedetail?id=682963a5-737a-46d2-bc9f-fa137b379ab5&lang=en
- Before first use - add 10ul beta-mercaptoethanol per ml RLT buffer, add 4 vol EtOH to RPE, make 70% EtOH and 80% EtOH. Note - use only RNAse free eppendorfs, barrier tips and water.
- Add 350ul RLT to Precellys tube
- Remove tissue samples in Precellys tubes from -80 and hold on dry ice
- Add 350ul RLT to Precellys tube
- Homogenise in Precellys homogeniser (Precellys)
- Microfuge max 3-5 min, carefully take supernatant, re-spin if needed
- Add supernatant to 350ul 70% EtOH, mix with pipette
- Add to spin column, spin 15s 8000g, discard flowthrough
- Add 350ul RW1, spin 15s 8000g, discard flowthrough
- Add 350ul RW1, spin 15s 8000g, transfer to new collection tube
- Add 500ul RPE, spin 15s 8000g, discard flow
- Add 500ul 80% EtOH, spin 2 min 8000g, transfer to new collection tube
- Spin max 5 min to dry
- Transfer column to 1.5ml tube, elute with 14ul RNAse free water by spin 1 min max

- Store at -80C
- EtOH/Ac precipitation:
- add 0.1 vol NaOAc 3M pH5.2 sterile filtered (1.5ul), 2.2 vol EtOH 100% (at -20C) (33ul)
- vortex, -20C, 20 min
- spin 5 min max, add 1ml 70% EtOH (4C), spin 1 min max
- remove supernatant, dry, resusp in 25 ul H2O
- Test absorbance with nanodrop spectrophotometer
- For nanodrop, use 1.2ul undiluted RNA
- The ratio A260/A280 should be 1.7 or greater, otherwise this indicates protein contamination. RatioA260/A230 should exceed 1.5 otherwise this indicates salt contamination.
- Failed samples are repeated or discarded

Labeling & Hybridisation

- RNA is reverse, transcribed, labeled with Cy3 fluorophore during in-vitro transcription and repurified according to Agilent protocol http://www.agilent.com/cs/library/usermanuals/Public/G4140-90040_GeneExpression_OneColor_6.9.pdf . Repurification employs Qiagen RNEasy Mini kit https://www.qiagen.com/gb/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en
- Incorporation of Cy3 is assessed with nanodrop spectrophotometer, requiring a minimum of 825ng cRNA and specific activity>6
- Failed samples are repeated or discarded.
- Microarray slides employed are Agilent Daphnia magna 8x60k GE Design ID 079797, details of probe sequences and layout can be made available through EArray https://earray.chem.agilent.com/earray/
- Hybridisation and washing of slides proceeds as specified in http://www.agilent.com/cs/library/usermanuals/Public/G4140-90040_GeneExpression_OneColor_6.9.pdf

Data acquisition

Microarray slide scanning & data acquisition

- Arrays are scanned . Slides were scanned using the G2565CA microarray scanner system (Agilent) at University of Birmingham Functional Genomics Laboratory. Data are captured using Agilent FE software http://www.agilent.com/cs/library/usermanuals/Public/G4140-90040_GeneExpression_OneColor_6.9.pdf

Data analysis

Microarray Data Analysis

- Microarray data analysis is not a standardised protocol, however several common software packages and web resources are usually employed:
- Agilent FE Feature Extraction Software
- Genespring (Agilent)
- MeV (http://www.tm4.org/mev.html)
- GE Workbench (http://wiki.c2b2.columbia.edu/workbench/index.php/Home)
- DAVID (https://david.ncifcrf.gov/)
- GSEA (http://software.broadinstitute.org/gsea/index.jsp)
- Cytoscape (http://www.cytoscape.org/)
- All data is also provided to University of Liverpool partners (Falciani & Antczak) for integrative analysis with other SOLUTIONS data types.

Quality control

QA/QC procedures :

- Quality and purity of RNA is assessed, as is labeling efficiency, samples failing are either discarded or repeated.
- Extensive QA/QC files are generated by the Agilent FE software. Failed hybridisations are repeated if sufficient subarrays remain, otherwise data is discarded.
- Further QC metrics are assessed on a probe-by-probe basis during data analysis, with data from probes showing low fluorescence or very high within-group variablility typically discarded.

Bioassay main features

Bioassay ID	WP12 bioassay_ChgH-GFP
Bioassay type	*In vivo*
Adverse Outcome Pathway	Estrogen receptor agonism leading to reproductive dysfunction Estrogen receptor antagonism leading to reproductive dysfunction Aromatase inhibition leading to reproductive dysfunction (in fish)
Test species	Medaka (*Oryzias latipes*)
Cell line/ cell strain	-
Transgene	ChgH-GFP

Measured endpoints/ molecular targets	**Measured via**
mortality	microscopic observation
choriogenin H transcription	fluroescence
aromatase enzymatic activity	fluroescence

Brief description of test

The described test uses early post-hatch medaka eleutheo-embryos of the ChgH-GFP line to quantify estrogen axis activity by fluorescence. Performing in parallel of a cotreatment with the sample of interest and a known concentration of testosterone allows the effects of compounds interferring with CYP450 aromatase activity to be taken into account in addition to upstream (eg. hypothalamic control, steroidogenesis) or downstream (eg. ER receptor agonists/ antagonists) events.

Document history

Version	**Modification**	**Author**	**Date**
A	creation	Andrew Tindall	03/09/2015

Regulatory aspects

The described test is carried out on eleuthero-embryo stages of development (day post hatch 0-2) excluded from the definition of a laboratory animal according to EU Directive EC86/609.

Experimental conditions

Exposure duration	24 h
Main determinant	EC50
Effect equivalent EQ	17α-ethinyl oestradiol
Unit of effect equivalent	ng/L
Concentration range in standard curve	15 - 171 ng/L (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	PMID: 26352216
Deviation from standard guideline?	No guideline available

Describe deviation from standard guideline
N/A

Assay format	6-well plate
Volume per well/ vessel	8 mL

Test media

Solvent	DMSO
Max solvent concentration in test media	0.5%
Reference media	Glass bottled Evian water

Composition of reference media
The composition can be downloaded from: http://evianwebsite.s3.amazonaws.com/website/files/evian-2014-AWQR-ENG.pdf Or click here :  Foxit Reader PDF Document

Renewal type	Static
Renewal frequency	None (24 h assay)

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	8
Age of organisms	DPH 0/1
Developmental stage of organisms	-
Feeding?	none
Frequency of feeding	-
Temperature controlled?	Yes
Temperature	26°C
Photoperiod	14:10 (light:dark)
Light intensity	700 – 2500 lux
Culturing conditions	26°C
Growth Medium	Animal facility water + methylene blue

Statistical parameters and quality control

Number of experimental repetitions	2
Number of replicates per treatment	1
Number of concentrations tested per sample	5
Positive control tested?	Yes
Positive control substance	17α-ethinyl estradiol (EE2)
Solvent control tested?	Yes
Other controls tested?	Yes – testosterone, fadrozole
pH of sample in test medium tested?	Yes
pH adjusted	Yes
DO of sample in test medium measured?	No
Conductivity of sample in test medium measured?	Not for single chemical/ artificial mixture testing
Ammonium/ nitrite content measured?	No

Experimental outline

Stock solution preparation:

- Sample extracts and controls are suspended in DMSO.
- Final DMSO concentration is constant across all experimental treatments.

Sample and control preparation:

- For the highest concentrations for each sample extract/ control, stock solutions are added to assay medium to give a final concentration of ≤ 0.5% DMSO.
- All extacts are tested in the presence and absence of testosterone (30 µg/L).
- They are then serial diluted as required in solvent control.

Plate preparation:

- 8 day post hatch zero (dph 0/1) ChgH-GFP fry per well, 8 mL per of sample/ control per well in six well plates TTP polystyrene (cat. Dominique Dutscher #009206).
- Place plates in incubator (14:10 light: dark, 26°C) for 24h.

Preparation for experimental read out:

- Fry from each of the experimenal groups are rinsed in assay media and placed into the corresponding well of a clean six well plate.

Experimental read out:

- Chgh-gfp fry are anaesthetised with 200 mg/L MS222 in assay medium and positioned dorsally on a black plastic plate for imaging.
- Dead fry are identified by a lack of heartbeat and are noted.
- Images of the ventral region of the abdomen of each fry are captured with a 0.3 s exposure time at 8x magnification using an Infinity 1-3C camera (Lumenera Corporation, Ottawa, ON, Canada) fitted to a Leica MZ10F stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany), illuminated with a 120W fluorescence source and ET-GFP long-pass filters (excitation 480/40, emission 510LP, Leica Microsystems GmbH, Germany).
- Images are stored as 8-bit colour jpg files.

Image analysis

An ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,) macro is used to carry out the following analysis:

- Colour images of medaka fry are separated into red, green and blue layers.
- The intensity of each pixel in the red layer is doubled and subtracted from the green layer.
- A threshold intensity of 10-255 is applied to the resulting images.
- The region containing the liver is manually selected and the intensity of all pixels within this region is summed.

Data analysis

Software used: Excel and GraphPad Prism

Lethality data analysis:

- Only concentrations below the EC10 for eleuthero-embryo mortality are considered.

Estrogen axis activity data analysis:

- Following image analysis the values obtained for the total fluorescence of the liver of the fry are transferred to Graphpad Prism.
- Total fluorescence values are normalised to the mean of the testosterone alone group.
- Concentrations are log converted.
- The log agonist vs response – find ECanything model is used to model and determine the EC50 of the response for the EE2 standard curve.
- EE2 equivalence values are read from this curve for each sample extract.
- The LOD and LOQ are determined as the mean of the solvent control + 3x its standard deviation or 10x its standard deviation respectively.
- A value for the testosterone control is also read from the EE2 standard curve.
- Values for testosterone spiked samples are read from the EE2 standard curve taking into account the fact that testosterone has been added to these groups.

Quality control

Pipettes:
Ensure that all pipettors are calibrated at least once a year.

Determine:
Between-runs (inter-assay) replication.

Assay replicates:
Minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels. If replicates vary more than a predetermined value, the assay is performed a third time.

Bioassay main features

Bioassay ID	WP12 bioassay_THbZIP-GFP
Bioassay type	*In vivo*
Adverse Outcome Pathway	N/A
Test species	Amphibiens (*Xenopus laevis*)
Cell line/ cell strain	-
Transgene	THbZIP-GFP

Measured endpoints/ molecular targets	Measured via
mortality	microscopic observation
THbZIP transcription	fluorescence

Brief description of test

The described test uses one week tadpole of the THbZIP-GFP line at the stage 55 according to NieuwKoop et Faber to quantify hypothalamo hypophyso thyroïd axis activity by fluorescence. Performing in parallel of a cotreatment with the sample of interest and a known concentration of the thyroid hormone triiodothyronine (T3) allows the effects of compounds interferring with THbZIP activity to be taken into account in addition to upstream (eg. CRH, TSH synthesis, thyroid hormone transport, interraction with thyroid recpetors, metabolism of thyroid hormone : deiodinase activity) events.

Document history

Version	Modification	Author	Date
A	creation	Andrew Tindall	03/09/2015

Regulatory aspects

The described test is carried out on NF stage 45 tadpoles excluded from the definition of a laboratory animal according to EU Directive EC86/609.

Experimental conditions

Exposure duration	48 h
Main determinant	EC50
Effect equivalent EQ	Triiodothyronine
Unit of effect equivalent	µg/L
Concentration range in standard curve	3.25-16.25 µg/L (serially diluted)
Bioassay performed to standard guideline?	No guideline available
Guideline no. or reference for published method	PMID:17874805
Deviation from standard guideline?	No guideline available

Describe deviation from standard guideline
N/A

Assay format	6-well plate
Volume per well/ vessel	8 mL

Test media

Solvent	DMSO
Max solvent concentration in test media	0.5%
Reference media	Glass bottled Evian water

Composition of reference media
The composition can be downloaded from: http://evianwebsite.s3.amazonaws.com/website/files/evian-2014-AWQR-ENG.pdf Or click here :  Foxit Reader PDF Document

Renewal type	Static
Renewal frequency	24h

Test organisms/ environmental conditions

Number of organisms/ cells per replicate	10
Age of organisms	One week
Developmental stage of organisms	45
Feeding?	none
Frequency of feeding	-
Temperature controlled?	Yes
Temperature	26°C
Photoperiod	obscurity
Light intensity	N/A
Culturing conditions	21°C
Growth Medium	Animal facility water + gentamicine

Statistical parameters and quality control

Number of experimental repetitions	2/3
Number of replicates per treatment	1
Number of concentrations tested per sample	5
Positive control tested?	Yes
Positive control substance	Triiodothyronine (T3)
Solvent control tested?	Yes
Other controls tested?	Yes – Triiodothyronine (T3) + thyroxine (T4)
pH of sample in test medium tested?	Yes
pH adjusted	Yes
DO of sample in test medium measured?	No
Conductivity of sample in test medium measured?	Not for single chemical/ artificial mixture testing
Ammonium/ nitrite content measured?	Not for single chemical/ artificial mixture testing

Experimental outline

Stock solution preparation:

- Sample extracts and controls are suspended in water or DMSO.
- Final DMSO concentration is constant across all experimental treatments.

Sample and control preparation:

- For the highest concentrations for each sample extract/ control, stock solutions are added to assay medium to give a final concentration of ≤ 0.5% DMSO.
- All extacts are tested in the presence and absence of Triiodothyronine (T3) 3.25 µg/L.
- They are then serial diluted as required in solvent control.

Plate preparation:

- Ten stage 45 tadpoles and 8 mL of sample/ control per well in six well TTP polystyrene plates (cat. Dominique Dutscher #009206).
- Place plates in incubator (obscurity, 26°C) for 48h.

Plate renewal :

- Remove solutions from each well of the 6-well plates and renew with the same solutions.

Preparation for experimental read out:

- Tadpoles from each of the experimenal groups are rinsed in evian and placed into the corresponding well of a clean 6-well plate.

Experimental read out:

- THbzip-GFP tadpoles are anaesthetised with 100 mg/L MS222 in assay medium and positioned dorsally, the head in the middle of the well on a black plastic plate for imaging.
- Dead tadpoles are identified by their color and are noted.
- Images of the ventral region of the head of each tadpole are captured with a 3 s exposure time at 1.25x magnification using an Hamamatsu Orca-ER camera (Hamamatsu, Japan) fitted to a Leica macrofluo binocular macroscope (Leica Microsystems GmbH, Wetzlar, Germany), illuminated with a 120W fluorescence source and GFP band-pass filters (excitation 470/40, DM500, emission 525/50, Leica Microsystems GmbH, Germany).
- Images are stored as 8-bit grey scale jpg files.

Image analysis

- The mean grey value is calculated for each image.

Data analysis

Software used: Excel and GraphPad Prism

Lethality data analysis:

- Only concentrations below the EC10 for eleuthero-embryo mortality are considered.

Thyroid axis activity data analysis:

- Following image analysis the values obtained for the mean grey value of the tadpole heads are transferred to Graphpad Prism.
- Mean fluorescence values are normalised to the mean of the evian group.
- Concentrations are log converted.
- The log agonist vs response – find ECanything model is used to model and determine the EC50 of the response for the T3 standard curve.
- T3 equivalence values are read from this curve for each sample extract.
- The LOD and LOQ are determined as the mean of the solvent control + 3x its standard deviation or 10x its standard deviation respectively.
- Values for T3 spiked samples are read from the T3 standard curve taking into account the fact that T3 has been added to these groups.

Quality control

Pipettes:
Ensure that all pipettors are calibrated at least once a year.

Determine:
Between-runs (inter-assay) replication.

Assay replicates:
Minimum 2 independent repeats of the assay. The results of replicates are compared to ensure that the variability does not exceed a pre-determined levels. If replicates vary more than a predetermined value, the assay is performed a third time.