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REPORT OF THE VALIDATION STUDY OF THE EASZY ASSAY IN TEST GUIDELINE 250

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NO. 335

REPORT OF THE VALIDATION STUDY OF THE EASZY ASSAY IN TEST GUIDELINE 250

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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Environment Directorate
ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT
Paris 2021

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FOREWORD

This document describes the design and results of the two phases of the validation effort for the EASZY assay, an assay for the detection of Endocrine Active Substances, acting through estrogen receptors, using transgenic cyp19a1b-GFP Zebrafish embrYos (EASZY), adopted and published in 2021 as OECD **Test Guideline 250**. The EASZY assay is a mechanism-based in vivo screening assay in which transgenic tg(cyp19a1b:GFP) Zebrafish embryos are exposed for 96 hours during the embryonic stages of development and the expression of the green fluorescent protein (GFP) driven by the cyp19a1b promoter is induced. The project to develop this assay was led by France.

The purpose of phase I of the validation was to determine whether the standard operating procedure (SOP) can be used reliably and reprodcibly across multiple laboratories. The purpose of the phase II validation was to generate a larger set of data to confirm the conclusions from phase I regarding the transferability of the assay and to set a definitive statistical protocol for the analysis of the results.

The EASZY assay has been validated through an international effort via the OECD and the Validation Management Group for Ecotoxicity (VMG-Eco) assisted the validation exercise to develop the XETA by evaluating the validation proposals as well as the validation exercise results.

The Working Party of the National Coordinators of the Test Guidelines Programme endorsed the validation report at its 33rd meeting in April 2021**.** This report is published under the responsibility of the Chemicals and Biotechnology Committee.

Table of contents

Tables

Figures

1 General Introduction

This document compiles the results of the inter-laboratory study conducted between 2014 and 2018 which aimed at validating the OECD standardized *in vivo* assay on transgenic *tg (cyp19a1b:GFP)* zebrafish embryos to detect endocrine active substances that have the potential to act as estrogens and to induce the expression of GFP driven by the estrogen receptors-regulated cyp19ab zebrafish promoter (EASZY assay).

The validation of the EASZY assay was organized in two consecutives validation phases.

The aims of the phase 1 validation were i) to evaluate the transferability of the transgenic model and the test method into different European laboratories ii) to experimentally report the capacity of the laboratories to identify active and inactive chemicals in different laboratories, to improve the on standard operating protocol (SOP) and to document the intra and inter-assay variability by measuring the estrogenic activity of selected ligands. In phase 1, seven test chemicals were selected: 17α-ethinylestradiol, 17β-estradiol, 4-tert-octylphenol, bisphenol A as ER agonist; testosterone as aromatizable androgen; spironolactone and dexamethasone as inactive chemicals.

The phase 2 validation was established to i) gain further information on the capacity of the EASZY assay to produce reliable and accurate data about the estrogenic activity of tested substances notably by experimentally assessing the possibility of false-positive and false-negative outcomes generated when testing a set of eleven active and inactive test chemicals under blindly conditions ii) characterize the concentration-effect relationships iii) take into account chemically measured concentrations of selected substances. The eleven test chemicals selected for the phase 2 validation were: 2,4-dihydroxybenzohenone, bisphenol S, bisphenol F, levonorgestrel, norethindrone, dihydrotestosterone, 17β-trenbolone as active chemicals; diclofenac, triclosan, benzophenone and 11-ketotestosterone as inactive chemicals.

The progress of the results of the phase 1 and phase 2 validation studies were presented at the occasion of various VMG-Eco meetings between 2014 and 2018. Based on the data collected and discussions with VMG-Eco experts, the standard operating protocol, the validity and acceptability criteria were improved and several refinements of the test design protocol and experimental procedure were adopted as well as improvement of the biological model by establishing a homozygote transgenic line which was not available at the initiation of the validation study.

2 Detection of Endocrine Active Substance, acting through estrogen receptors, using transgenic cyp19a1bGFP Zebrafish embrYos (EASZY)

II.1. Principle of EASZY

EASZY is an *in vivo* mechanism-based assay that uses transgenic zebrafish *tg(cyp19a1b:GFP)*embryos expressing GFP under the control of the zebrafish *cyp19a1b* promoter (see Figure 1). The cyp19a1b gene codes for the P450 brain aromatase B, the enzyme responsible for the biosynthesis of estrogens. The transgenic *tg(cyp19a1b:GFP)* zebrafish line was developed and characterized by Tong et *al.*, (2009). In this model, green fluorescent protein (GFP) expression is controlled by 3.4kb of the zebrafish *cyp19a1b* proximal promoter followed by the first exon, the first intron and the beginning of the second exon comprising the natural translation start site (see Figure 1 for the genetic construction).

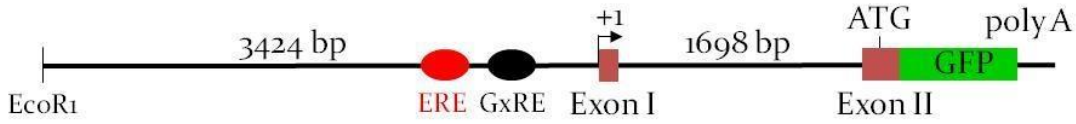


Figure 1 : Diagram showing the *cyp19a1b: GFP* construct that was used to produce transgenic founders. The construct contains 3,424 bp of the 5' flanking region, 54 bp of exon I (untranslated), 1,698 bp of intron I, and 20 bp of untranslated exon II followed by the natural translation start site. The GFP gene with the SV40 polyA sequence (poly A) was fused to the first 10 amino acids of CYP19A1B with a 9-aa linker (from Tong et al., 2009).

In the EASZY assay, newly fertilized zebrafish eggs are exposed to test substance for 96 hours with a total renewal of the medium each day. At the end of the experiment, the fluorescence of each fish is acquired using a fluorescence microscope. The intensity of fluorescence is then quantified using image analysis software. The data (expressed as fold induction above control) are analyzed to determine the estrogenic activity of chemical. Effective concentrations (EC_x) can be derive derived.

II.2. Main characteristics and outcomes of EASZY

EASZY assay

(1) Provides **mechanistic information** regarding the capacity of chemicals to activate the ER-signaling pathway *in vivo* in radial glial cells in the developing brain of fish, while considering the biodisponibility and pharmacodynamics of test chemicals.

(2) 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. *In vivo* imaging of transgenic *tg(cyp19a1b:GFP)*zebrafish embryos is achieved using a fluorescence microscope equipped with a fluorescence camera. Concentration-response curves can be modeled to derive effective concentrations (ECx) (e.g., EC_{10}, EC_{20}, EC_{50}).

(3) Is a rapid and cost-effective *in vivo* embryo fish screening assay for estrogenic activity of chemicals.

(4) Is the only assay providing information on the capacity of substances to target RGCs in the developing brain. Since RGCs act as stem cells giving birth to new neurons in vertebrates, the data provided by EASZY are unique.

II.3. Compounds detected in EASZY assay

A panel of natural and synthetics hormones as well as chemicals belonging to various chemical families have been tested for their capacity to affect GFP in the transgenic *tg(cyp19a1b:GFP)* embryo model (Brion et al., 2012, Cano-Nicolau et al., 2016, Le Fol et al., 2017a, Neale et al., 2017, Serra et al., 2018)

Based on these studies, the following conclusions can be made

In EASZY, GFP is induced in a ER-specific manner by:

- Compounds that bind directly to estrogen receptors as agonists. The assay detects and distinguishes ER agonist compounds from the strong (e.g., natural and synthetic estrogens) to the weak ER agonist compounds (e.g., alkylphenolics compounds, bisphenols).
- Compounds that need to be bio-transformed prior to elicit an induction of GFP. Among them, different categories can be distinguished.

Metabolism of the parent compound into estrogenic metabolites. An important feature of the EASZY assay relies on the metabolic capacity of the zebrafish embryo. There exist evidences showing that zebrafish embryos have biotransformation capacities catalyzing both Phase I and Phase II enzymatic reactions (Goldstone et al., 2010). Recently, the metabolic profiles of two estrogenic compounds BP2 and BPS have been characterized in 4-day zebrafish and compared to adult fish demonstrating the metabolic competence of the zebrafish embryos with no major qualitative difference between 4-day zebrafish and adults (Le Fol et al., 2017b).

Several compounds that require metabolic activation into estrogenic metabolites have been shown to induce GFP in an ER-dependent manner. This was exemplified by methoxychlor whose estrogenic activity is due to estrogenic metabolites such as 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane HPTE. Furthermore, several 19 nortestosterone synthetic progestins have been shown to induced GFP in ER-dependent manner in the developing brain of zebrafish

(Cano-Nicolau et al., 2016). While the metabolic profiles are not known in the zebrafish model, these compounds are known to be biotransformed into estrogenic derivatives in mammals.

Aromatizable androgens: aromatizable androgens such as testosterone and methyltestosterone have been shown to induce GFP. This androgenic regulation of the *cyp19a1b* is strictly mediated through ER and does not involve neither androgen receptor (AR) nor androgen response element (Mouriec et al., 2009) but is due to aromatization of testosterone and methyltestosterone into estradiol and methyltestradiol respectively.

Non Aromatizable androgens: in EASZY several non aromatizable androgens can induce GFP in an ER-dependent manner. It is the case of dihydrotestosterone (DHT), 17β-trenbolone and metribolone. DHT effect likely involves conversion into 5 α-androstane-3β,17β-diol, a metabolite of DHT with known estrogenic activity. Conversion of DHT into alpha-androstane-3β,17βdiol requires 5α reductase and 3β hydroxysteroid dehydorgenase, both of which are expressed in the developing brain of fish.

For 17β-trenbolone, its estrogenic activity likely reflects its capacity to bind to and activate ER at high concentrations. Indeed, in several *in silico* and *in vitro* ER transactivation assays, 17β-trenbolone has been identified as positive substance (Brown et al., 2015, OECD 455). Furthermore, *in vivo*, in rat, 17β-trenbolone has been shown to act at the brain level to alter expressions of proteins through both AR and ER-mediated process (Fucui Ma & Daicheng Liu, 2015).

In EASZY, anti-estrogenic activity of chemicals can be detected.

The estrogen induced-GFP can be inhibited by compounds that bind to ER as antagonist (e.g., pure antagonist of ER ICI 182-780) or by compounds that negatively interfere with the ER-signaling pathway (e.g., negative cross-talk between TCDD-induced AhR signaling pathway with the estrogen-induced GFP expression). Both ICI 182-780 and TCDD alone have no effect on the basal fluorescence driven by the cyp19a1b promoter while co-exposure of embryos to estrogen with ICI 182-780 and TCDD lead to an inhibition of the estrogen induced GFP revealing their anti-estrogenic activity.

The Figure 2 summarizes the different categories of substances that alter fluorescence in transgenic tg(cyp19a1b:GFP) and their mode of action.

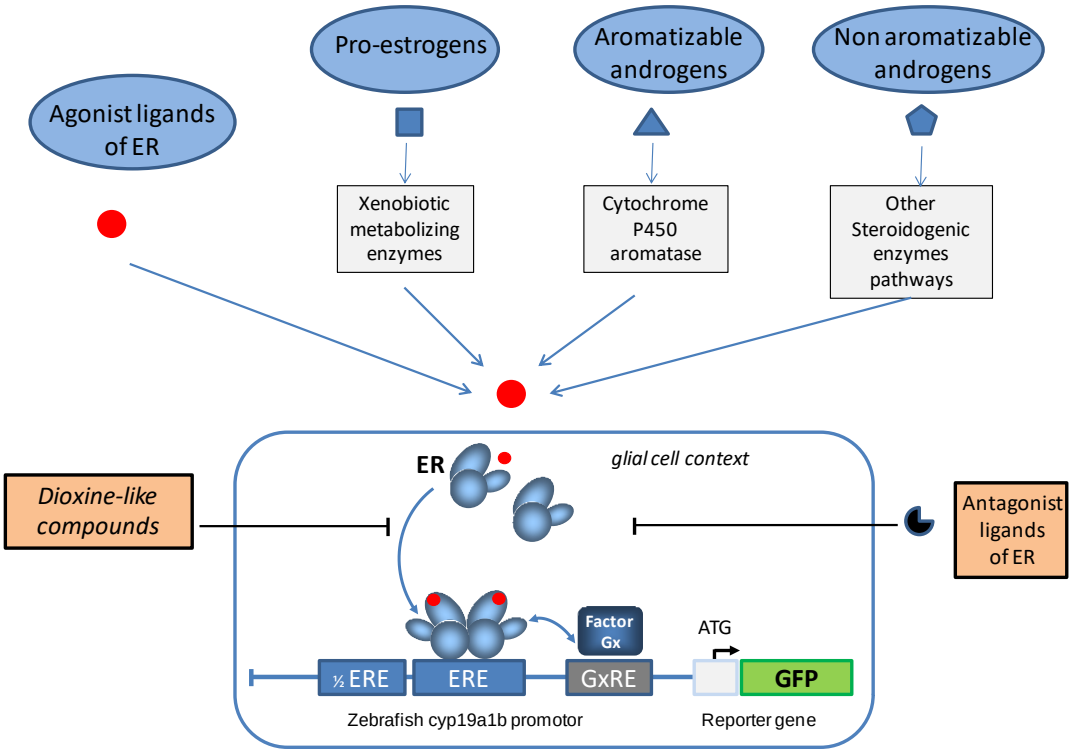


Figure 2 : Different categories of substances can modulate either positively or negatively the transcriptional activity of the zebrafish cyp19a1b promoter by interacting with ERs and/ or the ER signaling pathway leading to induction GFP or inhibition of the E2-induced fluorescence in transgenic embryos (from Brion et al., 2012)

II.4. Potential compounds not detected by EASZY

- In EASZY, compounds that do not bind to and activate ER, either directly or indirectly (i.e., after metabolic transformation), are not detected.
- Potentially, estrogenic compounds that are rapidly metabolized and excreted into inactive metabolites could result in a lack of GFP induction (no current example)
- Estrogenic compounds that could not reach their molecular targets within the brain could lead to a negative result (no current example).

III. Objectives and organization of the validation study

The objectives of the Phase 1 study are

i) to assess the transferability of the transgenic line and the test method into the participating laboratories

ii) to experimentally establish the relevance, the reliability of the EASZY assay by documenting the intra and inter-laboratory reproducibility based on standard operating protocol and to document its accuracy (sensitivity and specificity) by

evaluating the estrogenic activity of selected ligands (

iii) Table 1).

Table 1. List of selected chemicals for phase 1 validation

Name	CAS number	Categories	Expected response in EASZY
17α ethynilestradiol	57-63-6	Agonist ligand of ER	POS
17β-estradiol	50-28-2	Agonist ligand of ER	POS
4-tert-octylphenol	140-66-9	Agonist ligand of ER	POS
Bisphenol A	80-05-7	Agonist ligand of ER	POS
Testosterone	58-22-0	Aromatizable androgen	POS
Spironolactone	52-01-7	Antagonist ligand of MR	NEG
Dexamethasone	50-02-2	Agonist ligand of GR	NEG

The selected compounds include two strong reference estrogens (17α ethynilestradiol, 17β-estradiol); Bisphenol A and 4-tert-octylphenol selected for their estrogenic potencies two to five orders of magnitude lower than reference estrogens; Testosterone selected as a representative substance leading to estrogenic effect after biotransformation (aromatization); spironolactone (mineralocorticoid) and dexamethasone (glucocorticoid) selected as synthetic steroidal compounds being inactive in the EASZY assay.

After approval of the study design by WMG ECO (October 2013), the reference laboratory was in charge of sending the transgenic line (adults and embryos) to the participating laboratories.

The reference laboratory was also in charge of

i) purchasing and distributing the organic solvent (DMSO) and chemicals to each laboratory

ii) transferring the test protocol to expose the fish, to analyze fluorescent images

iii) collecting and analyzing the results iv) writing the report.

In addition to the reference laboratory (LAB A), three different laboratories from France, UK, Germany were involved at the initiation of the validation study. A fourth laboratory was involved later in the phase 1 (from 2016) and realized the EASZY assays for the phase 1 from 2016 to June 2017.

All the laboratories have the necessary equipments and skills for (zebra)fish husbandry and are all equipped with fluorescent microscopes.

They received the transgenic line from LAB A. No specific difficulties have been met regarding transferability and rearing the strain in these. They were trained to perform the EASZY and to analyze the fluorescent images.

Among the laboratories, two of them successfully tested at least one reference substance (E2 and /or EE2). The results of these assays were satisfactoriness as these laboratories were both able to measure an estrogenic activity. Unfortunately, these two laboratories were not able to perform additional assays to test the whole set of ligands. Therefore, only the data from laboratories having tested all the selected ligands (table 1) are reported herein.

LAB A, LAB B and LAB C are equipped with different microscope apparatus. The Table 2 lists the main characteristics of the microscope used by these laboratories to analyze the fluorescence of the fish.

Table 2 : Main characteristics of the fluorescent microscopes used by the participating laboratories in the phase 1 of the validation study.

	LAB A	LAB B	LAB C
microscope	Upright AxioImager Z1 (Zeiss)	Upright AxioImager Z1 (Zeiss)	Upright BX41 (Olympus)
objective	x10 PlanNeofluar	x10 PlanNeofluar	x10 UPlan FL N
camera	MRm High Resolution Fluorescence camera	MRm High Resolution Fluorescence camera	monochrome DMK 31AU03
light source	HBO lamp	HBO lamp	LED XCite Series 120Q
filter	GFP	GFP	GFP

3 Results of the phase 1

III. 1. Quantification of GFP using a unique set of images

At first, it was asked to each laboratory to analyze a unique set of fluorescent images using the ImageJ macro developed to quantify GFP. The whole-set of images was acquired by the reference laboratory.

The images represented varying levels of expression of GFP in the brain of fish from control to zebrafish exposed to increasing concentrations of EE2. In order to analyze the images, the Image J macro was distributed to each participating laboratory with a specific document explaining how to install, to run and to report the data.

The Figure 3 illustrates the results obtained regarding the quantification of GFP I, individual fish. A total of n=103 fluorescent images were analyzed and analyzed according to the concentration of exposure to EE2.

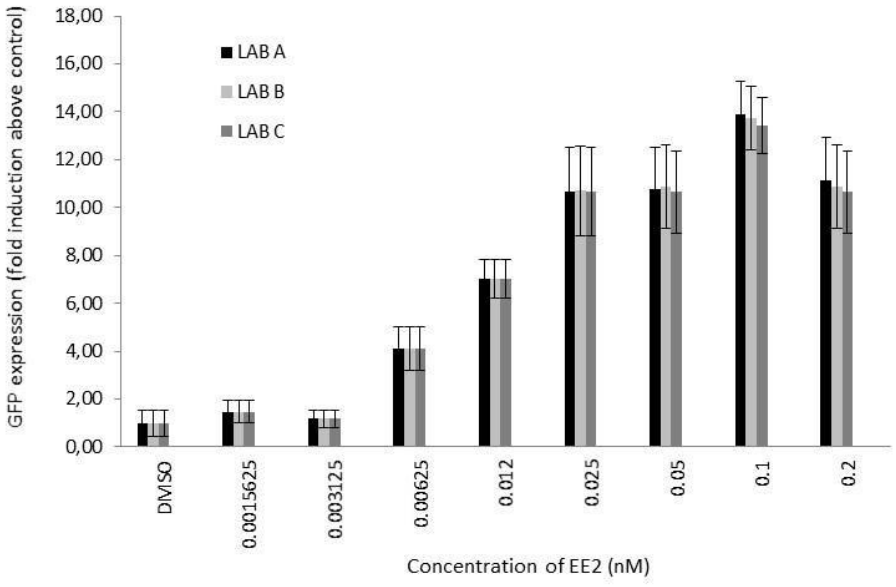


Figure 3: A unique set of fluorescent images generated by the reference laboratory (Lab A) was analyzed by each laboratory using the ImageJ macro developed to quantify fluorescence in the brain of zebrafish. EE2 concentrations were from 0.0015 nM to 0.2nM (0.44 ng/L to 59.8 ng/L)

Based on these data, it is concluded that each laboratory could use and run the Image J macro in a correct manner allowing a perfect quantification of GFP from individuals expressing varying levels of GFP.

III.2. Results of the EASZY assays

III.2.1. Control groups

- ***Positive control*:**

A positive control should be performed in each experiment to ensure that the batch of embryos used for an assay are able to respond to an estrogenic stimulation. For that purpose, a group of zebrafish embryos are exposed to a single concentration of EE2 0.05 nM (EE2 =14.8 ng/L).

The Figure 4 illustrates the effect of a single concentration of EE2 used as positive controls on GFP expression. Its shows that EE2 at a concentration of 0.05 nM (14.8 ng/L) led to a significant induction in all the assays performed by the 3 laboratories.

For Lab A, the mean fold of induction varied from 9.1 ± 3.5 (assay number 5) to 32.9± 9.4 (assay number 6) with a mean fold induction across assays of 21 ± 9 in agreement with previously collected data collected by this laboratory.

For LAB B, the mean fold of induction varied from 7.7 ± 3.4 (assay number 7) to 15.4± 1 (assay number 4) with a mean fold induction across assays of 10.9 ± 2.9

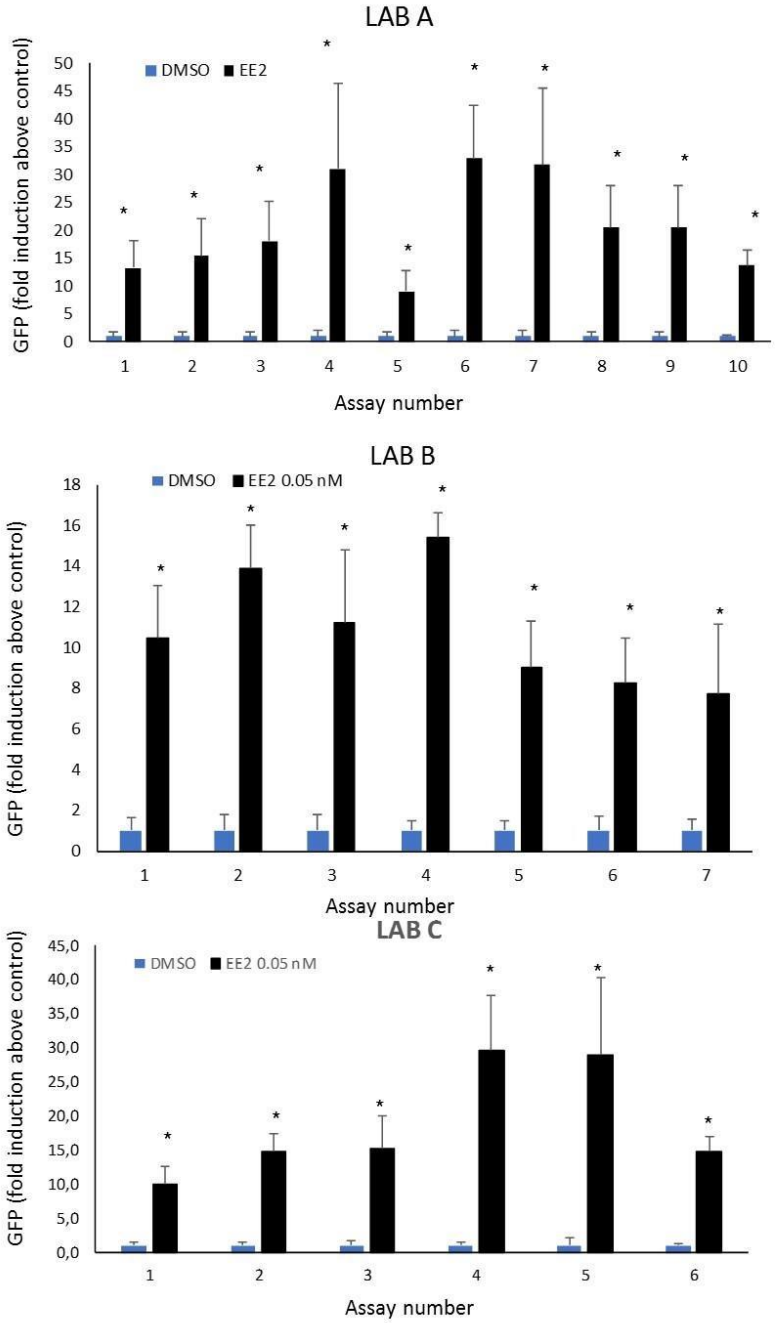


Figure 4: EE2 0.05 nM used as a positive control significantly inducted GFP as compared to the solvent groups in each assay conducted by the participating laboratories (data are mean ± SD).

For LAB C, the mean fold of induction varied from 9.9 ± 2.6 (assay number 1) to 29.6± 8 (assay number 4) with a mean fold induction across assays of 18.9 ± 8.3.

Overall, EE2 0.05 nM (14.8 ng/L) was efficient in significantly inducing GFP as compared to the solvent groups in all the assays conducted. The intensity of the effect mediated by EE2 at this concentration varied across assays. This can be attributed to the basal level of expression of GFP in non-exposed fish that varied from a batch to another. The effect of EE2 0.05 nM

(14.8 ng/L) on GFP induction measured by Lab C was also lower as compared to Lab A and C.

Based on this data, it can be preconized to perform a positive control (EE2 at 0.05nM or 14.8 ng/L). The positive control should lead to a mean fold induction higher or equal to 9 as compared to solvent control to validate the assay.

- Solvent versus water control.

A solvent control is performed if a solvent is required to dissolve the test substance. In that case, the effect of the solvent must be checked prior to run the test to ensure that it does not significantly alter GFP. Several organic solvents have been tested and among them none alter GFP as compared to water control within the concentration range tested (up to 1%) (Brion et al., 2012). Dimethylsulfoxide (DMSO) is usually employed at 0.01% (V:V) which is in compliance with OECD recommendation to limit the use of solvent to a maximum concentration of 0.01%.

The Figure 5 reports the GFP expression measured in solvent (DMSO) and water control groups across assays performed in the different laboratories. In all assay performed no significant difference was noticed between solvent (DMSO) and water control groups thereby confirming the absence of solvent effect on GFP expression.

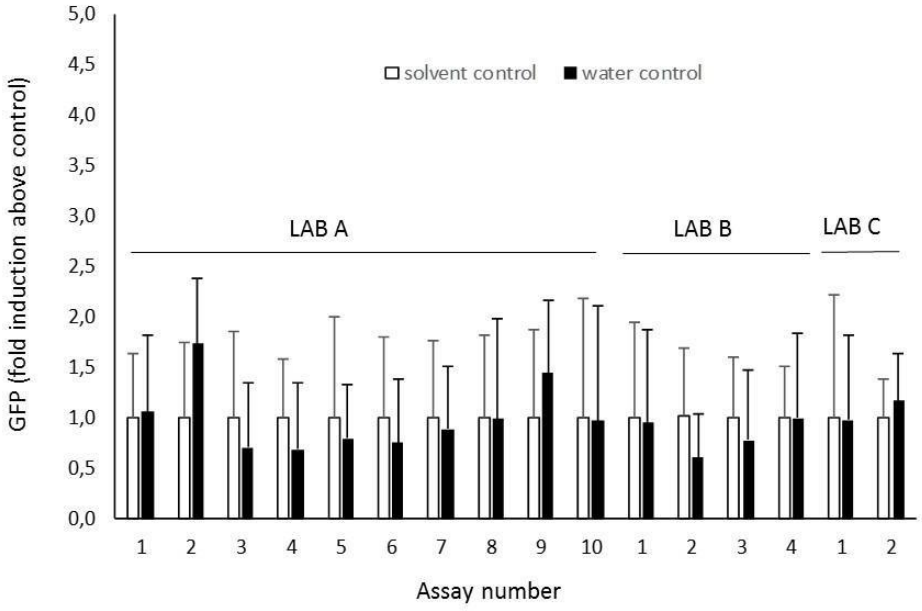


Figure 5 : Relative GFP expression between solvent (DMSO) and water control groups. No difference was noticed confirming the absence of effect of this solvent on GFP expression under the experimental conditions preconized.

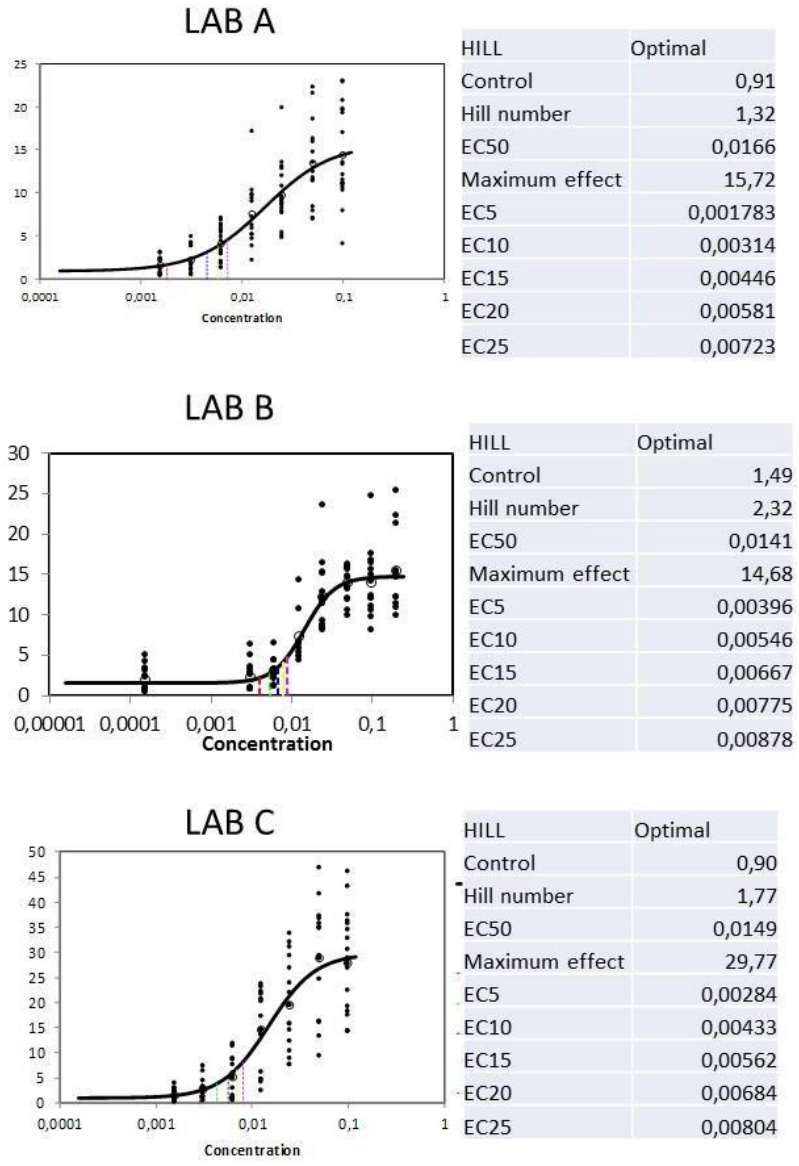
III.2.2. Effect of various selected ligands in the EASZY assay

Each laboratory tested the selected ligands (see

Table 1) in at least two independent experiments. The fluorescence intensities measured in the brain of 4-dpf zebrafish were expressed as as fold induction above solvent control. Data were then modeled using the Regtox 7.0.6 Microsoft Excel TM macro (http://www.normalesup.org/~vindimian/fr_index.html), which uses the Hill equation model and allows calculation of EC50. For a given chemical, EC50 was defined as the concentration inducing 50% of its maximal effect.

III.2.2.1. 17α-ethinylestradiol EE2)

The
Figure 6 illustrates typical concentration-responses curves obtained with EE2 in the different laboratories. Each table on the right of the modeled curves reports the Hill parameters and the ECx concentrations (expressed in nM and ng/L).



HILL	Optimal
Control	0,91
Hill number	1,32
EC50	0,0166
Maximum effect	15,72
EC5	0,001783
EC10	0,00314
EC15	0,00446
EC20	0,00581
EC25	0,00723

HILL	Optimal
Control	1,49
Hill number	2,32
EC50	0,0141
Maximum effect	14,68
EC5	0,00396
EC10	0,00546
EC15	0,00667
EC20	0,00775
EC25	0,00878

HILL	Optimal
Control	0,90
Hill number	1,77
EC50	0,0149
Maximum effect	29,77
EC5	0,00284
EC10	0,00433
EC15	0,00562
EC20	0,00684
EC25	0,00804

Figure 6 : Example of concentration-response curves obtained in EASZY for EE2 in LAB A, B and C. Each point represents the GFP expression measured in the brain of individual (expressed as fold GFP induction above solvent group). Data were modelled using the Hill

model. The tables on the right of modelized curves report the Hill parameters and the ECx (expressed in nM) derived.

The Table 3 summarizes the EC50 for EE2 measured for each assay realized in the different laboratories, the mean EC50 ± SD and the intra-laboratory coefficient of coefficient (CV expressed in %). For all the laboratories, EE2 induced a concentration-dependent induction of GFP in the brain with similar and reproducible EC50 between assays resulting in inter-assay CVs between 3.5% and 33%. For the LAB B, the mean EC50 is however higher than the mean EC50 reported for LAB A and LAB B and could be attributed to EC50 derived from assays 1 and 2. These two assays represented the two first concentration responses experiments performed by this laboratory which could explain the "deviation" of the EC50 measured at the occasion of these experiments. Despite this, the data obtained for EE2 are very consistent between assays and laboratories and in agreement with previously reported values for EE2 using EASZY (Brion et al., 2012, Cano-Nicolau et al., 2016).

Table 3: EC50 expressed in nM (ng/L) derived for 17α-EE2 in LAB A, B and C

Assays	LAB A	LAB B	LAB C
1	0.010 (2.96)	0.028 (8.29)	0.015 (4.44)
2	0.010 (2.96)	0.027 (8.00)	0.016 (4.74)
3	0.013 (3.85)	0.014 (4.14)	0.016 (4.74)
4	0.013 (3.85)	0.017 (5.03)	
5	0.010 (2.96)		
6	0.019 (5.63)		
7	0.010 (2.96)		
8	0.011 (3.26)		
9	0.008 (2.37)		
10	0.018 (5.33)		
Mean EC50	0.012 (3.62)	0.021 (6.37)	0.016 (4.64)
SD	0.004 (1.08)	0.007 (2.9	0.001 (0.17)
CV inter-assay%	30.70	32.29	3.48

III.2.2.2. 17β-estradiol (17β-E2)

In a similar way in EE2, E2 induced GFP in a concentration dependent manner (Figure 7) with comparable EC50 (Table 4) among assays and laboratories. As previously reported in Brion et al., 2012 the estrogenic activity of E2 is less pronounced than that of EE2, the relative estrogenic potency of EE2 being 100fold higher than that E2.

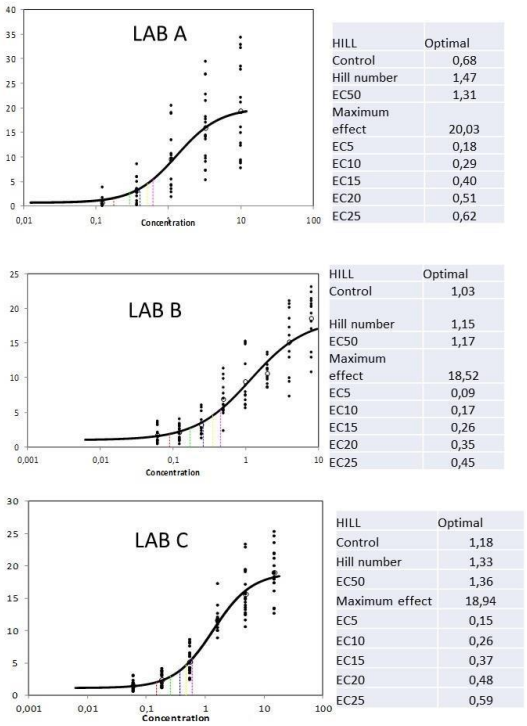


Figure 7: Example of concentration-response curves obtained in EASZY for E2 in LAB A, B and C

Table 4: EC50 expressed in nM (ng/L) derived for 17β-E2 in LAB A, B and C

Assays	LAB A	LAB B	LAB C
1	2.87 (781.7)	1.74 (473.9)	2.80 (762.7)
2	1.94 (528.4)	2.11 (574.7)	1.36 (37 5.9)
3	1.47 (400.4)	2.69 (732.7)	
4	2.05 (558.4)	1.11 (302.3)	
5	1.55 (422.2)		
Mean EC50	1.98 (538.2)	1.91 (520.9)	2.08 (569.3)
SD	0.56 (151.9)	0.66 (180.5)	1.02 (273.5)
CV inter-assay%	28.10	34.61	49.18

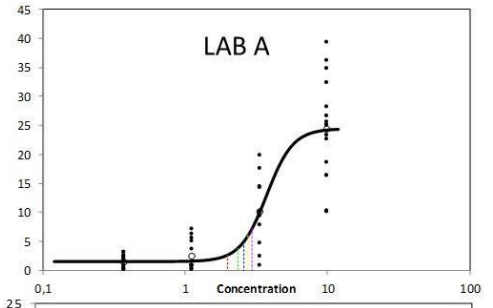
III.2.2.3. Bisphenol A (BPA)

The data collected for BPA are reported in the Table 5.

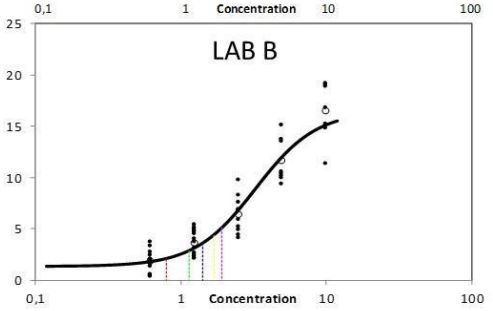
Table 5: EC50 expressed in µM (and µg/L) derived for BPA in LAB A, B and C

Assays	LAB A	LAB B	LAB C
1	3.7 (845)	5.1 (1164)	3.6 (822)
2	4.3 (982)	2.6 (594)	3.6 (822)
3	4.53 (982)	3.3 (753)	2.5 (571)
4	4.5 (1027)	3.5 (799)	
Mean EC50	4.25 (958)	3.61 (827)	3.22 (738)
SD	0.37 (79)	1.09 (241)	0.65 (145)

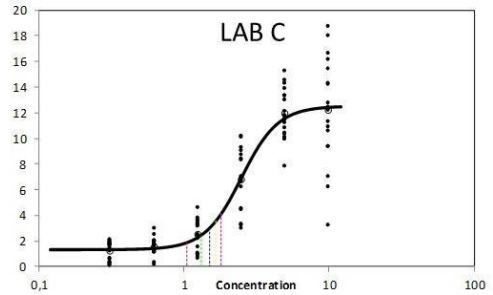
In each assay, BPA induced a full concentration-response curve (Figure 8) with a mean EC50 reported by the three laboratories varying between 3.2µM (738µg/L) and 4.25µM (958µg/L). The inter-assays coefficients of variation within each laboratory were also satisfactory (CV < 30%).



HILL	Optimal
Control	1,56
Hill number	4,69
EC50	3,72
Maximum effect	24,38
EC5	1,99
EC10	2,33
EC15	2,57
EC20	2,77
EC25	2,94



HILL	Optimal
Control	1,37
Hill number	2,07
EC50	3,28
Maximum effect	16,49
EC5	0,79
EC10	1,14
EC15	1,42
EC20	1,68
EC25	1,93



HILL	Optimal
Control	1,30
Hill number	3,44
EC50	2,48
Maximum effect	12,54
EC5	1,05
EC10	1,31
EC15	1,50
EC20	1,65
EC25	1,80

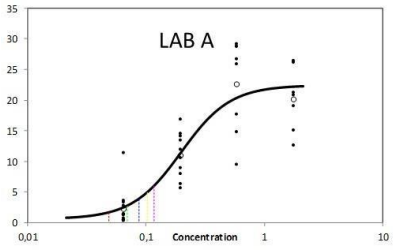
Figure 8: Example of concentration-response curves obtained in EASZY for BPA in LAB A, B and C. Data points represent the GFP expression measured in individuals (expressed as fold GFP induction above solvent group).

III.2.2.4. 4-ter-Octylphenol

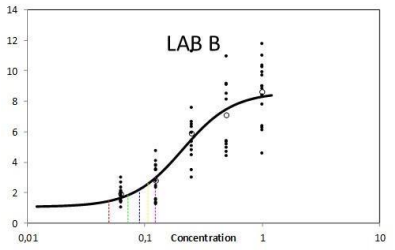
The data collected for4-ter-Octyphenol are reported in the Table 6. In each assay, 4-ter-Octyphenol induced a full concentration-response curve (Figure 9) with a mean EC50 reported by the three laboratories varying between 0.2µM (41.8 µg/L) and 0.29µM (60.5µg/L). The inter-assays coefficients of variation within each laboratory were also satisfactory.

Table 6: EC50 expressed in µM (µg/L) derived for 4-ter-Octylphenol in LAB A, LAB B and LAB C

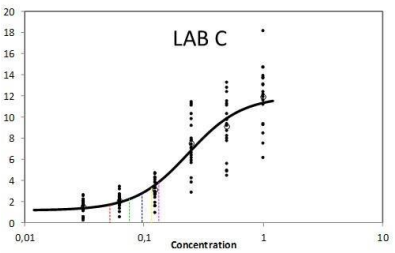
Assays	LAB A	LAB B	LAB C
1	0.44 (90.8)	0.18 (37.1)	0.22 (45.4)
2	0.24 (49.5)	0.24 (49.5)	0.36 (74.3)
3	0.20 (41.3)	0.18 (37.1)	0.24 (49.5)
4		0.21 (43.3)	
Mean EC50	0.29 (60.5)	0.20 (41.8)	0.27 (56.4)
SD	0.13 (26.5)	0.03 (5.9)	0.07 (15.6)
CV inter-assay%	44.07	14.26	27.17



HILL	Optimal
Control	0,57
Hill number	2,10
EC50	0,20
Maximum effect	22,36
EC5	0,05
EC10	0,07
EC15	0,09
EC20	0,10
EC25	0,12



HILL	Optimal
Control	1,09
Hill number	2,03
EC50	0,21
Maximum effect	8,61
EC5	0,05
EC10	0,07
EC15	0,09
EC20	0,11
EC25	0,12



HILL	Optimal
Control	1,16
Hill number	1,94
EC50	0,24
Maximum effect	11,96
EC5	0,05
EC10	0,08
EC15	0,10
EC20	0,12
EC25	0,13

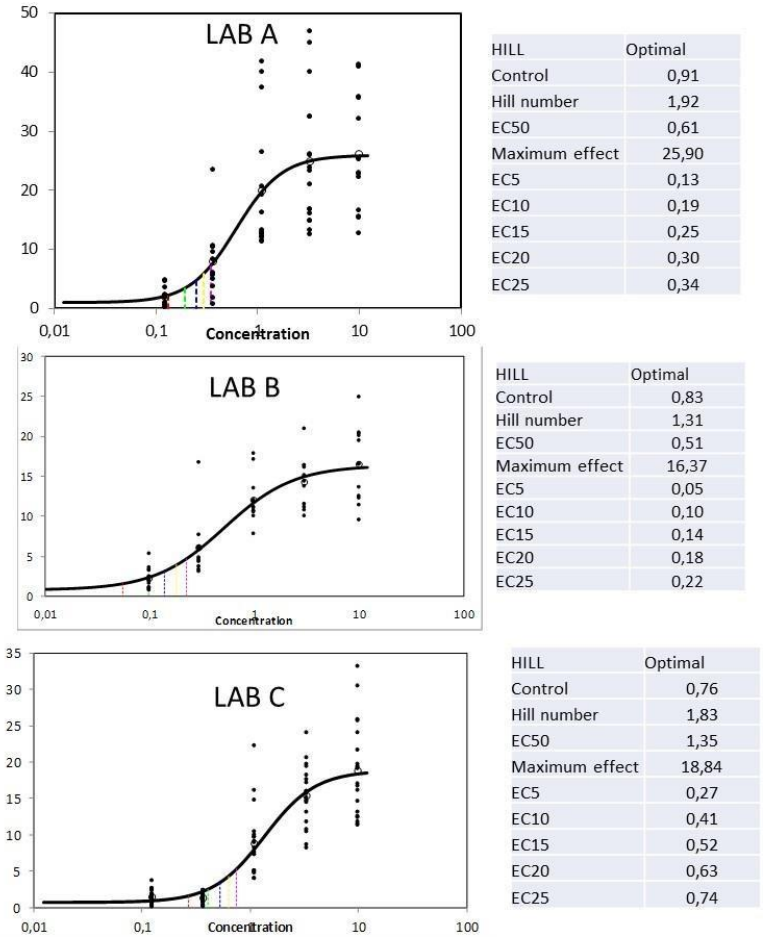
Figure 9 : Typical concentration-response curves obtained in EASZY for 4-terOctylphenol in LAB A, B and C

III.2.2.5. Testosterone

Among the selected ligands, the amortizable androgen testosterone was selected as it has been shown to induce the brain aromatase in an ER-dependent manner (Mouriec et al., 2009). The results obtained in the three laboratories confirmed the capacity of testosterone to induce GFP in a concentration dependent manner with full concentration-response curves obtained in all the laboratories (Figure 10) with similar EC50 values (Table 7)

Table 7 : EC50 expressed in µM derived for Testosterone in LAB A, B and C

Assays	LAB A	LAB B	LAB C
1	0.90 (259.6)	0.51 (147.1)	0.92 (265.3)
2	0.61 (175.9)	0.47 (135.6)	1.35 (389.4)
3	0.46 (132.7)	0.21 (60.6)	
Mean EC50	0.66 (189.4)	0.49 (114.4)	1.14 (327.4)
SD	0.22 (64.5)	0.03 (47)	0.30 (87.7)
CV inter-assay%	34.06	5.77	26.79



HILL	Optimal
Control	0,91
Hill number	1,92
EC50	0,61
Maximum effect	25,90
EC5	0,13
EC10	0,19
EC15	0,25
EC20	0,30
EC25	0,34

HILL	Optimal
Control	0,83
Hill number	1,31
EC50	0,51
Maximum effect	16,37
EC5	0,05
EC10	0,10
EC15	0,14
EC20	0,18
EC25	0,22

HILL	Optimal
Control	0,76
Hill number	1,83
EC50	1,35
Maximum effect	18,84
EC5	0,27
EC10	0,41
EC15	0,52
EC20	0,63
EC25	0,74

Figure 10 : Example of concentration-response curves obtained in EASZY for 4ter-Octylphenol in LAB A, B and C. Each point represents the GFP expression measured in the brain of individual (expressed as fold GFP induction above solvent group). Data were modelled using the Hill model. The tables on the right of modelized curves report the Hill parameters and the ECx (expressed in nM) derived.

III.2.2.6. Spironolactone and dexamethasone

Spironolactone and dexamethasone, two synthetic ligands of the mineralocorticoid and glucocorticoid receptor respectively, were unable to induce an effect in EASZY as revealed by the lack of GFP induction (Figure 11 and Figure 12 respectively) at the concentrations tested ranging from 10µM to 0.12µM, i.e. from 4166 µg/L to 50 µg/L for spironolactone and 3925µg/L to 35 µg/L for dexamethasone.

In one assay performed by LAB B, the mean measured fluorescent intensity in zebrafish exposed to dexamethasone 1µM was significantly higher than that of solvent control group (Figure 12C). However, this effect was not reproduced by the LAB B (Figure 12D).

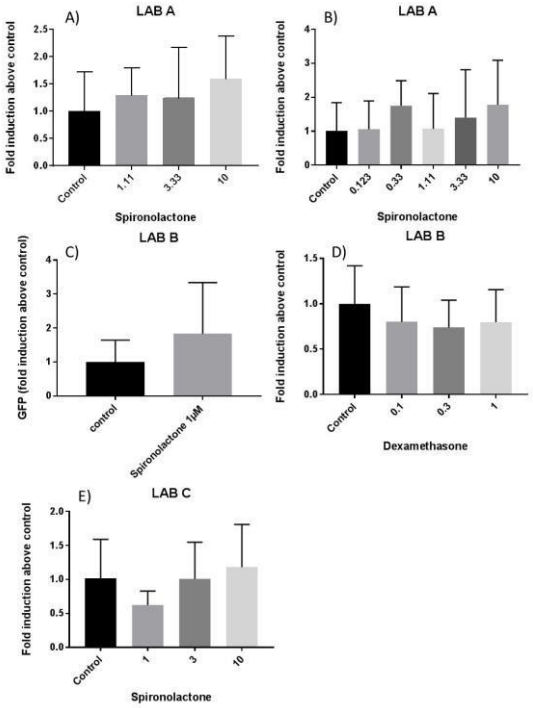


Figure 11 Effect of spironolactone (µM) on GFP expression in EASZY

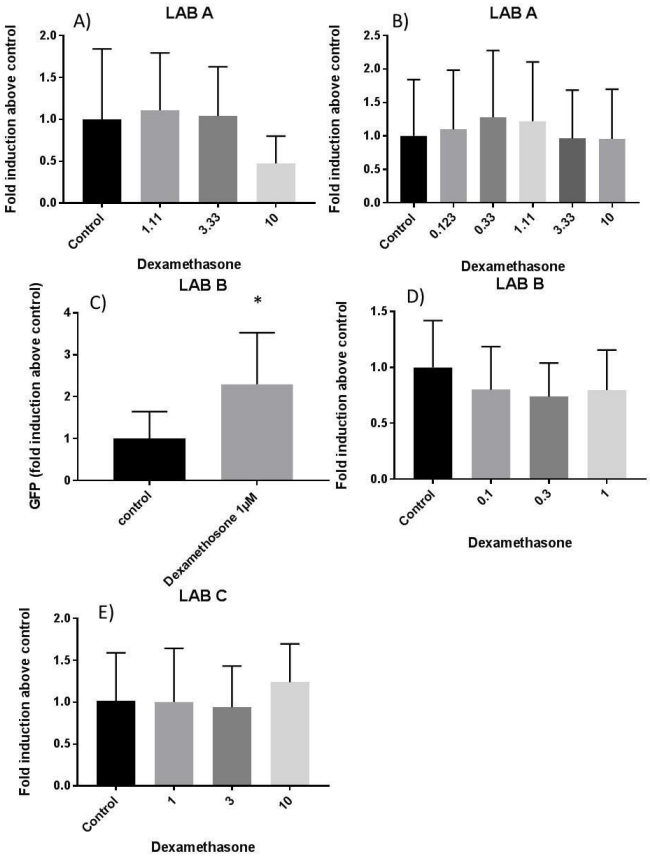


Figure 12: Effect of Dexamethasone (µM), a cortisol analogue, on GFP expression in EASZY

III. 3. Conclusions of the phase 1 validation and Perspectives.

During the phase 1 of the validation of EASZY the transfer of the transgenic *tg(cyp19a1b:GFP)* line and the EASZY method into different laboratories was successfully realized.

After a training period, the participating laboratories assessed the biological activity of a set of seven substances in the EASZY assay including steroidal estrogens used as reference compounds (EE2, E2), one bisphenol (BPA), one alkylphenolic compound (4-ter-OP), one aromatizable androgen (testosterone) as well as two negative substances (spironolactone and dexamethasone). All the substances were tested according to the description of the test methods provided to the laboratories.

The description of the test method was enough clear and informative to allow the laboratories to perform the EASZY without modifying the protocol. However, the LAB C decided to anesthetize the 4-dpf zebrafish prior to measure the fluorescence. The possibility to anesthetize the fish was therefore added in the description of the operating protocol. Furthermore, the LAB C used eggs collected from different spawns obtained by pair-crossing adult fish rather than mass-spawning.

Based on the data reported herein, the following conclusions can be made:

- all the laboratories were able detect and to quantify the estrogenic activity of the estrogenic ligands (EE2, E2, BPA, OP) and the aromatizable androgen, testosterone.

- the negative substances, Dexamethasone and Spironolactone were unable to produce any significant effect, albeit Dexamethasone 1µM significantly induced GFP in one assay.
- The active substances in EASZY induced GFP in a concentration dependent manner. The estrogenic activity of the active substances was similar among the laboratories resulting in an identical ranking of the estrogenic activity of the tested substances: EE2>> E2>> OP>testosterone>BPA.
- The EC50 derived were consistent between assays performed within a laboratory and between laboratories demonstrating that the data obtained with EASZY are reproducible within and between the laboratories.

Overall, the results from the phase 1 helped to strengthen the methodology of the EASZY assay and to define validity criteria.

During the next phase of the validation, three different laboratories will be involved among which two already participated to Phase 1 (LAB A and LAB C). An additional laboratory (LAB D) expressed the wish to participate in the phase

2. The transferability of the biological material into LAB D has been realized in 2017 and the persons in charge of realizing the EASZY assay have been trained by LAB A.

4 Results of the phase 2.

IV.1. Introduction phase 2

During the phase 1 of the validation of the EASZY assay, the transfer of the transgenic *tg(cyp19a1b:GFP)* line and the EASZY method into different laboratories was successfully realized. Based on the standard operating protocol (SOP), the biological activity of a set of seven substances in the EASZY assay was assessed. It included two steroidal estrogens (EE2, E2), one bisphenol (BPA), one alkylphenolic compound (4-ter-OP), one aromatizable androgen (testosterone) as well as two negative substances (spironolactone and dexamethasone). The results obtained in the phase 1 demonstrated the transferability of the assay and addressed the repeatability and the reproducibility of the EASZY assay within and between laboratories (see -ANNEX 4 for a summary of the quantification of the estrogenic activity of test chemicals in phase 1 validation and the coefficient of variation within and between laboratories)

All the data obtained during the phase 1 have been reported in a validation report and presented during the last VMG-ECO meeting held in October 2017 in Paris. The data were judged satisfactory and encouraging to allow the realization of the phase 2. However, some comments were made notably as regard to the number of test substances and the need to perform chemical analysis of the substance in the medium. The laboratory in charge of managing the validation therefore set-up the validation study from end of October 2017 to December 2017 to meet all the necessary criteria for an appropriate validation scheme. The phase 2 of the validation was thereafter conducted from January to July 2018.

IV.2. Objectives of the phase 2

The objectives of the phase 2 validation were i) to gain further information on the capacity of the EASZY assay to produce reliable and accurate data about the estrogenic activity of tested substances ii) to characterize the concentration-effect relationships iii) to taking into account chemically measured concentrations of selected substances.

Three laboratories tested blindly the estrogenic activity of the 11 selected substances. The set of substances was presented at the VMG-ECO meeting in October 2017.

IV.3. Selected substances for the phase 2

The selected substances for the phase 2 are reported in the Table 8. They encompassed either inactive (4 test chemicals) or active (7 test chemicals) chemicals on brain aromatase cyp19a1b gene.

Table 8: Selected substances for the phase 2 of the validation. All the substances have been blindly tested by three laboratories

Substances	CAS number	Expected activity in EASZY
Diclofenac	15307-86-5	NEG
Triclosan	3380-34-5	NEG
Benzophenone	119-61-9	NEG
11 Ketotestosterone	564-35-2	NEG
2,4-Dihydroxybenzophenone	131-56-6	POS
Bisphenol S	80-09-1	POS
Bisphenol F	620-92-8	POS
Levonorgestrel	797-63-7	POS
Norethindrone	68-22-4	POS
Dihydrotestosterone	521-18-6	POS
17β Trenbolone	10161-33-8	POS

The active substances were selected as they are known to exhibit a suitable range of estrogenic activity (from the nM range to the µM range) and cover different mechanisms of action to induce an estrogenic response in the EASZY assay, i.e. estrogen receptor agonists and substances requiring metabolization/biotransformation into estrogenic metabolites prior to elicit an estrogenic activity.

IV.4. Organization of the Phase 2

The Figure 13 illustrates the general organization of the phase 2 validation.

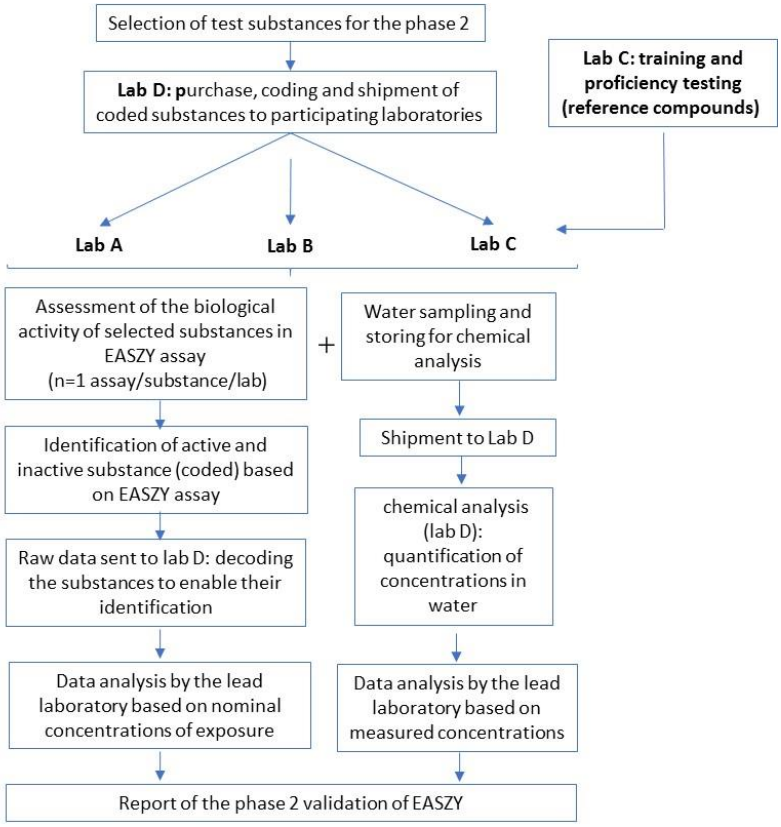


Figure 13: General scheme of the phase 2 validation of the EASZY assay. Each participating laboratory in charge of realizing the EASZY assays (Lab A, Lab B, Lab C) tested all the selected substances blindly

Four different laboratories were involved in the phase 2 of the validation.

One laboratory (labelled Lab D) was in charge of purchasing, coding and sending all the selected substances to the three laboratories responsible for realizing the EASZY assays. The substances were sent under powder form, stored in smoked glass containers. Detailed instructions were provided by Lab D to dilute the powder in an appropriate volume of solvent (dimethylsulfoxide DMSO).

A specific coding of the substances was carried out by the laboratory D so that no information could be exchanged between the laboratories in charge of realizing the assays (labelled Lab A, Lab B and Lab C). Lab D was also in charge of providing a sampling protocol to collect and store water samples. Appropriate vials were provided by Lab D for storing and shipping the water samples for subsequent chemical analysis of the substances in the medium.

Lab A-B and C were responsible for testing blindly the substances in the EASZY assay based on the standard operating protocol (SOP) defined after the phase 1 of the validation. After realizing all the testing, the laboratories sent their data with a conclusion about the estrogenic activity of each substance categorized as either active (significant induction of GFP) or inactive (no induction of GFP). Thereafter, the lab D decoded the substance to allow their identification and quantified the concentrations of substances in the medium.

Two out of the three laboratories were already involved in the phase 1. For the third one (Lab C), it was its first involvement. In late 2017, the transferability of the biological material into Lab C was performed and the persons in charge of realizing the EASZY assay have been trained by the leading laboratory (Lab A).

IV.5. Proficiency testing (Lab C):

Prior to testing unknown chemicals with the EASZY assay, Lab C demonstrated its proficiency to realize the EASZY assay. The proficiency testing was evaluated in early 2018 by measuring the estrogenic activity of a subset of reference substances, i.e. 17β-estradiol (N=4 independent assays) and 4-ter-Octyphenol (N=1 assay).

The Lab C successfully assessed the estrogenic activity of E2 and 4-ter- Octyphenol. Each compound led to concentration-dependant inductions of GFP in transgenic zebrafish embryos (Figure 14).

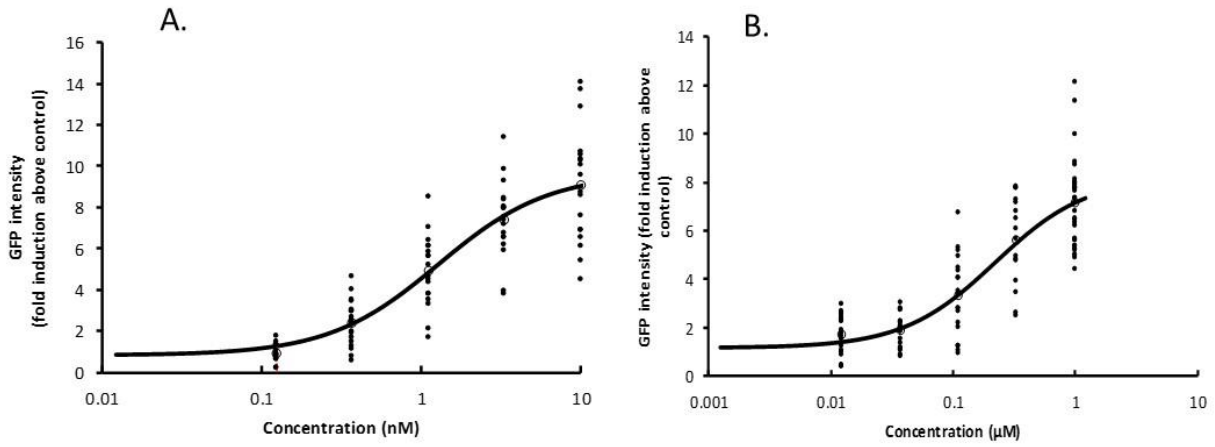


Figure 14: Example of concentration-response curves obtained by Lab C during the proficiency testing with (A) 17β-estradiol and (B) 4-ter-octylphenol. The EC50 derived for 17β-estradiol and 4 ter-octylphenol from these experiments were respectively 1.29 nM and 0.22 µM (based on nominal concentrations)

The EC50 values obtained by Lab C during the proficiency testing are reported in the Table 9.

Table 9 : Results of the proficiency testing of two reference substances (E2 and 4-ter-OP) by Lab C. $EC_{50}$are expressed in nM (ng/L) and µM (µg/L)

reference substance	Assay1	Assay 2	Assay 3	Assay 4	Mean	SD	CV (%)
EC_{50} E2 nM (ng/L)	1.97 (536.6)	3.19 (868.9)	1.53 (416.7)	1.29 (351.4)	2.0 (543.4)	0.8 (230.2)	42.3
EC_{50} 4-ter-OP µM (µg/L)	0.22 (45.4)	-	-	-	-	-	-

The reported EC50 values agree with those previously measured notably by Lab A and Lab B during the phase 1 of the validation (see ANNEX 4). Indeed, the mean EC50 values ± SD and the intra-assay coefficient of variation (CV%) for E2 reported by the 3 participating laboratories during the phase 1 were 1.98 nM ± 0.56 (538 ng/L ± 152) (CV 28%), 1.91 nM ± 0.66 (521 ng/L ± 180) (CV 34%) and 2.08 nM ± 1.02 (569 ng/L ± 273) (CV 49%) respectively. Furthermore, the inter-assay coefficient of variability found by Lab C for E2 was consistent with those previously reported for this compound as the CV inter-assay reported varied from 28% to 49%.

For 4-ter-octylphenol, only one assay has been conducted by lab C during the proficiency testing. The EC50 value for 4-ter-octylphenol (EC50=0.22 µM = 45.4 µg/L) was in perfect agreement with the EC50 values ± SD reported by the three participating laboratories during the phase 1, i.e. 0.29 µM ± 0.13 (60.5 µg/L ± 26.5), 0.20 µM ± 0.03 (41.8 µg/L ± 5.9) and 0.27 µM ± 0.07 (56.4 µg/L ± 15.6) respectively.

Although the proficiency testing was limited to two positive reference substances, the data provided by Lab C were sufficient to demonstrate its capacity in assessing the estrogenic activity of test substance using the EASZY assay.

IV.6. Assessment of the biological activity of coded substances using EASZY

IV.6.1. ***Compliance of the assays with the validity criteria of EASZY***

All the EASZY assays were performed from March to May 2018. For the laboratories A and B, the eleven substances were evaluated by performing three successive EASZY assays (n=4 or 3 substances per assay). For laboratory C, 4 different assays were performed to test all the eleven substances (n=2-3 substances per assay).

Appropriate controls were run according to the SOP, i.e. water control, solvent control (DMSO 0.01% V/V) and positive control (EE2 at 0.05 nM, or 14.8 ng/L). The mortality was evaluated in all experimental groups during the time course of the experiments and reported at the end of each experiment (expressed as cumulative mortality).

The compliance of the assays with the validity criteria of EASZY were checked and are summarized in Table 10.

Table 10: Compliance of the EASZY assays with the validity criteria

Proposed criteria	Fulfilment
Fertilization rates of the eggs collected from the batches >70%	100% (10/10)
Mortality in controls (water, solvent) ≤ 20% at the end of the test	90% (9/10) [a]
Mean measured fold induction of GFP induced by the reference substance (EE2 0.05 nM) ≥ 9 as compared to controls	100% (10/10)

[a] For one assay (Lab B, assay # 2; Substance 4 to Substance 7), an unexpected higher mortality rates in the water and solvent controls (30 and 35% respectively) was reported.

The Table 11 presents in detail the survival rate measured in controls. In almost all assays, the survival rates observed at the end of the experiments were comprised between 90 and 100% except for one assay performed in laboratory B for which an unexpected mortality rate was recorded in water and solvent controls. The reasons for this excessive mortality are not known especially as in the positive control group (EE2 0.05 nM; 14.8 ng/L) the survival rate was 100%.

Table 11: Survival rate (%) recorded in water and solvent control groups

	Laboratory A			Laboratory B			Laboratory C			
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 4
Water control	90	90	95	100	**65**	100	100	100	100	100
Solvent control	90	95	90	100	**70**	100	100	95	100	100

The Table 12 reports the mean measured ± SD GFP intensity expressed as fold induction above solvent control group.

In each EASZY assay, the range of mean induction levels (10.4 – 30.5) reported for the positive control (EE2 0.05 nM ; 14.8 ng/l) is relatively important, but was ≥ 9 as compared to control, fulfilling this criterion of validity.

Table 12: Mean GFP induction ± standard deviation (SD) measured in positive controls (EE2 at a nominal concentration of 0.05 nM). The survival rates (%) in the positive controls are also indicated.

	Laboratory A			Laboratory B			Laboratory C			
	assay 1	assay 2	assay3	assay 1	assay 2	assay 3	assay 1	assay 2	assay 3	assay 4
Mean GFP induction ± SD	17.19 ±4.87	24.05 ±8.41	14.87 ±3.65	30.5 ±9.03	15.00 ±4.17	22.32 ±8.29	10.49 ±3.89	10.4 0±2.89	16.72 ±5.4	16.87 ±4.68
Survival rate (%)	90	95	95	100	100	100	100	100	100	100

IV.6.2. Biological activity of the 11 substances tested in the three laboratories

IV.6.2.1. Survival rate in zebrafish embryos

At the end of experiments the survival rate was reported for all treatment groups

For most of the tested substances, the survival rates varied from 80 to 100% (see ANNEX 5).

Low survival rates were observed for some substances, but it did not reflect their intrinsic toxicity. For instance, lowest mortality rates were observed for 2.4 dihydroxybenzophenone in Lab A for two concentrations (40-65%) but the effect was not related to the concentration of exposure and was not found in Laboratory B and C. Similarly, the survival rates reported for benzophenone by the Lab B (75-85%) were higher than those found by the two other laboratories. GFP expression were measured in these treatment groups.

In contrast, triclosan and diclofenac elicited acute toxicity. Based on the cumulative mortality at 96 hours, the lethal concentration leading to 50% of mortality (LC50) was also estimated for each substance (Figure 15).

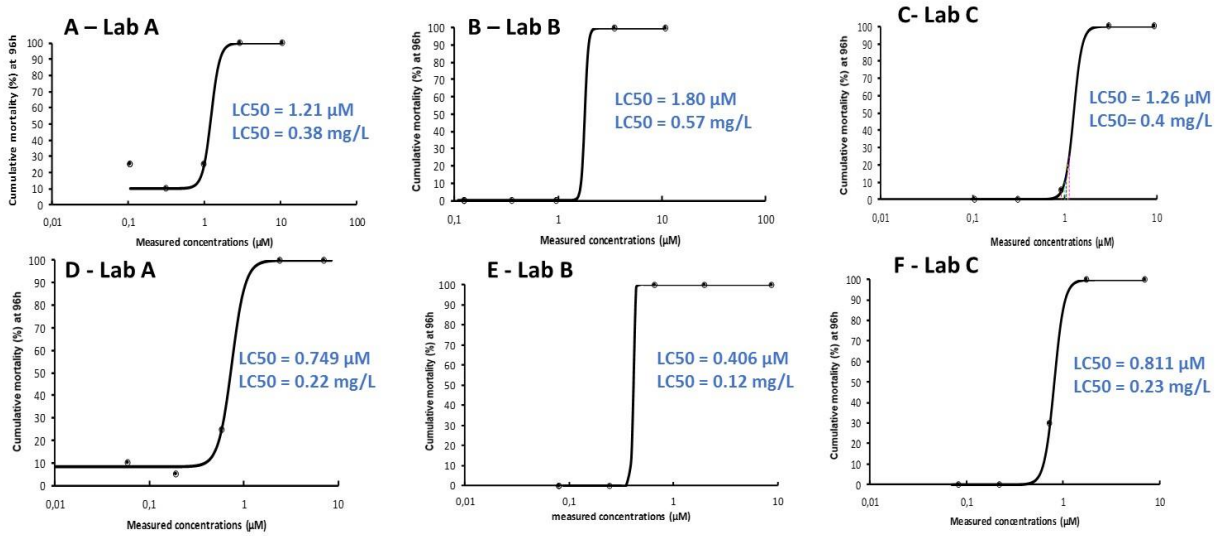


Figure 15: Cumulative mortality and LC50 (expressed in µM and mg/L) in transgenic zebrafish embryos at 96 hours for: (A-C) diclofenac and (D-F) tricolosan

Interestingly, the toxic responses were very similar from a laboratory to another. Furthermore, the LC50 reported herein in transgenic zebrafish embryos for triclosan and diclofenac are in agreement with those previously reported for zebrafish [1].

[1]Diclofenac EQS dossier 2011.pdf - CIRCABC
Triclosan : Norme de Qualité environnementale Triclosan– n° CAS : 3380-34-5 INERIS (https://substances.ineris.fr/fr/substance/2723)

IV.6.2.2. GFP intensity measured in solvent and water controls

A solvent control was performed. Dimethylsulfoxide (DMSO) was used as solvent to dissolve the test substances. In all treatment group the final concentration of DMSO was 0.01% (v:v). The Table 13 reports the mean measured GFP expression in water and solvent control groups. No significant difference was noticed between water and solvent groups confirming the lack of effect of DMSO on GFP expression. The variability measured in water and solvent controls is high but consistent among the different assays performed within and between laboratories. The variability measured in controls likely reflects the natural variation of the expression of the cyp19a1b gene in zebrafish embryos reported in literature as well as the method to measure *in vivo* the fluorescence. Nonetheless, due to the magnitude of the response observed in exposed zebrafish embryos and the number of individuals monitored, the statistical power of the EASZY assay is high reflecting its capacity to detect a difference between control and exposed groups.

Table 13: Mean GFP intensity ± standard deviation (SD) measured in negative control groups. No significant difference was measured (n= number of GFP measurement per treatment group)

			solvent control	water control	positive control
Lab A	1	mean	1.00	0.89	17.19
		SD	0.43	0.49	4.87
		n	17	16	17
	2	mean	1.00	0.71	24.05
		SD	0.58	0.51	8.41
		n	19	15	16
	3	mean	1.00	0.97	14.87
		SD	0.55	0.61	3.65
		n	16	17	18
Lab B	1	mean	1.00	1.17	30.50
		SD	0.72	0.69	9.03
		n	20	20	20
	2	mean	1.00	1.43	15.00
		SD	0.67	0.87	4.17
		n	14	11	15
	3	mean	1.00	1.44	22.32
		SD	1.08	1.12	8.29
		n	20	20	20
Lab C	1	mean	1.00	0.91	10.49
		SD	0.44	0.30	3.89
		n	20	20	20
	2	mean	1.00	0.92	10.40
		SD	0.32	0.37	2.89
		n	19	20	20
	3	mean	1.00	1.43	16.72
		SD	0.78	0.79	5.40
		n	17	20	20
	4	mean	1.00	1.21	16.87
		SD	0.65	0.56	4.68
		n	18	20	19

IV.6.2.3. Qualitative assessment of the estrogenic activity of the 11 substances

At a first step, each laboratory was requested to evaluate the potential estrogenic activity of the test substances.

They were categorized as either active or inactive. An active substance was defined as a substance inducing significantly the GFP expression as compared to solvent control while an inactive substance.did not significantly induce the GFP expression.

The ANNEX 6 reports the GFP profiles obtained for the coded substances in the three laboratories. Based on these data, each laboratory reports the biological activity of each substance categorized as active or inactive (Table 14).

There was complete agreement across the three laboratories in the experimentally determined biological activity of the selected compounds. . Based on these data, it can be concluded that the EASZY method accurately identify substances acting on the ER-regulated brain aromatase *cyp19a1b* gene without producing false positive or false negative response.

For the substance 1 tested in Lab B (i.e., triclosan), a low but significant induction was measured for the lowest concentration (0.08 µM) (see ANNEX 6). The mean measured GFP fold induction as compared to solvent was 1.7 ± 0.8. However, given the very low induction measured and the absence of concentration-response relationship, the substance was not identified as active. During the phase 1 of the validation of EASZY, a similar observation was made for dexamethasone, an agonist ligand of the glucocorticoid receptor. A mean measured two-fold induction was observed for dexamethasone 1µM. As this effect was not reproducible, the substance was identified as inactive. Altogether, the data obtained with triclosan and dexamethasone tend to suggest that the mean measured fold induction should be higher than 2 as compared to water or solvent control to be considered as significant from a biological point of view and to declare a substance as active in EASZY.

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Table 14: Biological activity of coded substances. Data were reported firstly as either inactive (in green) or active (in red) substance in the EASZY assay.

Lab A			Biological activity	
Assay #	coded substance	substance name	experimental	expected
1	LabA-Sub1	Diclofenac		
1	LabA-Sub2	Triclosan		
1	LabA-Sub3	2,4-Dihydroxybenzophenone		
2	LabA-Sub4	Bisphenol S		
2	LabA-Sub5	Benzophenone		
2	LabA-Sub6	Bisphenol F		
2	LabA-Sub7	Levonorgestrel		
3	LabA-Sub8	Norethindrone		
3	LabA-Sub9	Dihydrotestosterone		
3	LabA-Sub10	17b Trenbolone		
3	LabA-Sub11	11 Ketotestosterone		

Lab B			Biological activity	
Assay #	coded substance	substance name	experimental	expected
1	LabB-Sub1	Triclosan		
1	LabB-Sub2	2,4-Dihydroxybenzophenone		
1	LabB-Sub3	Diclofenac		
2	LabB-Sub4	Benzophenone		
2	LabB-Sub5	Bisphenol F		
2	LabB-Sub6	Bisphenol S		
2	LabB-Sub7	17b Trenbolone		
3	LabB-Sub8	11 Ketotestostérone		
3	LabB-Sub9	Levonorgestrel		
3	LabB-Sub10	Norethindrone		
3	LabB-Sub11	Dihydrotestosterone		

Lab C			Biological activity	
Assay #	coded substance	Name of substance	experimental	expected
1	Lab C-Sub1	2,4-Dihydroxybenzophenone		
1	Lab C-Sub2	Diclofenac		
2	Lab C-Sub3	Triclosan		
2	Lab C-Sub4	Bisphenol F		
2	Lab C-Sub5	Bisphenol S		
3	Lab C-Sub6	Benzophenone		
3	Lab C-Sub7	11 Ketotestosterone		
3	Lab C-Sub8	17b Trenbolone		
4	Lab C-Sub9	Norethindrone		
4	Lab C-Sub10	Dihydrotestosterone		
4	Lab C-Sub11	Levonorgestrel		

IV.6.2.4. Quantification of the estrogenic activity based on nominal concentrations of exposure

All the biological data were further analysed based on the nominal concentrations of exposure. The concentration-response curves were modelled using the Hill equation model The Regtox 7.0.6 Microsoft Excel TM macro was used[2].

It allows derivation of effective concentrations leading to 50% of effect (EC50). The Table 15 summarizes the EC50 expressed in µM and in (µg/L) for all active substances in the three laboratories.

Table 15: EC50 (expressed in µM and in (µg/L)) derived from the nominal concentration-response curves for the active substances in EASZY. For each compound, one assay was realized in each laboratory

	EC50 in µM and (µg/L) based on nominal concentrations of exposure		
Substances	Lab A	Lab B	Lab C
2,4-Dihydroxybenzophenone	1.5 (321)	1.49 (319)	1.3 (278)
Bisphenol S	>60 (>15016)	>60 (>15016)	23.8 (5956)
Bisphenol F	2.04 (408)	1.97 (394)	2.07 (414)
Levonorgestrel	0.13 (40.6)	0.38 (118.7)	0.22 (68.7)
Norethindrone	0.007 (2.1)	0.017 (5.1)	0.012 (3.6)
Dihydrotestosterone	1.59 (462)	0.4 (116)	0.44 (128)
17β-Trenbolone	0.45 (122)	0.96 (260)	0.67 (181)

The Table 16 summarizes the ability of the EASZY assay to rank the substances according to their estrogenic activity.

[2] http://www.normalesup.org/~vindimian/fr_index.html

Table 16: EC50 (expressed in µM and in (µg/L)) derived from the nominal concentration-response curves for the active substances in EASZY. For each compound, one assay was realized in each laboratory

	Rank of substances based on their estrogenic activity		
Substances	Lab A	Lab B	Lab C
Norethindrone	1	1	1
Levonorgestrel	2	2	2
17 β-Trenbolone	3	4	4
2,4-Dihydroxybenzophenone	4	5	5
Dihydrotestosterone	5	3	3
Bisphenol F	6	6	6
Bisphenol S	7	7	7

The data provided in Table 15 andTable 16 demonstrate an overall good agreement between the EC50 found in the three laboratories and the capacity of EASZY to rank the substances according to their estrogenic activities.

However, some differences were highlighted. For instance, BPS induced only weak estrogenic activities in Lab A and Lab B. It precluded the possibility to derive an EC50 (EC50 >60 µM (EC50> 15016 µg/L) in Lab A and Lab B). In contrast, a concentration-response curve was observed in lab C with an estimated EC50 = 23.8 µM (EC50=5956 µg/L).

A difference was also noticed for Dihydrotestosterone which appeared much less active in Lab A (EC50 = 1.59 µM; EC50 = 462 µg/L) as compared to Lab B (EC50 = 0.40 µM; EC50! 116 µg/L) and Lab C (EC50 = 0.44 µM); EC50= 128 µg/L).

Despite some differences were noticed about the quantification of the estrogenic activity of the substances, it can be concluded that the laboratory provides reliable data about the biological activity of the substances and their estrogenic activity.

IV.6.2.5. Chemical analysis of the substances in the medium.

During the phase 2, water samples were taken in all treatment groups to analysis the substances in freshly prepared solutions (T0h) and immediately prior to renewal of the medium (T24h). All the chemical analyses have been performed by the Lab D from June to end of July 2018. Almost 490 samples were analysed during this period.

The Table 17 reports the limit of detection and the analytical methods for each substance

Table 17: Overview of the analytical methods and their limits of detection

Compound	Limit of detection (ng/L)	Method
Diclofenac	9	HPLC system coupled to mass spectrometer
Triclosan	10	HPLC system coupled to mass spectrometer
2,4-dihydroxybenzophenone	73	HPLC system coupled to mass spectrometer
Norethindrone	26	HPLC system coupled to mass spectrometer
Levonorgestrel	97.7	HPLC system coupled to mass spectrometer
Bisphenol F	74.9	UPLC coupled to mass spectrometer
Bisphenol S	7	UPLC coupled to mass spectrometer
Benzophenone	167	HPLC system coupled to mass spectrometer
17b Trenbolone	21	HPLC system coupled to mass spectrometer
11 Ketotestosterone	28	HPLC system coupled to mass spectrometer
Dihydrotestosterone	184	HPLC system coupled to mass spectrometer
Ethinylestradiol	2.23	HPLC system coupled to mass spectrometer

The chemically determined concentrations measured in freshly prepared solutions (T0h) and immediately prior to renewal of the medium (T24h) are reported in ANNEX 7. These data led to the following conclusions.

In water and solvent controls, no contamination by any of the test substances was found.

For most substances, a good agreement between the nominal and the measured concentrations was observed. This was observed in the different laboratories and there was, in general, no marked differences between T0 and T24.

Some deviations between the nominal and the measured concentrations were however noticed for certain substances (see ANNEX 8).

It was the case for diclofenac (Lab C), triclosan (Lab A, B and C) and benzophenone (Lab C) with measured concentrations, below the nominal concentrations (up to 47%).

For bisphenol S, the measured concentrations in Lab C were consistently higher than the nominal concentrations (from 145 to 177% of the nominal concentrations at T0). It results in higher concentrations of exposure of zebrafish embryos to BPS as compared to laboratories A and B.

A major difference was also noticed for dihydrotestosterone with a rapid drop of the concentrations measured between T0h and T24h in all treatment groups. As a result, the concentrations of exposure to dihydrotestosterone in laboratory A were low as compared to laboratories B and C.

Overall, the chemical analysis demonstrated that the measured values were satisfactory to accept all the experiment. It shows that semi-static exposure system in glass crystalliser with a

total renewal of the water every day allow to expose the zebrafish embryos at the desired concentrations of test substances and avoid any contamination in the control groups indicating that the studies were valid for use to assess the relevance and the reliability of the data collected within the phase 2.

IV.6.2.6. Quantification of the estrogenic activity based on chemically measured concentrations of exposure.

Based on the chemical analysis, an estimation of the mean measured concentration of exposure has been extrapolated according to a method previously used for semi-static renewal system. The method used is based on the calculation of Time-weighted mean (ANNEX 8 of the OECD TG 211).

As measurement of water concentrations were not realized before and after each renewal of the medium, it was assumed that the variation observed between T0 and T24h at the occasion of one renewal reflected the behaviour of the test substance over the duration of the assay..

For each experiment, the mean measured concentrations of exposure was calculated (ANNEX 8). These data were used for analysing the GFP expression as a function of chemically determined concentrations.

The Figure 16 and Figure 17 represent the modelled concentration-responses curves based on measured concentrations.

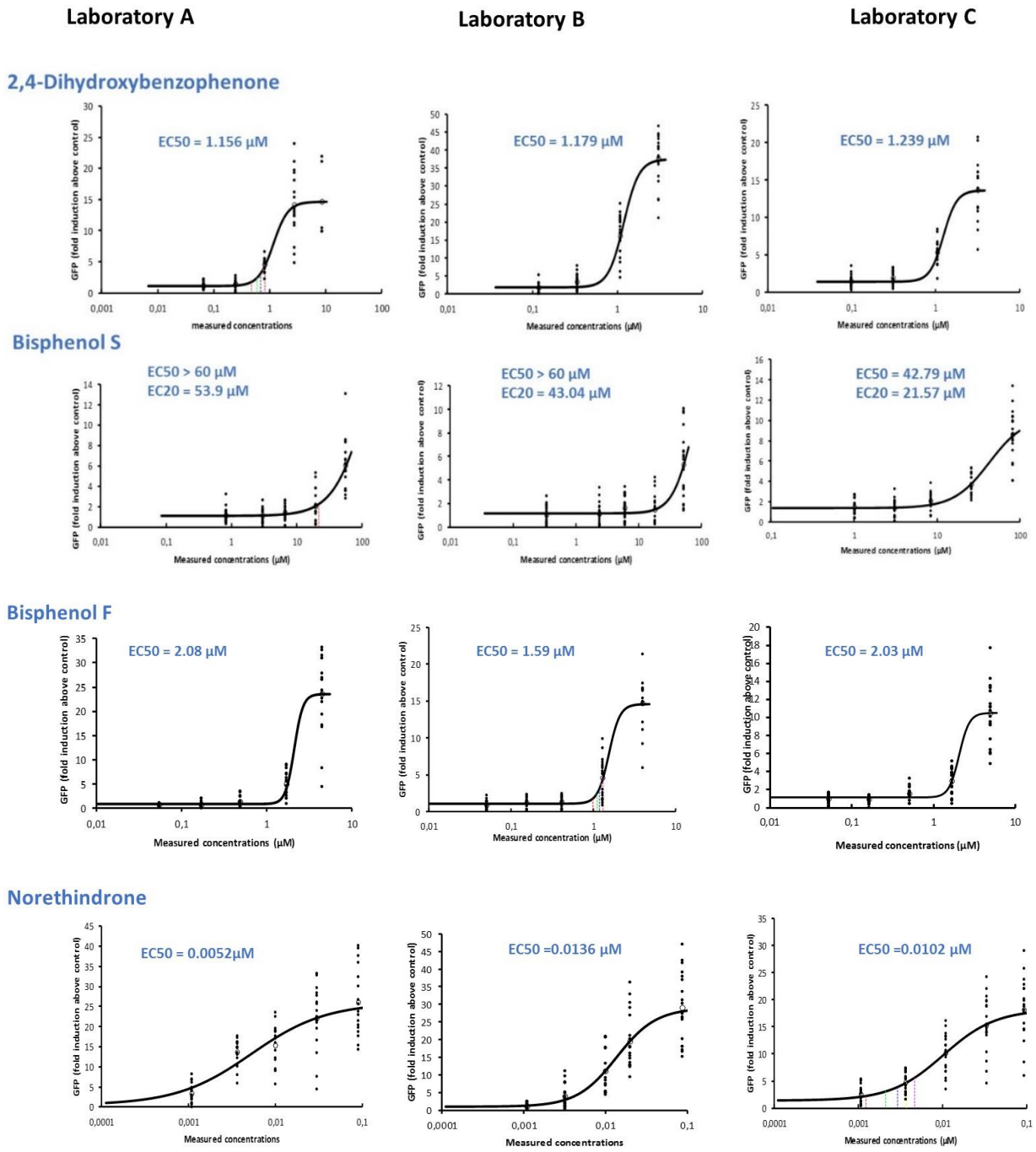

Figure 16: Concentration-response curves of GFP induction in transgenic tg(cyp19a1b:GFP) embryos by selected substances in the three laboratories. All the chemically measured concentrations are expressed in µM. Each data point represents the GFP intensity measured in zebrafish embryos after 4 days of exposure expressed as fold induction above solvent control. EC50 were derived from the Hill model.

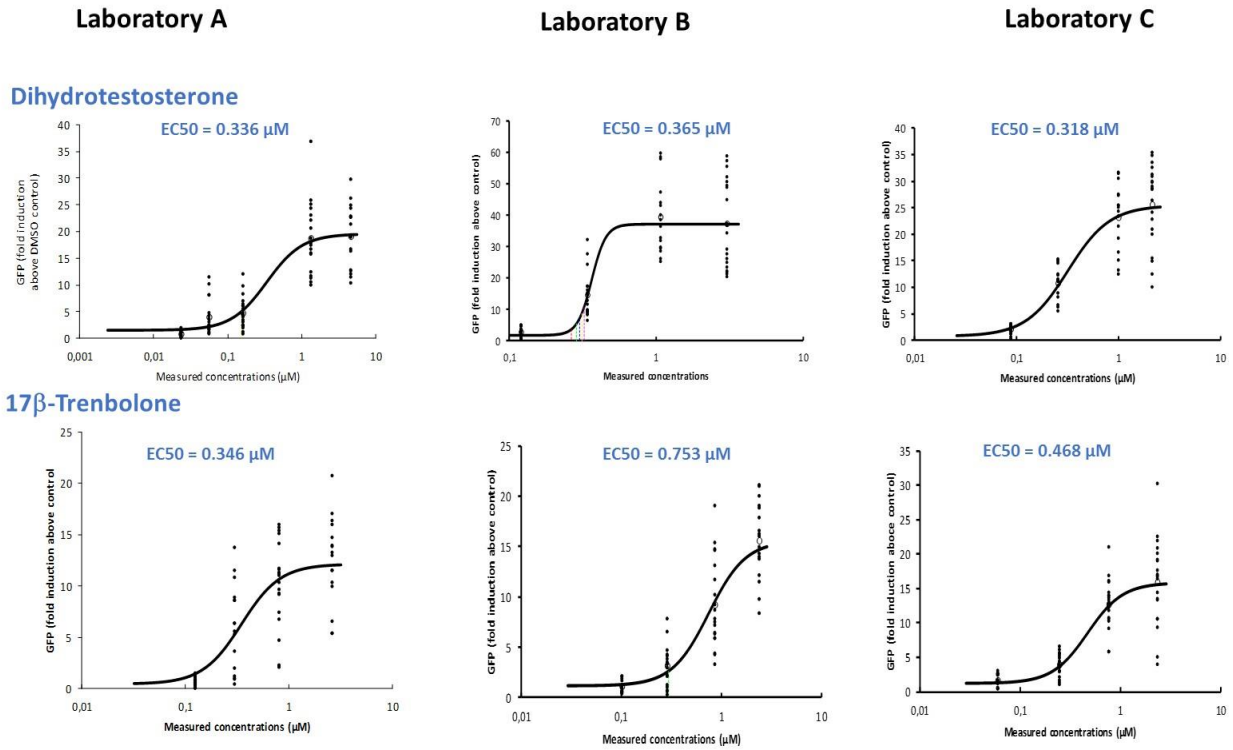

Figure 17: Concentration-response curves of GFP induction in transgenic tg(cyp19a1b:GFP)embryos by selected substances in the three laboratories. All the chemically measured concentrations are expressed in µM. Each data point represents the GFP intensity measured in zebrafish embryos after 4 days of exposure expressed as fold induction above solvent control. EC50 were derived from the Hill model

The Table 18 summarizes the EC50 derived for the test substances.

Table 18: EC_{50} (expressed in µM and µg/L) derived from chemically measured concentration-response curves for the active substances in EASZY. The coefficient of variation between laboratory (CV %) is indicated

EC_{50} substances		Lab A	Lab B	Lab C	Mean	SD	CV (%)
2,4-Dihydroxybenzophenone	µM	1.16	1.18	1.24	1.19	0.04	3.6
	µg/L	248	253	265	255	9	
Bisphenol S	µM	>60	>60	42.8	-	-	-
	µg/L	> 15016	> 15016	10711	-	-	
Bisphenol F	µM	2.08	1.55	2.03	1.89	0.29	15.5
	µg/L	416	310	406	378	59	
Levonorgestrel	µM	0.15	0.37	0.26	0.26	0.11	42.3
	µg/L	47.5	114.7	80.3	80.8	33.6	
Norethindrone	µM	0.005	0.014	0.010	0.010	0.005	46.6
	µg/L	1.49	4.18	2.98	2.88	1.35	
Dihydrotestosterone	µM	0.336	0.365	0.318	0.340	0.02	7.0
	µg/L	98	106	92	99	6.9	
17β-Trenbolone	µM	0.346	0.753	0.468	0.522	0.209	40
	µg/L	94	204	127	141	56	

IV.6.2.7. Comparison of the EC_{50} based on nominal concentrations and EC_{50} based on measured concentrations

The Table 19 compares the EC_{50} based on either nominal or chemically measured concentrations.

Table 19: Comparison of the EC_{50} expressed in µM and in µg/L based on either nominal or chemically measured concentrations of test substances

Substance			EC50 LAB A	EC50 LAB B	EC50 LAB C	Mean	SD	CV (%)
Norethindrone	µM	nominal	0.007	0.017	0.012	0.012	0.005	42
		measured	**0.005**	**0.014**	**0.010**	**0.010**	**0.005**	**47**
	µg/L	nominal	2.1	5.1	3.6	3.6	1.5	
		measured	**1.5**	**4.2**	**3.0**	**2.9**	**1.3**	
Levonorgestrel	µM	nominal	0.13	0.38	0.22	0.24	0.13	52
		measured	**0.15**	**0.37**	**0.26**	**0.26**	**0.11**	**42**
	µg/L	nominal	40.6	118.7	68.7	76.0	39.6	
		measured	**47.5**	**114,7**	**80.3**	**80.8**	**33.6**	
Dihydrotestosterone	µM	nominal	1.59	0.40	0.44	0.81	0.68	83
		measured	**0.34**	**0.37**	**0.32**	0.34	0.02	7
	µg/L	nominal	462	116	128	235	196	
		measured	**248**	**253**	**265**	**255**	**9**	
17β- Trenbolone	µM	nominal	0.45	0.96	0.67	0.69	0.26	37
		measured	**0.35**	**0.75**	**0.47**	0.52	0.21	40
	µg/L	nominal	122	260	181	188	69	
		measured	**94**	**204**	**127**	**141**	**56**	
2,4-Dihydroxybenzophenone	µM	nominal	1.50	1.49	1.30	1.43	0.11	8
		measured	**1.16**	**1.18**	**1.24**	**1.19**	**0.04**	**4**
	µg/L	nominal	321	319	278	306	24	
		measured	**248**	**253**	**265**	**255**	**9**	
Bisphenol F	µM	nominal	2.04	1.97	2.07	2.03	0.05	3
		measured	**2.08**	**1.55**	**2.03**	**1.89**	**0.29**	**16**
	µg/L	nominal	408	393	414	405	11	
		measured	**416**	**310**	**406**	**378**	**59**	
Bisphenol S	µM	nominal	>60	>60	23,8			
		measured	**>60**	**>60**	**42.8**			
	µg/L	nominal	>15016	>15016	5956			
		measured	**> 15016**	**> 15016**	**10711**			

In most cases, the EC50 derived using the chemically measured concentrations in the water were lowest or similar as compared to EC50 derived using nominal concentrations of exposure. As a result, the ratio between EC50 nominal and EC50 measured were >1 or similar, i.e. ratio ~ 1. It was the case for 2,4-dihydroxybenzophenone, norethindrone, 17b-trenbolone, levonorgestrel and bisphenol F.

There were however noticeable exceptions. For instance, marked difference were observed between the two EC50 reported for Dihydrotestosterone in laboratory A and Bisphenol S in laboratory C (Table 19).

Based on measured concentrations of Dihydrotestosterone in the medium, the EC50 derived was 4.6 times weakest as compared to the "nominal" EC50 (Table 19). It reflects that measured concentrations of the substance were below the nominal concentrations of exposure in all

exposure conditions (see ANNEX 7 and 8). Remarkedly, the "measured" EC50 for Lab A was very similar with EC50 from Lab B and C resulting in a very low inter-laboratory coefficient of variation.

For BPS in lab C, the "measured" EC50 was 2-fold higher as compared to the "nominal" EC50. This difference is explained by the higher measured concentrations of BPS as compared to nominal concentrations. Indeed, the chemically measured concentrations of BPS varied from 145% to 196% of the nominal concentrations (see ANNEX 8).

Altogether, these data demonstrated that chemical analyzes of substances in water can explain the observed differences about estrogenic activity of some substances between laboratories (i.e., dihydrotestosterone, bisphenol S). Furthermore, the chemical analysis allowed the refinement of the EC50 values thereby increasing the correlations between EC50 from the laboratories (Figure 18).

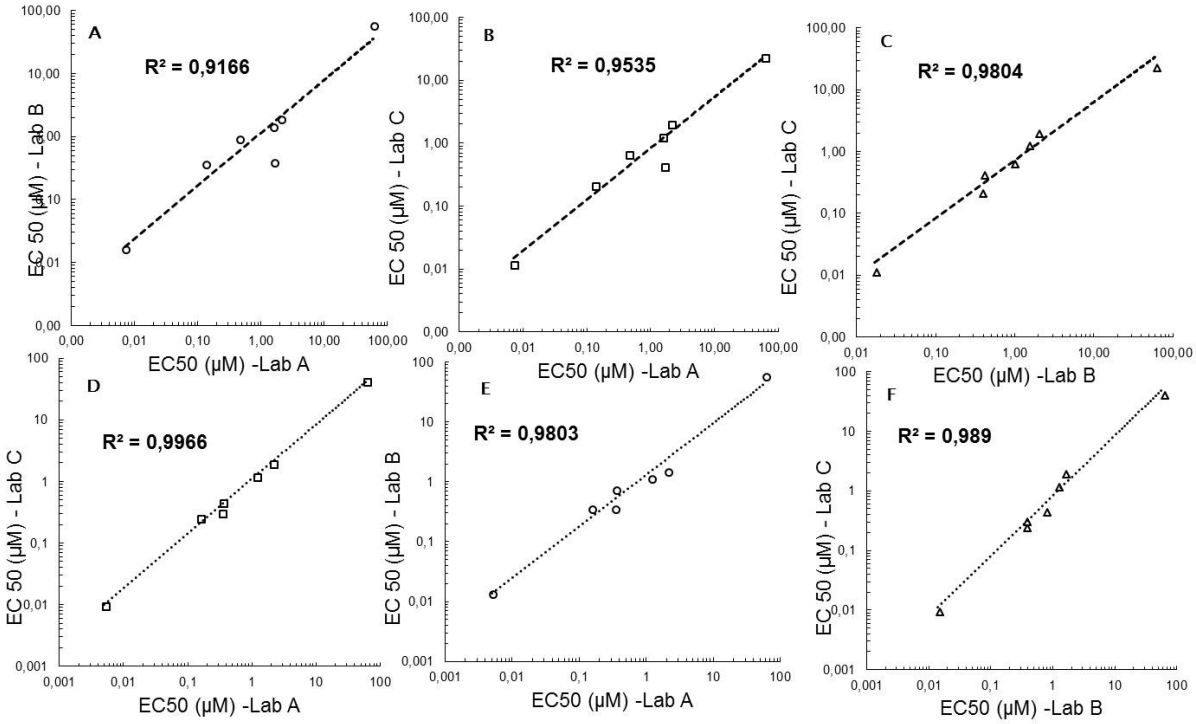

Figure 18 : Correlation between the EC50 values reported for the selected substances in the three laboratories. EC50 values were derived using (A-C) nominal concentrations of exposure or (D-F) chemically determined concentrations

IV.7. Conclusions of the phase 2 validation of EASZY.

From October 2017 to July 2018, the phase 2 validation study of the EASZY assay has been set-up and conducted. Eleven substances, encompassing either active or inactive substances, were selected and evaluated using the SOP defined after the phase 1.

Among the participating laboratories, one was naïve and important efforts have been made to transfer the assay method into this laboratory and allow it to manage the EASZY assay. After a training period, the demonstration of the laboratory proficiency was done as illustrated by the successful quantification of the estrogenic activity of reference ligands (17β-estradiol and 4-ter-octylphenol).

Eleven substances were tested blindly by three laboratories. The results obtained clearly demonstrated the capacity of the EASZY assay to accurately identify estrogenic substances from inactive ones.

Furthermore, the quantification of the estrogenic activity of the seven active substances demonstrated that the EASZY assay can reliably quantify the estrogenic activity of test substances.

A significant number of chemical analysis have been evaluated by the laboratory D (not involved in the biological testing) to further characterize the exposure conditions of the zebrafish embryos in the EASZY assay.

Based on chemical analysis we demonstrated that no contamination was found in negative controls as the test substances were below the limit of detection in all control groups. In general, the measured concentrations were in very good agreement with the expected nominal concentrations to which the fish have been exposed and no major differences were noticed between the participating laboratory. For two substances, the chemical analysis allowed to highlight some deviations between the nominal and the measured concentrations. By using chemically measured concentrations, the EC50 values were refined for all substances and corrected for two of them thereby reducing the coefficient of variation observed between the laboratories.

Altogether, the data reported in the phase 2 of the validation provide further evidence of the reliability, accuracy and robustness of the EASZY assay for screening the estrogenic activity of substances acting on the ER-regulated cyp19a1b gene in the developing brain of zebrafish.

5 Additional information following the WNT commenting rounds

V.1. Test design:

After the first commenting round, the lack of replication was outlined with a recommendation to distribute the embryos in different exposure vessels for each test condition. The test design was modified to include 3 replicates per test condition containing 7 embryos each (n=7*3 embryos per test condition).

The lead laboratory evaluated the estrogenic activity of BPF in EASZY assays run in parallel using either one replicate with 20 embryos (initial design) or three replicates with 7x 3 embryos. The results obtained are shown in Figure 19. It demonstrates that the change of the test design does not influence the outcome of the assay as the estrogenic activity of BPF with similar GFP induction profiles whatever the test design.

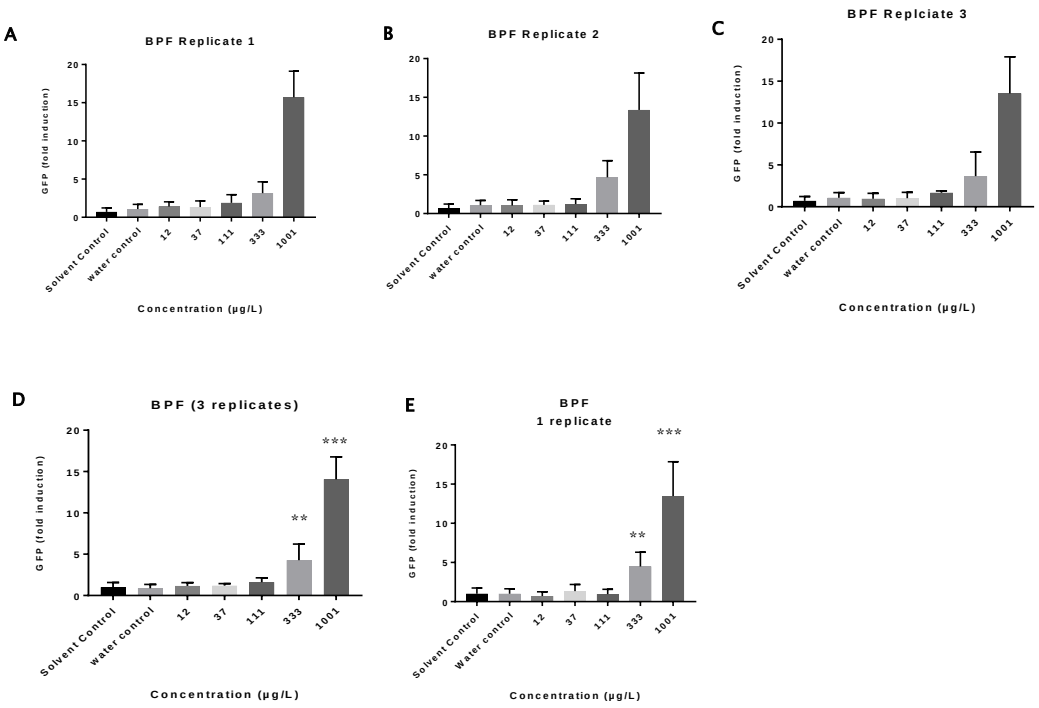

Figure 19; Estrogenic activity of BPF in EASZY using either three replicates with n=7 embryos or one replicate with n=20 embryos. The histograms A-C show the responses

obtained in the three replicates (from replicate 1 to replicate 3) each containing n=7 embryos. D. Mean of the means measured in the three replicates. E. Estrogenic activity of BPF in EASZY using a single replicate containing n=20 embryos

The comparison of the estrogenic activity of BPF with the data collected from the phase 2 validation study (BPF was blindly tested in three laboratories) (Figure 20) demonstrated that the estrogenic activity of BPF was similar further illustrating that the test design of the EASZY assay with three replicates does not influence the outcome of the assay.

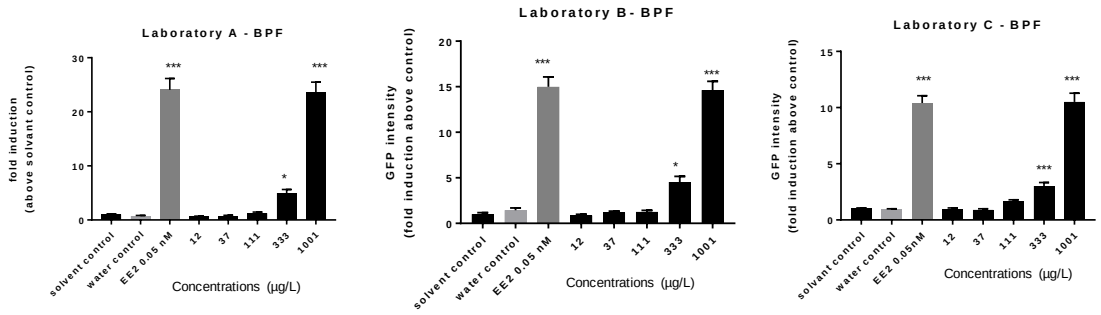

Figure 20 Estrogenic activity of BPF reported during the phase 2 validation study illustrating that the GFP expression profiles were similar as those reported with the new test design

V.2. Validity criteria.

Comments were made about the mortality and hatching rate in controls for a test to be valid. These validity criteria were changed while considering the test design with 3 replicates per test condition. The maximum mortality was set to 1 death per replicate (*i.e.*, 14.3%) and the overall hatching rate was set to 90% (*i.e.*, maximum two unhatched embryos)

The retrospective analysis of 13 independent EASZY assays performed in 2020 by the lead laboratory showed that the validity criterion for the mortality was met for all the control groups of each assay (

Table 20. The validity criterion for the hatching rate was also fulfilled as no more than two unhatched embryos (one per test container) was reported from all the control groups issued from these assays.

Table 20: Mortality rate observed at the end of EASZY assays performed in the lead laboratory. For each control condition (water, solvent, positive controls), the number of dead embryos out of the total number of embryos of each replicate is indicated (n/7).

	Water control			Solvent control			Positive control		
Assay	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
1	0/7	0/7	0/7	0/7	0/7	0/7	1/7	0/7	1/7
2	0/7	0/7	0/7	0/7	0/7	0/7	1/7	0/7	1/7
3	0/7	1/7	1/7	0/7	0/7	1/7	0/7	0/7	0/7
4	0/7	0/7	1/7	0/7	0/7	1/7	0/7	0/7	0/7
5	0/7	0/7	1/7	0/7	0/7	1/7	0/7	0/7	0/7
6	0/7	0/7	1/7	0/7	1/7	0/7	0/7	1/7	0/7
7	0/7	0/7	0/7	0/7	1/7	0/7	0/7	1/7	0/7
8	1/7	1/7	1/7	1/7	1/7	0/7	1/7	0/7	0/7
9	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7
10	1/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7
11	1/7	0/7	0/7	1/7	0/7	0/7	0/7	0/7	0/7
12	1/7	0/7	0/7	1/7	0/7	0/7	0/7	0/7	0/7
13	0/7	0/7	1/7	1/7	0/7	0/7	1/7	1/7	1/7

V.3. Acceptability criteria.

Comments were made regarding the mortality rate measured in exposed groups with a recommendation to align this acceptability criterion with other test guidelines in order to strengthen the quality of the EASZY assay. A retrospective analysis of the mortality rates recorded from 386 exposed group replicates highlighted that the mortality rate does not exceed one dead embryo in most cases with only 3% of the exposed-group replicates not meeting this acceptability criterion (Table 21).

Table 21 : Mortality rate measured in exposed group replicates. In more than 96% of the replicates analysed, the mortality does not exceed 1 dead embryo per replicate

	No death (0/7)	1 dead (1/7)	2 deads (2/7)	> 2 deads (> 2/7)
Number of replicates analysed (386)	304	70	8	4
100%	78.75%	18.1%	2.1%	1%

Therefore, the revised test guideline mentions that the mortality rate should not exceed one embryo per replicate at the end of the test.

V.4. Availability of the transgenic line

The *tg(cyp19a1b:GFP)* has been transferred in different european countries and is maintained in the lead laboratory (Ineris, France). Currently, Ineris ensure the maintenance and the provision of the *tg(cyp19a1b:GFP)* line (either as embryos or adults) for public laboratories or private companies upon material transfer agreement (MTA).

6 Summary and general conclusions of the 2-step validation study of the EASZY assay

The results reported herein were conducted as part of the VMG-ECO OECD working program to validate an OECD standardized *in vivo* assay on transgenic *tg (cyp19a1b:GFP)* zebrafish embryos to detect endocrine active substances acting as agonist ligands of ERs to induce the expression of GFP driven by the ER-regulated cyp19ab zebrafish promoter (EASZY assay).

During the validation, we first demonstrated the transferability of the transgenic *tg (cyp19a1b:GFP)* line and the EASZY method into different academic or industrial laboratories in France (3), UK (1) and Germany (1).

The EASZY Phase 1 validation has demonstrated that the protocol can be used to detect estrogenic effects of various test chemicals including ER agonist ligands (17α-ethinylestradiol, 17β-estradiol, bisphenol A, 4 tert- octylphenol) as well as aromatizable androgens, testosterone, which elicits an ER-dependent activation of the GFP after aromatization of testosterone into estradiol. We further demonstrated that dexamethasone (a glucocorticoid receptor agonist ligand) and spironolactone (an antagonist of the mineralocorticoid receptor) were unable to produce any significant effect highlighting the capacity of the test to discriminate active and inactive test chemicals in EASZY.

For the EASZY Phase 2 validation, a set of eleven test chemicals, including either active or inactive chemicals, were further evaluated in three laboratories including a naïve laboratory, not involved in the Phase 1. Its proficiency to perform the EASZY assay and to provide reliable data was evaluated using a set of "proficiency substances".

For the phase 2 validation, studies were conducted under code with a specific coding for each test chemical. This is important to ensure that the EASZY assay successfully identify active and inactive chemicals without *a priori* knowledge of the biological activity of the test chemicals. Another objective of the phase 2 validation of EASZY was to characterize the exposure conditions of the zebrafish embryos in the EASZY assay.

The measured concentrations in the water were in very good agreement with the expected nominal concentrations to which the fish have been exposed and no major differences were noticed between the participating laboratory demonstrating that the semi-static exposure system in glass vessels was satisfactory.

The results demonstrated that EASZY allowed to reliably identify active and inactive

chemicals when the assays were conducted under code. Thus, all the laboratories correctly identify the four inactive substances meaning that no false positive results were generated by the EASZY assay. The specificity of the test is important to consider also for minimizing the number of additional tests of higher level, including the animals used, that would be conducted based on false positive results in EASZY. Conversely, all the active chemicals were correctly identify demonstrating the specificity of the assay. This minimizes the risk of not identifying a chemical that would exhibit an estrogenic activity *in vivo*.

Altogether, the data collected demonstrated that EASZY is robust, specific and sensitive enough to reliably discriminate between test chemicals with estrogenic properties and test chemicals having no estrogenic activity.

During the VMG-meeting, the positioning of the EASZY assay within the actual conceptual framework, how and when use EASZY, was regularly questioned. The revised OECD GD 150 identifies EASZY as a level 3 assay of the actual conceptual framework, thus providing mechanistic information of the test chemicals *in vivo*. In addition to reliably identifies active chemicals acting on ERs to induce the brain aromatase expression, results of validation demonstrated that EASZY reliably quantify the estrogenic activity of test chemicals. Although quantification of the estrogenic activity is not the primary aim of the test and cannot be used for the risk assessment of chemicals, such information can be relevant to prioritize chemicals for additional tests of higher levels based on their estrogenic activities in EASZY. Quantification of the estrogenic activity in EASZY can also provide relevant information to target the concentration range for tests of higher levels.

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ANNEX 1: Description of the test method

Standard Operating Protocol (SOP) & validity criteria used for phase 1 and 2.

1. Apparatus and Materials

The following equipment is required:

- Binocular microscope
- Fluorescence microscope equipped with a 10X objective, GFP filter (excitation filter 470 nm [Band Path 450-490]; emission filter 525 nm [BP 500-550] and a fluorescence camera for fluorescence imaging
- Computer with Image Analysis Software
- Crystallizers (or Petri dish) made of chemically inert material (e.g., glass) in which the zebrafish are exposed. Crystallizers should be of suitable capacity in relation to the recommended loading (25 ml of water)
- Incubator with controlled temperature of 27°C ± 1°C. A control of the atmospheric humidity can be recommended as well as a control of the light/dark photoperiod (14hrs light :10hrs dark). - Glass Plate to cover crystallizers - Pipettus, pipette (25 ml).
- Pipettes: P10 , P200, P1000.
- Vessels to prepare stock solutions and the different test concentrations.
- Tanks with large volume of water for maintaining sexually mature transgenic fish used as breeding stocks. The breeding stock is composed of unexposed healthy male and female fish
- Spawn trap: Glass trays covered by a mesh (e.g., green plastic mesh)
- Sterile Pasteur Pipette for collecting eggs from the spawning tray, for transferring eggs into crystallizers.
- Glass Vessels to collect zebrafish embryos
- Multi-well fluorescence slides for GFP measurement in live embryos using a flurorescence microscope.
- Equipement for conductivity, pH, oxygen measurement.
- Bleach (2.6%)
- Solvent to dissolve the test substance (if solvent is required).

2. Culture of zebrafish tg(cyp19a1b:GFP) line

The transgenic *tg(cyp19a1b:GFP)* zebrafish is a heterozygous line that expresses GFP across generations with a stable rate of transmission of the transgene to progeny. To date, several generations of transgenic fish have been cultivated (F>10) and used to obtain transgenic animals without alterations of expression of the transgene across generations.

The culture conditions used for the transgenic *tg(cyp19a1b:GFP)* line are identical as for wild-type zebrafish. To ensure diversity of the genetic background of the *tg(cyp19a1b:GFP)*strain, transgenic male or female are regularly crossed with wildtype fish. By observing the constitutive expression of GFP in offsprings carrying the transgene, transgenic embryos are selected and used to maintain the strain.

The Figure 21 described the main key steps of EASZY, from egg production to data analysis, which are detailed in the following sections.

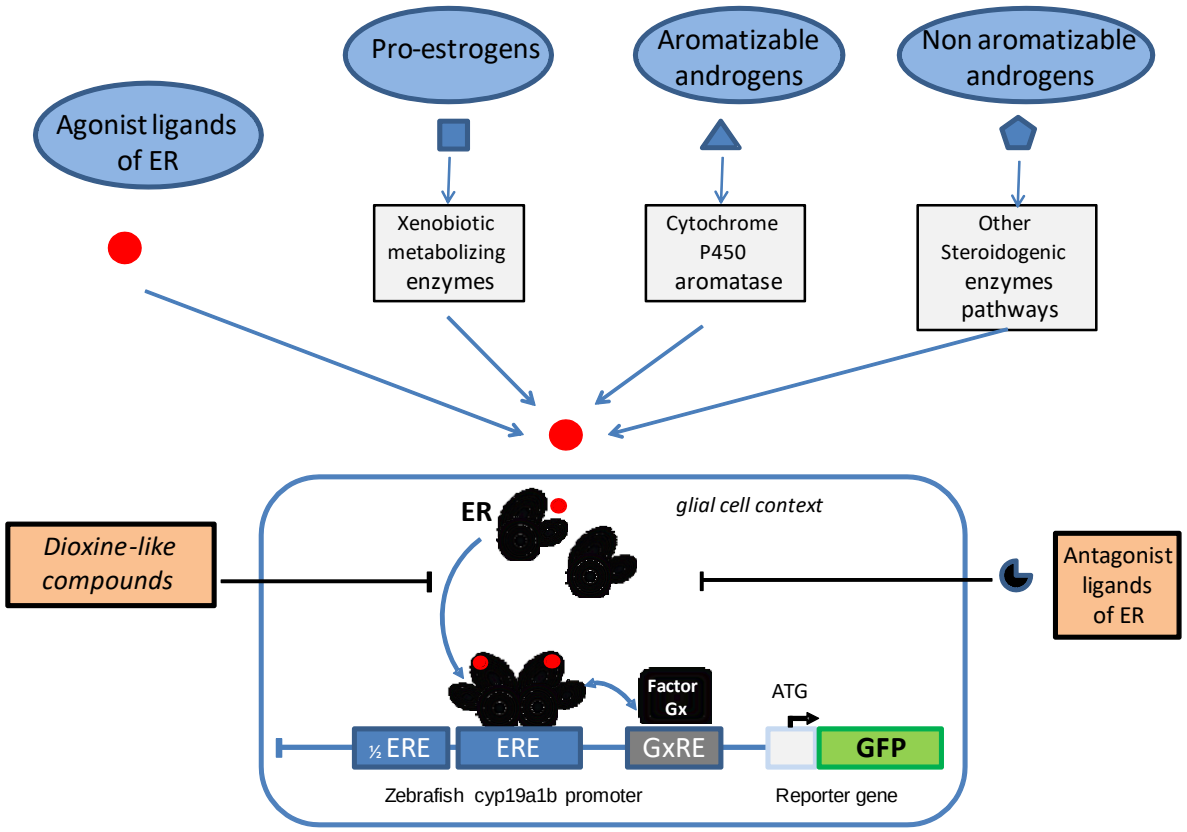


Figure 21 Scheme of the EASZY assay illustrating the main steps for its accomplishment

3. Maintenance of transgenic tg(cyp19a1b:GFP)brood fish and eggs production

Transgenic zebrafish eggs are produced via mass spawning in large tanks. Breeding fish are maintained in tanks, with a loading capacity of 100 L, subjected to a 14 hrs light: 10 hrs dark photoperiod. In the breeding tank, 60 to 100 transgenic adult males and females are

continuously held together (the male:female sex ratio varied from 1:1 to 2:1). Alternatively, eggs can be collected from different spawns obtained by pair-crossing adult fish rather than massspawning.
For breeding, the fish are fed three times daily: twice with granular diets and once with live *Artemia* nauplii. The day prior to the assay, a glass tray covered with a green mesh (made of inert material and of appropriate mesh size to protect eggs from predation by their parents) is placed in the tank. The next morning, spawning is stimulated by the onset of light. The glass tray containing eggs is carefully removed from the tank, 2 hours after the onset of light.
Eggs are transferred from the glass tray to a crystallizer containing clean water and the excess of organic matter is removed. Eggs are then cleaned with water supplemented with 200 µL of bleach solution (2.6%) for 100 ml of water during 5 minutes and rinsed 3x5min in clean water. Using a binocular microscope, fertilized eggs with normal development are selected and transferred into small aquaria (glass crystallizers).
The rate of fertilization should be > 70% otherwise the spawn is not considered as useful to subsequently perform the EASZY assay.
Since *tg(cyp19a1b:GFP)*line is heterozygous, crossing transgenic male and female does not produce 100% of transgenic embryos. The distinction between nontransgenic and transgenic embryos cannot be done at these very early stages of embryonic development but can be realized later by observing the constitutive expression of GFP from 72-hour-old post-fertilization embryos. As a result, the test is initiated with a mixed population of embryos composed of transgenic and non transgenic embryos. The percentage of transgenic embryos is however high (around 80%) and constant from one batch to another. To ensure, a sufficiently number of transgenic fish to analyse, 20 embryos per exposure condition are selected.

4. Exposure of transgenic *tg(cyp19a1b:GFP)* embryos

Twenty embryos (mixed population of transgenic and non transgenic embryos) are placed in each glass vessel appropriately and clearly labelled. The excess of water is then removed by taking care that embryos are always covered by a thin layer of water. Immediately after, 25 ml of freshly prepared test solution is added.
Glass vessels are then transferred into the incubator.
For each experiment, different controls and concentrations of test substance are performed:

- *Water control*:

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 $\mu S.cm^{-1}$) and temperature (28°C ± 1) and aerated to oxygen saturation. This water is also used as dilution water. Other well-characterized surface or well water can be used as well as reconstituted water (e.g., ISO water).

- *Positive control*:

Zebrafish embryos are exposed to EE2 at a final concentration of 0.05 nM, i.e. the lowest concentration of EE2 leading to a maximum fold induction of GFP. The mean measured fold induction of GFP induced by EE2 0.05 nM (14.8 ng/L) should be > or equal to 9 as compared to controls. If the effect of EE2 0.05 nM is below 9, the assay is invalid.

Test solution of EE2 0.05 nM (14.8 ng/L) is made from a 10^3X concentrated stock solution of EE2 solubilized in DMSO. The EE2 stock solution is renewed at regular interval (every month) to ensure reproducible results among experiments.

- *Solvent control*.

A solvent control is performed if a solvent is required to dissolve the test substance. In that case, the effect of the solvent must be checked prior to run the test to ensure that it does not significantly alter GFP. Several organic solvents have been tested and among them none alter GFP as compared to water control within the concentration range tested (up to 1%).

Dimethylsulfoxide (DMSO) is usually employed at 0.01% (v:v) which is in compliance with OECD recommendation to limit the use of solvent to a maximum concentration of 0.01%.

If a significant difference between GFP expression between the solvent and the water control groups is observed, then the EASZY assay is not considered as valid.

- *Test substance:*

In most cases, the test substance is dissolved in organic solvent (e.g., DMSO). A dilution range of test substance is carried out in organic solvent from stock solution (10^3x). The water is then supplemented with a constant volume of organic solvent samples from the different concentrated test concentrations, i.e.
2.5µl per 25ml of water (final concentration of DMSO 0.01%, volume/volume).

- *Test concentrations* (Figure 22*):*

Usually, five concentrations of test substance, spaced by a constant factor, are required. The highest concentration should result in a maximal effect on GFP expression and the lowest concentration should give no significant effect on fluorescence as compared to control. The concentrations of test substance should not induce mortality as compared to control. Without information on the ability of test substance to alter GFP, a range-finding study is recommended with at least 5 concentrations spaced by decadic logarithmic (log10) intervals. Based on these range-finding experiments, a suitable concentration range with five concentrations spaced by a constant factor of 3 (or 2) should then be used to refine the concentration-response curve.

During the exposure period, it is recommended to check for mortality and to remove, if any, the dead embryos. In absence of information on the chemical stability of the test substance, the medium is renewed each day.

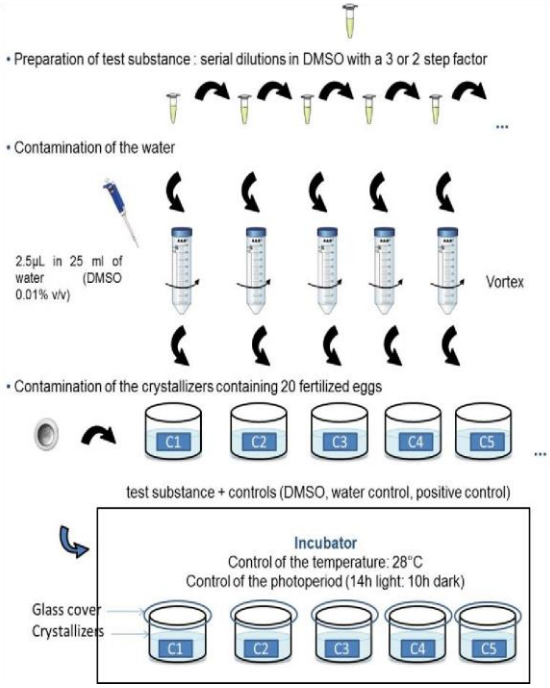


Figure 22: Contamination of the medium and exposure of zebrafish to different concentrations of the test substance. After contamination, the crystallizers are placed into an incubator with a constant temperature of 28°C and a controlled photoperiod. The glass covers avoid the evaporation of the medium. The medium is renewed every day

5. In vivo imaging of tg(cyp19a1b:GFP)zebrafish: wild-field fluorescence microscopy.

5.1. Some considerations on the main characteristics of the fluorescence microscope

Fluorescence imaging is realized using an upright fluorescence microscope equipped with a 10X objective, a GFP filter, an external light source (e.g., HBO lamp) and a fluorescence camera.
Setting the parameters for fluorescence acquisition are critical and depend on the main technical characteristics of each microscope. Once these parameters are set, they must be kept for all experiments.

- **Objective**: a 10X magnification objective allows to photograph the head of the larvae (defined as the region of interest or ROI) hence avoiding to photograph the yolk sac that fluoresces at similar wavelengths as GFP. The use of specific objective for fluorescence applications (e.g., Fluar) is highly recommended.

- **GFP filter:** the following characteristics are highly recommended for eGFP filter: excitation wavelength 470 nm [Band Path 450-490]; emission wavelength 525 nm [BP 505-545]. For each excitation and emission wavelength the band path is low with no overlap between the excitation and emission spectra.

- **External light source:** various light sources can be used. Very often, the light source is composed of an HBO lamp. During the lifetime of the lamp (which do not exceed 500 hours), the intensity should be constant. However, the stability of the external light source can be assessed before and after series of measurement using a fluorescence calibration slide.

- **Fluorescence camera:** the camera should have a high resolution and a highly sensitive charge-couple device (CCD) sensor. A monochrome (black and white) (CCD) digital camera is recommended as it increases the sensitivity and reduces the time of exposure as compared to color camera.

- **Time of exposure:** the time of exposure must be optimized for each microscope. To set this parameter, it is recommended to vary this setting and to evaluate its influence on the fluorescence emitted by both control and EE2-exposed fish. When properly set, it must allow to detect the fluorescence in both control and exposed-fish. If the time of exposure is too short, the basal fluorescence cannot be detected properly. In contrast, if time of exposure is too high, it will saturates the signal for larvae with high expression levels of GFP.

5.2. In vivo imaging

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 (a 21-well fluorescence slide allows to transfer all the larvae collected from the same exposure group).
At this stage, the zebrafish can be anesthetized usied benzocaine as anesthetic (Sigma #E1501). Benzocaine is prepared as a stock solution in absolute ethanol (100g.L-1). Larvae are anesthetized using benzocaine at a final concentration of 50 mg.L-1 (12.5 µL of the solution of benzocaine at 100g.L-1 diluted in 25 mL of medium). Anesthesia is performed directly in the exposure vessel and 5 minutes later, larvae are dispatched on observation slide. This mild anesthesia is not lethal for the duration and larvae recover quickly.

Each well of the glass slide containing live zebrafish larvae is observed. At this stage, it is easy to distinguish fish expressing GFP from those that do not express GFP (i.e., non transgenic fish, see above for details).

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. The Figure 23 illustrates the influence of positioning on the resulting fluorescence measurement. The fluorescence intensity was 62% lower as compared to fluorescence dorsal view. Therefore, in case of incorrect position (ventral or lateral view), the operator must re-orientate the larvae to observe it dorsally. When appropriately orientated, the head of each embryo is photographed under the same exposure parameters.

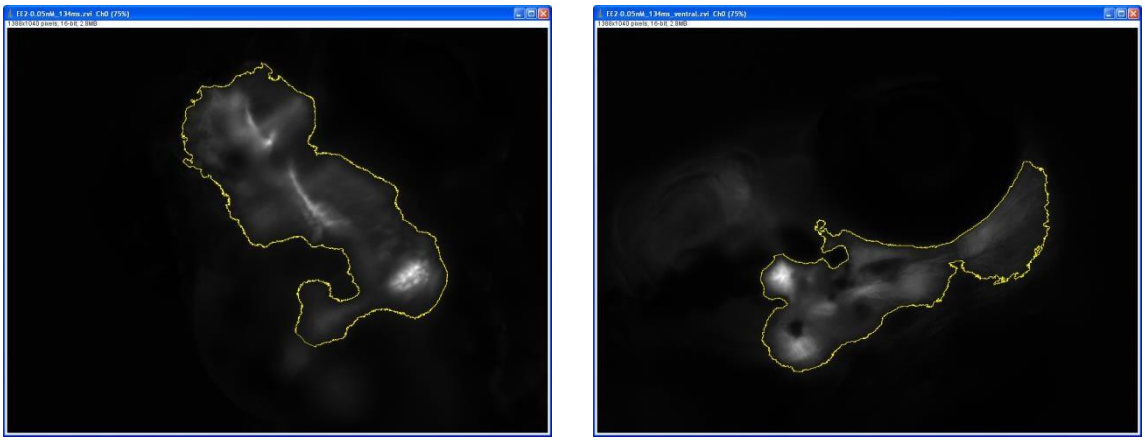


Figure 23: Influence of the positioning of fish on fluorescence measurement. The fluorescence intensity measured on the left image (dorsal view) is 35% higher as compared to the right image (ventral view). A correct positioning is mandatory to compare the fluorescence intensity among images

Each photograph is then named and saved in a specific folder for subsequent image analysis. The name of each picture should indicate:

- Name of the test substance,
- concentration of exposure, - time of exposure, - number of individual.
- Example: BPA-0.5µM-134ms_12.zvi. (test substance BPA- at a concentration of 0.5µM- time of exposure= 134ms- twelfth fish analyzed in the series)

Various image format exists and differs according to the device (e.g., zvi for Zeiss). It is important to determine if the output format of the photograph can be used by the image analysis software used to subsequently quantify GFP.

With well-trained operators (2 operators), 3 different substances (5 concentrations) plus controls are run in 4 hours. This generates approximately 350 photographs to analyze.

After the microscopic observation, the total number of dead embryos as well as the total number of transgenic and non transgenic fish is recorded. Additional observations such as non-hatched or malformed embryos are also reported.

5.3. Quantification of GFP by image analysis.

We present below a procedure to analyze each photograph by using freely available image analysis software (Image J available at http://rsb.info.gov/ij/).
A critical criterion for image analysis is the threshold grey level. The threshold grey level corresponds to a grey value which allows to distinguish the fluorescence due to the reporter fluorescent protein from the natural autofluorescence of fish (background fluorescence). To determine this parameter, the following procedure was established.

5.3.1. Determining the threshold level of grey level

1. Non transgenic zebrafish are photographed using the same parameters as previously defined (refer to the paragraph "*In vivo imaging"*).
2. Photographs are imported in the image analysis software.
3. A region of interest (ROI) is manually defined. The ROI corresponds to brain regions where expression of GFP is normally observed in transgenic *tg(cyp19a1b:GFP)*zebrafish (Figure 23).
4. Run the analysis for each ROI to obtain grey level of each pixel within the selected area.
5. The threshold is defined as the maximal grey level found in the ROI of non transgenic fish. To refine the threshold grey level, it is recommended to analyze several non transgenic fish.

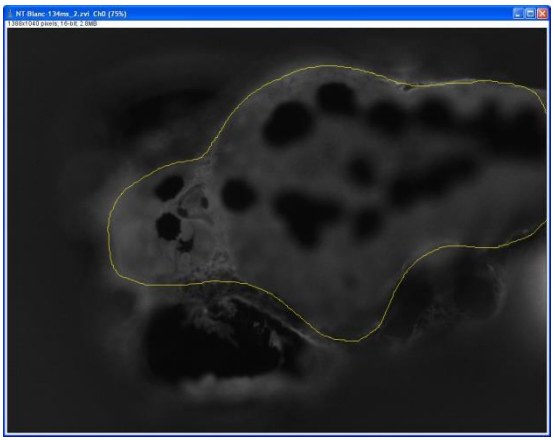


Figure 24: Fluorescence imaging of a non-transgenic zebrafish larvae to measure to background level of fluorescence (threshold level of grey value).

Once defined, only pixels having a grey level above the threshold will be analysed. In our case, the threshold for background grey level was set 290 from a maximum grey level 4095 (the grey scale varies according to the camera used to capture images and the resolution of the image, e.g., 8, 12, 16 bit/pixel).

This threshold grey level is stable over time but can be refined when changes on the fluorescent device are realized such as installation of a new HBO lamp.

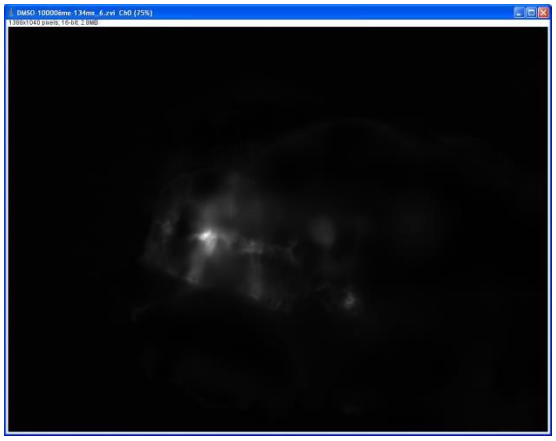
5.3.2. Quantification of GFP

In the following sections, a general scheme is presented to analyse and quantify GFP using Image J software.

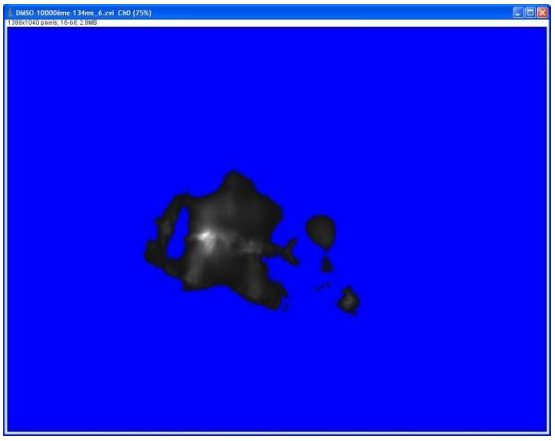
- Prior to image analysis it is important to specify in the dialogue box which parameters need to be record by the software.
  - **Area**: Area of the Region of Interest expressed in square pixels
  - **Mean gray value**: Average gray value within the ROI. This is the sum of the gray values of all the pixels in the selection divided by the number of pixels of the ROI.

- **Standard deviation**: Standard deviation of the gray values
- **Min & max gray level**: Minimum and maximum gray values measured within the ROI.
- **Integrated density**: The sum of the values of the pixels in the ROI. This is equivalent to the product of Area and Mean Gray Value.
- **Limit to threshold**: it corresponds to the threshold grey level (refer to paragraph 5.3.1)

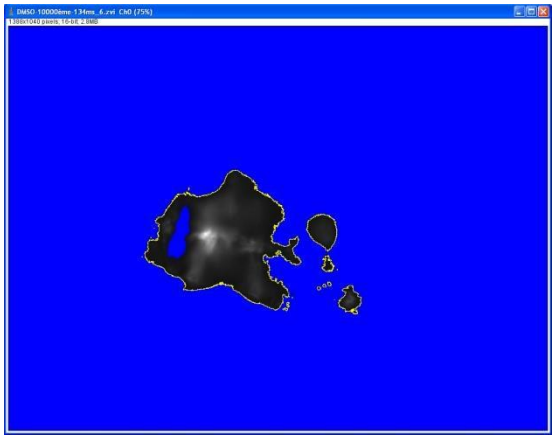
• Once the parameter are checked, each image are open for image analysis and appears as follows:



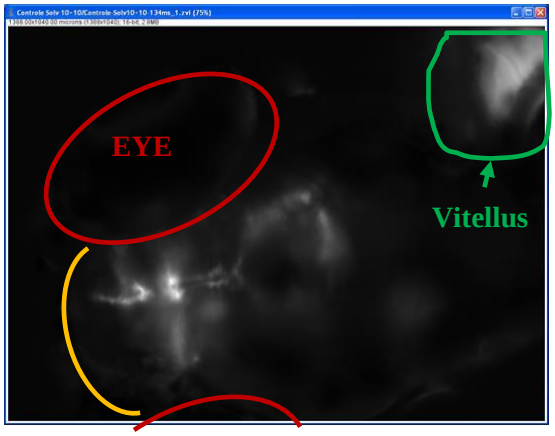
• The threshold grey level is applied to image: pixels having a grey value below the threshold level are masked and appeared in blue while pixels having grey levels equal or above the threshold grey level appeared in white.



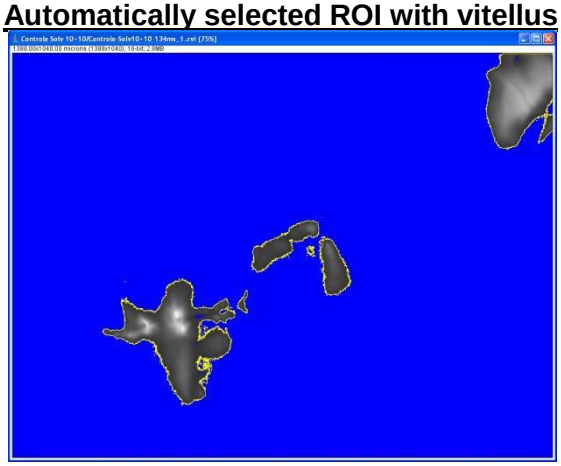
• The areas (ROI) corresponding to pixels with gray values higher than the threshold grey levels are selected.



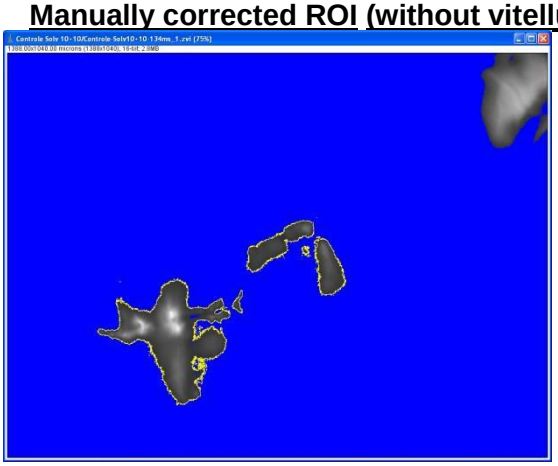
- Each image must be checked carefully in order to verify the automatically selected ROI. For instance, in some pictures part of the yolk sac can appear on the image. Given that vitellus fluoresce at a similar excitation/emission wavelengths as GFP, the ImageJ will integrate pixel gray values from this area leading to an over estimation of the integrated density. In that case, the ROI has to be corrected by deselecting the yolk sac area from the ROI. The following pictures illustrate how to proceed in that case.



Automatically selected ROI with vitellus



Manually corrected ROI (without vitellus)



Area	Mean	SD	Min	Max	**IntDen**
94157	468	178	290	1321	**44070123**

Area	Mean	SD	Min	Max	**IntDen**
61122	415	150	290	1321	**25373516**

As illustrated with this example, an incorrect definition of the ROI within the image leads to an over-estimation of the Integrated Density (IntDen) of 42%.

- Data image analysis can be export in a spreadsheet application for further analysis.
- In order to handle a large number of images and reduce repetitive actions from user, a specific ImageJ macro was developed for high-throughput image analysis and can be released upon request. In order to easily report pixel analysis of each image, a template for file naming was defined such as follows

[Treatment]-[Concentration]-[Exposure Time]_[Fish #].
Example : EE2-0.05nM-134ms_15.zvi

- For each assay, data are transfer in a new worksheet.

6. Analysis of the results.

- After image analysis of each individual fish, the results are reported in a worksheet.

- For each exposure condition, the quantification of the fluorescence, measured as integrated density (IntDens) in transgenic *tg(cyp19a1b:GFP)*zebrafish, is expressed as mean fold ± standard error of the mean (S.E.M) above water control or solvent control (if solvent control is used).

- Appropriate statistical tests methods can be applied to compare means using non-parametric or parametric ANOVA follow by post-hoc test.
- For each test chemical, concentration-response curve is modeled using a sigmoidal concentration-response curve (Hill model) which is a four parametric logistic model. Concentrations inducing 10, 20 or 50% of its maximal effect, i.e. EC_{10}, EC_{20}, EC_{50}, are derived. Maximum fold of induction is also reported.

ANNEX 2: Template used to collect the data

Excel template used to collect the data from ImageJ

	Label	Area	Mean	StdDev	Min	Max	IntDen	RawIntDen	Min Thr	Max Thr	Fold/Mean Ctrl IntDen	Mean Fold	SD Fold	Mean RawIntDen	SD RawIntDen
1	Solvent Control														
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12															
13															
14															
15															
16															
17															
18															
19															
20															
1	Water control														
2															
3															
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11															
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17															
18															
19															
20															
1	Positive control EE2 0.05 nM														
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ANNEX 3: GFP analysis using ImageJ

Release notes concerning GFP analysis of the EASZY assay using the specific ImageJ developed (macro v2.2).

General description

Minimum system requirements

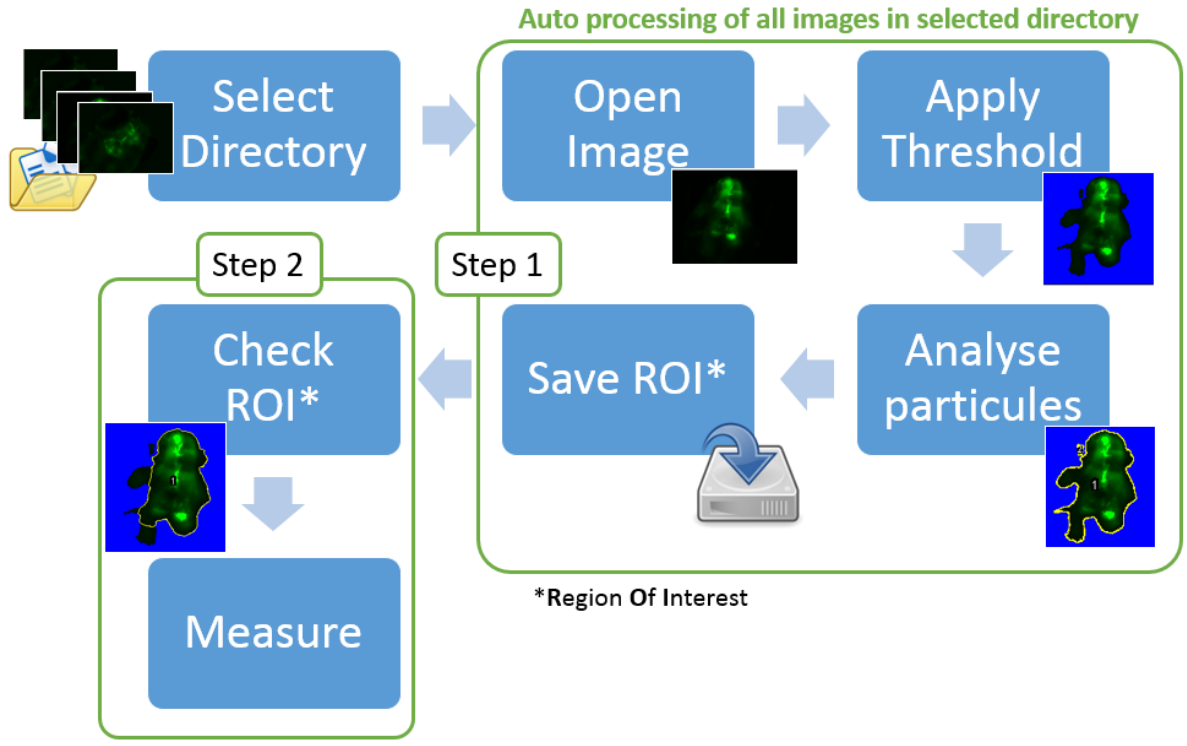
Software resources needed to use *tg(cyp19a1b:GFP)*

Analysis macro are:

- ImageJ version 1.49o or higher - http://rsbweb.nih.gov/ij/download.html
- Java 1.6 or higher - www.java.com
- Bio-Formats 5.0.1 or higher http://www.openmicroscopy.org/site (library for reading and writing life sciences image file formats)
- Cyp19a1b-GFP_Analysis.ijm file saved to the Plugins directory within ImageJ

If you can't use an installer because you lack administrator privileges, a ready to use package is available for Windows operating systems. Uncompress **ImageJ_1.50-win-jre6_EASZY.zip** archive on your hard disk and run ImageJ.exe.
More information on other operating systems can be found on the above-mentioned links.

Description of the Image processing pipeline of "Cyp19a1b-GFP_Analysis" macro:

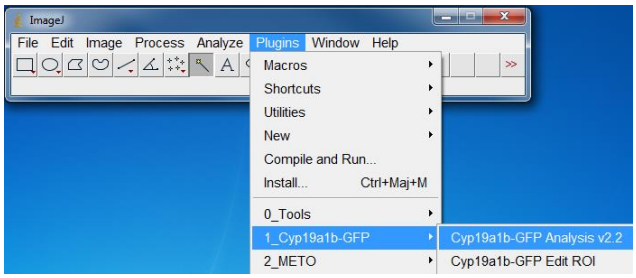


This ImageJ macro automates tasks (open, set threshold, save, measure) to process a large number of *tg (cyp19a1b:GFP)* Zebrafish images.

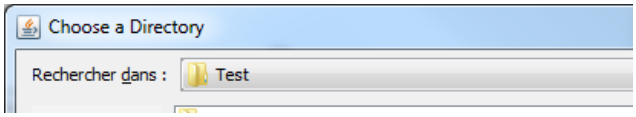
- Step 1 - Batch processing of selected directory
  - List all files containing user-defined extension (.czi - .zvi - .tif) within selected directory and sub-directories.
  - Apply the user-defined threshold (default 290) then analyse particles above the threshold.
  - Group pixels above the threshold in one Region Of Interest and save this ROI in a zip file in the image directory.
- Step 2 - Check selected Region Of Interest and measure
  - Open images one by one to check the auto-selected ROI.
  - User can confirm the ROI, modify it directly or remove image from further analysis.

Detailed description of image analysis steps

Run **ImageJ.exe**.
Launch the macro in ImageJ toolbar menu:
Plugins
1_Cyp19a1b-GFP
Cyp19a1b-GFP_Analysis



The macro prompts to select the directory containing image files.
The selected directory and all subdirectories will be processed for analysis.

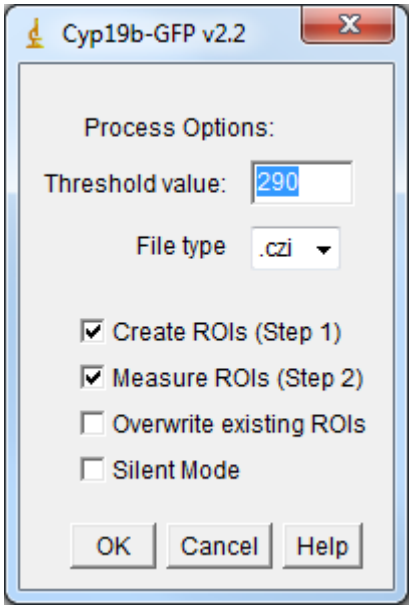


The macro displays a dialog box to set analysis options:

Threshold value – set lower threshold value for pixel intensity, segmenting the image into features of interest (above threshold) and background.
Threshold value has to be defined for each acquisition system (microscope + camera + exposure time).

File type – select image type between CZI, ZVI (Zeiss Vision Image) and TIFF (Tagged Image File Format).

Create ROIs – Step 1, Analyse pixels above the user-defined threshold value and automatically saves Regions Of Interest zip file in the image directory.



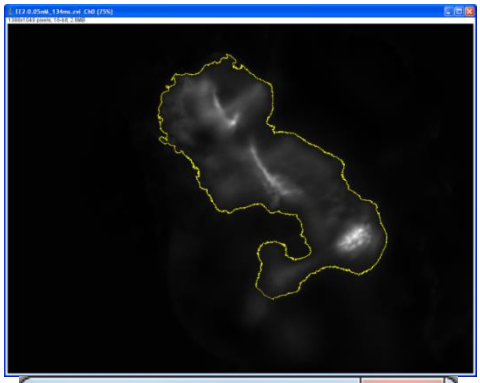
Measure ROIs – Skip Step 1 and proceed directly to Step 2 of image analysis to check individually and measure previously created ROIs.

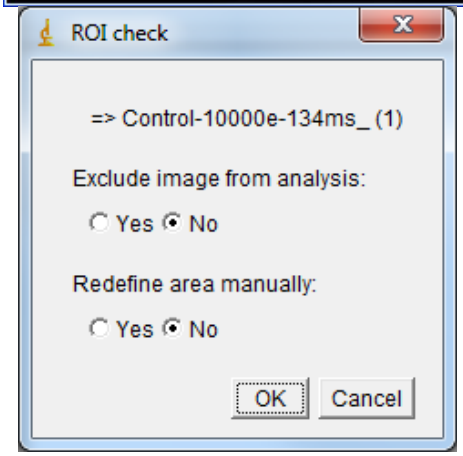
Overwrite existing ROIs – if selected, the macro will overwrite ROIs zip files without prompting the user many times.

Silent Mode - if selected, the macro will measure all images with their associated ROI without prompting the user many times.

After completion of Step 1 of image processing, images and their corresponding ROIs are checked by the user.

A dialog box is displayed to validate ROI. If the area is correct the user has to click OK to proceed with next image.
The dialog box allows to exclude current image from analysis (e.g. blurred image) **OR** to manually redefine selected area.
Just select Yes on the radio button option if needed then click OK or **ENTER↵** to continue

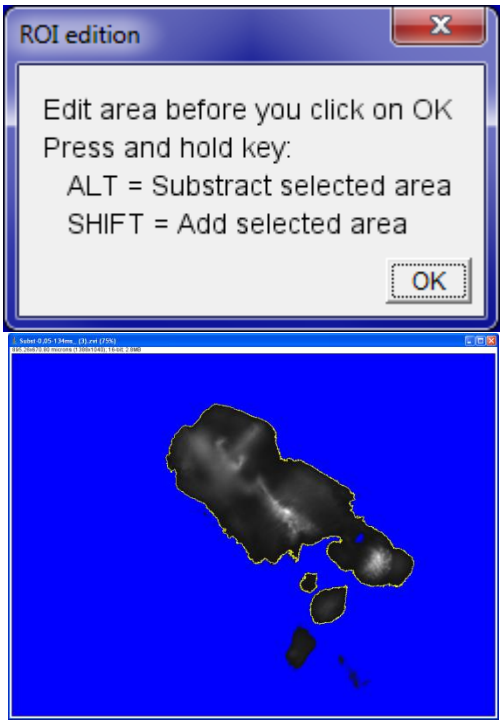




If you selected to redefine area manually, a new dialog box is displayed to get access to the ImageJ toolbar and modify the yellow selection. The user-defined threshold is then automatically applied.

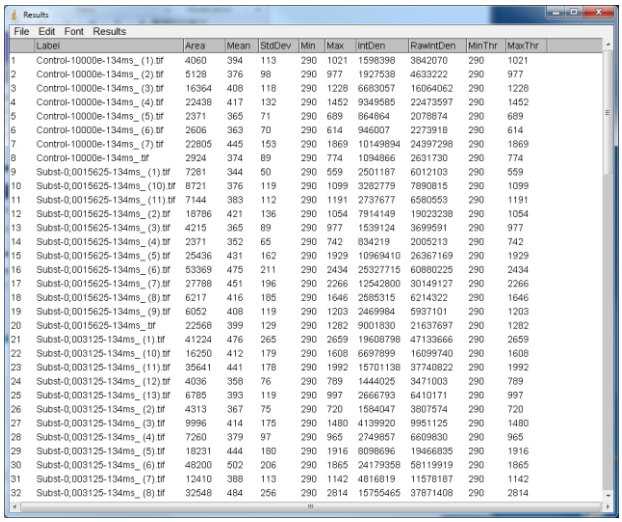
Select the appropriate tool in ImageJ toolbar:
 Freehand (default) OR  Wand tool
To remove non specific fluorescence (e.g. vitellus), use Freehand tool user and hold ALT key while selecting area to remove from ROI.
To add area to the previously selected ROI, hold SHIFT key while selecting new area to add.

Click  to validate the new ROI: image is then measured and zip file is automatically updated.



In case the selection is empty after manual correction the macro discard image from analysis.

After completion of Step 2, a list of all measured images with filename is displayed in the Result Table window of ImageJ.
This table can be automatically saved as a csv or xls file.



	Label	Area	Mean	StdDev	Min	Max	IntDen	RawIntDen	MinThr	MaxThr
1	Control-10000e-134ms_ (1).tif	4060	394	113	290	1021	1598398	3842070	290	1021
2	Control-10000e-134ms_ (2).tif	5128	376	98	290	977	1927538	4633222	290	977
3	Control-10000e-134ms_ (3).tif	16364	408	118	290	1228	6683057	16064062	290	1228
4	Control-10000e-134ms_ (4).tif	22438	417	132	290	1452	9349585	22473597	290	1452
5	Control-10000e-134ms_ (5).tif	2371	365	71	290	689	864864	2078874	290	689
6	Control-10000e-134ms_ (6).tif	2606	363	70	290	614	946007	2273918	290	614
7	Control-10000e-134ms_ (7).tif	22805	445	153	290	1869	10149894	24397298	290	1869
8	Control-10000e-134ms_.tif	2924	374	89	290	774	1094866	2631730	290	774
9	Subst-0,0015625-134ms_ (1).tif	7281	344	50	290	559	2501187	6012103	290	559
10	Subst-0,0015625-134ms_ (10).tif	8721	376	119	290	1099	3282779	7890815	290	1099
11	Subst-0,0015625-134ms_ (11).tif	7144	383	112	290	1191	2737677	6580553	290	1191
12	Subst-0,0015625-134ms_ (2).tif	18786	421	136	290	1054	7914149	19023238	290	1054
13	Subst-0,0015625-134ms_ (3).tif	4215	365	89	290	977	1539124	3699591	290	977
14	Subst-0,0015625-134ms_ (4).tif	2371	352	65	290	742	834219	2005213	290	742
15	Subst-0,0015625-134ms_ (5).tif	25436	431	162	290	1929	10969410	26367169	290	1929
16	Subst-0,0015625-134ms_ (6).tif	53369	475	211	290	2434	25327715	60880225	290	2434
17	Subst-0,0015625-134ms_ (7).tif	27788	451	196	290	2266	12542600	30149127	290	2266
18	Subst-0,0015625-134ms_ (8).tif	6217	416	185	290	1646	2585315	6214322	290	1646
19	Subst-0,0015625-134ms_ (9).tif	6052	408	119	290	1203	2469984	5937101	290	1203
20	Subst-0,0015625-134ms_.tif	22568	399	129	290	1282	9001830	21637697	290	1282
21	Subst-0,003125-134ms_ (1).tif	41224	476	265	290	2659	19608798	47133666	290	2659
22	Subst-0,003125-134ms_ (10).tif	16250	412	179	290	1608	6697899	16099740	290	1608
23	Subst-0,003125-134ms_ (11).tif	35641	441	178	290	1992	15701138	37740822	290	1992
24	Subst-0,003125-134ms_ (12).tif	4036	358	76	290	789	1444025	3471003	290	789
25	Subst-0,003125-134ms_ (13).tif	6785	393	119	290	997	2666793	6410171	290	997
26	Subst-0,003125-134ms_ (2).tif	4313	367	75	290	720	1584047	3807574	290	720
27	Subst-0,003125-134ms_ (3).tif	9996	414	175	290	1480	4139920	9951125	290	1480
28	Subst-0,003125-134ms_ (4).tif	7260	379	97	290	965	2749857	6609830	290	965
29	Subst-0,003125-134ms_ (5).tif	18231	444	180	290	1916	8098696	19466835	290	1916
30	Subst-0,003125-134ms_ (6).tif	48200	502	206	290	1865	24179358	58119919	290	1865
31	Subst-0,003125-134ms_ (7).tif	12410	388	113	290	1142	4816819	11578187	290	1142
32	Subst-0,003125-134ms_ (8).tif	32548	484	256	290	2814	15755465	37871408	290	2814

ANNEX 4: summary of the results of the phase 1

Summary of the phase 1 validation. EC50 values (expressed either in nM or µM) and coefficient of variability inter-assay (CV %) reported for the seven selected substances tested in the three participating laboratories during the phase 1 of the validation of the EASZY assay. SD= standard deviation, Neg= no effect

Chemical	Run	Lab A	Lab B	Lab C
EE2 (nM)	1	0.01	0.028	0.015
	2	0.01	0.027	0.016
	3	0.013	0.014	0.016
	4	0.013	0.017	
	5	0.01		
	6	0.019		
	7	0.01		
	8	0.011		
	9	0.008		
	10	0.018		
	mean	**0.0122**	**0.0215**	**0.0157**
	SD	0.004	0.007	0.001
	CV %	**29.9**	**32.8**	**3.7**
E2 (nM)		2.87	1.74	2.8
		1.94	2.11	1.36
		1.47	2.69	
		2.05	1.11	
		1.55		
	mean	**1.976**	**1.9125**	**2.08**
	SD	0.6	0.7	1.0
	CV %	**28.2**	**34.6**	**49.0**
Bisphenol A (µM)	1	3.7	5.1	3.6
	2	4.3	2.6	3.6
	3	4.3	3.3	2.5
	4	4.5	3.5	
	mean	**4.2**	**3.6**	**3.2**
	SD	0.3	1.1	0.6
	CV %	**8.2**	**29.1**	**19.6**
4-ter-octylphenol (µM)	1	0.44	0.18	0.22
	2	0.24	0.24	0.36
	3	0.2	0.18	0.24
	4		0.21	
	mean	**0.293**	**0.203**	**0.273**
	SD	0.129	0.029	0.076
	CV %	**43.8**	**14.2**	**27.7**
Testosterone (µM)	1	0.9	0.51	0.92
	2	0.61	0.47	1.35
	3	0.46	0.21	
	mean	**0.657**	**0.397**	**1.135**
	SD	0.224	0.163	0.304
	CV %	**34.1**	**41.1**	**26.8**
Dexamethasone	1	neg	neg	neg
Spironolactone	1	neg	neg	neg

ANNEX 4: summary of the results of the phase 1 (continued)

Summary of the phase 1 validation. EC50 values (expressed either in ng/L or µg/L) and coefficient of variability inter-assay (CV %) reported for the seven selected substances tested in the three participating laboratories during the phase 1 of the validation of the EASZY assay. SD= standard deviation, Neg= no effect

Chemical	Run	Lab A	Lab B	Lab C
EE2 (ng/L)	1	2.96	8.29	4.44
	2	2.96	8.00	4.74
	3	3.85	4.14	4.74
	4	3,85	5.03	
	5	2.96		
	6	5.63		
	7	2.96		
	8	3.26		
	9	2.37		
	10	5.33		
	mean	3.62	6.37	4.64
	SD	1.08	2.9	0.17
	CV %	29.9	32.8	3.7
E2 (ng/L)		781.7	473.9	762.7
		528.4	574.7	375.9
		400.4	732.7	
		558.4	302.3	
		422.2		
	mean	538.2	520.9	569.3
	SD	151.9	180.5	273.5
	CV %	28.2	34.6	48.0
Bisphenol A (µg/L)	1	845	1164	822
	2	982	594	822
	3	982	753	571
	4	1027	799	
	mean	958	827	738
	SD	79	241	145
	CV %	8.2	29.1	19.6
4-ter-octylphenol (µg/L)	1	90.8	37.1	45.4
	2	49.5	49.5	74.3
	3	41.3	37.1	49.5
	4		43.3	
	mean	60.5	41.8	56.4
	SD	26.5	5.9	15.6
	CV %	43.8	14.2	27.8
Testosterone (µg/L)	1	259.6	147,1	265.3
	2	175.9	135,6	389.4
	3	132.7	60.6	
	mean	189.4	114.4	327.4
	SD	64.5	47	87.7
	CV %	34.1	41.1	26.8
Dexamethasone	1	neg	neg	neg
Spironolactone	1	neg	neg	neg

ANNEX 5: survival rate of zebrafish embryos exposed to test chemicals

Survival rate (expressed in %) of 4-dpf zebrafish measured after 96 hours of exposure. For each substance, the chemically measured concentrations are reported.

	Laboratory A		Laboratory B		Laboratory C	
	concentrations µM (measured)	survival rate (%)	concentrations µM (measured)	survival rate (%)	concentrations µM (measured)	survival rate (%)
Diclofenac	10.45	0	10.87	0	9.55	0
	2.90	0	3.53	0	3.04	0
	0.98	75	0.96	100	0.93	95
	0.32	90	0.36	100	0.31	100
	0.10	75	0.12	100	0.10	100
Triclosan	7.11	0	8.74	0	7.06	0
	2.41	0	1.98	0	1.77	0
	0.59	75	0.67	0	0.73	70
	0.19	95	0.24	100	0.22	100
	0.06	90	0.08	100	0.08	100
2,4 dihydroxybenzophenone	8.64	40	9.20	96	10.62	90
	2.84	80	3.06	100	3.22	100
	0.83	65	1.08	100	1.07	100
	0.25	90	0.34	100	0.32	100
	0.07	85	0.12	100	0.10	100
Bisphenol S	56.85	95	52.03	100	82.40	100
	19.62	95	18.39	95	26.44	100
	6.68	95	6.15	92	8.50	100
	3.01	80	2.43	100	3.11	100
	0.84	100	0.35	95	1.03	100
Bisphenol F	4.54	100	4.02	95	4.89	95
	1.74	80	1.31	96	1.67	100
	0.50	100	0.42	95	0.50	100
	0.17	100	0.16	95	0.16	100
	0.06	100	0.05	83	0.05	100

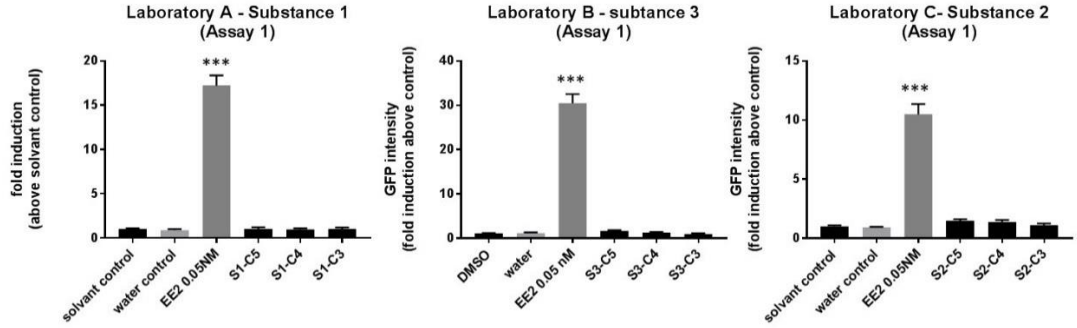
Annex 5: survival rate of zebrafish embryos exposed to test chemicals

	Laboratory A		Laboratory B		Laboratory C	
	concentrations µM (measured)	survival rate (%)	concentrations µM (measured)	survival rate (%)	concentrations µM (measured)	survival rate (%)
Benzophenone	9.94	85	4.62	75	4.62	95
	3.79	95	2.00	79	2.00	100
	0.81	95	0.69	96	0.69	100
	0.46	90	0.18	75	0.18	95
	0.12	95	0.08	86	0.08	100
Levonorgestrel	1.05	100	0.91	95	0.95	100
	0.54	90	0.32	100	0.32	100
	0.13	95	0.12	100	0.16	100
	0.04	100	0.04	100	0.05	100
	0.01	95	0.01	100	0.02	95
Norethindrone	0.09	90	0.09	100	0.09	95
	0.03	100	0.02	95	0.03	100
	0.01	95	0.01	100	0.01	100
	0.00	90	0.00	100	0.00	100
	0.00	100	0.00	100	0.00	100
Dihydrotestosterone	4.65	85	7.61	95	7.69	100
	1.34	90	2.56	100	2.15	100
	0.16	100	0.92	100	1.02	100
	0.06	90	0.31	100	0.26	100
	0.02	100	0.12	100	0.09	100
17B-trenbolone	8.27	80	8.20	36	6.66	90
	2.64	90	2.42	95	2.39	100
	0.82	95	0.87	95	0.78	100
	0.30	80	0.29	95	0.25	100
	0.12	100	0.10	88	0.06	100
11 -Ketotestosterone	1.02	90	0.91	100	1.02	100
	0.33	90	0.28	100	0.33	100
	0.12	75	0.10	100	0.12	100
	0.03	80	0.03	100	0.04	100

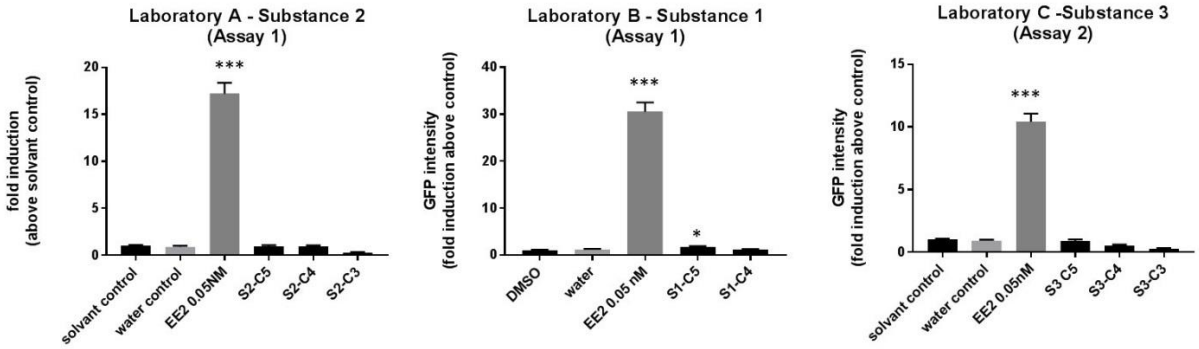
ANNEX 6: GFP expression in zebrafish embryos exposed to test chemicals

GFP expression in 4-dpf zebrafish embryos exposed for 96 hours to selected substances. Results are expressed as mean fold induction above control± SEM.

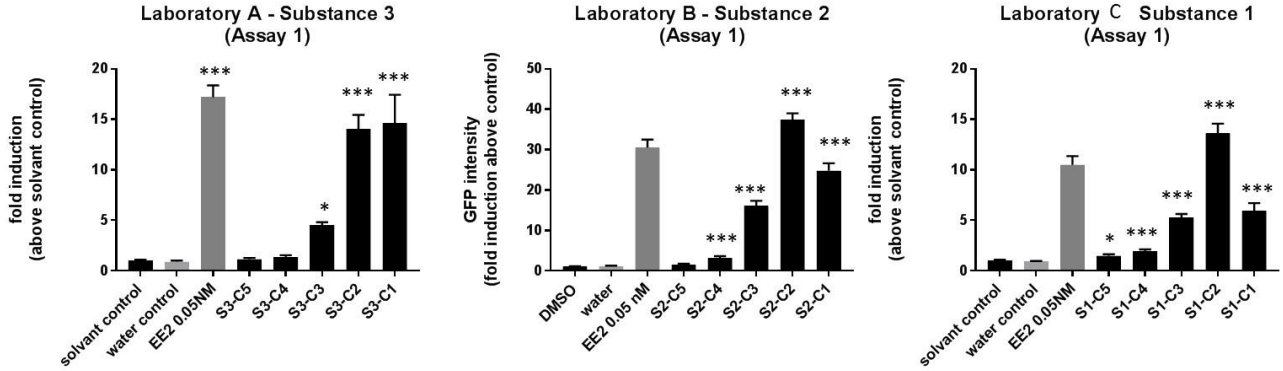
Diclofenac



Triclosan

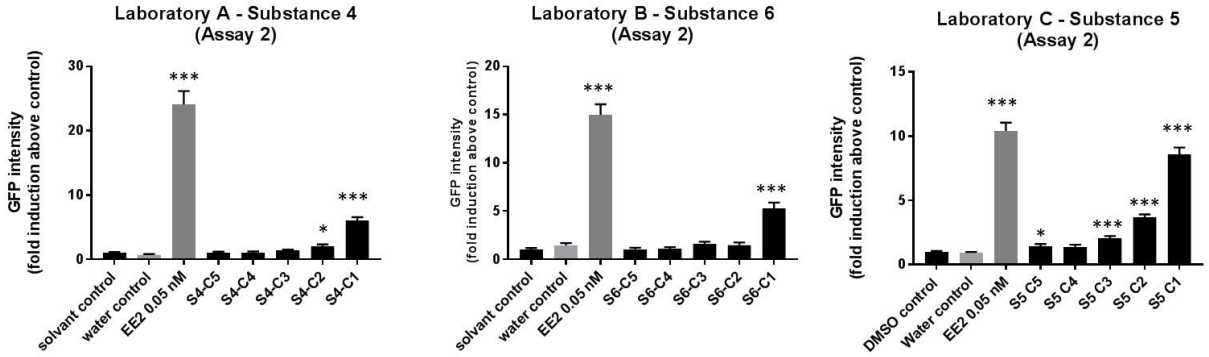


2,4-dihydrybenzophenone

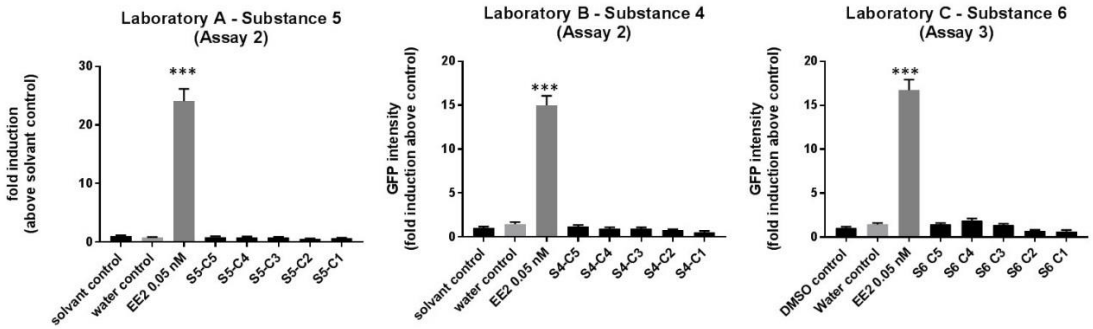


ANNEX 6: GFP expression in zebrafish embryos exposed to test chemicals (continued)

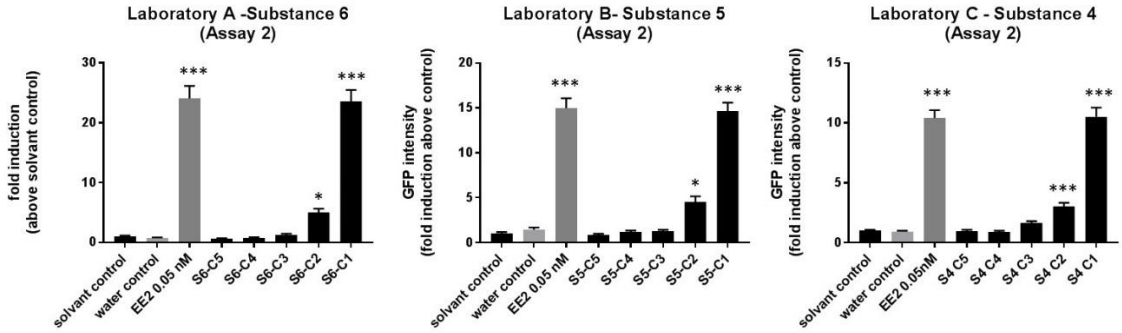
Bisphenol S



Benzophenone

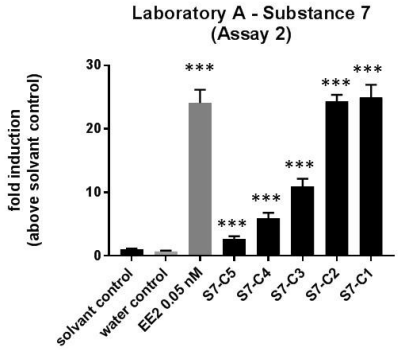


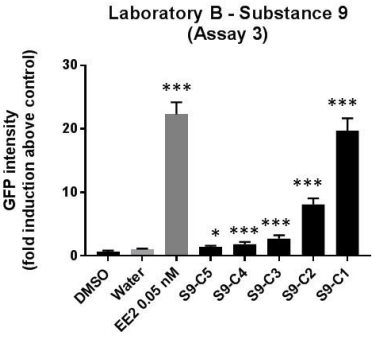
Bisphenol F

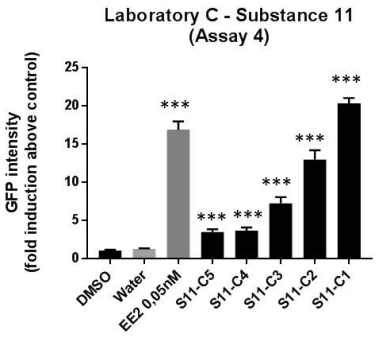


ANNEX 6: GFP expression in zebrafish embryos exposed to test chemicals (continued)

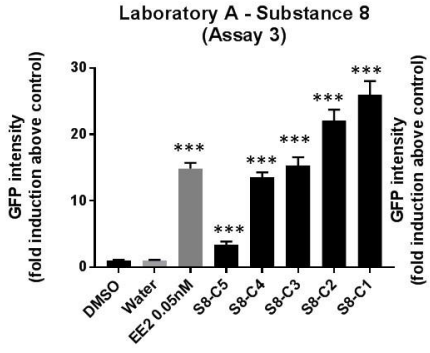
Levonorgestrel

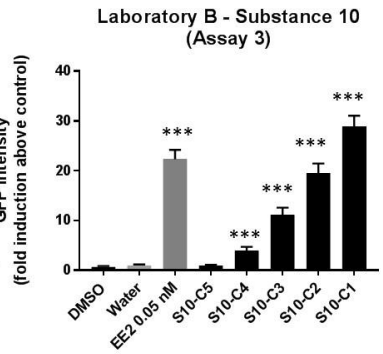


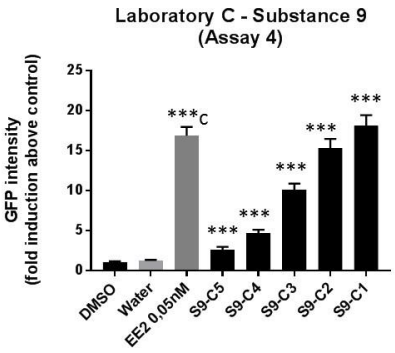




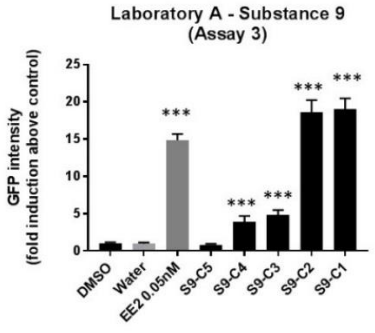
Norethindrone

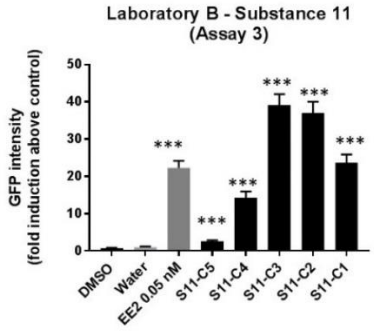


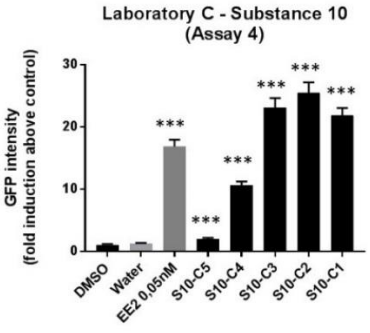




Dihydrotestosterone

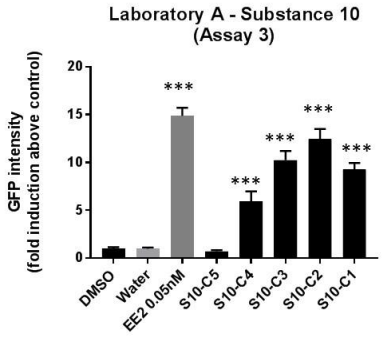


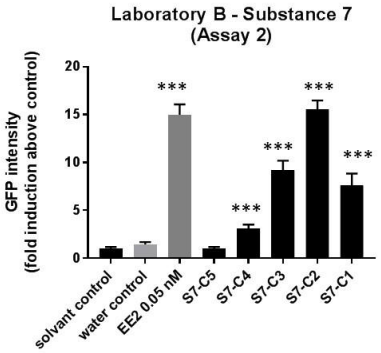


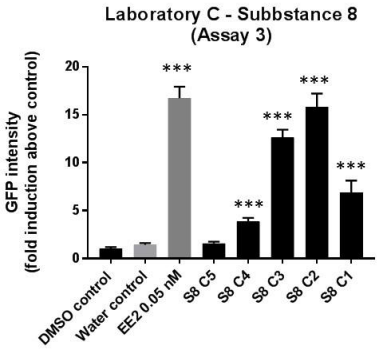


ANNEX 6: GFP expression in zebrafish embryos exposed to test chemicals (continued)

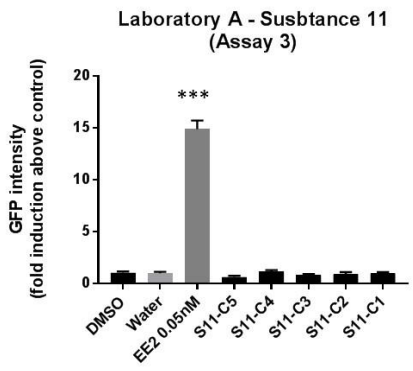
17β-trenbolone

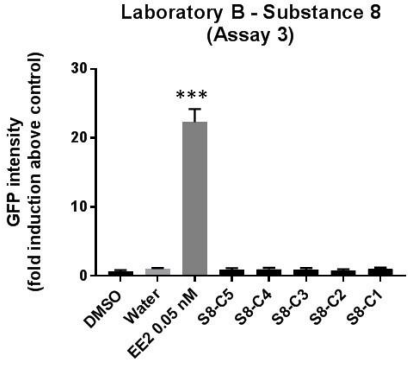


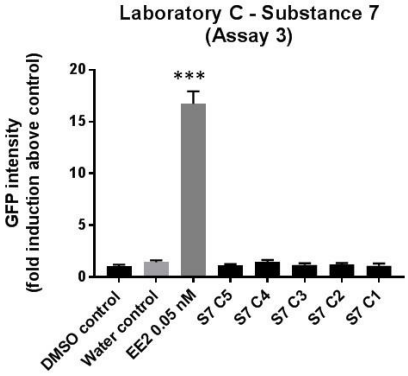




11-Ketotestosterone







ANNEX 7: chemical analysis

Chemical analysis: Measured concentrations (in µM and in mg/L) of test substances in the medium. For 17α-ethinylestradiol concentrations are expressed in nM and ng/L

Triclosan - Concentration (µM)

Laboratory A

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabA-Sub2 C1	10.00	0.995	9.95	6.34	±0.44	7.95	±0.56
LabA-Sub2 C2	3.00	0.995	2.99	2.53	±0.18	2.30	±0.16
LabA-Sub2 C3	1.00	0.995	1.00	0.65	±0.05	0.53	±0.04
LabA-Sub2 C4	0.30	0.995	0.30	0.24	±0.02	0.15	±0.01
LabA-Sub2 C5	0.10	0.995	0.10	0.07	±0.005	0.05	±0.003
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabB-Sub1 C1	10.02	0.995	9.97	8.97	±0.63	8.52	±0.60
LabB-Sub1 C2	3.00	0.995	2.99	2.41	±0.17	1.61	±0.11
LabB-Sub1 C3	1.00	0.995	1.00	0.74	±0.05	0.60	±0.04
LabB-Sub1 C4	0.30	0.995	0.30	0.28	±0.02	0.22	±0.02
LabB-Sub1 C5	0.10	0.995	0.10	0.09	±0.01	0.07	±0.005
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabC-Sub3 C1	9.79	0.995	9.74	7.07	±0.49	7.05	±0.49
LabC-Sub3 C2	2.94	0.995	2.92	1.87	±0.13	1.67	±0.12
LabC-Sub3 C3	0.98	0.995	0.97	0.76	±0.05	0.71	±0.05
LabC-Sub3 C4	0.29	0.995	0.29	0.25	±0.02	0.20	±0.01
LabC-Sub3 C5	0.10	0.995	0.10	0.10	±0.01	0.07	±0.005
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Triclosan - Concentration (mg/L)

Laboratory A

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabA-Sub2 C1	2.90	0.995	2.88	1.83	±0.13	2.30	±0.16
LabA-Sub2 C2	0.87	0.995	0.86	0.73	±0.05	0.67	±0.05
LabA-Sub2 C3	0.29	0.995	0.29	0.19	±0.01	0.15	±0.01
LabA-Sub2 C4	0.09	0.995	0.09	0.07	±0.005	0.04	±0.003
LabA-Sub2 C5	0.03	0.995	0.03	0.02	±0.001	0.01	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabB-Sub1 C1	2.90	0.995	2.89	2.60	±0.18	2.47	±0.17
LabB-Sub1 C2	0.87	0.995	0.87	0.70	±0.05	0.47	±0.03
LabB-Sub1 C3	0.29	0.995	0.29	0.21	±0.01	0.17	±0.01
LabB-Sub1 C4	0.09	0.995	0.09	0.08	±0.006	0.06	±0.004
LabB-Sub1 C5	0.03	0.995	0.03	0.03	±0.002	0.02	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabC-Sub3 C1	2.83	0.995	2.82	2.05	±0.14	2.04	±0.14
LabC-Sub3 C2	0.85	0.995	0.85	0.54	±0.04	0.48	±0.03
LabC-Sub3 C3	0.28	0.995	0.28	0.22	±0.02	0.21	±0.01
LabC-Sub3 C4	0.09	0.995	0.08	0.07	±0.005	0.06	±0.004
LabC-Sub3 C5	0.03	0.995	0.03	0.03	±0.002	0.02	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Diclofenac Concentration (µM)

Laboratory A

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µM)	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabA-Sub1 C1	10.03	0.985	9.88	10.90	±0.327	10.03	±0.301
LabA-Sub1 C2	3.01	0.985	2.97	3.00	±0.09	2.79	±0.084
LabA-Sub1 C3	1.00	0.985	0.99	1.03	±0.031	0.93	±0.028
LabA-Sub1 C4	0.30	0.985	0.30	0.32	±0.009	0.32	±0.01
LabA-Sub1 C5	0.10	0.985	0.10	0.11	±0.003	0.10	±0.003
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µM)	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabB-Sub3 C1	10.01	0.985	9.86	11.03	±0.331	10.70	±0.321
LabB-Sub3 C2	3.00	0.985	2.96	3.66	±0.11	3.39	±0.102
LabB-Sub3 C3	1.00	0.985	0.99	1.06	±0.032	0.87	±0.26
LabB-Sub3 C4	0.30	0.985	0.30	0.35	±0.01	0.38	±0.011
LabB-Sub3 C5	0.10	0.985	0.10	0.12	±0.004	0.13	±0.004
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µM)	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabC-Sub2 C1	9.80	0.985	9.66	9.09	±0.273	10.02	±0.301
LabC-Sub2 C2	2.94	0.985	2.90	3.23	±0.097	2.86	±0.086
LabC-Sub2 C3	0.98	0.985	0.97	0.94	±0.028	0.91	±0.027
LabC-Sub2 C4	0.29	0.985	0.29	0.30	±0.009	0.32	±0.01
LabC-Sub2 C5	0.10	0.985	0.10	0.11	±0.003	0.10	±0.003
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Diclofenac Concentration (mg/L)

Laboratory A

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by purity	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabA-Sub1 C1	2.97	0.985	2.93	3.23	±0.097	2.97	±0.089
LabA-Sub1 C2	0.89	0.985	0.88	0.89	±0.027	0.83	±0.025
LabA-Sub1 C3	0.30	0.985	0.29	0.31	±0.009	0.28	±0.008
LabA-Sub1 C4	0.09	0.985	0.09	0.09	±0.003	0.09	±0.003
LabA-Sub1 C5	0.03	0.985	0.03	0.03	±0.001	0.03	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by purity	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabB-Sub3 C1	2.96	0.985	2.92	3.27	±0.098	3.17	±0.095
LabB-Sub3 C2	0.89	0.985	0.88	1.09	±0.033	1.00	±0.03
LabB-Sub3 C3	0.30	0.985	0.29	0.31	±0.009	0.26	±0.008
LabB-Sub3 C4	0.09	0.985	0.09	0.10	±0.003	0.11	±0.003
LabB-Sub3 C5	0.03	0.985	0.03	0.04	±0.001	0.04	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by purity	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabC-Sub2 C1	2.90	0.985	2.86	2.69	±0.081	2.97	±0.089
LabC-Sub2 C2	0.87	0.985	0.86	0.96	±0.029	0.85	±0.025
LabC-Sub2 C3	0.29	0.985	0.29	0.28	±0.008	0.27	±0.008
LabC-Sub2 C4	0.09	0.985	0.09	0.09	±0.003	0.09	±0.003
LabC-Sub2 C5	0.03	0.985	0.03	0.03	±0.001	0.03	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

2,4-dihydroxybenzophenone Concentration (µM)

Laboratory A

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µM)	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabA-Sub3 C1	10.01	0.99	9.91	8.13	±0.164	9.18	±0.185
LabA-Sub3 C2	3.00	0.99	2.97	2.87	±0.058	2.80	±0.057
LabA-Sub3 C3	1.00	0.99	0.99	0.83	±0.017	0.82	±0.017
LabA-Sub3 C4	0.30	0.99	0.30	0.25	±0.005	0.26	±0.005
LabA-Sub3 C5	0.10	0.99	0.10	0.07	±0.001	0.07	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µM)	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabB-Sub2 C1	10.00	0.99	9.90	9.10	±0.184	9.31	±0.188
LabB-Sub2 C2	3.00	0.99	2.97	3.17	±0.064	2.95	±0.06
LabB-Sub2 C3	1.00	0.99	0.99	1.08	±0.022	1.09	±0.022
LabB-Sub2 C4	0.30	0.99	0.30	0.35	±0.007	0.33	±0.007
LabB-Sub2 C5	0.10	0.99	0.10	0.13	±0.003	0.12	±0.002
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µM)	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabC-Sub1 C1	9.86	0.99	9.76	10.77	±0.218	10.48	±0.212
LabC-Sub1 C2	2.96	0.99	2.93	3.46	±0.07	3.00	±0.061
LabC-Sub1 C3	0.99	0.99	0.98	1.12	±0.023	1.03	±0.021
LabC-Sub1 C4	0.30	0.99	0.29	0.33	±0.007	0.30	±0.006
LabC-Sub1 C5	0.10	0.99	0.10	0.10	±0.002	0.10	±0.002
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

2,4-dihydroxybenzophenone Concentration (mg/L)

Laboratory A

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by purity	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabA-Sub3 C1	2.14	0.99	2.12	1.74	±0.035	1.97	±0.040
LabA-Sub3 C2	0.64	0.99	0.64	0.61	±0.012	0.60	±0.012
LabA-Sub3 C3	0.21	0.99	0.21	0.18	±0.004	0.18	±0.004
LabA-Sub3 C4	0.06	0.99	0.06	0.05	±0.001	0.06	±0.001
LabA-Sub3 C5	0.02	0.99	0.02	0.01	±0.000	0.01	±0.000
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by purity	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabB-Sub2 C1	2.14	0.99	2.12	1.95	±0.039	1.99	±0.040
LabB-Sub2 C2	0.64	0.99	0.64	0.68	±0.014	0.63	±0.013
LabB-Sub2 C3	0.21	0.99	0.21	0.23	±0.005	0.23	±0.005
LabB-Sub2 C4	0.06	0.99	0.06	0.08	±0.002	0.07	±0.001
LabB-Sub2 C5	0.02	0.99	0.02	0.03	±0.001	0.02	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by purity	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabC-Sub1 C1	2.11	0.99	2.09	2.31	±0.047	2.24	±0.045
LabC-Sub1 C2	0.63	0.99	0.63	0.74	±0.015	0.64	±0.013
LabC-Sub1 C3	0.21	0.99	0.21	0.24	±0.005	0.22	±0.004
LabC-Sub1 C4	0.06	0.99	0.06	0.07	±0.001	0.07	±0.001
LabC-Sub1 C5	0.02	0.99	0.02	0.02	±0.000	0.02	±0.000
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

19-Norethindrone Concentration (nM)

Laboratory A

Sample	Nominal concentration (nM)	Standard purity	Nominal Value with correction by purity (nM)	T 0h		T 24h	
				Measured concentration (nM)		Measured concentration (nM)	
LabA-Sub8 C1	101.43	0.98	99.40	82.89	±1.25	97.98	±1.48
LabA-Sub8 C2	30.43	0.98	29.82	29.73	±0.45	30.86	±0.47
LabA-Sub8 C3	10.14	0.98	9.94	10.21	±0.15	10.10	±0.15
LabA-Sub8 C4	3.04	0.98	2.98	3.63	±0.05	3.75	±0.06
LabA-Sub8 C5	1.01	0.98	0.99	1.11	±0.02	1.10	±0.02
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (nM)	Standard purity	Nominal Value with correction by purity (nM)	T 0h		T 24h	
				Measured concentration (nM)		Measured concentration (nM)	
LabB-Sub10 C1	100.11	0.98	98.11	91.28	±1.38	86.60	±1.31
LabB-Sub10 C2	30.03	0.98	29.43	29.69	±0.45	13.01	±0.20
LabB-Sub10 C3	10.01	0.98	9.81	9.95	±0.15	10.18	±0.15
LabB-Sub10 C4	3.00	0.98	2.94	3.28	±0.05	3.23	±0.05
LabB-Sub10 C5	1.00	0.98	0.98	1.10	±0.02	1.11	±0.02
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (nM)	Standard purity	Nominal Value with correction by purity (nM)	T 0h		T 24h	
				Measured concentration (nM)		Measured concentration (nM)	
LabC-Sub9 C1	98.03	0.98	96.07	112.73	±2.24	77.44	±1.54
LabC-Sub9 C2	29.41	0.98	28.82	34.99	±0.70	33.26	±0.66
LabC-Sub9 C3	9.80	0.98	9.61	13.04	±0.26	9.19	±0.18
LabC-Sub9 C4	2.94	0.98	2.88	3.98	±0.08	3.36	±0.07
LabC-Sub9 C5	0.98	0.98	0.96	1.21	±0.02	0.97	±0.02
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

19-Norethindrone Concentration (µg/L)

Laboratory A

Sample	Nominal concentration (µg/L)	Standard purity	Nominal Value with correction by purity (µg/L)	T 0h Measured concentration (µg/L)		T 24h Measured concentration (µg/L)	
LabA-Sub8 C1	30.27	0.98	29.66	24.74	±0.37	29.24	±0.44
LabA-Sub8 C2	9.08	0.98	8.90	8.87	±0.13	9.21	±0.14
LabA-Sub8 C3	3.03	0.98	2.97	3.05	±0.05	3.01	±0.05
LabA-Sub8 C4	0.91	0.98	0.89	1.08	±0.02	1.12	±0.02
LabA-Sub8 C5	0.30	0.98	0.30	0.33	±0.01	0.33	±0.005
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µg/L)	Standard purity	Nominal Value with correction by purity (µg/L)	T 0h Measured concentration (µg/L)		T 24h Measured concentration (µg/L)	
LabB-Sub10 C1	29.87	0.98	29.28	27.24	±0.41	25.84	±0.39
LabB-Sub10 C2	8.96	0.98	8.78	8.86	±0.13	3.88	±0.06
LabB-Sub10 C3	2.99	0.98	2.93	2.97	±0.04	3.04	±0.05
LabB-Sub10 C4	0.90	0.98	0.88	0.98	±0.01	0.96	±0.01
LabB-Sub10 C5	0.30	0.98	0.29	0.33	±0.005	0.33	±0.005
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µg/L)	Standard purity	Nominal Value with correction by purity (µg/L)	T 0h Measured concentration (µg/L)		T 24h Measured concentration (µg/L)	
LabC-Sub9 C1	29.25	0.98	28.67	33.64	±0.67	23.11	±0.46
LabC-Sub9 C2	8.78	0.98	8.60	10.44	±0.21	9.92	±0.20
LabC-Sub9 C3	2.93	0.98	2.87	3.89	±0.08	2.74	±0.05
LabC-Sub9 C4	0.88	0.98	0.86	1.19	±0.02	1.00	±0.02
LabC-Sub9 C5	0.29	0.98	0.29	0.36	±0.01	0.29	±0.01
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

17b Trenbolone Concentration (µM)

Laboratory A

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µM)	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabA-Sub10 C1	10.07	0.93	9.37	9.52	±0.56	7.13	±0.42
LabA-Sub10 C2	3.02	0.93	2.81	2.76	±0.16	2.52	±0.15
LabA-Sub10 C3	1.01	0.93	0.94	0.91	±0.05	0.73	±0.04
LabA-Sub10 C4	0.30	0.93	0.28	0.33	±0.02	0.28	±0.02
LabA-Sub10 C5	0.10	0.93	0.09	0.15	±0.01	0.10	±0.01
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µM)	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabB-Sub7 C1	9.99	0.93	9.29	8.71	±0.51	7.71	±0.45
LabB-Sub7 C2	3.00	0.93	2.79	2.51	±0.15	2.32	±0.14
LabB-Sub7 C3	1.00	0.93	0.93	0.87	±0.05	0.87	±0.05
LabB-Sub7 C4	0.30	0.93	0.28	0.26	±0.02	0.33	±0.02
LabB-Sub7 C5	0.10	0.93	0.09	0.10	±0.01	0.11	±0.01
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µM)	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabC-Sub8 C1	9.89	0.93	9.19	8.46	±0.50	5.14	±0.30
LabC-Sub8 C2	2.97	0.93	2.76	2.82	±0.17	2.00	±0.12
LabC-Sub8 C3	0.99	0.93	0.92	0.94	±0.05	0.65	±0.04
LabC-Sub8 C4	0.30	0.93	0.28	0.28	±0.02	0.23	±0.01
LabC-Sub8 C5	0.10	0.93	0.09	0.07	±0.004	0.05	±0.003
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

17b Trenbolone Concentration (mg/L)

Laboratory A

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by purity (mg/L)	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabA-Sub10 C1	2.72	0.93	2.53	2.57	±0.15	1.93	±0.11
LabA-Sub10 C2	0.82	0.93	0.76	0.75	±0.04	0.68	±0.04
LabA-Sub10 C3	0.27	0.93	0.25	0.25	±0.01	0.20	±0.01
LabA-Sub10 C4	0.08	0.93	0.08	0.09	±0.01	0.08	±0.004
LabA-Sub10 C5	0.03	0.93	0.03	0.04	±0.002	0.03	±0.002
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by purity (mg/L)	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabB-Sub7 C1	2.70	0.93	2.51	2.36	±0.14	2.08	±0.12
LabB-Sub7 C2	0.81	0.93	0.75	0.68	±0.04	0.63	±0.04
LabB-Sub7 C3	0.27	0.93	0.25	0.23	±0.01	0.24	±0.01
LabB-Sub7 C4	0.08	0.93	0.08	0.07	±0.004	0.09	±0.01
LabB-Sub7 C5	0.03	0.93	0.03	0.03	±0.002	0.03	±0.002
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction by purity (mg/L)	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabC-Sub8 C1	2.67	0.93	2.49	2.29	±0.13	1.39	±0.08
LabC-Sub8 C2	0.80	0.93	0.75	0.76	±0.04	0.54	±0.03
LabC-Sub8 C3	0.27	0.93	0.25	0.25	±0.01	0.18	±0.01
LabC-Sub8 C4	0.08	0.93	0.07	0.08	±0.004	0.06	±0.004
LabC-Sub8 C5	0.03	0.93	0.02	0.02	±0.001	0.01	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

11 Ketotestosterone Concentration (nM)

Laboratory A

Sample	Nominal concentration (nM)	Standard purity	Nominal Value with correction by purity (nM)	T 0h		T 24h	
				Measured concentration (nM)		Measured concentration (nM)	
LabA-Sub11 C1	1004.93	0.98	984.83	967.53	±39.67	1069.62	±43.85
LabA-Sub11 C2	301.48	0.98	295.45	322.84	±13.24	338.90	±13.89
LabA-Sub11 C3	100.49	0.98	98.48	133.79	±5.49	103.99	±1.14
LabA-Sub11 C4	30.15	0.98	29.54	44.96	±1.84	23.63	±0.97
LabA-Sub11 C5	10.05	0.98	9.85	14.82	±0.61	5.89	±0.24
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (nM)	Standard purity	Nominal Value with correction by purity (nM)	T 0h		T 24h	
				Measured concentration (nM)		Measured concentration (nM)	
LabB-Sub8 C1	999.97	0.98	979.97	936.21	±10.30	884.68	±9.73
LabB-Sub8 C2	299.99	0.98	293.99	334.94	±3.68	223.42	±2.46
LabB-Sub8 C3	100.00	0.98	98.00	100.65	±1.11	101.89	±1.12
LabB-Sub8 C4	30.00	0.98	29.40	37.67	±0.41	32.28	±0.36
LabB-Sub8 C5	10.00	0.98	9.80	10.24	±0.11	11.88	±0.13
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (nM)	Standard purity	Nominal Value with correction by purity (nM)	T 0h		T 24h	
				Measured concentration (nM)		Measured concentration (nM)	
LabC-Sub7 C1	967.72	0.98	948.36	1115.48	±45.73	929.05	±38.09
LabC-Sub7 C2	290.32	0.98	284.51	319.85	±13.11	340.80	±13.97
LabC-Sub7 C3	96.77	0.98	94.84	159.74	±6.55	92.22	±3.78
LabC-Sub7 C4	29.03	0.98	28.45	36.45	±1.49	53.67	±2.20
LabC-Sub7 C5	9.68	0.98	9.48	12.83	±0.53	12.86	±0.53
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

11 Ketotestosterone Concentration (µg/L)

Laboratory A

Sample	Nominal concentration (µg/L)	Standard purity	Nominal Value with correction by purity (µg/L)	T 0h Measured concentration (µg/L)		T 24h Measured concentration (µg/L)	
LabA-Sub11 C1	303.90	0.98	297.82	292.59	±12.00	323.46	±13.26
LabA-Sub11 C2	91.17	0.98	89.35	97.63	±4.00	102.49	±4.20
LabA-Sub11 C3	30.39	0.98	29.78	40.46	±1.66	31.45	±0.35
LabA-Sub11 C4	9.12	0.98	8.93	13.60	±0.56	7.15	±0.29
LabA-Sub11 C5	3.04	0.98	2.98	4.48	±0.18	1.78	±0.07
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µg/L)	Standard purity	Nominal Value with correction by purity (µg/L)	T 0h Measured concentration (µg/L)		T 24h Measured concentration (µg/L)	
LabB-Sub8 C1	302.40	0.98	296.35	283.12	±3.11	267.54	±2.94
LabB-Sub8 C2	90.72	0.98	88.91	101.29	±1.11	67.56	±0.74
LabB-Sub8 C3	30.24	0.98	29.64	30.44	±0.33	30.81	±0.34
LabB-Sub8 C4	9.07	0.98	8.89	11.39	±0.13	9.76	±0.11
LabB-Sub8 C5	3.02	0.98	2.96	3.10	±0.03	3.59	±0.04
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µg/L)	Standard purity	Nominal Value with correction by purity (µg/L)	T 0h Measured concentration (µg/L)		T 24h Measured concentration (µg/L)	
LabC-Sub7 C1	292.65	0.98	286.79	337.33	±13.83	280.95	±11.52
LabC-Sub7 C2	87.79	0.98	86.04	96.73	±3.97	103.06	±4.23
LabC-Sub7 C3	29.26	0.98	28.68	48.31	±1.98	27.89	±1.14
LabC-Sub7 C4	8.78	0.98	8.60	11.02	±0.45	16.23	±0.67
LabC-Sub7 C5	2.93	0.98	2.87	3.88	±0.16	3.89	±0.16
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Benzophenone Concentration (µM)

Laboratory A

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabA-Sub5 C1	9.97	0.997	9.94	8.26	±1.29	11.84	±1.85
LabA-Sub5 C2	2.99	0.997	2.98	3.33	±0.52	4.30	±0.67
LabA-Sub5 C3	1.00	0.997	0.99	0.74	±0.12	0.88	±0.14
LabA-Sub5 C4	0.30	0.997	0.30	0.49	±0.08	0.44	±0.07
LabA-Sub5 C5	0.10	0.997	0.10	0.14	±0.02	0.10	±0.02
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabB-Sub4 C1	10.01	0.997	9.98	12.62	±1.97	9.34	±1.46
LabB-Sub4 C2	3.00	0.997	2.99	3.49	±0.54	2.66	±0.42
LabB-Sub4 C3	1.00	0.997	1.00	1.17	±0.18	1.04	±0.16
LabB-Sub4 C4	0.30	0.997	0.30	0.55	±0.09	0.46	±0.07
LabB-Sub4 C5	0.10	0.997	0.10	0.11	±0.02	0.11	±0.02
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabC-Sub6 C1	9.83	0.997	9.80	4.62	±0.72	4.61	±0.72
LabC-Sub6 C2	2.95	0.997	2.94	2.23	±0.35	1.78	±0.28
LabC-Sub6 C3	0.98	0.997	0.98	0.76	±0.12	0.64	±0.10
LabC-Sub6 C4	0.29	0.997	0.29	0.19	±0.03	0.17	±0.03
LabC-Sub6 C5	0.10	0.997	0.10	0.08	±0.01	0.07	±0.01
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Benzophenone Concentration (mg/L)

Laboratory A

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabA-Sub5 C1	1.82	0.997	1.81	1.51	±0.23	2.16	±0.34
LabA-Sub5 C2	0.55	0.997	0.54	0.61	±0.09	0.78	±0.12
LabA-Sub5 C3	0.18	0.997	0.18	0.14	±0.02	0.16	±0.03
LabA-Sub5 C4	0.05	0.997	0.05	0.09	±0.01	0.08	±0.01
LabA-Sub5 C5	0.02	0.997	0.02	0.02	±0.004	0.02	±0.003
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabB-Sub4 C1	1.82	0.997	1.82	2.30	±0.36	1.70	±0.27
LabB-Sub4 C2	0.55	0.997	0.55	0.64	±0.10	0.48	±0.08
LabB-Sub4 C3	0.18	0.997	0.18	0.21	±0.03	0.19	±0.03
LabB-Sub4 C4	0.05	0.997	0.05	0.10	±0.02	0.08	±0.01
LabB-Sub4 C5	0.02	0.997	0.02	0.02	±0.003	0.02	±0.003
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabC-Sub6 C1	1.79	0.997	1.79	0.84	±0.13	0.84	±0.13
LabC-Sub6 C2	0.54	0.997	0.54	0.41	±0.06	0.32	±0.05
LabC-Sub6 C3	0.18	0.997	0.18	0.14	±0.02	0.12	±0.02
LabC-Sub6 C4	0.05	0.997	0.05	0.04	±0.01	0.03	±0.005
LabC-Sub6 C5	0.02	0.997	0.02	0.02	±0.002	0.01	±0.002
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Bisphenol S Concentration (µM)

Laboratory A

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabA-Sub4 C1	58.95	0.980	57.77	63.67	±3.95	50.54	±3.13
LabA-Sub4 C2	17.68	0.980	17.33	21.56	±1.34	17.79	±1.10
LabA-Sub4 C3	5.89	0.980	5.78	6.30	±0.39	7.08	±0.44
LabA-Sub4 C4	1.77	0.980	1.73	3.23	±0.20	2.81	±0.17
LabA-Sub4 C5	0.59	0.980	0.58	0.89	±0.06	0.80	±0.05
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabB-Sub6 C1	59.85	0.980		52.54	±3.26	51.52	±3.19
LabB-Sub6 C2	17.95	0.980		20.25	±1.26	16.64	±1.03
LabB-Sub6 C3	5.98	0.980		6.74	±0.42	5.60	±0.35
LabB-Sub6 C4	1.80	0.980		3.11	±0.19	1.86	±0.12
LabB-Sub6 C5	0.60	0.980		0.09	±0.01	0.87	±0.05
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction by purity (µg/L)	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabC-Sub5 C1	57.31	0.980	56.16	83.29	±5.16	81.52	±5.05
LabC-Sub5 C2	17.19	0.980	16.85	26.03	±1.61	26.84	±1.66
LabC-Sub5 C3	5.73	0.980	5.62	8.86	±0.55	8.15	±0.51
LabC-Sub5 C4	1.72	0.980	1.68	3.05	±0.19	3.17	±0.20
LabC-Sub5 C5	0.57	0.980	0.56	0.94	±0.06	1.12	±0.07
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Bisphenol S Concentration (mg/L)

Laboratory A

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabA-Sub4 C1	14.75	0.980	14.46	15.93	±0.99	12.65	±0.78
LabA-Sub4 C2	4.43	0.980	4.34	5.40	±0.33	4.45	±0.28
LabA-Sub4 C3	1.48	0.980	1.45	1.58	±0.10	1.77	±0.11
LabA-Sub4 C4	0.44	0.980	0.43	0.81	±0.05	0.70	±0.04
LabA-Sub4 C5	0.15	0.980	0.14	0.22	±0.01	0.201	±0.01
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabB-Sub6 C1	14.98	0.980	14.68	13.15	±0.82	12.89	±0.80
LabB-Sub6 C2	4.49	0.980	4.40	5.07	±0.31	4.17	±0.26
LabB-Sub6 C3	1.50	0.980	1.47	1.69	±0.10	1.40	±0.09
LabB-Sub6 C4	0.45	0.980	0.44	0.78	±0.05	0.47	±0.03
LabB-Sub6 C5	0.15	0.980	0.15	0.02	±0.001	0.219	±0.01
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabC-Sub5 C1	14.34	0.980	14.06	20.85	±1.29	20.40	±1.27
LabC-Sub5 C2	4.30	0.980	4.22	6.52	±0.40	6.72	±0.42
LabC-Sub5 C3	1.43	0.980	1.41	2.22	±0.14	2.04	±0.13
LabC-Sub5 C4	0.43	0.980	0.42	0.76	±0.05	0.79	±0.05
LabC-Sub5 C5	0.14	0.980	0.14	0.23	±0.01	0.281	±0.02
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Bisphenol F Concentration (µM)

Laboratory A

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabA-Sub6 C1	5.02	0.980	4.92	4.23	±0.31	4.87	±0.36
LabA-Sub6 C2	1.51	0.980	1.48	1.81	±0.13	1.68	±0.12
LabA-Sub6 C3	0.50	0.980	0.49	0.48	±0.04	0.52	±0.04
LabA-Sub6 C4	0.15	0.980	0.15	0.18	±0.01	0.17	±0.01
LabA-Sub6 C5	0.05	0.980	0.05	0.06	±0.004	0.05	±0.004
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabB-Sub5 C1	5.01	0.980	4.91	3.96	±0.29	4.09	±0.30
LabB-Sub5 C2	1.50	0.980	1.47	1.35	±0.10	1.27	±0.09
LabB-Sub5 C3	0.50	0.980	0.49	0.44	±0.03	0.40	±0.03
LabB-Sub5 C4	0.15	0.980	0.15	0.16	±0.01	0.16	±0.01
LabB-Sub5 C5	0.05	0.980	0.05	0.04	±0.003	0.06	±0.004
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabC-Sub4 C1	4.88	0.980	4.79	5.26	±0.38	4.54	±0.33
LabC-Sub4 C2	1.46	0.980	1.44	1.53	±0.11	1.83	±0.13
LabC-Sub4 C3	0.49	0.980	0.48	0.51	±0.04	0.50	±0.04
LabC-Sub4 C4	0.15	0.980	0.14	0.16	±0.01	0.17	±0.01
LabC-Sub4 C5	0.05	0.980	0.05	0.06	±0.004	0.04	±0.003
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Bisphenol F Concentration (mg/L)

Laboratory A

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabA-Sub6 C1	1.00	0.980	0.98	0.85	±0.06	0.98	±0.07
LabA-Sub6 C2	0.30	0.980	0.30	0.36	±0.03	0.34	±0.02
LabA-Sub6 C3	0.10	0.980	0.10	0.10	±0.007	0.10	±0.008
LabA-Sub6 C4	0.03	0.980	0.03	0.04	±0.003	0.03	±0.003
LabA-Sub6 C5	0.01	0.980	0.01	0.01	±0.001	0.011	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabB-Sub5 C1	1.00	0.980	0.98	0.79	±0.06	0.82	±0.06
LabB-Sub5 C2	0.30	0.980	0.30	0.27	±0.02	0.25	±0.02
LabB-Sub5 C3	0.10	0.980	0.10	0.09	±0.006	0.08	±0.006
LabB-Sub5 C4	0.03	0.980	0.03	0.03	±0.002	0.03	±0.002
LabB-Sub5 C5	0.01	0.980	0.01	0.01	±0.001	0.012	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabC-Sub4 C1	0.98	0.980	0.96	1.05	±0.08	0.91	±0.07
LabC-Sub4 C2	0.29	0.980	0.29	0.31	±0.02	0.37	±0.03
LabC-Sub4 C3	0.10	0.980	0.10	0.10	±0.007	0.10	±0.007
LabC-Sub4 C4	0.03	0.980	0.03	0.03	±0.002	0.03	±0.002
LabC-Sub4 C5	0.01	0.980	0.01	0.01	±0.001	0.009	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Dihydrotestosterone Concentration (µM)

Laboratory A

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabA-Sub9 C1	10.01	0.982	9.83	10.60	±0.64	1.49	±0.09
LabA-Sub9 C2	3.00	0.982	2.95	2.57	±0.15	0.58	±0.035
LabA-Sub9 C3	1.00	0.982	0.98	1.16	±0.07	<LQ	-
LabA-Sub9 C4	0.30	0.982	0.29	0.34	±0.02	<LQ	-
LabA-Sub9 C5	0.10	0.982	0.10	0.12	±0.007	<LQ	-
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabB-Sub11 C1	9.99	0.982	9.81	6.61	±0.40	8.71	±0.52
LabB-Sub11 C2	3.00	0.982	2.94	2.76	±0.17	2.36	±0.14
LabB-Sub11 C3	1.00	0.982	0.98	0.94	±0.06	0.89	±0.05
LabB-Sub11 C4	0.30	0.982	0.29	0.35	±0.021	0.28	±0.017
LabB-Sub11 C5	0.10	0.982	0.10	0.12	±0.007	0.11	±0.007
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (µM)		Measured concentration (µM)	
LabC-Sub10 C1	9.82	0.982	9.64	7.59	±0.46	7.79	±0.47
LabC-Sub10 C2	2.94	0.982	2.89	2.16	±0.13	2.14	±0.13
LabC-Sub10 C3	0.98	0.982	0.96	1.31	±0.08	0.77	±0.05
LabC-Sub10 C4	0.29	0.982	0.29	0.26	±0.016	0.26	±0.016
LabC-Sub10 C5	0.10	0.982	0.10	0.09	±0.005	0.09	±0.005
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Dihydrotestosterone Concentration (mg/L)

Laboratory A

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabA-Sub9 C1	2.91	0.982	2.86	3.08	±0.18	0.43	±0.026
LabA-Sub9 C2	0.87	0.982	0.86	0.75	±0.04	0.17	±0.010
LabA-Sub9 C3	0.29	0.982	0.29	0.34	±0.02	<LQ	-
LabA-Sub9 C4	0.09	0.982	0.09	0.10	±0.006	<LQ	-
LabA-Sub9 C5	0.03	0.982	0.03	0.03	±0.002	<LQ	-
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabB-Sub11 C1	2.90	0.982	2.85	1.92	±0.12	2.53	±0.15
LabB-Sub11 C2	0.87	0.982	0.85	0.80	±0.05	0.69	±0.04
LabB-Sub11 C3	0.29	0.982	0.28	0.27	±0.02	0.26	±0.02
LabB-Sub11 C4	0.09	0.982	0.09	0.10	±0.006	0.08	±0.005
LabB-Sub11 C5	0.03	0.982	0.03	0.04	±0.002	0.033	±0.002
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h		T 24h	
				Measured concentration (mg/L)		Measured concentration (mg/L)	
LabC-Sub10 C1	2.85	0.982	2.80	2.20	±0.13	2.26	±0.14
LabC-Sub10 C2	0.86	0.982	0.84	0.63	±0.04	0.62	±0.04
LabC-Sub10 C3	0.29	0.982	0.28	0.38	±0.02	0.22	±0.01
LabC-Sub10 C4	0.09	0.982	0.08	0.08	±0.005	0.08	±0.005
LabC-Sub10 C5	0.03	0.982	0.03	0.03	±0.002	0.02	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Levonorgestrel Concentration (µM)

Laboratory A

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabA-Sub7 C1	1.01		1.01	1.12	±0.07	0.97	±0.06
LabA-Sub7 C2	0.30		0.30	0.35	±0.02	0.81	±0.05
LabA-Sub7 C3	0.10		0.10	0.13	±0.01	0.13	±0.01
LabA-Sub7 C4	0.03		0.03	0.04	±0.003	0.04	±0.003
LabA-Sub7 C5	0.01		0.01	0.01	±0.001	0.02	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabB-Sub9 C1	1.00		1.00	0.91	±0.06	0.91	±0.06
LabB-Sub9 C2	0.30		0.30	0.31	±0.02	0.33	±0.02
LabB-Sub9 C3	0.10		0.10	0.11	±0.01	0.12	±0.01
LabB-Sub9 C4	0.03		0.03	0.04	±0.003	0.04	±0.003
LabB-Sub9 C5	0.01		0.01	0.01	±0.001	0.01	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (µM)	Standard purity	Nominal Value with correction	T 0h Measured concentration (µM)		T 24h Measured concentration (µM)	
LabC-Sub11 C1	0.99		0.99	0.92	±0.06	0.99	±0.06
LabC-Sub11 C2	0.30		0.30	0.29	±0.02	0.35	±0.02
LabC-Sub11 C3	0.10		0.10	0.16	±0.01	0.15	±0.01
LabC-Sub11 C4	0.03		0.03	0.05	±0.003	0.05	±0.003
LabC-Sub11 C5	0.01		0.01	0.02	±0.001	0.02	±0.001
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Levonorgestrel Concentration (mg/L)

Laboratory A

Sample	Nominal concentration (µg/L)	Standard purity	Nominal Value with correction	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabA-Sub7 C1	0.315	1.000	0.315	0.350	±0.023	0.304	±0.020
LabA-Sub7 C2	0.095	1.000	0.095	0.108	±0.007	0.252	±0.016
LabA-Sub7 C3	0.032	1.000	0.032	0.041	±0.003	0.040	±0.003
LabA-Sub7 C4	0.009	1.000	0.009	0.013	±0.001	0.013	±0.001
LabA-Sub7 C5	0.003	1.000	0.003	0.004	±0.0003	0.005	±0.0003
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabB-Sub9 C1	0.313	1.000	0.313	0.283	±0.018	0.285	±0.019
LabB-Sub9 C2	0.094	1.000	0.094	0.098	±0.006	0.103	±0.007
LabB-Sub9 C3	0.031	1.000	0.031	0.035	±0.002	0.037	±0.002
LabB-Sub9 C4	0.009	1.000	0.009	0.013	±0.001	0.012	±0.001
LabB-Sub9 C5	0.003	1.000	0.003	0.004	±0.0003	0.004	±0.0003
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (mg/L)	Standard purity	Nominal Value with correction	T 0h Measured concentration (mg/L)		T 24h Measured concentration (mg/L)	
LabC-Sub11 C1	0.308	1.000	0.308	0.286	±0.019	0.309	±0.020
LabC-Sub11 C2	0.092	1.000	0.092	0.090	±0.006	0.109	±0.007
LabC-Sub11 C3	0.031	1.000	0.031	0.050	±0.003	0.048	±0.003
LabC-Sub11 C4	0.009	1.000	0.009	0.017	±0.001	0.016	±0.001
LabC-Sub11 C5	0.003	1.000	0.003	0.005	±0.0003	0.005	±0.0004
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

17α-Ethinylestradiol Concentration (nM)

Laboratory A

Sample	Nominal concentration (nM)	Standard purity	Nominal Value with correction	T 0h Measured concentration (nM)		T 24h Measured concentration (nM)	
Lab A EE2 S1,2,3	0.05	0.98	0.05	0.05	±0.002	0.05	±0.003
Lab A EE2 S4,5,6,7	0.05	0.98	0.05	0.05	±0.003	0.06	±0.003
Lab A EE2 S8,9,10,11	0.05	0.98	0.05	0.06	±0.003	0.08	±0.004
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (nM)	Standard purity	Nominal Value with correction	T 0h Measured concentration (nM)		T 24h Measured concentration (nM)	
Lab B EE2 S1,2,3	0.05	0.98	0.05	0.06	±0.003	0.05	±0.003
Lab B EE2 S4,5,6,7	0.05	0.98	0.05	0.04	±0.002	0.03	±0.002
Lab B EE2 S8,9,10,11	0.05	0.98	0.05	0.04	±0.002	0.04	±0.002
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (nM	Standard purity	Nominal Value with correction	T 0h Measured concentration (nM)		T 24h Measured concentration (nM)	
Lab C EE2 S1,2	0.05	0.98	0.05	0.06	±0.003	0.06	±0.003
Lab C EE2 S3,4,5	0.05	0.98	0.05	0.06	±0.003	0.05	±0.003
Lab C EE2 S6,7,8	0.05	0.98	0.05	0.05	±0.003	0.06	±0.003
Lab C EE2 S9,10,11	0.05	0.98	0.05	0.05	±0.003	0.09	±0.005
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

17α-Ethinylestradiol Concentration (ng/L)

Laboratory A

Sample	Nominal concentration (ng/L)	Standard purity	Nominal Value with correction	T 0h Measured concentration (ng/L		T 24h Measured concentration (ng/L)	
Lab A EE2 S1,2,3	14.74	0.98	14.44	14.12	±0.73	15.10	±0.79
Lab A EE2 S4,5,6,7	14.74	0.98	14.44	15.38	±0.80	16.64	±0.87
Lab A EE2 S8,9,10,11	14.74	0.98	14.44	16.61	±0.86	24.23	±1.26
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory B

Sample	Nominal concentration (ng/L)	Standard purity	Nominal Value with correction	T 0h Measured concentration (ng/L		T 24h Measured concentration (ng/L)	
Lab B EE2 S1,2,3	14.90	0.98	14.60	16.34	±0.85	14.93	±0.78
Lab B EE2 S4,5,6,7	14.90	0.98	14.60	12.08	±0.63	10.17	±0.53
Lab B EE2 S8,9,10,11	14.90	0.98	14.60	12.91	±0.67	13.00	±0.68
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

Laboratory C

Sample	Nominal concentration (ng/L)	Standard purity	Nominal Value with correction	T 0h Measured concentration (ng/L		T 24h Measured concentration (ng/L)	
Lab C EE2 S1,2	14.65	0.98	14.35	16.63	±0.86	19.06	±0.99
Lab C EE2 S3,4,5	14.65	0.98	14.35	18.29	±0.95	16.06	±0.84
Lab C EE2 S6,7,8	14.65	0.98	14.35	15.15	±0.79	17.34	±0.90
Lab C EE2 S9,10,11	14.65	0.98	14.35	15.93	±0.83	26.34	±1.37
Control	0	-	-	< LD	-	< LD	-
Control DMSO	0	-	-	< LD	-	< LD	-

ANNEX 8: nominal vs measured concentrations

Percent of the target nominal concentrations measured at T0 and T24 (prior to the renewal of the medium) and calculation of the time-weighted mean concentrations.

			% Nominal		
		Nominal concentration	T0	T24	time-weighted mean conc.
Diclofenac (µM)	Lab A	10.03	109	100	10.45
		3.01	100	93	2.90
		1.00	103	93	0.98
		0.30	105	106	0.32
		0.10	106	102	0.10
	Lab B	10.02	90	85	8.74
		3.00	80	53	1.98
		1.00	74	60	0.67
		0.30	92	72	0.24
		0.10	91	71	0.08
	Lab C	9.79	72	72	7.06
		2.94	64	57	1.77
		0.98	78	72	0.73
		0.29	84	67	0.22
		0.10	100	71	0.08
Triclosan (µM)	Lab A	10.00	63	80	7.11
		3.00	84	77	2.41
		1.00	65	53	0.59
		0.30	79	50	0.19
		0.10	71	48	0.06
	Lab B	10.02	90	85	8.74
		3.00	80	53	1.98
		1.00	74	60	0.67
		0.30	92	72	0.24
		0.10	91	71	0.08
	Lab C	9.79	72	72	7.06
		2.94	64	57	1.77
		0.98	78	72	0.73
		0.29	84	67	0.22
		0.10	100	71	0.08
2,4-dihydroxybenzophenone (µM)	Lab A	10.01	81	92	8.64
		3.00	95	93	2.84
		1.00	83	82	0.83
		0.30	83	86	0.25
		0.10	68	66	0.07
	Lab B	10.00	91	93	9.20
		3.00	106	98	3.06
		1.00	108	109	1.08
		0.30	118	110	0.34
		0.10	126	116	0.12
	Lab C	9.86	109	106	10.62
		2.96	117	102	3.22
		0.99	114	104	1.07
		0.30	113	103	0.32
		0.10	105	98	0.10

ANNEX 8: nominal vs measured concentrations (continued)

			% Nominal		
		Nominal concentration	T0	T24	time-weighted mean conc.
Norethindrone (nM)	Lab A	101.43	82	97	90.22
		30.43	98	101	30.29
		10.14	101	100	10.16
		3.04	119	123	3.69
		1.01	110	108	1.10
	Lab B	100.11	91	87	88.92
		30.03	99	43	20.22
		10.01	99	102	10.06
		3.00	109	108	3.25
		1.00	110	110	1.10
	Lab C	98.03	115	79	93.98
		29.41	119	113	34.11
		9.80	133	94	11.00
		2.94	135	114	3.66
		0.98	123	99	1.09
17b Trenbolone (µM)	Lab A	10.07	95	71	8.27
		3.02	91	83	2.64
		1.01	91	72	0.82
		0.30	108	93	0.30
		0.10	148	101	0.12
	Lab B	9.99	87	77	8.20
		3.00	84	78	2.42
		1.00	87	87	0.87
		0.30	88	109	0.29
		0.10	95	111	0.10
	Lab C	9.89	86	52	6.66
		2.97	95	67	2.39
		0.99	95	66	0.78
		0.30	94	77	0.25
		0.10	74	50	0.06
11 Ketotestosterone (nM)	Lab A	1004.93	96	106	1017.72
		301.48	107	112	330.81
		100.49	133	103	118.27
		30.15	149	78	33.16
		10.05	148	59	9.68
	Lab B	999.97	94	88	910.20
		299.99	112	74	275.43
		100.00	101	102	101.27
		30.00	126	108	34.91
		10.00	102	119	11.04
	Lab C	967.72	115	96	1019.42
		290.32	110	117	330.21
		96.77	165	95	122.90
		29.03	126	185	44.50
		9.68	133	133	12.84

ANNEX 8: nominal vs measured concentrations (continued)

			% Nominal		
		Nominal concentration	T0	T24	time-weighted mean conc.
Benzophenone (µM)	Lab A	9.97	83	119	9.94
		2.99	111	144	3.79
		1.00	75	88	0.81
		0.30	163	147	0.46
		0.10	137	97	0.12
	Lab B	10.01	126	93	10.90
		3.00	116	89	3.06
		1.00	117	104	1.10
		0.30	182	153	0.50
		0.10	114	105	0.11
	Lab C	9.83	47	47	4.62
		2.95	76	60	2.00
		0.98	77	65	0.69
		0.29	65	57	0.18
		0.10	84	73	0.08
Bisphenol S (µM)	Lab A	58.95	108	86	56.85
		17.68	122	101	19.62
		5.89	107	120	6.68
		1.77	183	159	3.01
		0.59	151	136	0.84
	Lab B	59.85	88	86	52.03
		17.95	113	93	18.39
		5.98	113	94	6.15
		1.80	173	104	2.43
		0.60	15	146	0.35
	Lab C	57.31	145	142	82.40
		17.19	151	156	26.44
		5.73	155	142	8.50
		1.72	177	185	3.11
		0.57	163	196	1.03
Bisphenol F (µM)	Lab A	5.02	84	97	4.54
		1.51	120	111	1.74
		0.50	96	104	0.50
		0.15	117	116	0.17
		0.05	113	108	0.06
	Lab B	5.01	79	82	4.02
		1.50	90	85	1.31
		0.50	88	79	0.42
		0.15	106	103	0.16
		0.05	86	118	0.05
	Lab C	4.88	108	93	4.89
		1.46	104	125	1.67
		0.49	104	102	0.50
		0.15	106	114	0.16
		0.05	125	88	0.05

ANNEX 8: nominal vs measured concentrations (continued)

			% Nominal		
		Nominal concentration	T0	T24	time-weighted mean conc.
Dihydrotestosterone (µM)	Lab A	10.01	106	15	4.65
		3.00	86	19	1.34
		1.00	116	0	0.16
		0.30	113	0	0.06
		0.10	118	1	0.02
	Lab B	9.99	66	87	7.61
		3.00	92	79	2.56
		1.00	95	89	0.92
		0.30	116	94	0.31
		0.10	123	112	0.12
	Lab C	9.82	77	79	7.69
		2.94	73	73	2.15
		0.98	133	79	1.02
		0.29	88	88	0.26
		0.10	93	88	0.09
Levonorgestrel (µM)	Lab A	1.01	111	96	1.05
		0.30	114	266	0.54
		0.10	131	126	0.13
		0.03	133	137	0.04
		0.01	142	149	0.01
	Lab B	1.00	91	91	0.91
		0.30	105	110	0.32
		0.10	113	120	0.12
		0.03	136	126	0.04
		0.01	137	142	0.01
	Lab C	0.99	93	100	0.95
		0.30	97	118	0.32
		0.10	163	155	0.16
		0.03	181	177	0.05
		0.01	168	177	0.02
Ethinylestradiol (nM)	Lab A	0.05	96	103	0.049
		0.05	104	113	0.054
		0.05	113	164	0.068
	Lab B	0.05	110	100	0.053
		0.05	81	68	0.037
		0.05	87	87	0.044
	Lab C	0.05	114	130	0.060
		0.05	125	110	0.058
		0.05	103	118	0.055
		0.05	109	180	0.070