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Validation of the Rapid Androgen Disruption Activity (RADAR) assay for the detection of androgen axis active substances, described in OECD Test Guideline 251

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Validation Report of the Androgen Disruption Activity Reporter Assay

FOREWORD

This document describes the design and results of the validation exercise for the Rapid Androgen Disruption Activity Reporter (RADAR) assay. This method was developed for the detection of androgen axis active substances. It is performed in 6-well plate format and can serve as a quick screen for potential androgen axis disrupting substances. The purpose of the validation exercise was to determine whether the standard operating procedure (SOP) could be successfully transferred across laboratories, to determine variability between laboratories and to verify the absence of false positives by testing compounds presumed to be inert.

The RADAR assay is being validated through an international effort via the OECD. The OECD has been working with member countries on the validation and harmonization of testing methods for the detection of chemicals that interfere with the estrogen, androgen and thyroid pathways.

The WNT approved the TG 251 on the RADAR assay and endorsed the validation report in its 34th meeting in April 2022, on the basis of a project led by France. This document is published under the responsibility of the Chemicals and Biotechnology Committee.

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4 ABREVIATIONS AND DEFINITIONS

17MT: 17α-Methyltestosterone.

AFSS: Androgenised female stickleback screen.

AR: Androgen receptors.

DPH: Day post hatch.

CEFAS: Centre for Environment, Fisheries and Aquaculture Science, UK.

E2: 17β-estradiol.

EE2: 17α-ethinyl estradiol.

Eleutheroembryo: The eleutheroembryonic life stage is post-hatch, but before the embryo is capable of independently feeding on exogenous food supplies and is a stage of on-going embryonic development. Applying this definition to *O. latipes* positions this period of development from stage 39 (hatching stage) to stage 42 (formation of structures required for prey capture including the teeth of the upper jaw, the otolith, and the shape of all fins) (Iwamatsu, 2004).

FIWI: Zentrum für Fisch und Wildtiermedizin, Switzerland

Fraunhofer: Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Germany.

GFP: Green fluorescent protein.

Idea: Idea Consulting, Japan.

LC50: Median of a test chemical that is estimated to be lethal to 50% of the test organisms within the test duration.

LPL: Laboratoires des Pyrénées et des Landes.

LOEC: The lowest observed effect concentration is the lowest tested concentration at which the test chemical is observed to have a statistically significant effect (at $p < 0.05$).

mDHT: 17α-Methyl-5α-dihydrotestosterone; mestanolone.

MS222: Tricaine methanesulfonate

NOEC: The no observed effect concentration is the tested concentration immediately below the LOEC.

SEM: Standard error of the mean.

Run: A run is defined here as an experiment performed using independent solutions.**Spiked mode:** Part of aRADAR assay performed in the presence of 3 µg/L of 17MT.

***spg1-gfp*:** Transgenic medaka line harbouring a genetic construction consisting of a 4159 base pairs of the three-spined stickleback *spiggin 1* gene promoter upstream of GFP coding sequence.

Test medium: The medium used for the assay, this could be any water permitting normal growth and development of *O. latipes* including medaka medium, glass bottled still mineral water, spring water, well water and charcoal-filtered tap water.

Unspiked mode: Part of the RADAR assay performed in the absence of 17MT.

UVCB: Substances of unknown or variable composition, complex reaction products or biological materials.

WF: Laboratoire WatchFrog, France.

5 ACKNOWLEDGEMENTS

This work is the collaborative effort of five laboratories which generously performed the experiments described here.

The following laboratories and their staff took part in the RADAR assay interlaboratory validation exercise:

- Institute of Environmental Ecology, IDEA Consultants Inc., Japan: Misa Toda, Yu Totsuka and Tetsuro Okamura performed the experiments. Prof. Taisen Iguchi (Nanobioscience, Yokohama City University) coordinated the work in Japan.
- Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Schmallenberg, Germany: Azora Konig performed the experiments. Dr Lena Kosak and Dr Elke Eilebrecht supervised the experiments.
- Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth, UK: Dr Marion Sebire, Ellen Blaker, Jessica Elphinstone Davis, David Hartnell and Sally Latham performed the experiments. Dr Marion Sebire and Dr Ioanna Katsiadaki supervised the experiments.
- Centre for Fish and Wildlife Health (FIWI), University of Bern, Switzerland: Jessica Rieder performed and coordinated the experiments, Prof. Helmut Segner supervised the experiments.
- Laboratoire WatchFrog, Evry, France: Marthe Chabanon, Sarah Bancel and Mannon Bouix performed the experiments. Dr Andrew Tindall supervised the experiments at WatchFrog and designed and coordinated the validation study.

Andrew Tindall (tindall@watchfrog.fr), wrote the validation report and the draft test guideline.

1. Introduction

1.1. Objectives of the Validation Study

The overall objective of the validation exercise for the RADAR was to establish the relevance of the assay to detect any potential androgen axis activity of compounds acting at different points within the androgen axis and via different modes of action. A second aim was to assess the transferability and reproducibility of the assay by comparing results obtained by a variety of laboratories in five different countries and two different continents.

1.2. Assay Development/Background

Development of the transgenic model, as well as initial experiments to determine the androgen responsiveness of the model, were carried out in the laboratory of Prof. Iguchi in Okazaki, Japan.

A characterisation of the model was then carried out in France, in particular focussing on the response of eleutheroembryos immediately after hatch. This work was published in Environmental Science and Technology (Sébillot et al., 2014) and involved the development of a test protocol as well as its characterisation.

Notably, this work demonstrated the androgen-specific response of the model by comparing the response to reference androgens to that of high concentrations of reference agonists for the progesterone, glucocorticoid and mineralocorticoid receptors. In addition, the ability of the reference anti-androgen, flutamide, to block this response was demonstrated. Likewise, the ability of the pharmaceutical aromatase inhibitor anastrozole to prevent signal loss from an aromatisable androgen by blocking its conversion to an estrogen was demonstrated.

The sensitivity of the assay to the anti-androgen flutamide was then determined and the three anti-androgenic pesticides tested during the OECD validation of the Androgenised Female Stickleback Screen (OECD, 2010; OECD, 2011a) were tested to allow a comparison of the sensitivity of the two assays. This comparison is shown in the table below:

Table 1: Comparison of the lowest observed effect concentrations of four anti-androgens tested in the RADAR assay (Sébillot et al., 2014) and the AFSS (OECD, 2010).

	RADAR assay	AFSS
Flutamide	138 µg/L	10 −250 µg/L
Linuron	249 µg/L	250 µg/L
Vinclozolin	143 µg/L	100 µg/L
Fenitrothion	277 µg/L	60 µg/L

The sensitivity of the RADAR assay is entirely compatible with the detection of androgen active chemicals and is even compatible with the determination of androgen activity of unconcentrated surface water samples contaminated with these androgen active chemicals. Examples of flutamide equivalents found in surface water are: UK wastewater treatment plant effluent: 21.3 − 1231 µg FL eq/L, River Ock, UK negative control site: >150 µg FL eq/L, River Lambro, Italy: 370 − 4723 µg FL eq/L (Grover et al., 2011; Lange et al., 2015; Urbatzka et al., 2007).

An extensive study was carried out comparing the response of the RADAR assay and the AFSS to a number of estrogens. It had previously been noted *in vitro* and *in vivo* (Jolly et al., 2009a; Katsiadaki et al., 2006a) that estrogens can exert an anti-androgenic effect, possibly via induction of aromatase enzyme (Guiguen et al, 2010; Scholz & Gutzeit, 2000) or by direct interaction with androgen receptors (AR). This phenomenon had also been observed during the characterisation of the RADAR assay (Sébillot et al., 2014). In order to further confirm that this effect is reproducible and is common to a wide range of estrogens, we carried out concentration response experiments in five different laboratories, four using the AFSS and one using the RADAR assay. The experiments were carried out with 17α-ethinyl estradiol (EE2), estradiol (E2), estrone and the weakly estrogenic compound nonylphenol (NP). All four estrogens/estrogenic chemicals inhibited androgen axis activity as measured by determination of the abundance of spiggin protein by ELISA (AFSS) or by quantification of the activity of the spiggin 1 promoter (RADAR assay). In all cases the RADAR assay showed a good predictability for the result obtained with the AFSS, although the RADAR assay was less sensitive to the anti-androgenic effects of EE2 and estrone (manuscript in preparation).

Together these characterisation and validation experiments demonstrate the ability of this model to detect androgen axis disruption and the close agreement of the results of this assay with those of the AFSS.

The following information supported the transferability of this protocol to the participating laboratories:

1. Medaka fish (*Oryzias latipes*) are already a widely used model organism across OECD countries. They are also widely accepted and validated as a test species in numerous OECD test guidelines including: OECD TG 203 (Fish Acute Toxicity Test; OECD, 2019a), OECD TG 210 (Fish Early Life Stage Toxicity Test; OECD, 1992a), OECD TG 212 (Fish Short Term

Toxicity Test on Embryo and Sac-fry Stages; OECD, 1998), OECD TG 229 (Fish Short-Term Reproduction Assay; OECD, 2012), OECD TG 230 (21-day Fish Assay; OECD, 2009), OECD TG 234 (Fish Sexual Development Test; OECD, 2011) and OECD TG 240 (Medaka Extended One Generation Reproduction Test; OECD, 2015).

2. An additional advantage of medaka is that they are reared in conditions that are almost identical to those of zebrafish. Laboratories with previous zebrafish husbandry experience were able to successfully rear and reproduce medaka (FIWI, Switzerland).
3. Founders, adult homozygous medaka for breeding embryos for on-site testing, were made available to participants in the ring test.
4. Embryos were available for shipping from a breeding/production site to another testing site.

1.3. Test organism

The medaka fish, *O. latipes*, is the test species for the RADAR. This species of fish is a well-established small model organism, having been extensively studied since the beginning of the twentieth century when Aida (1921) linked sex to body colouration in certain strains of medaka. Medaka is an ideal model for studying the vertebrate sex steroid axes. Sexual differentiation has been extensively studied (Kondo et al., 2009) and medaka estrogen receptors and androgen receptors (AR) show conformational conservation when compared to human receptors (Cui et al., 2009). In addition, steroidogenesis pathways are highly conserved among vertebrates, with a high concordance in the identification of endocrine active chemicals between fish and rat assays carried out in the context of the U.S. Environmental Protection Agency Endocrine Disruptor Screening Program (Ankley and Gray, 2013). Medaka were also the first vertebrate species after humans in which the master sex determining gene (*dmy*) was identified (Masuyama et al., 2012; Matsuda et al., 2002). This fact, besides its clear importance in itself, also highlights the extent to which the genetic basis of sex determination has been studied in medaka and allows definitive determination of the genetic sex of medaka.

As with mammals, medaka possess a XX/ XY sex determination system (Aida, 1921; Yamamoto, 1958, 1955). It is also possible to determine the phenotypic sex of medaka morphologically due to a dimorphism in their dorsal and anal fins. Due in part to these characteristics which allow clear confirmation of sex reversal due to the action of endocrine disrupting chemicals (EDCs), the effects of exogenous androgens and anti-androgens have been extensively studied in medaka. It has been well demonstrated that exposure to androgens during development can cause genetically female (XX) medaka to develop a male phenotype (Iwamatsu et al., 2006; Papoulias et al., 2000). In addition, androgen exposure has been linked to decreased gonado-somatic index and decreased vitellogenin production in female medaka and increased spermatozoa in the testis lumen of male medaka (Orn et al., 2006; Papoulias et al., 2000). In contrast, anti-androgens have been shown to decrease the number of papillary processes (Horie et al., 2017; Nakamura et al., 2014), induce intersex gonads and inhibit spermatogenesis in male medaka as well as reducing fecundity and fertility of mating couples (Kang et al., 2006; Kiparissis et al., 2003).

1.4. Genetic construct

The assay is transcription-based and uses a transgenic medaka line harbouring the *spg1-gfp* genetic construct. This genetic construct comprises of the promoter of the Spiggin 1 gene coupled to a reporter gene for Green Fluorescent Protein (GFP). The *spg1-gfp* transgenic line used in the RADAR assay harbours 4.159 kb of the three spined stickleback (*Gasterosteus aculeatus*) spiggin 1 gene promoter immediately upstream of the start codon driving expression of GFP coding sequence. The *spg1-gfp*

transgene faithfully replicates the tissue specificity of the spiggin 1 gene as observed in three-spined stickleback, with expression of GFP strictly limited to the developing kidney (mesonephros). The promoter region present in the transgene has been shown to contain three putative androgen response elements (ARE) and the expression of the transgene has been demonstrated to be significantly modulated in the presence of AR agonists, antagonists and compounds inducing or inhibiting steroidogenic enzymes (Sébillot et al., 2014). As activation of the *spiggin 1* promoter is a terminal step in androgen axis signalling, the quantity of Spiggin protein produced in a three-spined stickleback or the quantity of GFP produced in the *spg1-gfp* medaka model represents the overall or net effects of both endogenous and exogenous factors altering androgen axis signalling (alterations in production, transport, metabolism and excretion of hormones as well as activation and inhibition of AR).

The Spg1-GFP transgenic line used in the RADAR assay harbours 4.159 kb of the three spined stickleback *spiggin 1* gene promoter immediately upstream of the start codon driving expression of Green Fluorescent Protein (GFP) coding sequence (Figure 1).

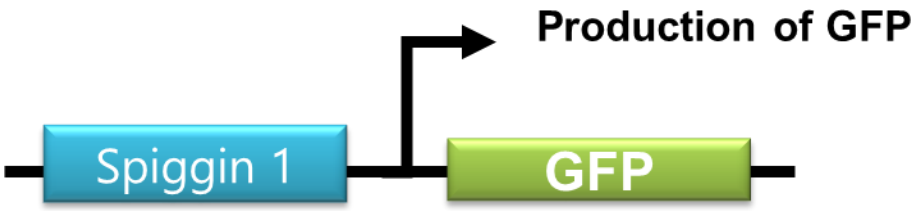

Figure 1: The transgene present in the Spg1-GFP medaka line used in the RADAR assay.

The *spg1-gfp* transgene faithfully replicates the tissue specificity of the *spiggin1* gene as observed in three-spined stickleback, with expression of *gfp* strictly limited to the developing kidney (mesonephros).

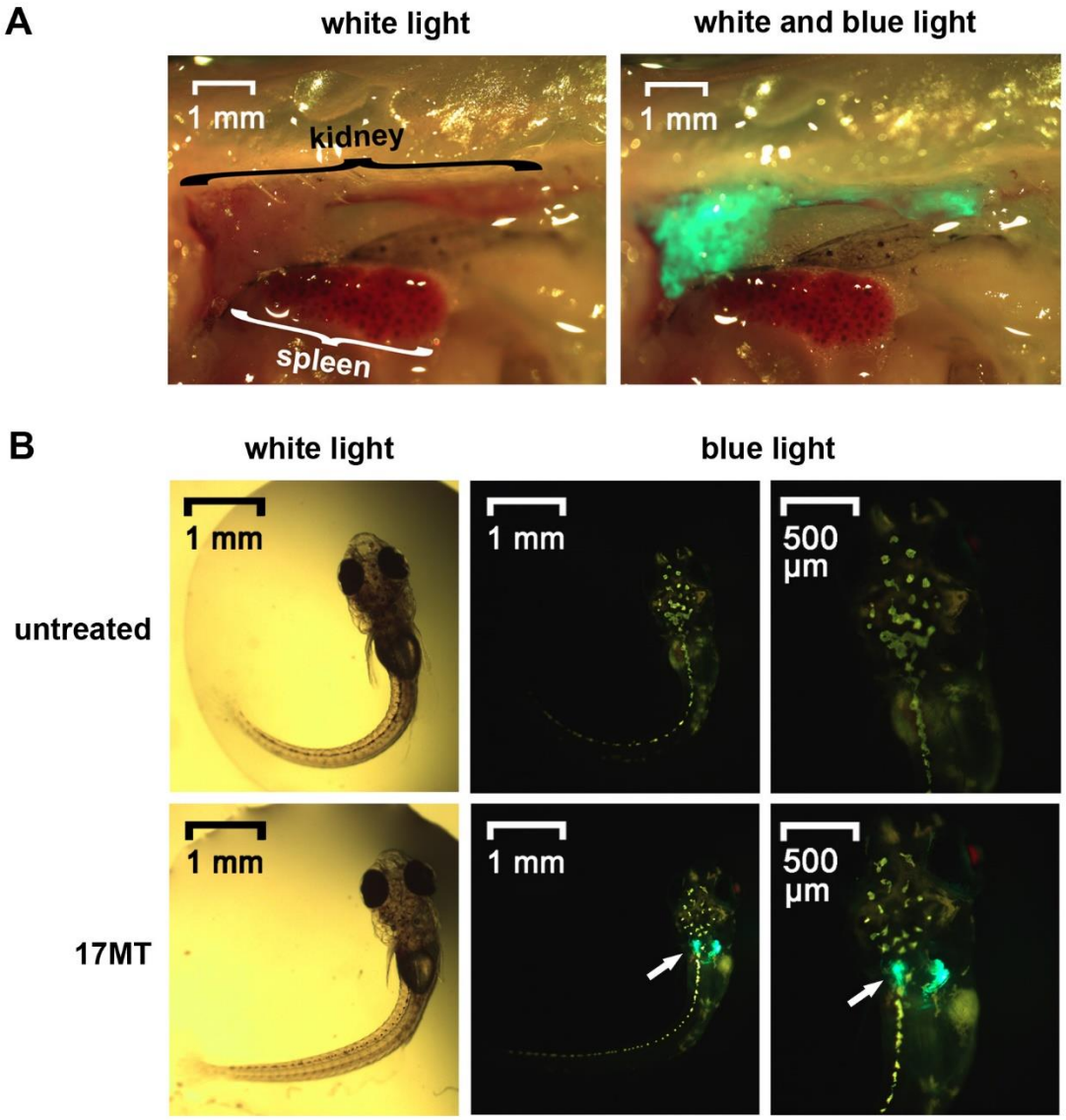

Figure 2 : Spg1-GFP medaka.

A) Dissected untreated adult male medaka observed under white light with or without additional blue light from a fluorescence source. B) Day post hatch 4 spg1.22-gfp eleutheroembryos treated for 4 days from day post hatch 0 with vehicle or 17α-methyltestosterone (302 µg/L) visualized under white or blue light. Arrows point to gfp expression in the developing mesonephros. Reprinted with permission from Sébillot et al. Environ Sci Technol. 48(18):10919-28 (2014). Copyright 2014 American Chemical Society.

2. Purpose and objectives

2.1. Purpose of the assay

The impact of endocrine disruptors on the health of humans and wildlife is now undeniable. In 1998, the OECD initiated a programme of work to develop new test guidelines or updates to existing test guidelines for the screening and testing of endocrine disruptors.

Currently, there is one new *in vitro* AR test guideline (TG 458) (OECD, 2020), and further tests are in the pipeline. However, at present, following *in vitro* testing the next tier for androgen disruption is the direct use of animal models at protected life stages. In addition, the aquatic tests using laboratory animals are only ratified for a small number of species, notably excluding zebrafish due to the lack of secondary sexual characteristics.

This development of the RADAR follows on from the work led by the UK CEFAS, on the OECD validation of the AFSS (GD 148) (OECD, 2011a).

The RADAR assay provides a rapid (72 hour) OECD Tier 3 *in vivo* screening test using eleutheroembryos. The purpose of the proposed RADAR assay is to provide a rapid test to measure the response of eleutheroembryonic, transgenic medaka to potential androgenic chemicals as an alternative to longer OECD Tier 3 *in vivo* fish studies such as the AFSS (OECD, 2011a) and the Fish Short-Term Reproduction Assay (TG 229) (OECD, 2012). Use of the RADAR assay will rapidly screen for (anti-)androgenic chemicals and reduce costs related to testing as well as potentially the number of OECD Tier 3 *in vivo* studies carried out with protected life stages.

2.2. Major characteristics of the assay

The RADAR assay involves aqueous exposures of medaka eleutheroembryos in a multi-well format to detect modulation of androgen axis signalling by potential androgen active chemicals. The assay is transcriptional-based and uses a transgenic medaka line containing the *spg1-gfp* genetic construct (see Figure 1 above) to detect the activity of pro-androgenic compounds such as AR agonists and compounds decreasing aromatase activity as well as anti-androgenic compounds that act through various mechanisms (including activation of metabolic elimination of androgens and AR antagonism). The assay measures the ability of a chemical to activate or inhibit transcription of the genetic construct, whether directly through binding to the AR or modifying the binding of a reference androgen (17α-methyltestosterone, 17MT) to the AR. The endpoint measured is the fluorescence of the transgenic eleutheroembryos. When transcription of the genomic construct is activated or inhibited following chemical exposure, the eleutheroembryos express more or less GFP and therefore emit more or less fluorescence compared to unexposed eleutheroembryos.

The assay measures GFP fluorescence in the transgenic *spg1-gfp* eleutheroembryos by fluorescence imaging using a fluorescence microscope. An automated ImageJ macro is then used to remove fluorescence generated by endogenous pigments in the medaka eleutheroembryos (melanophores, iridiphores, xanthophores, leucophores) (Braasch et al., 2009; Loire et al., 2013; Wakamatsu et al., 2001). The automated macro produces an Excel sheet containing a numerical value of the GFP signal in each eleutheroembryo.

Control eleutheroembryos are maintained in medaka medium, and at this developmental stage GFP signal is not expected in these control eleutheroembryos due to the low level of endogenous androgen axis signalling. If a pro-androgenic chemical is present in the exposure media of the test groups, an increase in fluorescence signal is expected.

A second set of exposures are carried out in the presence of 17MT, with the groups exposed to media containing the test chemical spiked with 17MT being compared to a 17MT alone control group. The aim of these exposures is to activate the androgen axis allowing the identification of anti-androgenic chemicals acting either through AR antagonism, other mechanisms of alteration of the ability of the AR to bind 17MT, modulation of aromatase expression or activity or via downregulation of AR expression. In addition, pro-androgenic chemicals can be identified acting through direct AR agonism, modulation of the AR to increase its affinity for 17MT, upregulation of AR expression or modulation of aromatase activity.

Results can be evaluated in terms of the lowest observable effect concentration (LOEC). They can also be evaluated as 17MT or flutamide equivalence by comparing the induction or inhibition of fluorescence to the 17MT or flutamide controls included in each experiment. However, the main aim of the RADAR assay is to simply categorise test chemicals as either androgen axis active or inactive.

Overview of schedule:
Day -1: Agitation of crystallizers containing the embryos if necessary to induce hatching. Eleutheroembryos hatch at day post fertilisation 10 under our conditions (26°C). **Day 0:** Prepare exposure solutions including controls. Add 5 eleutheroembryos (day post hatch 0) to each well. Remove the maximum amount of liquid without drying the eleutheroembryos (maximum remaining volume 800 µL). Fill each well of 6-well plates with 8 mL of each exposure solution (4 wells per exposure condition) under a chemical hood starting with plate 1 and finishing with the last plate. Incubate the plates at 26 °C in the dark. Do not feed the embryos during the experiment. **Day 1 and 2 (+ or - 60 min):** Note mortality and evacuate any dead eleutheroembryos. Prepare new exposure solutions including controls. Remove the maximum amount of liquid without drying the eleutheroembryos (maximum remaining volume 800 µL). Fill each well again with 8ml of their respective exposure solution. Replace the plates at 26 °C in the dark. Do not feed. Place the liquid waste in the appropriate waste container. **Day 3 (+ or- 60 min):** Note mortality and evacuate any dead eleutheroembryos. Rinse the eleutheroembryos by transfering them to new annotated 6-well plates with 8 mL of dechlorinated water or mineral water per well. Anesthetise the eleutheroembryos with MS222 at 200 mg/L by adding 2 mL/well of MS222 1 g/L. Place each eleutheroembryo on the reverse side of a black 96-well plate so that they are in an individual drop. Orient the eleutheroembryos for reading of their dorsal region under a fluorescent microscope. Each eleutheroembryo is photographed with the appropriate image capture software for the microscope used. Euthanise eleutheroembryos in MS222 at 1 g/L. Dispose of all waste in appropriate waste containers.

2.3. General experimental design

The assay is performed to determine the potential of a test chemical to modulate the androgen axis under sublethal concentrations. For the validation process a five concentrations test design was used (5 eleutheroembryos per well x 4 wells = 20 eleutheroembryos exposed per concentration). In the test guideline a minimum of three concentrations is recommended allowing to keep the same sensitivity. Newly hatched (day post hatch zero; DPH0) *spg1-gfp* medaka eleutheroembryos are used for the RADAR assay. The test is terminated at DPH3 after 72 h of exposure. They are not fed before or during the test as the test is terminated at stage 40 (Iwamatsu, 2004). Yolk is still present until stage 41/42 and is used as the source of energy for the development of the eleutheroembryo.

The test is run in two modes “spiked” and “unspiked” i.e. with and without the addition of 17MT. In spiked mode, all groups are spiked with 3 µg/L of 17MT. Eleutheroembryonic life stages of medaka do not synthesise enough androgen to generate a GFP signal. Therefore, spiking with 17MT is necessary in order to detect chemicals acting on androgen distribution, metabolization, degradation and AR antagonists.

The control groups include:

- Test medium and/ or solvent control: 4 wells with 5 organisms/well are exposed to test medium. This control defines the basal fluorescence level in the test medium. If a solvent is used, then this group exposed to test medium plus the solvent used at the same concentration as all other groups. In some cases, such as a solvent being used with no historical data available, both groups may be required.
- 17MT 3 µg/L: 4 wells with 5 organisms/well are exposed to 3 µg/L of 17MT. This control establishes the fluorescence level for a 17MT concentration of 3 µg/L. This control can serve as part of the 17MT standard curve if the optional controls described below are included, allowing a 17MT equivalence value to be read off the standard curve for any pro-androgenic test chemicals, this is optional.
- 17MT 10 µg/L: 4 wells with 5 organisms/well are exposed to 10 µg/L of 17MT. This control establishes the fluorescence level for a 17MT concentration of 10 µg/L. This control can serve as part of the 17MT standard curve if the optional controls described below are included, allowing a 17MT equivalence value to be read off the standard curve for any pro-androgenic test chemicals, this is optional.
- 17MT 3 µg/L + Flutamide 500 µg/L: 4 wells with 5 organisms/well are exposed to 500 µg/L of flutamide in the presence of 3 µg/L of 17MT. This control establishes the fluorescence inhibition for a flutamide concentration of 500 µg/L compared to the 17MT 3 µg/L alone control group. This control serves as part of the flutamide standard curve if the optional controls described below are included, allowing a flutamide, as an AR antagonist, equivalence value to be read off the standard curve for any test chemicals displaying anti-androgenic activity in spiked mode (presence of 3 µg/L of 17MT).

The calculation of equivalence values is not required and is for informative purposes only as the result of the assay is that the test chemical is active or inactive only. If equivalence values are to be calculated, the optional controls below should be included in each run.

The following additional control groups are optional, but are recommended for calibration of reading parameters in naïve laboratories as well as for quality control purposes. For this validation exercise, all control (obligatory and optional) were included in all experiments.

- 17MT 1.5 µg/L: 4 wells with 5 organisms/well are exposed to 1.5 µg/L of 17MT. This control establishes the fluorescence level for a 17MT concentration of 1.5 µg/L. This control serves as part of the 17MT standard curve, allowing a 17MT equivalence value to be read off the standard curve for any chemicals inducing androgen axis signalling.
- 17MT 3 µg/L + Flutamide 167 µg/L: 4 wells with 5 organisms/well are exposed to 167 µg/L of flutamide in the presence of 3 µg/L of 17MT. This control establishes the fluorescence inhibition for a flutamide concentration of 167 µg/L compared to the 17MT 3 µg/L alone control group. This control serves as part of the flutamide standard curve.
- 17MT 3 µg/L + Flutamide 55.6 µg/L: 4 wells with 5 organisms/well are exposed to 55.6 µg/L of flutamide in the presence of 3 µg/L of 17MT. This control establishes the fluorescence inhibition for a flutamide concentration of 55.6 µg/L compared to the 17MT 3 µg/L alone control group. This control serves as part of the flutamide standard curve.

17MT 3 µg/L + Flutamide 18.5 µg/L: 4 wells with 5 organisms/well are exposed to 18.5 µg/L of flutamide in the presence of 3 µg/L of 17MT. This control establishes the fluorescence inhibition for a flutamide concentration of 18.5 µg/L compared to the 17MT 3 µg/L alone control group. This control serves as part of the flutamide standard curve.

After 72 hours of exposure, the eleutheroembryos are imaged with a colour camera and GFP long pass filters. An image of the dorsal region including the kidneys of each organism is captured. Image analysis software is then used to quantify the GFP signal to allow androgen axis activity to be compared between different controls and exposure groups.

2.4. Experimental runs

One test is composed of three independent and valid runs using 4 x 5 organisms/treatment group/run (Figure 3). Each run should be performed using independent solutions. The runs should be ideally conducted sequentially but could be conducted in parallel. The raw data for a given test chemical is obtained by pooling the data from the three runs to obtain n=60 fluorescence values in each treatment group.

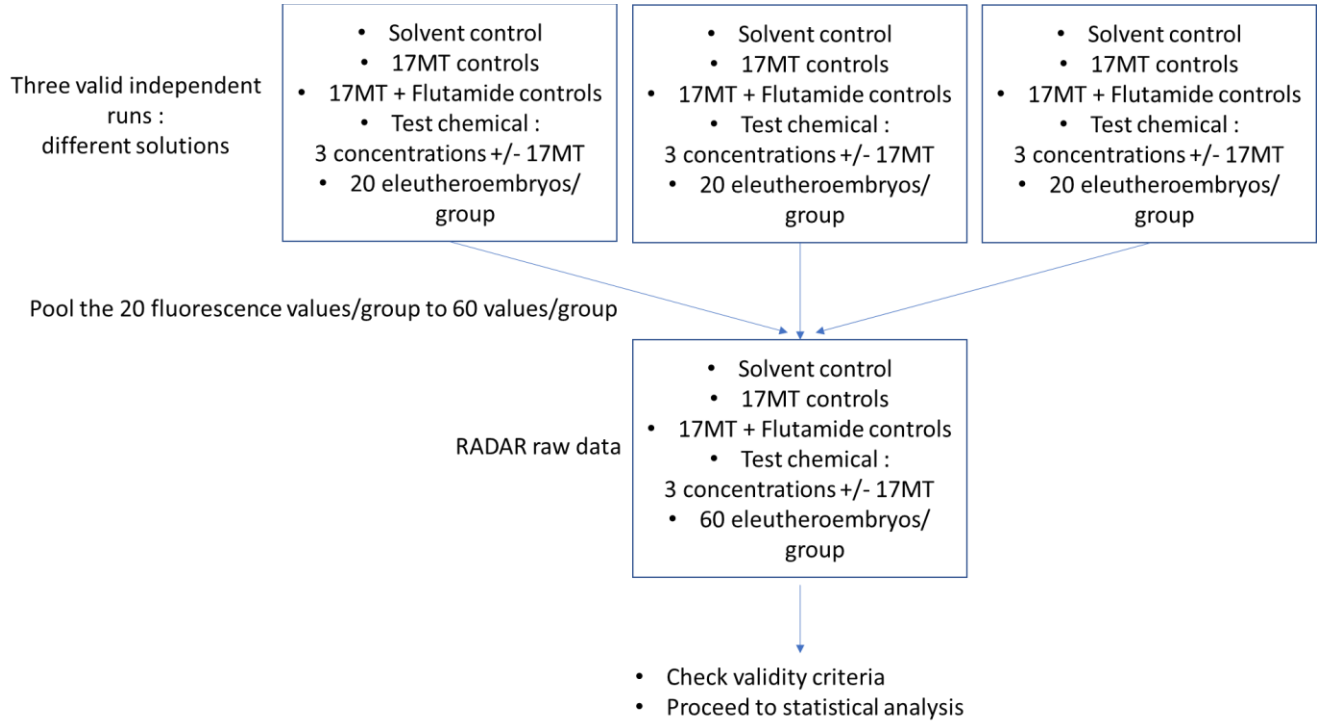

Figure 3: Overview of the RADAR assay.

"+/- 17MT" refers to spiked and unspiked groups. A RADAR assay is composed of three independent runs and utilises 840 eleutheroembryos in total. A full set of controls should be performed in each run.

3. Validation study

3.1. Specific goals

1. Each laboratory performed two calibration experiments with 17MT and flutamide in order to adjust their image capture settings to obtain the optimal sensitivity in fluorescence readings.
2. An interlaboratory validation study was then carried out with the five participating laboratories and intra- and inter-laboratory variability was determined. Concentration ranges of 17MT and flutamide were included in every run. Five additional androgen axis active chemicals were tested in every laboratory except Fraunhofer where only three were tested. The chemicals were chosen to cover a range of modes of action on the androgen axis. All also tested three expected inert chemicals, except Fraunhofer where only two were tested, to obtain sufficient information on the reliability, reproducibility, and sensitivity of the assay. An additional eight androgen axis active chemicals and two expected inerts were tested in the lead laboratory. Both 17MT and flutamide served as positive controls in all experiments and all test chemicals were tested in the presence and absence of 3 µg/L of 17MT.
3. Performance was compared between the participating laboratories. Reliability, reproducibility within and across laboratories, and sensitivity of the assay were determined.

3.2. Overview of Test Conditions

WatchFrog provided two of the participating laboratories with homozygous *spg1-gfp* medaka eggs in advance of the validation study to allow them to begin breeding colonies. These two laboratories were IDEA (Japan) and FIWI (Switzerland). All experiments carried out by IDEA were performed using eleutheroembryos bred in their laboratory. The majority of the experiments carried out by FIWI were also performed with eleutheroembryos bred within their department. A small number of experiments performed by FIWI at the end of the project used eleutheroembryos raised from eggs sent by the lead laboratory (WatchFrog). This was necessary because it was considered too time intensive to breed a new generation of fish in house and the initial breeding stock had begun to reach the end of their useful reproductive lifespan.

For the interlaboratory validation, all participating laboratories were asked to test five androgen axis active chemicals and three expected inert chemicals. The five active chemicals were selected to cover a range of modes of action expected to increase or decrease androgen axis activity. All of these chemicals were tested at five concentrations in the presence and absence of 3 µg/L 17MT. In addition, three concentrations of 17MT and four concentrations of flutamide in the presence of 3 µg/L 17MT were evaluated in each experimental run. These are shown in Table 2 below.

Table 2: Pro-androgenic, anti-androgenic and presumed inert chemicals tested by multiple laboratories within the interlaboratory exercise.

Chemicals in blue were included as controls in all runs of the RADAR assay.

	Test chemicals	Mode of action
Pro-androgenic	17MT	AR agonist
	mDHT	AR agonist
	Anastrozole	aromatase enzyme inhibitor
Anti-androgenic	Flutamide	AR antagonist
	Vinclozolin	M1 and M2 metabolites are AR antagonists
	Linuron	inhibition of steroidogenesis, AR antagonist
	Fenitrothion	AR antagonist
Presumed inert	Cromolyn	mast cell stabilizer
	Cefuroxime	cephalosporin antibiotic
	Amantadine	antiviral, antiparkinsonian

An additional eight androgen axis active chemicals and two chemicals expected to be inert were tested in the lead laboratory (Table 3).

Table 3: Pro-androgenic, anti-androgenic, chemicals with multiple modes of action on the androgen axis and presumed inert chemicals tested uniquely by the lead laboratory.

	Test chemicals	Mode of action
Pro-androgenic	Prochloraz	aromatase transcription inhibitor
	17β-trenbolone	AR agonist
Anti-androgenic	17α-ethinyl estradiol	aromatase transcription inducer
	17β-estradiol	aromatase transcription inducer
	Estrone	aromatase transcription inducer
	Nonylphenol	aromatase transcription inducer
Multiple modes of action	Dutasteride	5α-reductase inhibitor, AR antagonist
	Cyproterone acetate	AR antagonist, anti-progestin
Presumed inert	L-Dopa	antiparkinsonian
	Arabinose	monosaccharide

Preliminary experiments were performed in the lead laboratory using the proposed substances to determine appropriate testing concentrations. In concordance with validated OECD test guidelines such as the XETA assay (TG248; OECD, 2019b) the maximum test concentration was set to the lowest concentration among the solubility limit, the maximum tolerated concentration or 100 mg/L. The maximum tolerated concentration was defined as the highest concentration resulting in less than 10% combined mortality and sublethal effects such as malformation or immobility.

For 17α-methyl-5α-dihydrotestosterone (mDHT), EE2, E2, estrone and nonylphenol lower concentrations were tested as following the above guidance would have led to all test concentrations inducing the maximal fluorescence observable, saturating the system.

The five concentrations tested for each chemical are shown below in Table 4. All participating laboratories assayed each test chemical from the same batch and lot number, in the presence and absence of 3 µg/L 17MT.

The testing began with a calibration experiment. The goal of the calibration steps was to ensure that all laboratories attain a similar amplitude of response and sensitivity to the reference compounds 17MT and flutamide despite differences in imaging equipment used to read the experiment. The calibration required two steps:

4. Finding the best imaging settings to obtain a satisfactory amplitude of GFP induction with a concentration of 50 µg/L 17MT.
5. Applying these settings for the quantitation of three runs of a concentration-response experiment with 17MT and flutamide to check the amplitude of induction using increasing concentrations of 17MT and flutamide as well as the lowest concentration of 17MT and flutamide that elicited a detectable GFP response.

Once a laboratory demonstrated its ability to run the calibration experiments with an expected dynamic concentration response for 17MT and flutamide, it then obtained the agreement of the lead laboratory to begin to test the chemicals of interest.

The conditions for the interlaboratory validation exercise are summarized in Table 4 below:

Table 4: Conditions of the Rapid Androgen Disruption ActivityReporter (RADAR) assay

Test organism		Spg1-gfp medaka (*Oryzias latipes*) embryo
Exposure period		Day post hatch (DPH) 0 for 3 days (72h)
Criteria for selecting test individuals		Primary criteria were developmental stage and health of animal (alive and no-malformations)
Solvent control		Medaka medium 0.2% DMSO
17α-methyltestosterone (17MT) standard curve		17MT (1.5, 3, 10 µg/L)
Flutamide standard curve		Flutamide (18.5, 55.6, 167, 500 µg/L)
Concentration of obligatory test chemicals	mDHT	16, 8, 4, 2, 1 µg/L
	Anastrozole	9, 3, 1, 0.33, 0.11 mg/L
	Vinclozolin	300, 100, 33.3, 11.1, 3.7 µg/L
	Linuron	2.5, 1.25, 0.625, 0.31, 0.16 mg/L
	Fenitrothion	500, 250, 125, 62.5, 31.25 µg/L
	Cromolyn	1000, 100, 10, 1, 0.1 µg/L
	Cefuroxime	10, 1, 0.1, 0.01, 0.001 mg/L
	Amantadine	10, 1, 0.1, 0.01, 0.001 mg/L
Concentration of optional test chemicals	Prochloraz	10, 2, 0.4, 0.08, 0.016 mg/L
	17β-trenbolone	1000, 333.3, 111.1, 37, 12.3 µg/L
	17α-ethinyl estradiol	300, 90, 30, 9, 3 ng/L
	17β-estradiol	800, 200, 80, 20, 8 ng/L
	Estrone	20, 10, 2.5, 1, 0.5 µg/L
	Nonylphenol	200, 100, 50, 25, 12.5 µg/L
	Dutasteride	1000, 500, 250, 125, 62.5 µg/L
	Cyproterone acetate	100, 10, 1, 0.1, 0.01 µg/L
	L-Dopa	1000, 200, 40, 8, 1.6 µg/L
	Arabinose	10, 1, 0.1, 0.01, 0.001 mg/L
Exposure regime		Renewal every 24 hours for 72 hours (2 renewals). No feeding.
Endpoints		Total fluorescence of the kidney (mesonephros) of eleutheroembryos imaged dorsally
Eleutheroembryos per test condition		Five eleutheroembryos per well (6-well plate) x 4 wells (total of 20 eleutheroembryos per concentration)
Volume of test medium		8 mL per well
Test medium		Medaka medium (see “Preparation of Stock Solutions”)
Replication		Four wells per test concentration
Incubation conditions during exposure		26^{O}C +/- 1°C, in the dark
Measurement time		72 hours
Number of runs per experiment		Three runs were performed for each chemical. Each run was performed on separate days or weeks.

A sample assay design included the following chemicals and test concentrations as outlined in Table 5 below. *Note: Not more than two chemicals have been run per assay per week.*

Table 5: Assay Design with one test chemical

Test Group	Exposure Medium	Number of wells (5 eleutheroembryos/well)	Number of eleutheroembryos
Solvent control	Medaka medium + solvent	4	20
Test chemical	Medaka medium + solvent + test chemical (5 concentrations)	4 per concentration (20 per test chemical)	100
17MT (1.5 µg/L)	Medaka medium + solvent + 17MT	4	20
17MT control (3 µg/L)	Medaka medium + solvent + 17MT	4	20
17MT (10 µg/L)	Medaka medium + solvent + 17MT	4	20
17MT (3 µg/L) + Flutamide (18.5 µg/L)	Medaka medium + solvent + Flutamide	4	20
17MT (3 µg/L) + Flutamide (55.6 µg/L)	Medaka medium + solvent + Flutamide	4	20
17MT (3 µg/L) + Flutamide (167 µg/L)	Medaka medium + solvent + Flutamide	4	20
17MT (3 µg/L) + Flutamide (500 µg/L)	Medaka medium + solvent + Flutamide	4	20
17MT (3 µg/L) + test chemical	Medaka medium + solvent + 17MT + test chemical (5 concentrations)	4 per concentration (20 per chemical)	100
	TOTAL	72	360
	TOTAL - three replicate experiments	216	1080

3.2.1. *Test medium*

Medaka Medium was chosen as the test medium as this has been shown to be suitable for the growth of medaka embryos and eleutheroembryos.

The stock solution of 10x Medaka Medium has the following composition:

- NaCl 5 g/L
- $CaCl_2$ 0.151 g/L
- $MgSO_4$ 0.098 g/L
- KCl 0.15 g/L
- NaOH 1N 1.25 mL/L

This solution was diluted ten-fold with reverse osmosis water to obtain the 1x working solution. The pH was then adjusted between 7.2-8.0 with a solution of 1N NaOH.

3.2.2. *Test and control solutions*

Test solutions of the chosen concentrations were prepared by dilution of a stock solution prepared in DMSO. The final concentration of DMSO was 0.2% in all test and control solutions.

Test and control solutions were prepared each day that they were required from aliquots of stock solutions in DMSO stored at -20°C. Any remaining thawed stock solution was discarded and was not refrozen.

Although all solutions were prepared freshly each day that they were required, data from the lead laboratory shows that the control solutions can be prepared on the first day of a run and stored at 4°C and used at each renewal for a single run (Annex 1). They should be allowed to reach room temperature before being placed in contact with the eleutheroembryos.

3.2.3. ***Test validity***

RADAR experiments were judged valid during the validation exercise if the following criteria were met.

For each experimental run:

- A statistically significant induction of fluorescence was measured between the solvent control group and the 17MT 10 µg/L control group. The mean fluorescence of the 17MT 10 µg/L control group was at least 300% the mean of fluorescence of the solvent control group.
- The mortality did not exceed 15% in each control group.
- The percentage of malformed organisms did not exceed 15% in each control group.

For the pool of the three runs:

- An induction of fluorescence was measured between the 17MT 3 µg/L control group and the 17MT 10 µg/L control group. The mean fluorescence of the 17MT 10 µg/L control group was at least 10% higher than the mean of fluorescence of the 17MT 3 µg/L control group.

These validity criteria were applied after trimming or picture quality control if trimming or a picture quality control was performed.

3.2.4. ***Training***

Personnel from the participating laboratories were not trained in person and performed the assay based on a written protocol. Videos of a key step (eleutheroembryo positioning for imaging) were provided to one of the participating laboratories.

3.2.5. ***Equipment***

The following fluorescence imaging systems were used by the participating laboratories.

Table 6: Imaging systems used for the interlaboratory validation study.

Partner laboratory	Microscope	Objective	Fluorescent filters	Fluorescence source	Camera	Software
FIWI	Leica MZ10 F	Plan 1.0x M-Series	Leica ET GFP LP (ex480/40 emLP510)	Leica - Kübler C0DIX	Leica MC170 HD	Leica LAS
Fraunhofer	Leica DMI6000B	5.0x0.15 DRY	Leica K-CUBE I3 (ex470/40 emLP515 dichroic 510)	Leica EL6000 120W	Leica DFC420	Leica LAS AF
CEFAS	Olympus IX-83	4x; UPLFLN4XPH/0.13	Olympus CUBE U-F19002 GFP AT LP (ex475/40 em515LP dichroic 505)	CoolLED PE-300 LED illuminator	Olympus XC50	Olympus Cellsens Dimension
IDEA	Leica MZ10 F	Plan apo 1.0x; 8x zoom	Leica ET-GFP2 (ex480/40 emLP510)	Leica EL6000	Tucsen TrueChrome	Tucsen Tcapture
WatchFrog	Leica MZ10 F	Plan apo 1.0x; 8x zoom	Leica ET-GFP2 (ex480/40 emLP510)	Prior L200/D 200W	Baumer TXD-14C	Micromanager

3.3. Results of the interlaboratory validation study

3.3.1. *Statistical methodology*

The lead laboratory proposed a statistical method, this statistical decision tree was discussed with an independent statistical expert (Zhenglei Gao, Bayer) who has given her agreement that this statistical workflow is valid for the analysis of data from the RADAR assay.

The applied statistical workflow began with an image quality check to remove any images of badly positioned eleutheroembryos or other images that are not expected to provide an accurate measurement of fluorescence in the kidneys of the eleutheroembryos. A 10% trim of each test concentration group could be performed, omitting the highest 10% and lowest 10% of the fluorescence values from each group. A trim less than 10% may also be used based on expert judgement. Data trimming, is, however, optional. It should be noted that none of the data from any of the chemicals tested across multiple laboratories was trimmed.

Data were then analysed following the directives of the OECD for the analysis of ecotoxicology experiments (OECD, 2006). Sample data was examined and variance was found to be homogenous as determined by Levene's test. Each experimental group was then analysed to determine whether there was a normal distribution of values. If the values followed a normal distribution, an analysis of variance (ANOVA) was conducted, followed by a parametric post-hoc test (Dunnett's post-test). If the values of one or more experimental groups were not normally distributed, a variance test (Kruskal-Wallis) was conducted, followed by a non-parametric post-hoc test (Dunn's post-hoc test) to compare the groups with each other. Statistical significance was shown as: * : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$; ns : not significant $p > 0.05$.

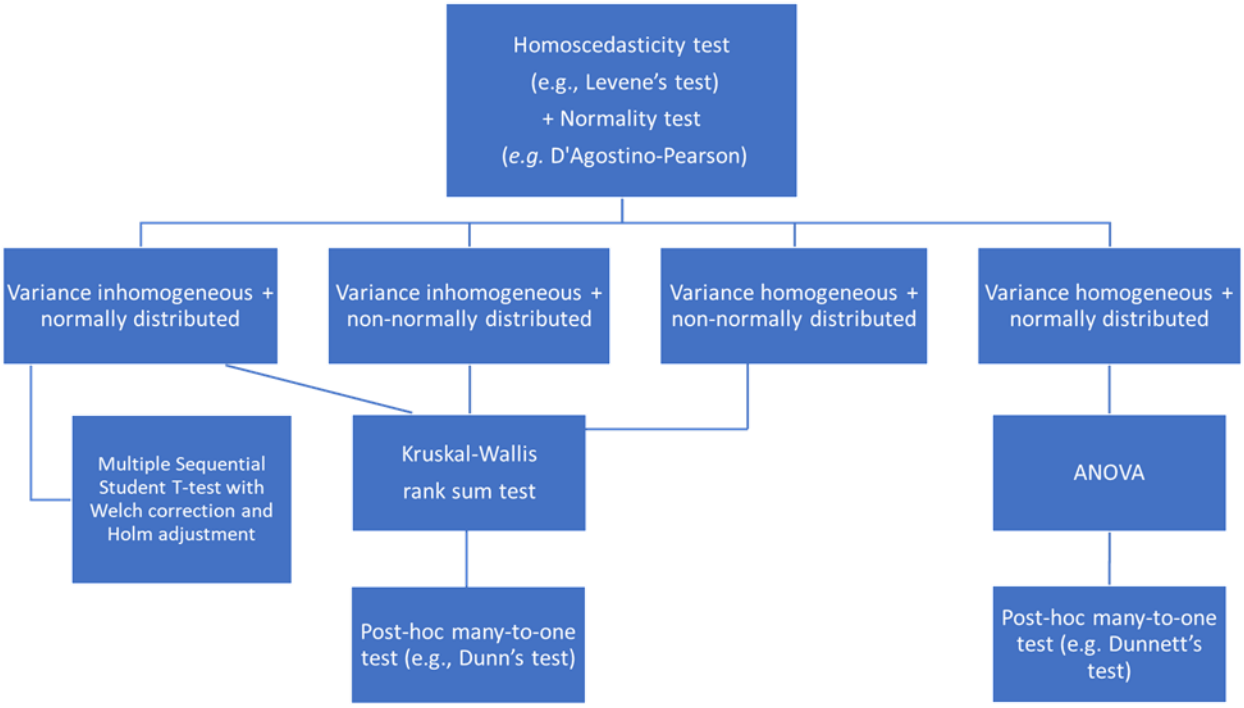

Figure 4: Flow Chart for the Statistical Analysis of Measured Fluorescence

3.3.2. *False positive rate*

The statistical tests used control the false positive results at the 5% level.

3.3.3. *Establishing a decision logic*

The RADAR assay is intended to be used as a screening assay. The result obtained with will, therefore, likely influence decisions regarding further testing with additional assays.

A decision logic was developed for the RADAR assay to provide logical assistance in the conduct and interpretation of the result of the bioassay (Figure 5). This decision logic is based on three valid runs pooled for statistical analysis (see Figure 3). A test chemical is considered to give a positive result in the RADAR assay if at least one concentration tested is active in either unspiked or 17MT spiked mode and a concentration-response relationship is observed.

- In unspiked mode an active concentration is defined as a concentration giving a statistically significant fluorescence increase compared to the test medium control.
- In 17MT-spiked mode an active concentration is defined as a concentration giving a statistically significant fluorescence increase or decrease compared to the 3 µg/L 17MT control.

Fluorescence decreases in unspiked mode are not expected as the eleutheroembryos do not synthesise detectable levels of androgens at this development stage. If a statistically significant fluorescence decrease is observed in unspiked mode, it could indicate that the RADAR assay is not appropriate for the test chemical, or a potential problem with the organisms or the test conditions which may require further investigations. Individual runs should be considered to determine if the statistically significant fluorescence decrease is present in the three runs and best professional judgement should then be used to decide between repeating: none of the runs, only one run using a new batch of organisms; a complete RADAR, possibly using a lower concentration range; or performing a different androgen axis activity test.

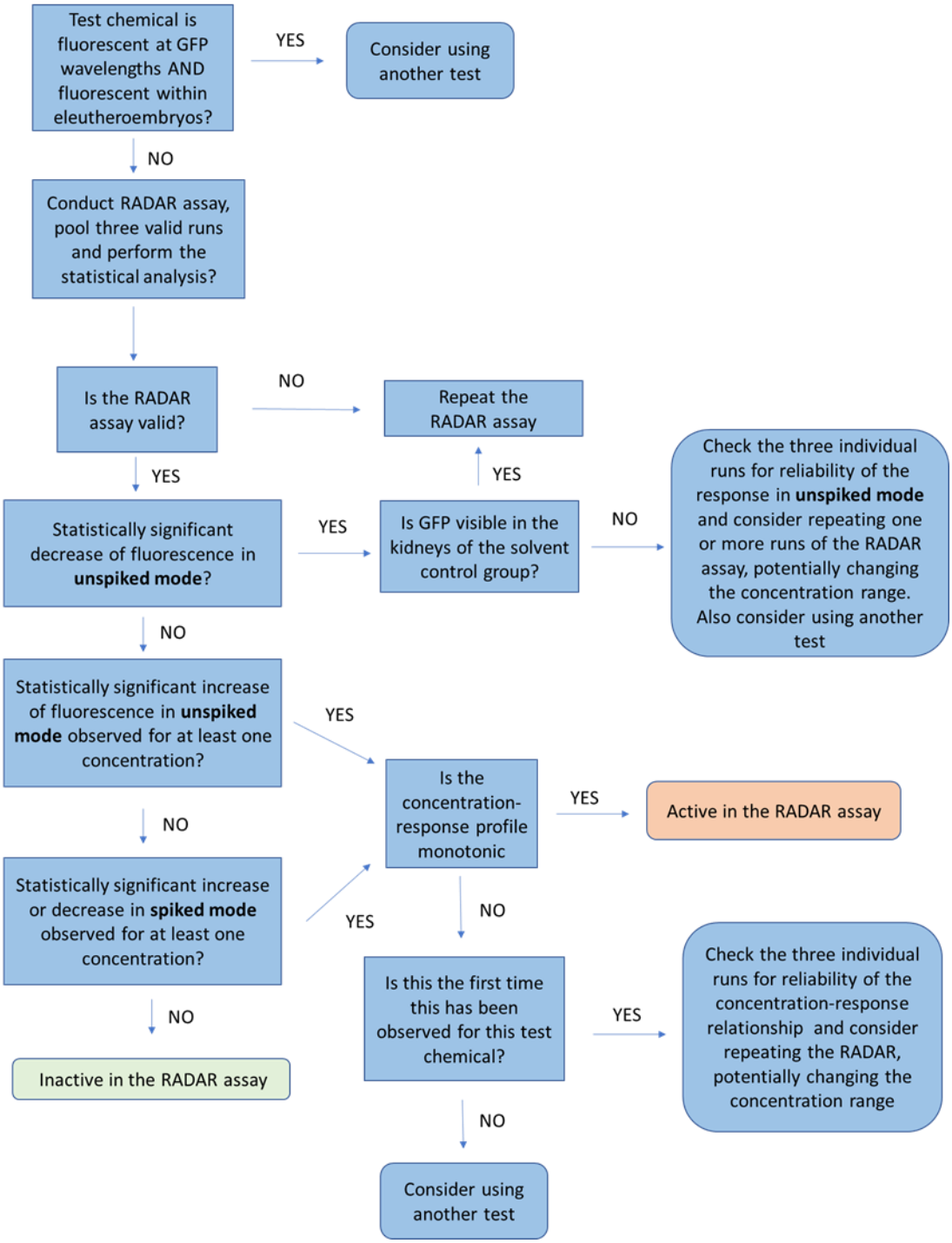

Figure 5: Decision logic for the conduct of the RADAR assay

3.3.4. *Establishing NOEC and LOEC*

The result of the RADAR assay is intended to be a classification of the test chemicals into potentially "androgen axis active" or "androgen axis inactive". The results of the RADAR assay are expressed here

in terms of LOEC and NOEC to allow the comparison of the results between the participating laboratories as a decisional aid for the possible validation of the assay.

The LOEC is defined as the lowest concentration found to be active either in unspiked or spiked mode. The NOEC is defined as the concentration tested immediately below the LOEC.

3.4. Results of Analyses

The results presented here are the results obtained with the statistical approach and decision logic described above. Due to the fact that staff involved in this interlaboratory validation study were not experienced with the RADAR assay or handling medaka eleutheroembryos, a higher tolerance for mortality and exclusion of inadequate images was employed. This criterion has now been refined in the

=test guideline to no less than 90% of the expected number of values per group, for controls and interpretable test chemical groups, following removal of data due to dead, malformed or immobile eleutheroembryos as well as images of poorly positioned eleutheroembryos.

Had the 10% limit for excluded data been applied to this data set, a small number of individual runs would have been excluded due to violation of the 10% limit by one or more control groups. These individual runs were: one run of the calibration experiment and one run of the mDHT experiment performed by FIWI; one run of the calibration experiment and one run of the linuron experiment performed by Fraunhofer; one run performed by WatchFrog in which both cefuroxime and vinclozolin were tested in the same run and one run performed by WatchFrog in which both fenitrothion and linuron were tested in the same run. All data from CEFAS and Idea would have passed the revised validity criteria. Tests were performed in a in a blind fashion at CEFAS, with none of the team performing the experiment and generating the data aware of the identity of the chemicals they were testing. During the validation study, one of the laboratories (CEFAS) preincubated their plates with test chemicals, the other laboratories did not.

3.4.1. ***Calibration***

Selecting image capture settings

Each laboratory performed an initial experiment to determine the optimal imaging settings to allow a satisfactory amplitude of GFP induction to be obtained. This involved exposing 50 eleutheroembryos to 50 µg/L of 17MT and adjusting parameters relating to image capture (white balance, gain, exposure time). These parameters were then fixed for all future experiments. This step had already been performed a number of years previously at the lead laboratory and, therefore, was not repeated.

Determining linearity and sensitivity to 17MT and Flutamide

Applying the image capture parameters that were determined in the previous calibration experiment, three runs of the RADAR assay were carried out by each participating laboratory. No test chemical was included in these three runs, which were limited to control groups only.

The first aim of this experiment was to generate data to allow an image analysis workflow to be selected which allowed background (non-GFP) fluorescence signal to be minimised prior to quantification of the images produced by each laboratory. The second aim was to verify that the fluorescence values obtained for a set of control solutions passed validity criteria when using the image capture parameters

that had previously been determined. The lead laboratory did not perform this step as they had previously optimised and tested the image capture parameters for their imaging system.

Figure 6 shows the results of the second calibration experiment. All four naïve laboratories obtained results showing increasing fluorescence with increasing concentrations of 17MT and fluorescence values for flutamide spiked with 3 µg/L of 17MT which were inversely proportional to the concentration of flutamide.

All four laboratories detected 3 and 10 µg/L of 17MT as active as shown in Table 7. Likewise, all four laboratories detected 167 and 500 µg/L of flutamide as active. CEFAS and Idea obtained a slightly higher sensitivity for increased androgen axis activity also detecting 1.5 µg/L of 17MT as active. FIWI demonstrated a slightly increased sensitivity for androgen axis inhibition evaluating 55.6 µg/L of flutamide as active.

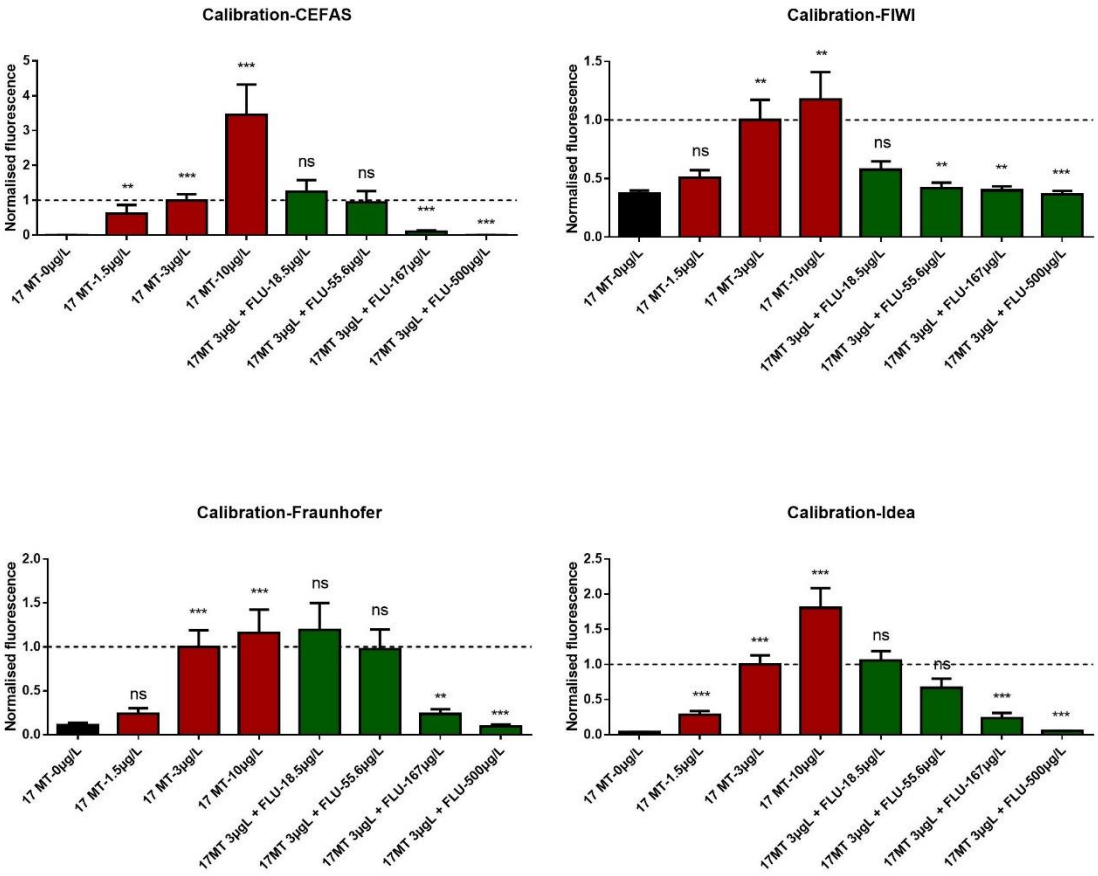

Figure 6: Mean and SEM of fluorescence for 17MT and flutamide controls employed within the RADAR assay obtained during calibration.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

Table 7: Summarised statistical results for the second step of the calibration experiment.

Results corresponding to a statistically significant variation of fluorescence are highlighted in green.

Laboratory	17MT (µg/L)			Flutamide (µg/L)			
	1.5	3	10	18.5	55.6	167	500
CEFAS	**	***	***	ns	ns	***	***
FIWI	ns	**	**	ns	**	**	***
Idea	***	***	***	ns	ns	***	***
Fraunhofer	ns	***	***	ns	ns	**	***

3.5. Results for androgen axis active chemicals

3.5.1. *Anastrozole Results*

Anastrozole is a pharmaceutical aromatase enzyme inhibitor. As such, it was expected that by blocking conversion of androgens to estrogens, it would increase androgen axis activity and therefore fluorescence in the presence of 17MT. In the absence of appreciable levels of 17MT (unspiked mode) it was expected that anastrozole would have no effect on fluorescence levels.

Figure 7 shows the results obtained when testing anastrozole. A summary of the statistical analysis is provided in Table 8. It can be noted that all four laboratories that tested anastrozole obtained an identical LOEC for the 17MT (1.5 µg/L) and flutamide (167 µg/L) controls. No statistically significant variation in fluorescence was observed in unspiked mode in any of the laboratories. An increase in fluorescence that was proportional to the concentration of anastrozole was observed in the presence of 17MT in all four laboratories. All four laboratories detected a statistically significant increase in fluorescence with 9 mg/L of anastrozole. However, the LOEC varied from 0.33 mg/L to 9 mg/L

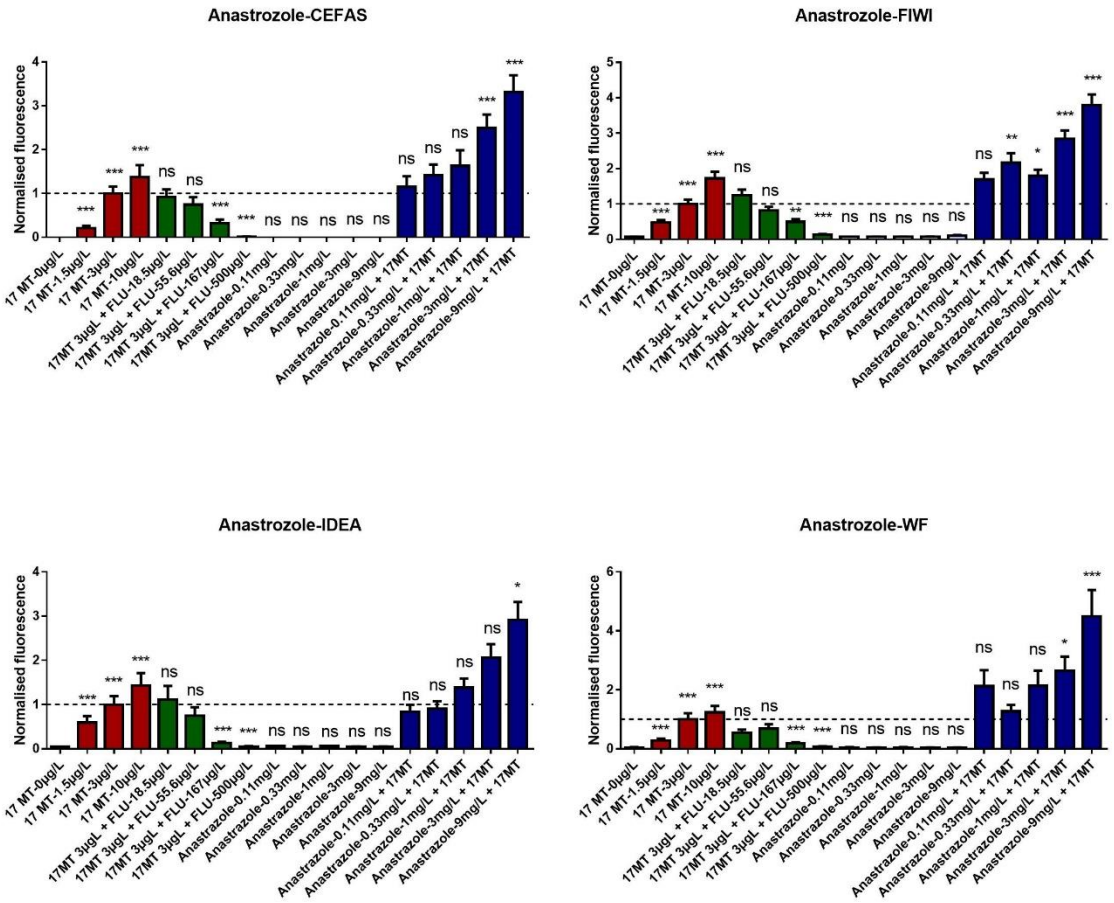

Figure 7: Mean and SEM of measured fluorescence for anastrozole.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

Table 8: Summarised statistical results for the anastrozole experiments.

Results corresponding to a statistically significant variation of fluorescence are highlighted in green.

Laboratory	17MT (µg/L)			Flutamide (µg/L)			
	1.5	3	10	18.5	55.6	167	500
CEFAS	***	***	***	ns	ns	***	***
FIWI	***	**	**	ns	ns	**	***
Idea	***	***	***	ns	ns	***	***
WatchFrog	***	***	***	ns	ns	***	***

Laboratory	Anastrozole (mg/L)					Anastrozole + 17MT (mg/L)				
	0.11	0.33	1	3	9	0.11	0.33	1	3	9
CEFAS	ns	ns	ns	ns	ns	ns	ns	ns	***	***
FIWI	ns	ns	ns	ns	ns	ns	**	*	***	***
Idea	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
WatchFrog	ns	ns	ns	ns	ns	ns	ns	**	*	***

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3.5.2. ***Fenitrothion Results***

Fenitrothion is an organophosphate insecticide that has previously been demonstrated to act as an AR antagonist (Horie et al., 2017; Tamura et al., 2001). It was used as a model antiandrogen during validation of the AFSS (OECD, 2010).

Figure 8 below shows the mean and SEM for each concentration of fenitrothion in each laboratory. A consistent decrease in fluorescence was obtained in each laboratory with increasing concentrations of fenitrothion in the presence of 17MT when compared to the 17MT 3 µg/L control. In the absence of 17MT, no appreciable increase in fluorescence can be observed. However, slight increases in fluorescence that were statistically significant were obtained in unspiked mode in the experiment performed by IDEA. These increases do not show a concentration-response relationship occurring at the lowest (31.25 µg/L) and middle (125 µg/L) concentrations (Table 9). It should be noted that a statistically significant decrease in fluorescence was observed in unspiked mode at 62.5 µg/L in the experiment performed by WatchFrog. This result is noted as “nc” in Table 9 for “not considered” as no GFP is visible in untreated control eleutheroembryos in unspiked mode, therefore, a decrease in GFP signal is impossible and any statistically significant decreases in fluorescence in unspiked mode are not considered as indicating androgen axis activity.

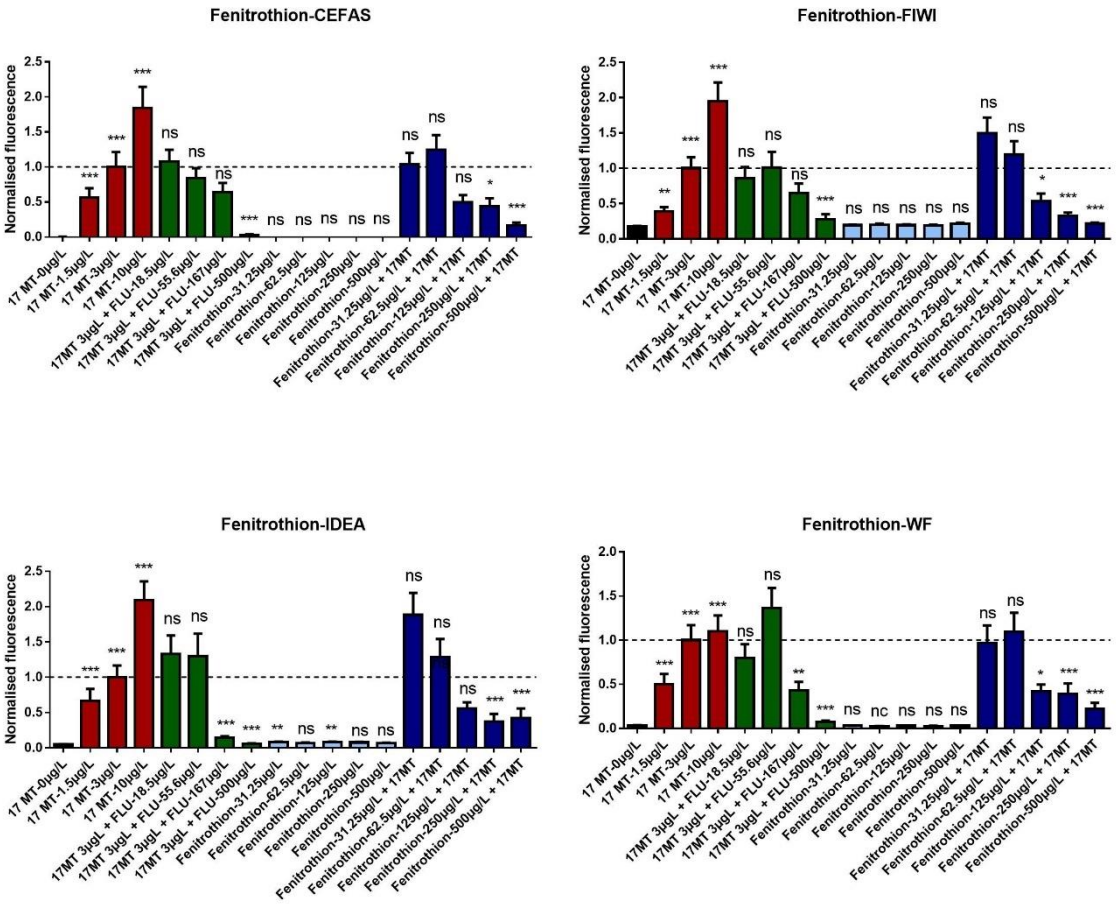


Figure 8: Mean and SEM of measured fluorescence for fenitrothion.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

Table 9: Summarised statistical results for the fenitrothion experiments.

Results corresponding to a statistically significant variation of fluorescence are highlighted in green.

Laboratory	17MT (µg/L)			Flutamide (µg/L)			
	1.5	3	10	18.5	55.6	167	500
CEFAS	***	***	***	ns	ns	ns	***
FIWI	**	**	**	ns	ns	ns	***
Idea	***	***	***	ns	ns	***	***
WatchFrog	***	***	***	ns	ns	**	***

Laboratory	Fenitrothion (µg/L)					Fenitrothion + 17MT (µg/L)				
	31.25	62.5	125	250	500	31.25	62.5	125	250	500
CEFAS	ns	ns	ns	ns	ns	ns	ns	ns	*	***
FIWI	ns	ns	ns	ns	ns	ns	ns	*	***	***
Idea	**	ns	**	ns	ns	ns	ns	ns	***	***
WatchFrog	ns	nc	ns	ns	ns	ns	ns	*	***	***

3.5.3. *Linuron Results*

Linuron is a is a phenylurea herbicide and a demonstrated AR antagonist (Lambright et al., 2000; Spirhanzlova et al., 2017). It was used as a model antiandrogen during validation of the AFSS (OECD, 2010).

Figure 9 below shows the mean and SEM for each concentration of linuron in each laboratory. As expected, no effect was recorded with linuron in the absence of 17MT in any of the laboratories except a slight but statistically significant increase in fluorescence recorded at an intermediate concentration by WatchFrog (Table 10). In the presence of 17MT, a concentration-dependent decrease in fluorescence was recorded by all participating laboratories. The LOEC was 0.625 mg/L for three laboratories, 0.313 mg/L for the lead laboratory (WatchFrog) and 1.25 mg/L for FIWI. The higher LOEC recorded by FIWI is likely to be due to the fact that one of the runs performed did not pass validity criteria, the data from the remaining two runs are shown here which showed similar results.

It should be noted that a statistically significant decrease in fluorescence was observed in unspiked mode for two intermediate concentrations in the experiment performed by FIWI. These results are noted as “nc” in Table 9 for “not considered” as no GFP is visible in untreated control eleutheroembryos in unspiked mode, therefore a decrease in GFP signal is impossible and any statistically significant decreases in fluorescence in unspiked mode are not considered as indicating androgen axis activity.

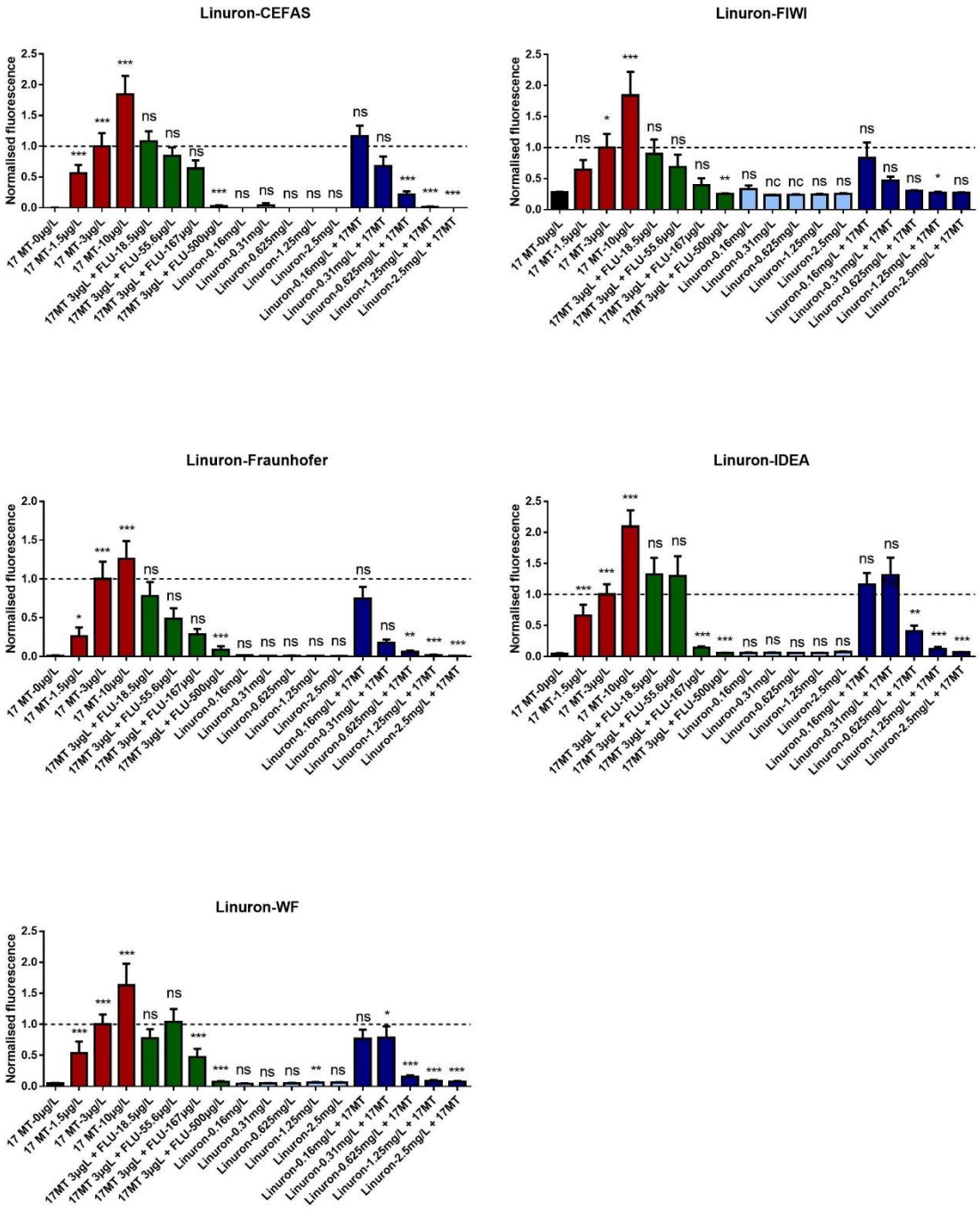


Figure 9: Mean and SEM of measured fluorescence for linuron.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

Table 10: Summarised statistical results for the linuron experiments.

Results corresponding to a statistically significant variation of fluorescence are highlighted in green.

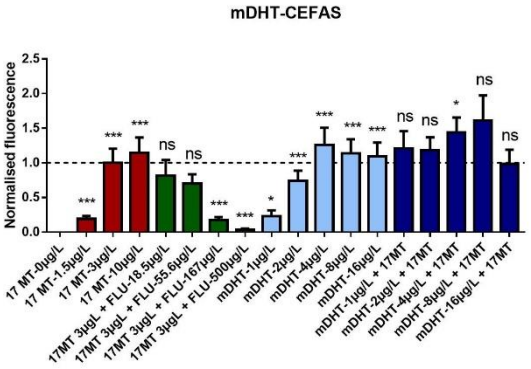
Laboratory	17MT (µg/L)			Flutamide (µg/L)			
	1.5	3	10	18.5	55.6	167	500
CEFAS	***	***	***	ns	ns	ns	***
FIWI	ns	*	***	ns	ns	ns	**
Fraunhofer	*	***	***	ns	ns	ns	***
Idea	***	***	***	ns	ns	***	***
WatchFrog	***	***	***	ns	ns	***	***

Laboratory	Linuron (mg/L)					Linuron + 17MT (mg/L)				
	0.16	0.31	0.625	1.25	2.5	0.16	0.31	0.625	1.25	2.5
CEFAS	ns	ns	ns	ns	ns	ns	ns	***	***	***
FIWI	ns	nc	nc	ns	ns	ns	ns	ns	*	ns
Fraunhofer	ns	ns	ns	ns	ns	ns	ns	**	***	***
Idea	ns	ns	ns	ns	ns	ns	ns	**	***	***
WatchFrog	ns	ns	ns	**	ns	ns	*	***	***	***

3.5.4. *mDHT Results*

The results obtained when testing the synthetic AR agonist mDHT are shown below in Figure 10. A summary of the statistical analysis is given in Table 11 and shows that all five laboratories obtained an identical LOEC for the 17MT and flutamide controls of 1.5 µg/L and 167 µg/L, respectively.

Apart from one intermediate concentration in the experiment performed by FIWI, no statistically significant results were obtained in 17MT spiked mode. In unspiked mode, a concentration-dependent increase in fluorescence was recorded, as expected, by all five laboratories, with three laboratories obtaining a LOEC of 1 µg/L and two laboratories obtaining a LOEC of 2 µg/L. Interestingly, four of the five laboratories observed a decrease in fluorescence at the highest concentration tested (16 µg/L).


mDHT-CEFAS
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
mDHT-1µg/L
mDHT-2µg/L
mDHT-4µg/L
mDHT-8µg/L
mDHT-16µg/L
mDHT-1µg/L + 17MT
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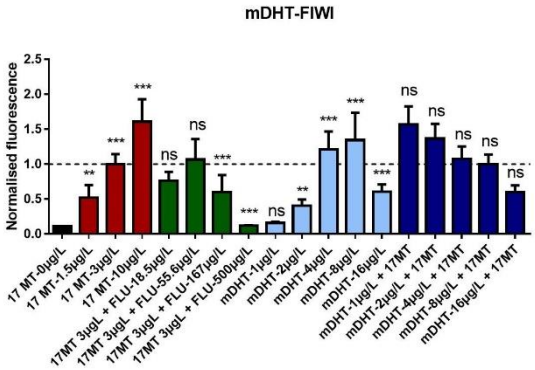
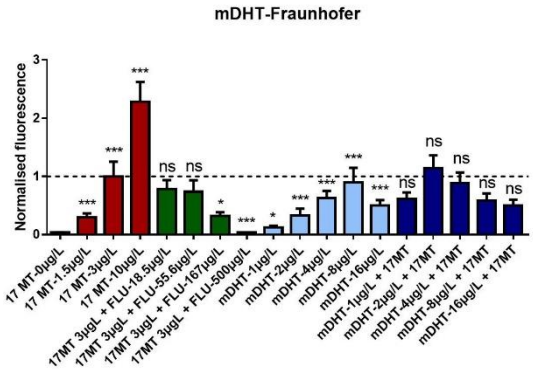
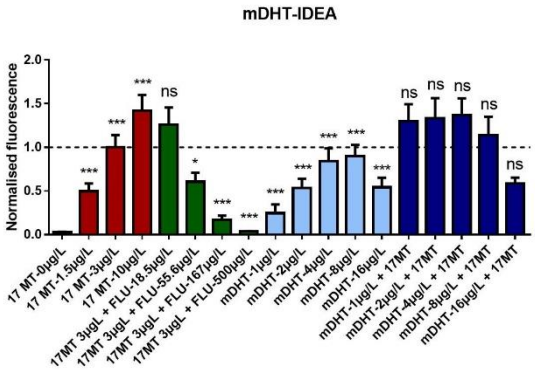
mDHT-4µg/L + 17MT
mDHT-8µg/L + 17MT
mDHT-16µg/L + 17MT


mDHT-FIWI
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
mDHT-1µg/L
mDHT-2µg/L
mDHT-4µg/L
mDHT-8µg/L
mDHT-16µg/L
mDHT-1µg/L + 17MT
mDHT-2µg/L + 17MT
mDHT-4µg/L + 17MT
mDHT-8µg/L + 17MT
mDHT-16µg/L + 17MT


mDHT-Fraunhofer
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
mDHT-1µg/L
mDHT-2µg/L
mDHT-4µg/L
mDHT-8µg/L
mDHT-16µg/L
mDHT-1µg/L + 17MT
mDHT-2µg/L + 17MT
mDHT-4µg/L + 17MT
mDHT-8µg/L + 17MT
mDHT-16µg/L + 17MT


mDHT-IDEA
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
mDHT-1µg/L
mDHT-2µg/L
mDHT-4µg/L
mDHT-8µg/L
mDHT-16µg/L
mDHT-1µg/L + 17MT
mDHT-2µg/L + 17MT
mDHT-4µg/L + 17MT
mDHT-8µg/L + 17MT
mDHT-16µg/L + 17MT

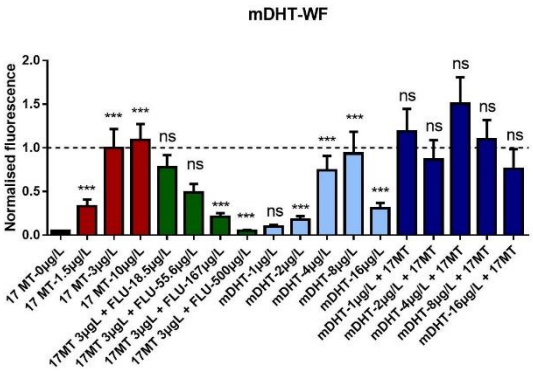

mDHT-WF
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
mDHT-1µg/L
mDHT-2µg/L
mDHT-4µg/L
mDHT-8µg/L
mDHT-16µg/L
mDHT-1µg/L + 17MT
mDHT-2µg/L + 17MT
mDHT-4µg/L + 17MT
mDHT-8µg/L + 17MT
mDHT-16µg/L + 17MT

Figure 10: Mean and SEM of measured fluorescence for mDHT.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

Table 11: Summarised statistical results for the mDHT experiments.

Results corresponding to a statistically significant variation of fluorescence are highlighted in green.

Laboratory	17MT (µg/L)			Flutamide (µg/L)			
	1.5	3	10	18.5	55.6	167	500
CEFAS	***	***	***	ns	ns	***	***
FIWI	**	***	***	ns	ns	***	***
Fraunhofer	***	***	***	ns	ns	*	***
Idea	***	***	***	ns	ns	***	***
WatchFrog	***	***	***	ns	ns	***	***

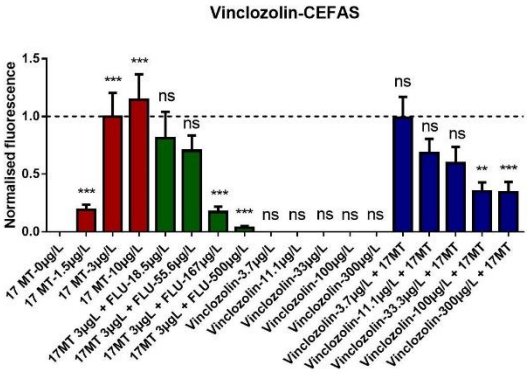
Laboratory	mDHT (µg/L)					mDHT + 17MT (µg/L)				
	1	2	4	8	16	1	2	4	8	16
CEFAS	*	***	***	***	***	ns	ns	*	ns	ns
FIWI	ns	**	***	***	***	ns	ns	ns	ns	ns
Fraunhofer	*	***	***	***	***	ns	ns	ns	ns	ns
Idea	***	***	***	***	***	ns	ns	ns	ns	ns
WatchFrog	ns	***	***	***	***	ns	ns	ns	ns	ns

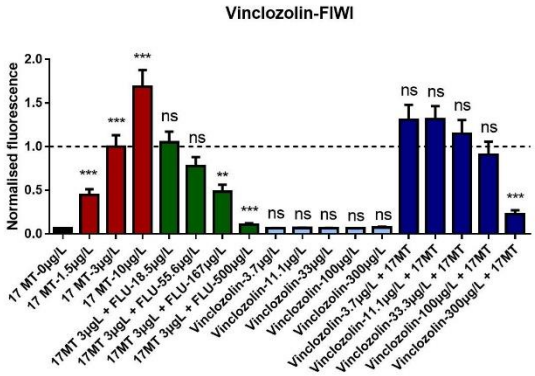
3.5.5. ***Vinclozolin Results***

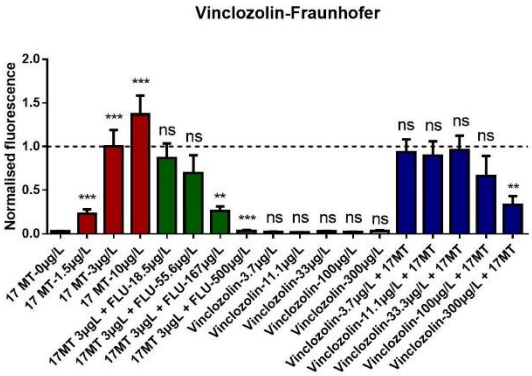
The dicarboximide fungicide vinclozolin was tested as a model anti-androgen during the validation of the AFSS assay. It inhibits androgen axis signalling, with its M1 and M2 metabolites acting as more powerful AR antagonists than the parent molecule (Kavlock and Cummings, 2005).

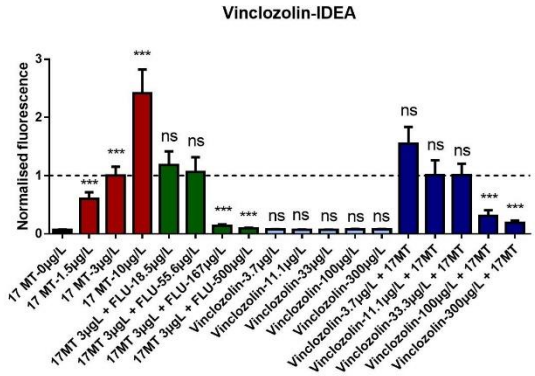
The results obtained during the interlaboratory validation exercise are shown in Figure 11. Table 12 shows that all five laboratories obtained a LOEC of 1.5 µg/L for the 17MT control groups. A LOEC of 167 µg/L was obtained for the flutamide controls in all laboratories except WatchFrog which recorded a LOEC of 500 µg/L.

No statistically significant deviation in fluorescence was recorded by any of the five laboratories when eleutheroembryos were exposed to vinclozolin in the absence of 17MT. In the presence of 3 µg/L 17MT, all five laboratories recorded a concentration-dependent decrease in fluorescence compared to the 17MT 3 µg/L control. A LOEC of 100 µg/L was obtained by two laboratories with the other three recording a LOEC of 300 µg/L.


Vinclozolin-CEFAS
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Vinclozolin-3.7µg/L
Vinclozolin-11.1µg/L
Vinclozolin-33µg/L
Vinclozolin-100µg/L
Vinclozolin-300µg/L
Vinclozolin-3.7µg/L + 17MT
Vinclozolin-11.1µg/L + 17MT
Vinclozolin-33.3µg/L + 17MT
Vinclozolin-100µg/L + 17MT
Vinclozolin-300µg/L + 17MT


Vinclozolin-FIWI
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Vinclozolin-3.7µg/L
Vinclozolin-11.1µg/L
Vinclozolin-33µg/L
Vinclozolin-100µg/L
Vinclozolin-300µg/L
Vinclozolin-3.7µg/L + 17MT
Vinclozolin-11.1µg/L + 17MT
Vinclozolin-33.3µg/L + 17MT
Vinclozolin-100µg/L + 17MT
Vinclozolin-300µg/L + 17MT


Vinclozolin-Fraunhofer
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Vinclozolin-3.7µg/L
Vinclozolin-11.1µg/L
Vinclozolin-33µg/L
Vinclozolin-100µg/L
Vinclozolin-300µg/L
Vinclozolin-3.7µg/L + 17MT
Vinclozolin-11.1µg/L + 17MT
Vinclozolin-33.3µg/L + 17MT
Vinclozolin-100µg/L + 17MT
Vinclozolin-300µg/L + 17MT


Vinclozolin-IDEA
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Vinclozolin-3.7µg/L
Vinclozolin-11.1µg/L
Vinclozolin-33µg/L
Vinclozolin-100µg/L
Vinclozolin-300µg/L
Vinclozolin-3.7µg/L + 17MT
Vinclozolin-11.1µg/L + 17MT
Vinclozolin-33.3µg/L + 17MT
Vinclozolin-100µg/L + 17MT
Vinclozolin-300µg/L + 17MT

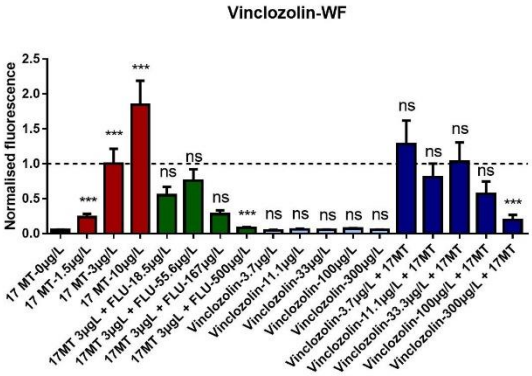

Vinclozolin-WF
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Vinclozolin-3.7µg/L
Vinclozolin-11.1µg/L
Vinclozolin-33µg/L
Vinclozolin-100µg/L
Vinclozolin-300µg/L
Vinclozolin-3.7µg/L + 17MT
Vinclozolin-11.1µg/L + 17MT
Vinclozolin-33.3µg/L + 17MT
Vinclozolin-100µg/L + 17MT
Vinclozolin-300µg/L + 17MT

Figure 11: Mean and SEM of measured fluorescence for vinclozolin.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

Table 12: Summarised statistical results for the vinclozolin experiments.

Results corresponding to a statistically significant variation of fluorescence are highlighted in green.

Laboratory	17MT (µg/L)			Flutamide (µg/L)			
	1.5	3	10	18.5	55.6	167	500
CEFAS	***	***	***	ns	ns	***	***
FIWI	***	***	***	ns	ns	**	***
Fraunhofer	***	***	***	ns	ns	**	***
Idea	***	***	***	ns	ns	***	***
WatchFrog	***	***	***	ns	ns	ns	***

Laboratory	Vinclozolin (µg/L)					Vinclozolin + 17MT (µg/L)				
	3.7	11.1	33.3	100	300	3.7	11.1	33.3	100	300
CEFAS	ns	ns	ns	ns	ns	ns	ns	ns	**	***
FIWI	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Fraunhofer	ns	ns	ns	ns	ns	ns	ns	ns	ns	**
Idea	ns	ns	ns	ns	ns	ns	ns	ns	***	***
WatchFrog	ns	ns	ns	ns	ns	ns	ns	ns	ns	***

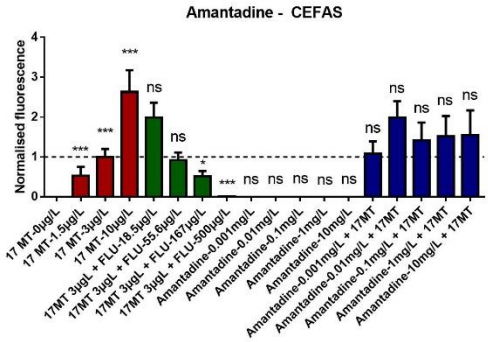
3.6. Results for expected inert chemicals

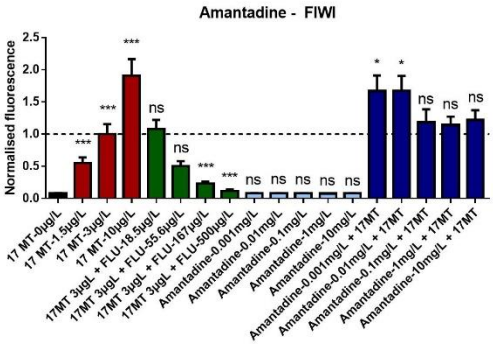
3.6.1. *Amantadine Results*

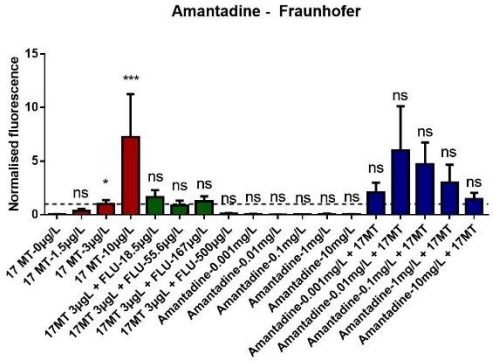
Amantadine was previously used as an antiviral medication to treat influenza caused by type A influenza virus. It is still commonly used to treat Parkinson's disease. It was expected to be inert with respect to androgen axis signalling.

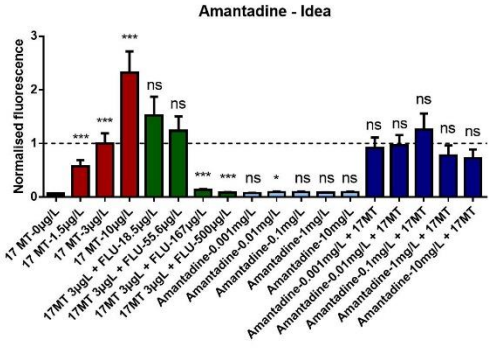
The results obtained during the interlaboratory validation exercise are shown in Figure 12. Table 13 shows that all five laboratories obtained a LOEC of 1.5 µg/L for the 17MT control group except for Fraunhofer which measured a LOEC of 3 µg/L. A LOEC of 167 µg/L was obtained for the flutamide controls in all laboratories except Fraunhofer where none of the tested concentrations of flutamide were statistically significantly different to the 17MT 3 µg/L control.

A statistically significant deviation in fluorescence was recorded in only one of the five laboratories when eleutheroembryos were exposed to amantadine in the absence of 17MT. The second lowest concentration of amantadine was statistically significantly different to the unspiked control (P=0.047). In the presence of 3 µg/L 17MT, none of the five laboratories recorded a statistically significant change in fluorescence compared to the 17MT 3 µg/L control except FIWI. The effect observed by FIWI is not considered as indicating androgen axis activity as it is non-monotonic with the two lowest tested concentrations (0.001 and 0.01 mg/L) statistically significantly different to the 17MT control. In cases where a non-monotonic concentration response is observed, the results of the three individual runs are studied to determine whether the response is reproducible. In this case, no response was observed in the first run, a response with 0.001 mg/L in the second run and a response with 0.01 mg/L in the third run.


Amantadine - CEFAS
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17 MT-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Amantadine-0.001mg/L
Amantadine-0.01mg/L
Amantadine-0.1mg/L
Amantadine-1mg/L
Amantadine-10mg/L
Amantadine-0.001mg/L + 17MT
Amantadine-0.01mg/L + 17MT
Amantadine-0.1mg/L + 17MT
Amantadine-1mg/L + 17MT
Amantadine-10mg/L + 17MT


Amantadine - FIWI
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17 MT-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Amantadine-0.001mg/L
Amantadine-0.01mg/L
Amantadine-0.1mg/L
Amantadine-1mg/L
Amantadine-10mg/L
Amantadine-0.001mg/L + 17MT
Amantadine-0.01mg/L + 17MT
Amantadine-0.1mg/L + 17MT
Amantadine-1mg/L + 17MT
Amantadine-10mg/L + 17MT


Amantadine - Fraunhofer
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17 MT-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Amantadine-0.001mg/L
Amantadine-0.01mg/L
Amantadine-0.1mg/L
Amantadine-1mg/L
Amantadine-10mg/L
Amantadine-0.001mg/L + 17MT
Amantadine-0.01mg/L + 17MT
Amantadine-0.1mg/L + 17MT
Amantadine-1mg/L + 17MT
Amantadine-10mg/L + 17MT


Amantadine - Idea
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17 MT-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Amantadine-0.001mg/L
Amantadine-0.01mg/L
Amantadine-0.1mg/L
Amantadine-1mg/L
Amantadine-10mg/L
Amantadine-0.001mg/L + 17MT
Amantadine-0.01mg/L + 17MT
Amantadine-0.1mg/L + 17MT
Amantadine-1mg/L + 17MT
Amantadine-10mg/L + 17MT

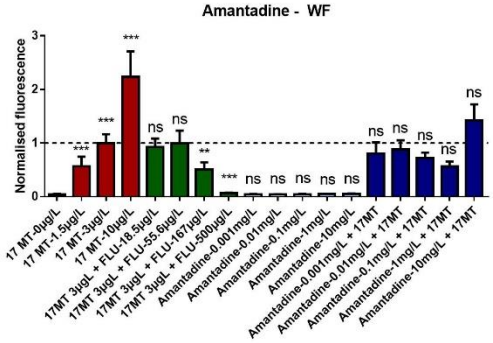

Amantadine - WF
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17 MT-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Amantadine-0.001mg/L
Amantadine-0.01mg/L
Amantadine-0.1mg/L
Amantadine-1mg/L
Amantadine-10mg/L
Amantadine-0.001mg/L + 17MT
Amantadine-0.01mg/L + 17MT
Amantadine-0.1mg/L + 17MT
Amantadine-1mg/L + 17MT
Amantadine-10mg/L + 17MT

Figure 12: Mean and SEM of measured fluorescence for amantadine.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

Table 13: Summarised statistical results for the amantadine experiments.

Results corresponding to a statistically significant variation of fluorescence are highlighted in green.

Laboratory	17MT (µg/L)			Flutamide (µg/L)			
	1.5	3	10	18.5	55.6	167	500
CEFAS	***	***	***	ns	ns	*	***
FIWI	***	***	***	ns	ns	***	***
Fraunhofer	ns	*	***	ns	ns	ns	ns
Idea	***	***	***	ns	ns	***	***
WatchFrog	***	***	***	ns	ns	**	***

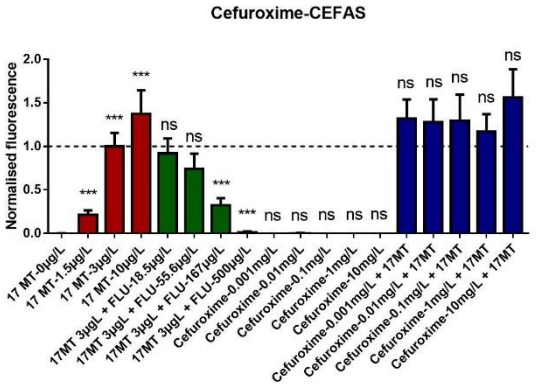
Laboratory	Amantadine (mg/L)					Amantadine + 17MT (mg/L)				
	0.001	0.01	0.1	1	10	0.001	0.01	0.1	1	10
CEFAS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
FIWI	ns	ns	ns	ns	ns	*	*	ns	ns	ns
Fraunhofer	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Idea	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
WatchFrog	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

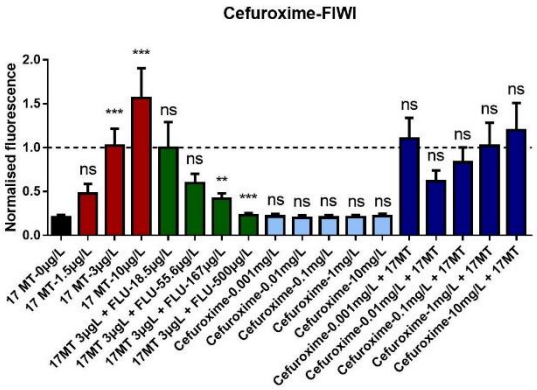
3.6.2. *Cefuroxime Results*

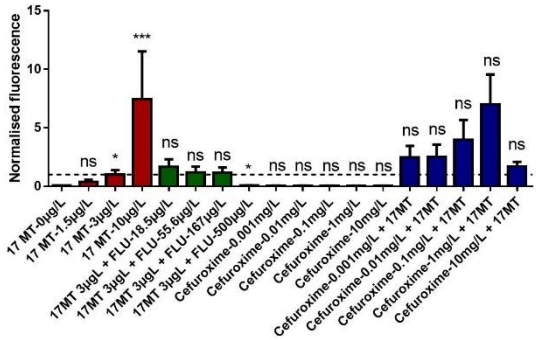
Cefuroxime is a cephalosporin antibiotic and was expected to be inert with regards to androgen axis activity.

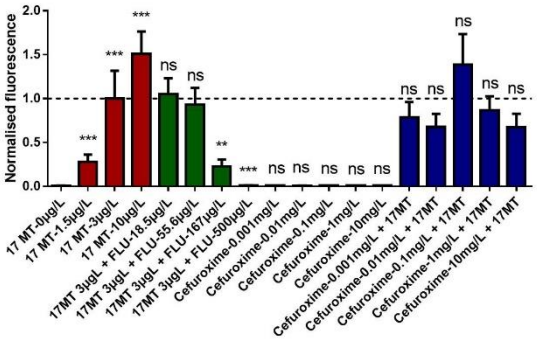
Figure 13 shows that a concentration-dependent response was observed for the two control chemicals 17MT and flutamide in all laboratories. The LOEC for each control chemical was between 1.5 and 3 µg/L for 17MT and between 167 and 500 µg/L for flutamide (Table 14).

No statistically significant deviation in fluorescence was recorded by any of the five laboratories when eleutheroembryos were exposed to cefuroxime in either the presence or absence of 17MT. In the presence of 3 µg/L 17MT, a large increase in fluorescence was observed by Fraunhofer with one of the intermediate concentrations (1 mg/L). This increase was not statistically significant and was due to a small number of eleutheroembryos with abnormally high levels of GFP as demonstrated by the large error bar and the lack of statistical significance. One of the runs performed at FIWI and one of the runs performed at Idea did not pass validity criteria, the data from the remaining two runs are shown here.


Cefuroxime-CEFAS
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Cefuroxime-0.001mg/L
Cefuroxime-0.01mg/L
Cefuroxime-0.1mg/L
Cefuroxime-1mg/L
Cefuroxime-10mg/L
Cefuroxime-0.001mg/L + 17MT
Cefuroxime-0.01mg/L + 17MT
Cefuroxime-0.1mg/L + 17MT
Cefuroxime-1mg/L + 17MT
Cefuroxime-10mg/L + 17MT


Cefuroxime-FIWI
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Cefuroxime-0.001mg/L
Cefuroxime-0.01mg/L
Cefuroxime-0.1mg/L
Cefuroxime-1mg/L
Cefuroxime-10mg/L
Cefuroxime-0.001mg/L + 17MT
Cefuroxime-0.01mg/L + 17MT
Cefuroxime-0.1mg/L + 17MT
Cefuroxime-1mg/L + 17MT
Cefuroxime-10mg/L + 17MT


Cefuroxime-Fraunhofer
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Cefuroxime-0.001mg/L
Cefuroxime-0.01mg/L
Cefuroxime-0.1mg/L
Cefuroxime-1mg/L
Cefuroxime-10mg/L
Cefuroxime-0.001mg/L + 17MT
Cefuroxime-0.01mg/L + 17MT
Cefuroxime-0.1mg/L + 17MT
Cefuroxime-1mg/L + 17MT
Cefuroxime-10mg/L + 17MT


Cefuroxime-IDEA
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Cefuroxime-0.001mg/L
Cefuroxime-0.01mg/L
Cefuroxime-0.1mg/L
Cefuroxime-1mg/L
Cefuroxime-10mg/L
Cefuroxime-0.001mg/L + 17MT
Cefuroxime-0.01mg/L + 17MT
Cefuroxime-0.1mg/L + 17MT
Cefuroxime-1mg/L + 17MT
Cefuroxime-10mg/L + 17MT

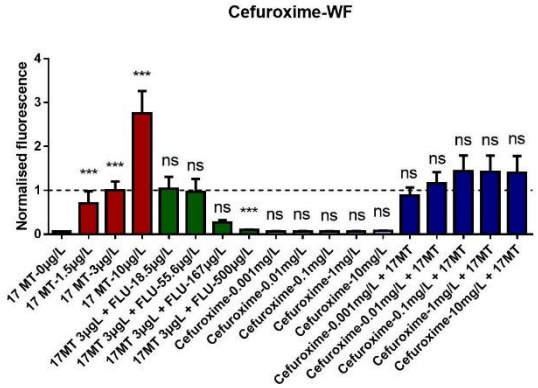

Cefuroxime-WF
Normalised fluorescence
17 MT-0µg/L
17 MT-1.5µg/L
17 MT-3µg/L
17 MT-10µg/L
17MT 3µgL + FLU-18.5µg/L
17MT 3µgL + FLU-55.6µg/L
17MT 3µgL + FLU-167µg/L
17MT 3µgL + FLU-500µg/L
Cefuroxime-0.001mg/L
Cefuroxime-0.01mg/L
Cefuroxime-0.1mg/L
Cefuroxime-1mg/L
Cefuroxime-10mg/L
Cefuroxime-0.001mg/L + 17MT
Cefuroxime-0.01mg/L + 17MT
Cefuroxime-0.1mg/L + 17MT
Cefuroxime-1mg/L + 17MT
Cefuroxime-10mg/L + 17MT

Figure 13: Mean and SEM of measured fluorescence for cefuroxime.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

Table 14: Summarised statistical results for the cefuroxime experiments.

Results corresponding to a statistically significant variation of fluorescence are highlighted in green.

Laboratory	17MT (µg/L)			Flutamide (µg/L)			
	1.5	3	10	18.5	55.6	167	500
CEFAS	***	***	***	ns	ns	***	***
FIWI	ns	***	***	ns	ns	**	***
Fraunhofer	ns	*	***	ns	ns	ns	*
Idea	***	***	***	ns	ns	**	***
WatchFrog	***	***	***	ns	ns	ns	***

Laboratory	Cefuroxime (mg/L)					Cefuroxime + 17MT (mg/L)				
	0.001	0.01	0.1	1	10	0.001	0.01	0.1	1	10
CEFAS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
FIWI	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Fraunhofer	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Idea	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
WatchFrog	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

3.6.3. *Cromolyn Results*

Cromolyn is a pharmaceutical mast cell stabilizer and was expected to be inert with regards to androgen axis activity.

Figure 14 shows that a concentration dependent response was observed for the two control chemicals 17MT and flutamide in all laboratories. The LOEC for the control chemicals was 1.5 µg/L for 17MT and 167 µg/L for flutamide (Table 15).

As expected, no statistically significant deviation in fluorescence was recorded by any of the five laboratories when eleutheroembryos were exposed to cromolyn in either the presence or absence of 17MT. One of the runs performed at Idea did not pass validity criteria, the data from the remaining two runs are shown here.

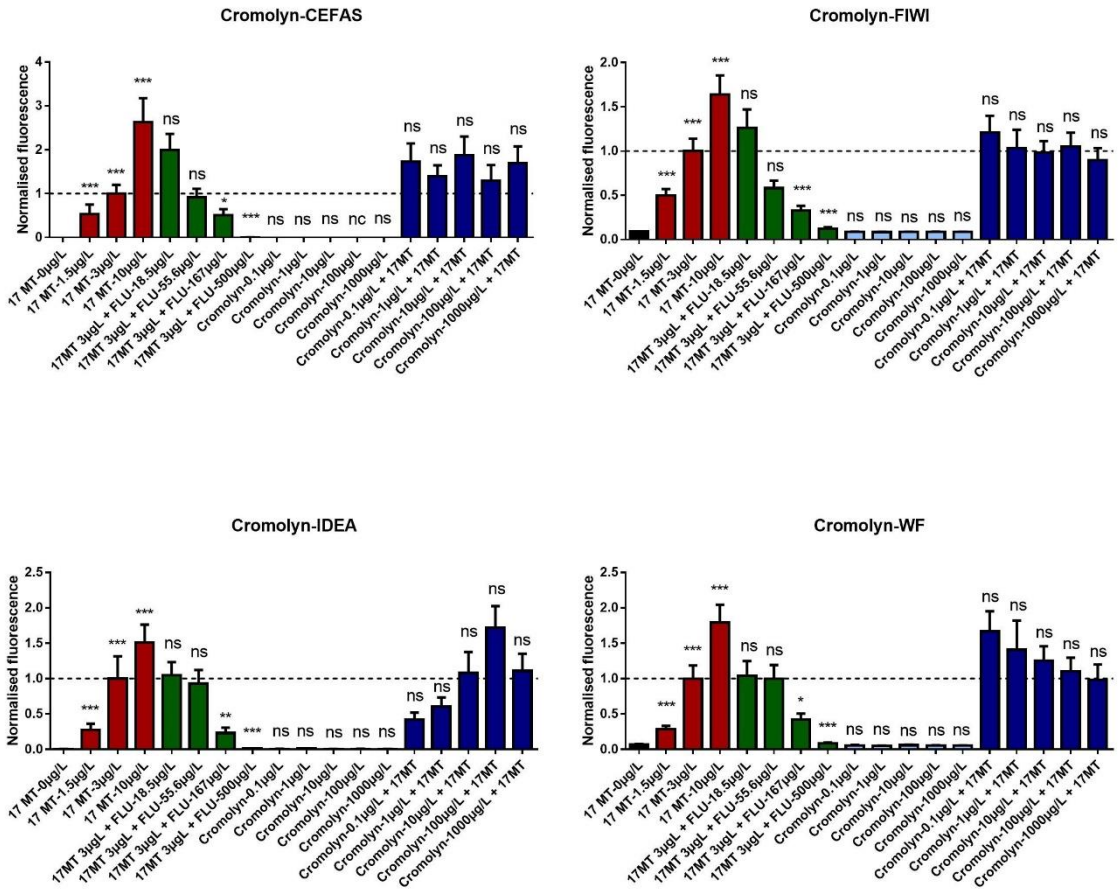


Figure 14: Mean and SEM of measured fluorescence for cromolyn.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

Table 15: Summarised statistical results for the cromolyn experiments.

Results corresponding to a statistically significant variation of fluorescence are highlighted in green.

Laboratory	17MT (µg/L)			Flutamide (µg/L)			
	1.5	3	10	18.5	55.6	167	500
CEFAS	***	***	***	ns	ns	*	***
FIWI	***	***	***	ns	ns	***	***
Idea	***	***	***	ns	ns	**	***
WatchFrog	***	***	***	ns	ns	*	***

Laboratory	Cromolyn (µg/L)					Cromolyn + 17MT (µg/L)				
	0.1	1	10	100	1000	0.1	1	10	100	1000
CEFAS	ns	ns	ns	nc	ns	ns	ns	ns	ns	ns
FIWI	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Idea	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
WatchFrog	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

3.7. Chemical Analyses

Selected samples were retained and frozen until quantitative chemical analysis. This was performed in order to ensure that the laboratories were able to accurately prepare the chemicals used in the assay at the correct concentrations and also to measure the loss of the chemicals between initial contact with the eleutheroembryos and media renewal after 24 h.

Due to the high number of participating laboratories and test chemicals, in order to reduce costs, analytical verification was performed for test solutions from three of the five laboratories taking part in the OECD validation excluding the lead laboratory (WatchFrog). The three laboratories selected were CEFAS, UK; IDEA Consulting, Japan and FIWI, Switzerland. This selection includes a government institute (CEFAS), a private company (IDEA Consulting) and an academic institution (FIWI).

Analysis of the highest and lowest test concentrations for each of the selected test chemicals was performed to inform on the actual concentrations for the entire concentration range. In order to reduce costs, intermediate concentrations was not be tested, as a dilution series was performed and it can be reasonably expected that the intermediate concentrations will be in line with the highest and lowest concentrations.

Three runs of the assay were performed for each test chemical. In order to reduce costs, analytical verification was performed for the first run only for chemicals expected to be active in the assay (17MT, Flutamide, Anastrazole, mDHT, Fenitrothion, Linuron and Vinclozolin). As these chemicals were expected to elicit a biological response, the biological response from run one could be compared with runs two and three to inform on the presence of the test chemical. The test solutions at T0 (exposure solution prior to contact with the eleutheroembryos) and at T24 (the same solution after contact with the eleutheroembryos for 24 hours) were analysed. The T24 sample informs on loss of the test chemical between daily renewals of the test solutions.

For the chemicals expected to be inert (Cromolyn, Cefuroxime and Amantadine) the analytical verification was carried out for the T0 samples for all three runs to ensure that the test chemical was present in the system. This was of particular importance for the inert chemicals as no biological

response is expected in the assay. However, in order to reduce costs, the highest and lowest test concentrations were only verified for run one. For runs two and three, only the highest test concentration was verified. In addition, as for the chemicals expected to be active, T24 samples are suggested for analytical verification for run one only to inform on loss of the test chemical between daily renewals of the test solutions.

All solutions were stored at -20° and sent to the contract analytical chemistry laboratories of Laboratoires des Pyrénées et des Landes (LPL) in France by the participating laboratories. The detection and quantification limits obtained by LPL are given in Table 16.

Table 16: Detection and quantification limits for the chemical analysis

Chemical	Detection limit (µg/L)	Quantification limit (µg/ml)
17MT	0.3	1.0
Flutamide	4	13
Anastrozole	9	32
Fenitrothion	8	25
Linuron	3	10
mDHT	0.6	2.0
Vinclozolin	0.75	2.5
Amantadine	0.15	0.5
Cefuroxime	8	25
Cromolyn	21	70

3.7.1. ***17MT***

The measured concentrations of 17MT were close to the nominal values prior to exposure for both the lowest tested concentration of 1.5 µg/L (73-85% nominal) except for the sample from Idea and also the highest tested concentration of 10 µg/L (61-71% nominal) as shown in Table 17. For the 1.5 µg/L sample from Idea, 17MT was detected, but not quantifiable, indicating an actual concentration of between 0.3 and 1.0 µg/L.

Following 24 h of exposure the actual concentration of 17MT in the nominal 1.5 µg/L samples was between 0.3 and 1.0 µg/L. The measured concentrations for the nominally 10 µg/L samples ranged from 41-66% of nominal (Table 18). This indicates that approximately 50% of the nominal 17MT concentration was actually present after 24 h contact with the eleutheroembryos.

Table 17 : Nominal and measured concentrations for 17MT before exposure

Laboratory	Run	Nominal 17MT Concentration (µg/L)	Measured 17MT Concentration (µg/L)	Measured/Nominal (%)
CEFAS	R1	1.5	1.10	73
	R1	10	7.14	71
FIWI	R1	1.5	1.28	85
	R1	10	6.42	64
Idea	R1	1.5	0.3 - 1.0	-
	R1	10	6.05	61

Table 18 : Nominal and measured concentrations for 17MT after 24 h of exposure

Laboratory	Run	Nominal 17MT Concentration (µg/L)	Measured 17MT Concentration (µg/L)	Measured/Nominal (%)
CEFAS	R1	1.5	0.3 - 1.0	-
	R1	10	5.67	57
FIWI	R1	1.5	0.3 - 1.0	-
	R1	10	6.59	66
Idea	R1	1.5	0.3 - 1.0	-
	R1	10	4.06	41

3.7.2. ***Flutamide***

The measured concentrations of flutamide were close to the nominal values prior to exposure for both the lowest tested concentration of 0.0185 mg/L (detected but unquantifiable-130% nominal) and also the highest tested concentration of 0.5 mg/L (72-102% nominal) as shown in Table 19. The fact that several of the lower concentration samples tested indicated the presence of flutamide below the quantification limit can be explained by the fact that the quantification limit was 13 µg/L (70% of nominal).

Following 24 h of exposure, the measured concentrations ranged from detected but unquantifiable-151% of nominal for the lowest tested concentration and from 66-137% of nominal for the highest tested concentration (Table 20). This indicates that there was no appreciable loss of test chemical between media renewals.

Table 19: Nominal and measured concentrations for flutamide before exposure

Laboratory	Run	Nominal Flutamide Concentration (mg/L)	Measured Flutamide Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.0185	0.013 - 0.004	-
	R1	0.5	0.43	86
FIWI	R1	0.0185	0.013 - 0.004	-
	R1	0.5	0.359	72
Idea	R1	0.0185	0.024	130
	R1	0.5	0.511	102

Table 20: Nominal and measured concentrations for flutamide after 24 h of exposure

Laboratory	Run	Nominal Flutamide Concentration (mg/L)	Measured Flutamide Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.0185	0.013 - 0.004	-
	R1	0.5	0.388	78
FIWI	R1	0.0185	0.013 - 0.004	-
	R1	0.5	0.322	66
Idea	R1	0.0185	0.028	151
	R1	0.5	0.687	137

3.7.3. *Anastrozole*

The measured concentrations of anastrozole were very close to the nominal values prior to exposure for both the lowest tested concentration of 0.11 mg/L (89-95% nominal) and also the highest tested concentration of 9 mg/L (96-99% nominal) as shown in Table 21.

Following 24 h of exposure, the measured concentrations ranged from 90-102% of nominal for the lowest tested concentration and from 87-94% of nominal for the highest tested concentration (Table 22). This indicates that there was no appreciable loss of test chemical between media renewals.

Table 21 : Nominal and measured concentrations for anastrozole before exposure

Laboratory	Run	Nominal Anastrozole Concentration (mg/L)	Measured Anastrozole Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.11	0.098	89
	R1	9	8.658	96
FIWI	R1	0.11	0.100	91
	R1	9	8,798	98
Idea	R1	0.11	0.105	95
	R1	9	8.930	99

Table 22 : Nominal and measured concentrations for anastrozole after 24 h of exposure

Laboratory	Run	Nominal Anastrozole Concentration (mg/L)	Measured Anastrozole Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.11	0.099	90
	R1	9	8.322	92
FIWI	R1	0.11	0.112	102
	R1	9	8.418	94
Idea	R1	0.11	0.100	91
	R1	9	7.837	87

3.7.4. *Fenitrothion*

The measured concentrations of fenitrothion were reasonably close to the nominal values prior to exposure for the highest tested concentration of 0.5 mg/L (69-85% nominal) as shown in Table 23. None of the samples analysed for the lowest tested concentration of 0.031 mg/L were quantifiable, although fenitrothion was detected in the sample from CEFAS. The quantification limit was 25 µg/L,

which was 81% of nominal for the lowest tested concentration, the detection limit was 8 µg/L or 26% of nominal.

Following 24 h of exposure, the measured concentrations ranged from 51-66% of nominal for the highest tested concentration (Table 24). This indicates that there was no major loss of test chemical between media renewals.

Table 23 : Nominal and measured concentrations for fenitrothion before exposure

Laboratory	Run	Nominal Fenitrothion Concentration (mg/L)	Measured Fenitrothion Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.031	0.008 - 0.025	-
	R1	0.5	0.377	75
FIWI	R1	0.031	< 0.008	-
	R1	0.5	0.427	85
Idea	R1	0.031	< 0.008	-
	R1	0.5	0.344	69

Table 24 : Nominal and measured concentrations for fenitrothion after 24 h of exposure

Laboratory	Run	Nominal Fenitrothion Concentration (mg/L)	Measured Fenitrothion Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.031	< 0.008	-
	R1	0.5	0.3	60
FIWI	R1	0.031	< 0.008	-
	R1	0.5	0.328	66
Idea	R1	0.031	< 0.008	-
	R1	0.5	0.254	51

3.7.5. *Linuron*

The measured concentrations of linuron were very close to the nominal values prior to exposure for both the lowest tested concentration of 0.16 mg/L (99-101% nominal) and also the highest tested concentration of 2.5 mg/L (83-114% nominal) as shown in Table 25.

Following 24 h of exposure the measured concentrations range from 88-98% of nominal for the lowest tested concentration and from 64-87% of nominal for the highest tested concentration (Table 26). This indicates that there was a marginal (lowest test concentration) and moderate (highest test concentration) loss of test chemical between media renewals.

Table 25 : Nominal and measured concentrations for linuron before exposure

Laboratory	Run	Nominal Linuron Concentration (mg/L)	Measured Linuron Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.16	0.161	101
	R1	2.5	2.089	84
FIWI	R1	0.16	0.16	100
	R1	2.5	2.856	114
Idea	R1	0.16	0.159	99
	R1	2.5	2.064	83

Table 26 : Nominal and measured concentrations for linuron after 24 h of exposure

Laboratory	Run	Nominal Linuron Concentration (mg/L)	Measured Linuron Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.16	0.141	88
	R1	2.5	2.177	87
FIWI	R1	0.16	0.156	98
	R1	2.5	1.61	64
Idea	R1	0.16	0.145	91
	R1	2.5	1.926	77

3.7.6. *mDHT*

Prior to exposure, the measured concentrations of mDHT were low compared to nominal values, particularly for Idea. The measured values for the highest tested concentration of 16 µg/L were 52-77% of nominal as shown in Table 27. It is difficult to judge the actual concentrations of mDHT for the lowest test concentration as the expected concentration was 1 µg/L and the quantification limit was 2 µg/L. As expected, none of the samples were above this limit which was 200% of nominal.

Following 24 h of exposure approximately 50% of the initial mDHT had been lost with 34-51% of nominal present in the different laboratories (Table 28).

Table 27 : Nominal and measured concentrations for mDHT before exposure

Laboratory	Run	Nominal mDHT Concentration (µg/L)	Measured mDHT Concentration (µg/L)	Measured/Nominal (%)
CEFAS	R1	1	< 0.6	-
	R1	16	11.2	70
FIWI	R1	1	0.6 - 2.0	-
	R1	16	12.3	77
Idea	R1	1	0.6 - 2.0	-
	R1	16	8.3	52

Table 28 : Nominal and measured concentrations for mDHT after 24 h of exposure

Laboratory	Run	Nominal mDHT Concentration (µg/L)	Measured mDHT Concentration (µg/L)	Measured/Nominal (%)
CEFAS	R1	1	< 0.6	-
	R1	16	5.5	34
FIWI	R1	1	< 0.6	-
	R1	16	8.2	51
Idea	R1	1	0.6 - 2.0	-
	R1	16	5.8	36

3.7.7. *Vinclozolin*

The measured concentrations of vinclozolin were below expected values prior to exposure for both the lowest tested concentration of 3.7 µg/L (detected but unquantifiable) and also the highest tested concentration of 300 µg/L (55-75% nominal) as shown in Table 29. The quantification limit was 2.5 µg/L or 68% of nominal for the lowest tested concentration.

Following 24 h of exposure, a high degree of loss of the test chemical had occurred (Table 30). Only one of the samples from the highest test concentration was quantifiable and showed 22% of the expected concentration of vinclozolin.

Table 29 : Nominal and measured concentrations for vinclozolin before exposure

Laboratory	Run	Nominal Vinclozolin Concentration (µg/L)	Measured Vinclozolin Concentration (µg/L)	Measured/Nominal (%)
CEFAS	R1	3.7	0.75 - 2.5	-
	R1	300	225	75
FIWI	R1	3.7	0.75 - 2.5	-
	R1	300	140	47
Idea	R1	3.7	0.75 - 2.5	-
	R1	300	165	55

Table 30 : Nominal and measured concentrations for vinclozolin after 24 h of exposure

Laboratory	Run	Nominal Vinclozolin Concentration (µg/L)	Measured Vinclozolin Concentration (µg/L)	Measured/Nominal (%)
CEFAS	R1	3.7	0.75 - 2.5	-
	R1	300	< 7.5	-
FIWI	R1	3.7	< 0.75	-
	R1	300	7.5 - 25	-
Idea	R1	3.7	0.75 - 2.5	-
	R1	300	65	22

3.7.8. *Amantadine*

The measured concentrations of amantadine were very close to the nominal values prior to exposure for both the lowest tested concentration of 1 µg/L (102-104% nominal) and also the highest tested concentration of 10 mg/L across all three runs (82-107% nominal) for CEFAS and FIWI as shown in Table 31. Something unexpected seems to have happened with the dilution of amantadine for the experiments performed by Idea. The 10 mg/L samples contain approximately 1 mg/L of amantadine and the 1 µg/L sample was measured as 6.55 µg/L.

Following 24 h of exposure, the samples from the highest test concentration show very little difference to their initial values. This indicates that there was no appreciable loss of test chemical between media renewals (Table 32). The measured concentrations for the lowest test concentration seem quite variable, with 90% nominal recorded for the sample from CEFAS, 351% of nominal recorded for the sample from FIWI (approximately 3.5 times more amantadine than was present in the same sample 24 h earlier) and a concentration below the quantification limit recorded for Idea.

Table 31 : Nominal and measured concentrations for amantadine before exposure

Laboratory	Run	Nominal Amantadine Concentration (µg/L)	Measured Amantadine Concentration (µg/L)	Measured/Nominal (%)
CEFAS	R1	1	1.02	102
	R1	10000	9983	100
	R2	10000	9315	93
	R3	10000	8805	88
FIWI	R1	1	1.04	104
	R1	10000	10701	107
	R2	10000	8175	82
	R3	10000	10259	103
Idea	R1	1	6.55	655
	R1	10000	1095	11
	R2	10000	1043	10
	R3	10000	918	9

Table 32 : Nominal and measured concentrations for amantadine after 24 h of exposure

Laboratory	Run	Nominal Amantadine Concentration (µg/L)	Measured Amantadine Concentration (µg/L)	Measured/Nominal (%)
CEFAS	R1	1	0.90	90
	R1	10000	10102	101
FIWI	R1	1	3.51	351
	R1	10000	10140	101
Idea	R1	1	0.15 - 0.5	-
	R1	10000	886	9

3.7.9. *Cefuroxime*

The measured concentrations of cefuroxime were very close to the nominal values prior to exposure for both the highest tested concentration of 10 mg/L (88-108% nominal) across all three runs and all laboratories (Table 33).

Following 24 h of exposure, the measured concentrations for the highest test concentration were still very close to nominal values ranging from 86-91% for FIWI and Idea (Table 34). This indicates that there was no appreciable loss of test chemical between media renewals. Cefuroxime was not detectable in the T24 sample from CEFAS indicating perhaps an error during sampling or analysis.

No cefuroxime was detected in any of the samples for the lowest test concentration, this is not surprising since the detection limit was 8 times higher than the nominal concentration.

Table 33 : Nominal and measured concentrations for cefuroxime before exposure

Laboratory	Run	Nominal Cefuroxime Concentration (mg/L)	Measured Cefuroxime Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.001	< 0.008	-
	R1	10	9.41	94
	R2	10	8.79	88
	R3	10	9.34	93
FIWI	R1	0.001	< 0.008	-
	R1	10	10.79	108
	R2	10	10.75	108
	R3	10	9.34	93
Idea	R1	0.001	< 0.008	-
	R1	10	10.25	103
	R2	10	10.09	101

Table 34 : Nominal and measured concentrations for cefuroxime after 24 h of exposure

Laboratory	Run	Nominal Cefuroxime Concentration (mg/L)	Measured Cefuroxime Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.001	< 0.008	-
	R1	10	< 0.008	-
FIWI	R1	0.001	< 0.008	-
	R1	10	9.07	91
Idea	R1	0.001	< 0.008	-
	R1	10	8.62	86

3.7.10. ***Cromolyn***

The measured concentrations of cromolyn were very close to the nominal values prior to exposure for both the highest tested concentration of 1 mg/L (98-115% nominal) across all three runs and all laboratories (Table 35).

Following 24 h of exposure, the measured concentrations for the highest test concentration were still very close to nominal values ranging from 104-111% (Table 36). This indicates that there was no appreciable loss of test chemical between media renewals.

No cromolyn was detected in any of the samples for the lowest test concentration, this is not surprising since the detection limit was 210 times higher than the nominal concentration.

Table 35 : Nominal and measured concentrations for cromolyn before exposure

Laboratory	Run	Nominal Cromolyn Concentration (mg/L)	Measured Cromolyn Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.0001	< 0.021	-
	R1	1	1.153	115
	R2	1	1.124	112
	R3	1	1.072	107
FIWI	R1	0.0001	< 0.021	-
	R1	1	1.088	109
	R2	1	1.043	104
	R3	1	1.122	112
Idea	R1	0.0001	< 0.021	-
	R1	1	0.981	98
	R2	1	1.008	100

Table 36 : Nominal and measured concentrations for cromolyn after 24 h of exposure

Laboratory	Run	Nominal Cromolyn Concentration (mg/L)	Measured Cromolyn Concentration (mg/L)	Measured/Nominal (%)
CEFAS	R1	0.0001	< 0.021	-
	R1	1	1.109	111
FIWI	R1	0.0001	< 0.021	-
	R1	1	1.063	106
Idea	R1	0.0001	< 0.021	-
	R1	1	1.035	104

3.8. Results for chemicals tested uniquely in the lead laboratory

Having demonstrated the reliability of the RADAR assay to generate reproducible results across the participating partner laboratories, a number of additional chemicals were tested with the RADAR assay in the lead laboratory only. These additional chemicals were tested to increase the number of expected active and inert chemicals tested within the validation exercise and also to cover a wider range of modes of action on the androgen axis. Unfortunately, due to time and financial constraints, these additional chemicals could not be tested in all participating laboratories.

3.8.1. *Androgen axis active chemicals*

Cyproterone acetate is a pharmaceutical anti-androgen and anti-progestin. It acts on the androgen axis by antagonising AR (Neumann, 1994). As would be expected for an AR antagonist, it showed no activity in the RADAR assay in unspiked mode and decreased fluorescence signal in a concentration dependent manner in spiked mode (Figure 15A). The LOEC obtained by the lead laboratory was 10 µg/L.

Dutasteride is a pharmacological inhibitor of type I and II 5α-reductase which has also been shown to act as an AR antagonist in certain cell lines (Chhipa et al., 2013). An interesting profile was obtained for this chemical with the RADAR assay (Figure 15B). As expected, no statistically significant variation in fluorescence was observed in unspiked mode. In spiked mode, a statistically significant increase in fluorescence was observed at the lower concentrations of 62.5 and 125 µg/L. Inversely, a statistically significant decrease in fluorescence was observed with the higher concentrations of 500 and 1000 µg/L.

The imidazole fungicide prochloraz has been shown to inhibit the expression of aromatase enzyme (Higley et al., 2010). It would, therefore, be expected to increase androgen axis activity by inhibiting the conversion of androgens to estrogens. An increase in fluorescence was recorded with the two highest tested concentrations of prochloraz 500 and 1000 µg/L in unspiked mode and with the three lowest concentrations tested in spiked mode (Figure 15C). A statistically significant decrease in fluorescence was observed with the highest tested concentration in spiked mode.

The synthetic androgen 17β-trenbolone was also assessed with the RADAR assay. As would be expected, it generated a strong and concentration dependent increase in fluorescence in both unspiked and spiked mode (Figure 15D). The LOEC was 37 µg/L in unspiked mode and 111 µg/L in spiked mode.

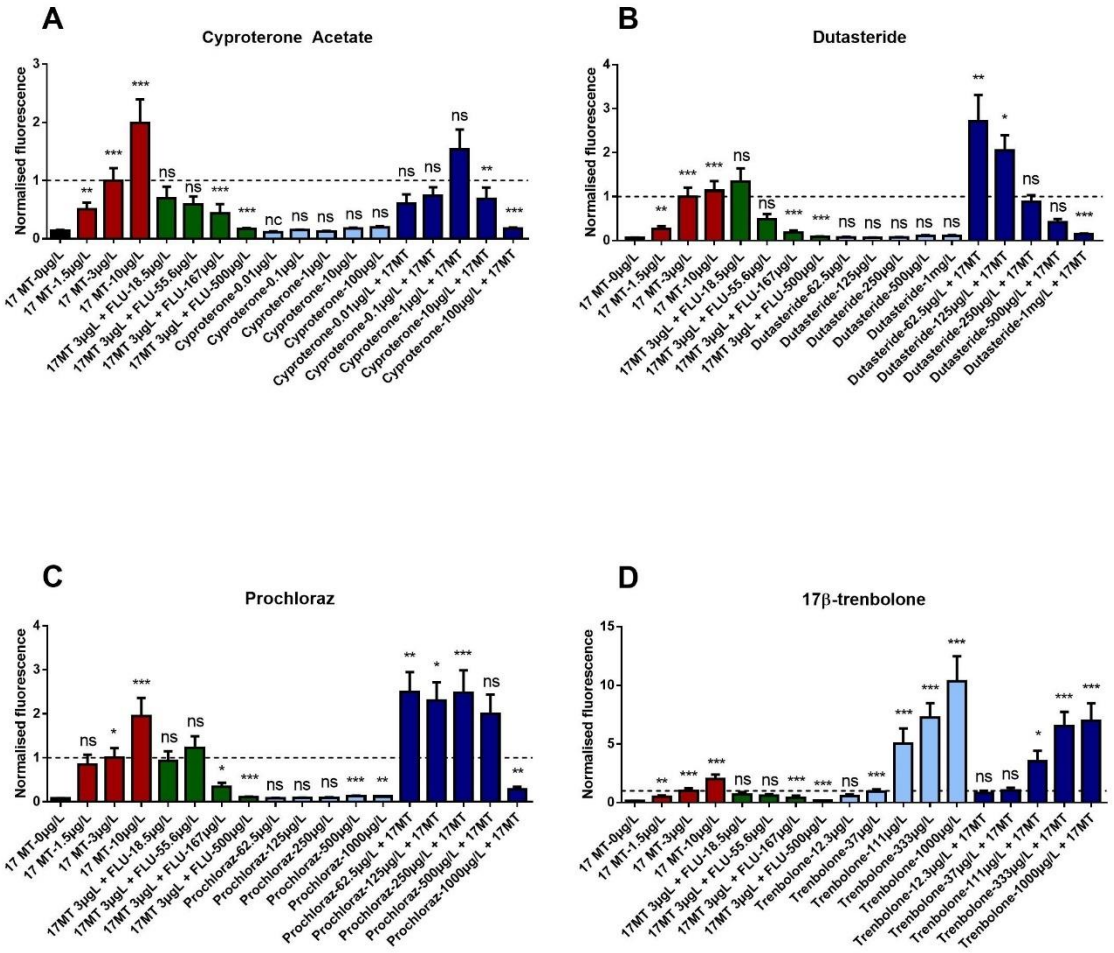


Figure 15: Mean and SEM of measured fluorescence for androgen axis active chemicals tested in the lead laboratory.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

3.8.2. *Estrogenic chemicals*

It has previously been noted *in vitro* and *in vivo* (Jolly et al., 2009b; Katsiadaki et al., 2006b) that estrogens can exert an anti-androgenic effect, possibly via increased expression of aromatase enzyme. This phenomenon was also observed during the characterisation of the RADAR assay (Sébillot et al., 2014). In order to further confirm that this effect is reproducible and is common to a wide range of estrogens, we carried out concentration-response experiments using the RADAR assay. The experiments were carried out with E2, EE2, estrone and the weakly estrogenic compound nonylphenol (Figure 16). All four chemicals inhibited androgen axis activity as measured by the RADAR assay in a

concentration dependent manner in spiked mode. None of the tested estrogens displayed activity in unspiked mode.

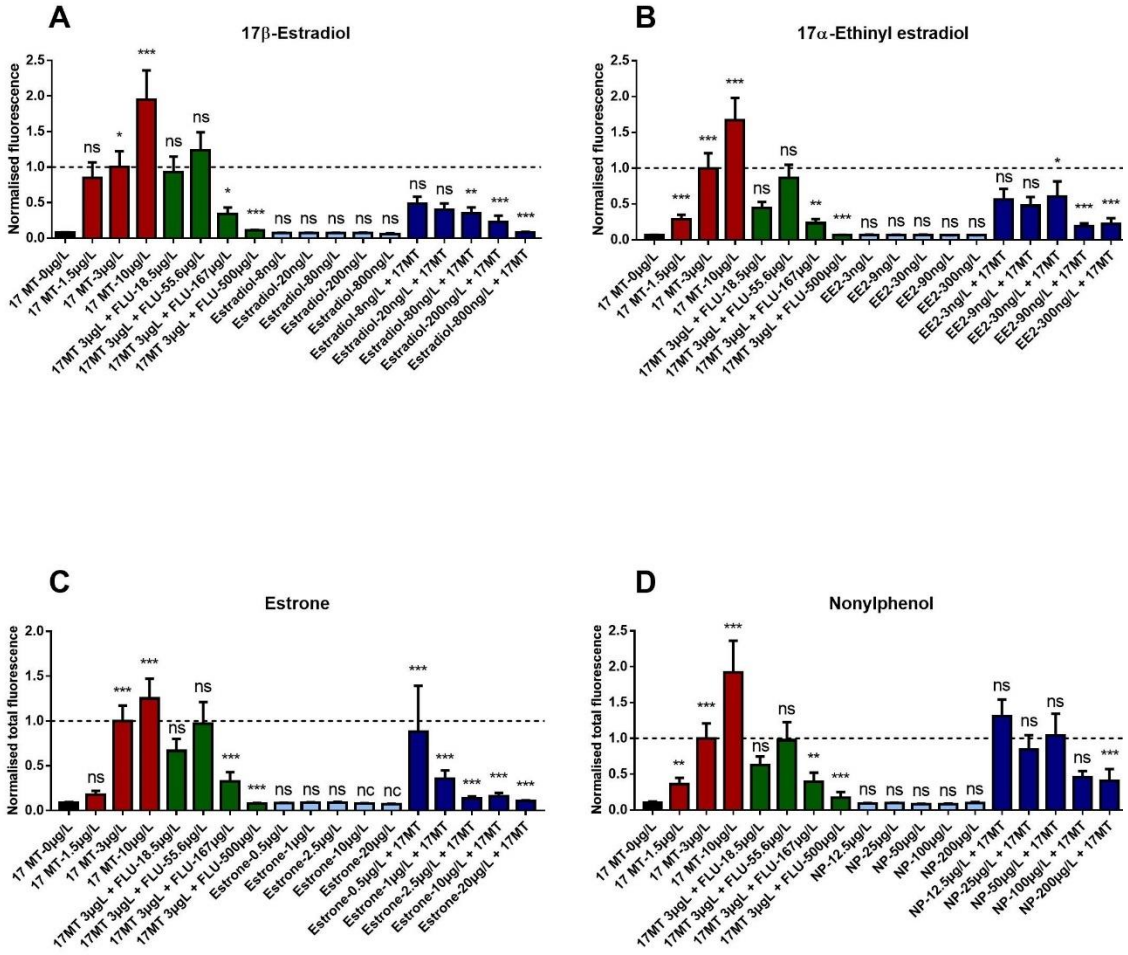


Figure 16: Mean and SEM of measured fluorescence for estrogenic chemicals tested in the lead laboratory.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

3.8.3. *Expected inert chemicals*

To complement the date generated by the participating laboratories with the three expected inert chemicals (amantadine, cefuroxime and cromolyn), two additional chemicals were tested by the lead laboratory which were expected to be inactive on the androgen axis. These two chemicals were the monosaccharide arabinose and the anti-parkinsonian medication L-dopa (Figure 17). Neither of these chemicals gave a statistically significant variation in fluorescence in either unspiked or spiked modes.

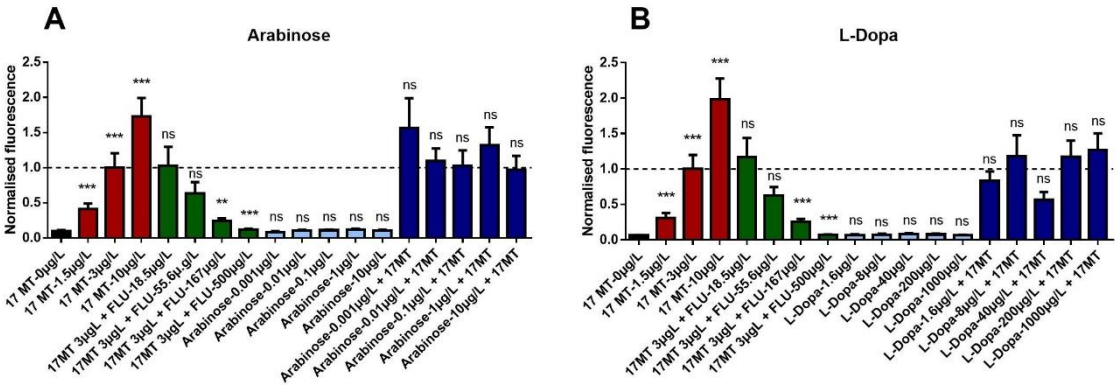


Figure 17: Mean and SEM of measured fluorescence for expected inert chemicals tested in the lead laboratory.

Fluorescence values were normalised to the mean of the 17MT 3 µg/L group, the value of this group is indicated with a dashed line.

3.9. Discussion

The data generated during the interlaboratory validation exercise of the RADAR assay demonstrated that the assay is robust and produced reliable and reproducible data across five laboratories. The five laboratories used a range of different fluorescence imaging systems to read the experiments and either bred the spg1-gfp eleutheroembryos in house or raised them from shipped embryos. Despite this, all five laboratories were able to perform the assay with the expected sensitivity. All expected active test chemicals were identified as active and gave similar results in all participating laboratories. Likewise, all expected inert chemicals were determined to be inert by all partner laboratories. Individual chemical results are discussed in detail below.

3.9.1. *Calibration*

The calibration exercise was not performed by the lead laboratory (WatchFrog) as they had previously calibrated their image acquisition parameters for use with the RADAR assay. All four naïve laboratories performed the calibration exercise and following obtained data showing a concentration-response relationship for both 17MT and flutamide. With an acceptable sensitivity. The LOECs for 17MT and flutamide ranged from 1.5 to 3 µg/L and from 55.6 to 167 µg/L, respectively.

Based on these data, all four naïve laboratories were advised to fix their image acquisition settings used in the calibration exercise and to proceed to testing of the expected active and inert chemicals.

3.9.2. *Anastrozole*

As expected, all participating laboratories observed a lack of effect in unspiked mode and a clear concentration dependent increase in fluorescence in spiked mode. Whilst the effect was extremely reproducible across laboratories, the LOECs varied from 0.33-9 mg/L.

3.9.3. *Fenitrothion*

As an AR antagonist, fenitrothion was expected to cause a concentration dependent decrease in fluorescence in spiked mode. This effect was observed in a highly reproducible manner across all participating laboratories. In addition to this, the LOECs obtained by each laboratory were also very similar, ranging from 125 to 250 µg/L.

3.9.4. *Linuron*

Linuron is an AR antagonist as is fenitrothion. It was, therefore, expected to give similar results in the RADAR assay. As with fenitrothion, a reproducible concentration-dependent decrease in fluorescence was obtained across all five laboratories. Most laboratories recorded a LOEC of 625 µg/L with one laboratory detecting activity at 1.25 mg/L and one at 313 µg/L.

3.9.5. *mDHT*

The synthetic AR agonist mDHT was tested by all laboratories. This chemical which is a strong inducer of androgen mediated transcription was expected to induce a high degree of fluorescence in unspiked mode. This induction was observed in all five laboratories with three of the laboratories detecting an increase in fluorescence for all tested concentrations (1-16 µg/L). The remaining two laboratories detected all concentrations from 2-16 µg/L. Except for one intermediate concentration of mDHT that was detected by one laboratory, mDHT did not induce a statistically significant increase in fluorescence in spiked mode. This was possibly due to saturation of the system by the combined effects of 17MT and mDHT, both powerful AR agonists.

3.9.6. *Vinclozolin*

As with the AR antagonists fenitrothion and linuron, vinclozolin induced a concentration dependent decrease in fluorescence in spiked mode in all laboratories. A reproducible LOEC of 100-300 µg/L was obtained by all laboratories. Antagonism of AR by vinclozolin is generally attributed to its M1 and M2 metabolites which are more powerful AR antagonists than the parent molecule. The reproducible detection of vinclozolin demonstrates to some extent the metabolic capability of the eleutheroembryos.

3.9.7. *Amantadine (inert compound)*

Amantadine was selected as an inert chemical for the validation of the RADAR assay as it is biologically active but a literature search failed to identify any known activity on the androgen axis. Slight activity was noted by IDEA and FIWI at intermediate concentrations, however, this activity was not reproducible

across the three experimental runs and did not demonstrate a concentration-response relationship and, therefore, amantadine is classified as inactive in the RADAR assay.

3.9.8. ***Cefuroxime (inert compound)***

Cefuroxime was selected as an inert chemical for the validation of the RADAR assay as it is biologically active but to date, it is not known to be active on the androgen axis. No active concentrations were detected by the five laboratories; therefore, it is categorized as an androgen axis inactive molecule by the RADAR assay.

3.9.9. ***Cromolyn (inert compound)***

Cromolyn was selected as the third inert chemical for the interlaboratory validation based on the same criterion as the other inert chemicals, a lack of published data showing androgen axis activity. No active concentrations were detected by any of the four laboratories, cromolyn is, therefore, considered as androgen axis inactive by the RADAR assay.

3.9.10. *Chemical analysis*

Chemical analysis was performed on key samples from the RADAR assay interlaboratory validation study. The vast majority of measured concentrations were either extremely close or reasonably close to the expected values.

A notable exception was the expected inert chemical amantadine, which was at a ten-fold lower than expected concentration in all experiments performed by Idea. It was, however, at the correct concentration in the experiments performed by CEFAS and FIWI.

Another exception was vinclozolin, for which there was a combined effect that it was measured at roughly 50% of the expected concentration at T0 and also appeared to suffer some loss during the 24 h exposure period prior to media renewal. This meant that the concentrations measured after 24 h exposure were undetectable for CEFAS and FIWI and 22% of nominal for Idea. Despite this, vinclozolin was correctly identified as androgen axis active by all participating laboratories.

A major technical problem with the chemical analysis was the quantification and detection limits which were in some cases higher or considerably higher than the lowest test concentration. This made interpretation of the results for the lowest test concentration difficult in some cases and entirely impossible in others.

3.9.11. *Chemicals tested uniquely in the lead laboratory*

Following the confirmation of reproducible classification of chemicals as pro-androgenic, anti-androgenic or inert across all participating laboratories, additional chemicals were tested in the lead laboratory only using the RADAR assay.

The two additional expected inert chemicals proved to be inert when assessed with the RADAR assay.

All four estrogenic chemicals showed a concentration dependent decrease in fluorescence in spiked mode. This is believed to be via induction or aromatase expression, resulting in a reduced circulating concentration of 17MT and, therefore, reduced androgen axis activity and fluorescence. Ranking the four estrogenic chemicals by anti-androgenic activity based on the recorded LOECs, their anti-androgenic strength appears to be EE2>E2>nonylphenol>estrone, although it is difficult to judge the LOEC of estrone as all tested concentrations were active.

With regards to the additional androgen axis active chemicals, the results obtained for the AR antagonist cyproterone and anti-progestin cyproterone acetate and the synthetic AR agonist 17β-trenbolone were as expected. However, interesting results were obtained for prochloraz and dutasteride.

Prochloraz is known to inhibit aromatase expression and displayed the expected increase in androgen axis activity at the lower tested concentrations. The highest tested concentration of prochloraz (1 mg/L) showed a statistically significant decrease in fluorescence in spiked mode. Although the validity criteria were met for this concentration with <15% mortality both in unspiked and spiked mode and no abnormal behaviour noted. It was noted by the person performing the assay that when pipetting the eleutheroembryos exposed to this concentration, they felt softer and stuck to the pipette. This is believed to indicate that 1 mg/L prochloraz induced non-androgen specific general physiological effects on the eleutheroembryos which may have resulted in the observed reduction in fluorescence.

Androgen axis activity in the presence of the pharmaceutical 5α-reductase inhibitor, dutasteride, showed a non-monotonic concentration-response relationship in spiked mode. The lowest two concentrations tested showed a statistically significant increase in fluorescence. The two intermediate

concentrations showed no statistically significant activity on the androgen axis and the highest concentration tested showed an almost complete inhibition of androgen axis signalling. The increase in fluorescence observed at low test concentrations is believed to be due to alteration in the relative presence of 17MT and mDHT, due to inhibition of 5α-reductase blocking the conversion of the 17MT, used as a cotreatment in spiked mode, into mDHT. As the concentration of dutasteride increased, it is believed that androgen axis signalling progressively decreased due to the ability of dutasteride to antagonise the AR at high concentrations (Chhipa et al., 2013).

3.10. Conclusions

The RADAR assay interlaboratory validation exercise demonstrated that the assay provides the expected results with the chemicals tested and is reproducible across laboratories. The data generated in the five laboratories matched the expected response profiles and the test chemicals were correctly classified as androgen axis active or inactive in each laboratory. The column labelled “Expected” indicates the expected result based on published data concerning the mode of action of the test chemical. The column labelled “AR model” shows the predicted activity of each test chemical based on computational modelling of the results of 11 *in vitro* high throughput screening assays (Kleinstreuer et al., 2017). It should be noted that the results obtained in this study are largely in agreement with those obtained with the AR model, although the AR model does not cover effects on steroidogenesis, preventing it from identifying effects such as aromatase inhibition (e.g. anastrozole) and 5α-reductase inhibition (dutasteride/ finasteride).

Table 37 : Summary of expected and experimental results for the RADAR assay from the validation exercise.

* indicates that dutasteride was not evaluated with the AR model, but finasteride which has the same mode of action was evaluated and predicted to be inert.

	CAS	Expected	WF	CEFAS	FIWI	IDEA	Fraunhofer	AR model
17MT	58-18-4	active	active	active	active	active	active	active
Flutamide	13311-84-7	active	active	active	active	active	active	active
Amantadine	665-66-7	inert	inert	inert	inert	inert	inert	
Cefuroxime	56238-63-2	inert	inert	inert	inert	inert	inert	
Cromolyn	15826-37-6	inert	inert	inert	inert	inert		
Arabinose	5328-37-0	inert	inert					
L-Dopa	53587-29-4	inert	inert					
Anastrozole	120511-73-1	active	active	active	active	active		inert
Fenitrothion	122-14-5	active	active	active	active	active		
Linuron	330-55-2	active	active	active	active	active	active	active
mDHT	521-11-9	active	active	active	active	active	active	
Vinclozolin	50471-44-8	active	active	active	active	active	active	active
Cyproterone Acetate	427-51-0	active	active					active
Dutasteride	164656-23-9	active	active					*
Prochloraz	67747-09-5	active	active					
17β-Trenbolone	10161-33-8	active	active					active
17β-Estradiol	50-28-2	active	active					active
17α-Ethinyl Estradiol	57-63-6	active	active					active
Estrone	53-16-7	active	active					active
Nonylphenol	104-40-5	active	active					

The validation exercise successfully evaluated not only the RADAR assay itself, but the optimisation and validation steps required to set up the assay in a naïve laboratory using image acquisition equipment which has not previously been employed for this assay.

Once the image acquisition and treatment steps had been optimised, the experimental protocol for the assay as well as the data treatment, statistical approach and a decision logic for classifying the test chemical as androgen axis active or inactive were evaluated and validated. The protocol was successfully transferred to five laboratories from different OECD countries.

In all cases, once the experimental protocol, data analysis procedure and decision logic had been applied, the expected results were obtained for all test chemicals. The RADAR assay was shown to be sensitive to a range of different modes of androgen axis activity including: AR agonism, AR antagonism, inhibition of aromatase enzyme at the protein level, inhibition or activation of aromatase expression, 5α-reductase enzyme inhibition as well as chemicals requiring metabolic activation. By following the described decision logic, none of the expected inert chemicals were identified as androgen axis active during this validation exercise.

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6 Annex 1: storage of control solutions during an experimental run

In order to determine that it was possible to prepare control solutions on the first day of a run and store them at 4°C for the duration of a single run of the RADAR assay, the lead laboratory prepared these solutions and sampled them each day directly from the glass bottle that they were stored in. The solutions were sampled at 0 h, 24 h, 48 h, 72 h and 96 h. Each day the bottles of control solutions were placed at room temperature for 2 h prior to sampling, to mimic the effects of allowing the bottles to reach room temperature prior to renewing an experiment and also the time taken to renew the control solutions of an experiment. The bottles were then placed at 4°C until the next sampling point.

It should be noted that the 96 h time point is 24 h after completion of the RADAR assay. Both the 72 h time point and the 96 h time point are after the last use of the control solutions for a renewal of media in a RADAR which occurs after 48 h.

Table 38 shows the measured concentrations of 17MT for expected values of 1.5 µg/L and 10 µg/L after storage at 4°C. A loss of around 20% can be noted for the lower initial concentration of 1.5 µg/L. A high degree of variability in the measure can be noted by the fact that a loss of 21% of the initial concentration of 17MT can be noted after 48 h storage, but only 12 % of the initial concentration was lost after 72 h storage. As the measurements indicates a 19% loss after 24 h and only 20 % loss after 96 h, this suggests that the initial measurement at T0 might be higher than the actual concentration. In any case, the loss is around 20% at all time points and is, therefore, considered to be within an acceptable range.

The percentage loss of 17MT for the higher initial concentration of 10 µg/L ranges from 0.14% after 24 h to 5.6 % after 96 h. Due to imprecision in the measurements (-15% loss after 48 h) this indicates that no appreciable loss occurred over the 96 h period.

Table 38: Measured concentrations of 17MT for expected values of 1.5 µg/L and 10 µg/L after storage at 4°C.

Time after preparation (h)	Measured concentration 17MT for expected 1.5 (µg/L)	Loss (%)	Measured concentration 17MT for expected 10 (µg/L)	Loss (%)
0	1.31	-	7.34	-
24	1.06	19	7.33	0.14
48	1.03	21	8.43	-15
72	1.15	12	7.68	-4.6
96	1.05	20	6.93	5.6

Table 39 shows the measured concentrations of flutamide for expected values of 18.5 µg/L and 500 µg/L after storage at 4°C. No appreciable loss occurred for the higher concentration of 500 µg/L with a loss of between 0.22% (96 h) and 2.6% (72 h). It is difficult to judge the percentage loss of flutamide whilst storing the lower concentration of 18.5 µg/L at 4°C as the quantification limit was 13 µg/L. However, after 48 h storage, which corresponds to the moment when the last renewal of control solutions in a RADAR assay would be performed, the loss is only 13 %.

Table 39: Measured concentrations of flutamide for expected values of 18.5 µg/L and 500 µg/L after storage at 4°C.

Time after preparation (h)	Measured concentration flutamide for expected 18.5 (µg/L)	Loss (%)	Measured concentration flutamide for expected 500 (µg/L)	Loss (%)
0	16	-	464	-
24	0.4-13	-	462	0.43
48	14	13	460	0.86
72	0.4-13	-	452	2.6
96	0.4-13	-	463	0.22

Overall, these results indicate that it is possible to prepare the control solutions for a RADAR assay on the first day of the assay and store them at 4°C until the last renewal for the same run which is performed 48 h after the beginning of the test. Different control and test solutions should still be used for different runs.