Network of reference laboratories and related organisations for monitoring and bio-monitoring of emerging environmental pollutants

Case Study 1 (Research Level)

Oestrogens in Sewage Treatment Effluents

WP leader: The Environment Agency, United Kingdom – Science Group (UK EA)

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Sixth Framework Programme

Environment Agency

- **Task 1: Overview of the 3 types of methods, list of potential laboratories (IVM lead).**

Review of existing methods

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Application of criteria for method selection as defined by validation V1 criteria.

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Ranking of methods

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Identification of research laboratories able to participate

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1 participant per method type (in vitro bioassays that detect oestrogenic activity, in vivo bioassays that detect oestrogenic activity and in vitro direct compound measurement assays)

Deliverable C1.1. Report submitted August 2006

Selection of Methods for the Three Types

- Type 1 (in-vitro bioassay that detects direct estrogenic activity)
  - E-Screen (human mammary carcinoma cell proliferation assay) applied rivers, ponds, wetlands and municipal wastewater effluent

- Type 2 (in-vivo bioassay that detects oestrogenic activity)
  - Direct homologous quantitative sandwich monoclonal ELISA for fathead Minnow vitellogenin in blood plasma.

- Type 3 (in-vitro direct measurement assay for measurement of quantities of oestrogenic target compounds)
  - Monoclonal ELISA detecting 17β-oestradiol in municipal wastewaters following SPE extraction

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- **Task 2: Preparation and performance of the intra-laboratory comparison exercises (UK EA lead)**

Meeting of participants (London, January 2007)

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Samples sent to Type 1 and 3 participants and for chemical analysis (January 2007)

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Fish plasma samples sent to Type 2 participants (May 2007)

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Results received back from all participants by June 2007

- **Task 3: Evaluation and assessment of the results (UK EA lead)**

Results sent to IVM for an evaluation of the results from the CASE1 study (July 2007)

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Evaluation of results sent to laboratory participants (September 2007)

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2nd meeting of participants (workshop in Bergen, October 2007)

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Actions from workshop completed (January, 2008)

- **Task 3: Evaluation and assessment of the results (UK EA lead) - continued**

Feedback on the applicability of the validation protocols to CASE 1(April 2008)

Deliverable C1.3

Report on the analytical chemistry, bioassays and the intra-laboratory study (October 2008)

Deliverable C1.2

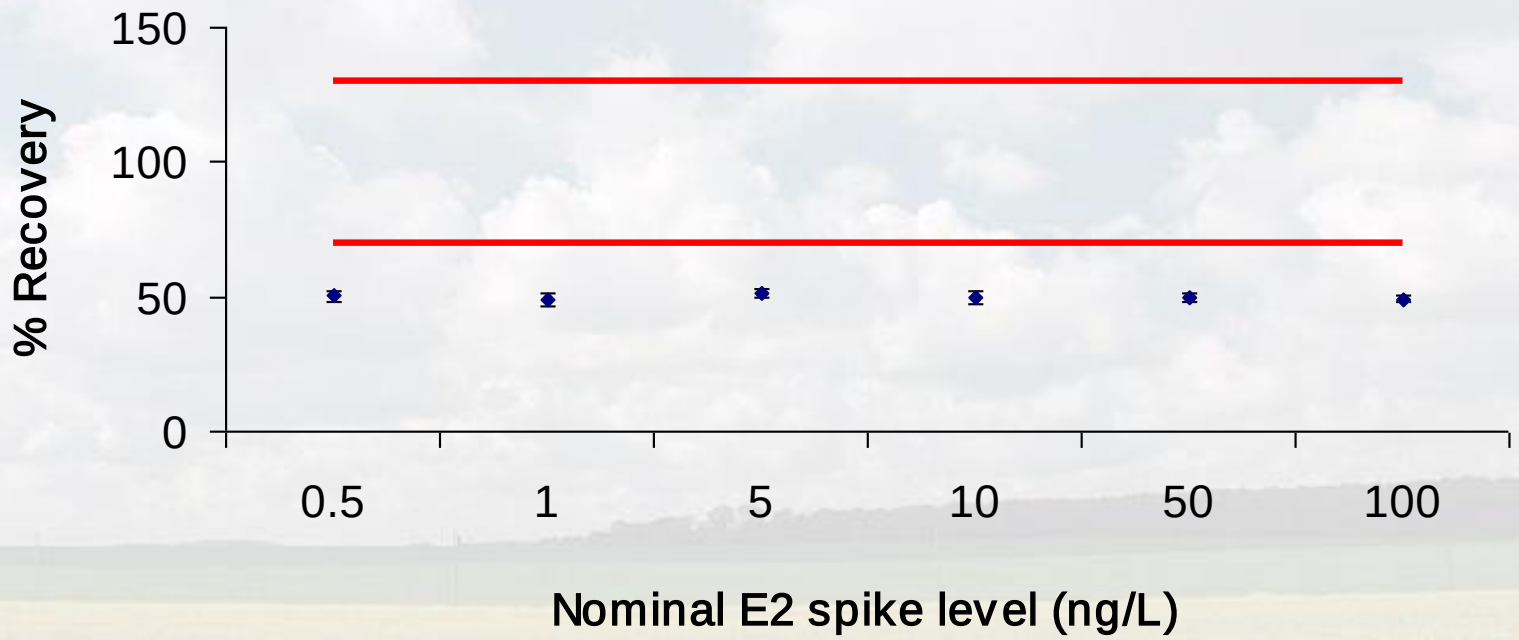
Validation Protocol (V1)

- Stage 1: Assessment of accuracy, precision, linearity and range (Types 1,2 & 3)
- Stage 2: Assessment of negative response and linearity (Types 1, 2 & 3)
- Stage 3: Assessment of specificity and discriminative ability in environmental matrices (Types 1, 2 & 3)
- Stage 4: Assessment of relative potency of oestrogenic compounds (Type 1 only)

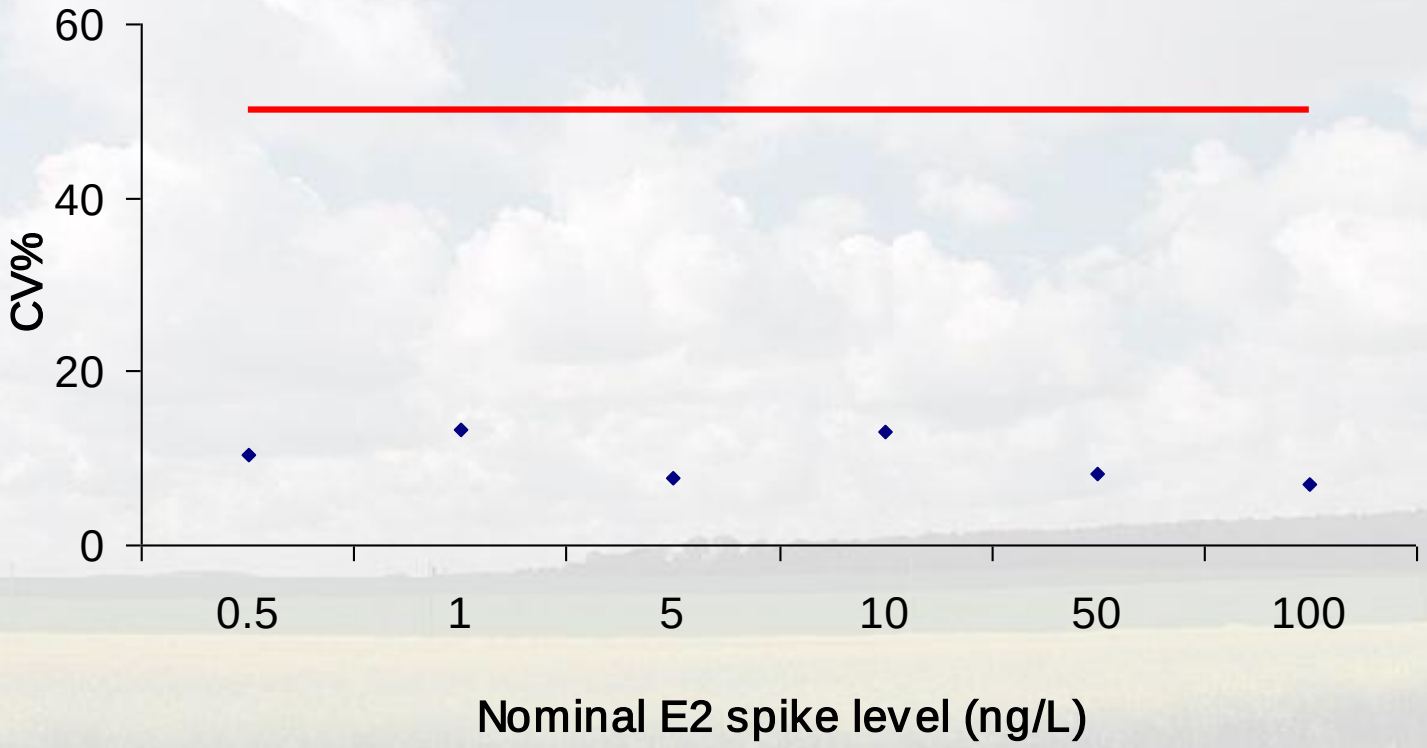
Type 1: Stage 1 Assessment of Accuracy, Precision, Sensitivity, Linearity and Range

- UKEA prepared six independent stocks and from this serial dilutions of 0.5, 1, 5, 10, 50 and 100 ng E2. Sent to method laboratory alongside 250 ml volumes of RO (E2 ng/L).
- Mean recovery: +/- 30%
- CV: <50%
- Calibration curve adhere to a recognised curvilinear model
- Sufficient range and sensitivity

Stage 1 Accuracy: percentage recovery from water Type 1 Method (E-screen)



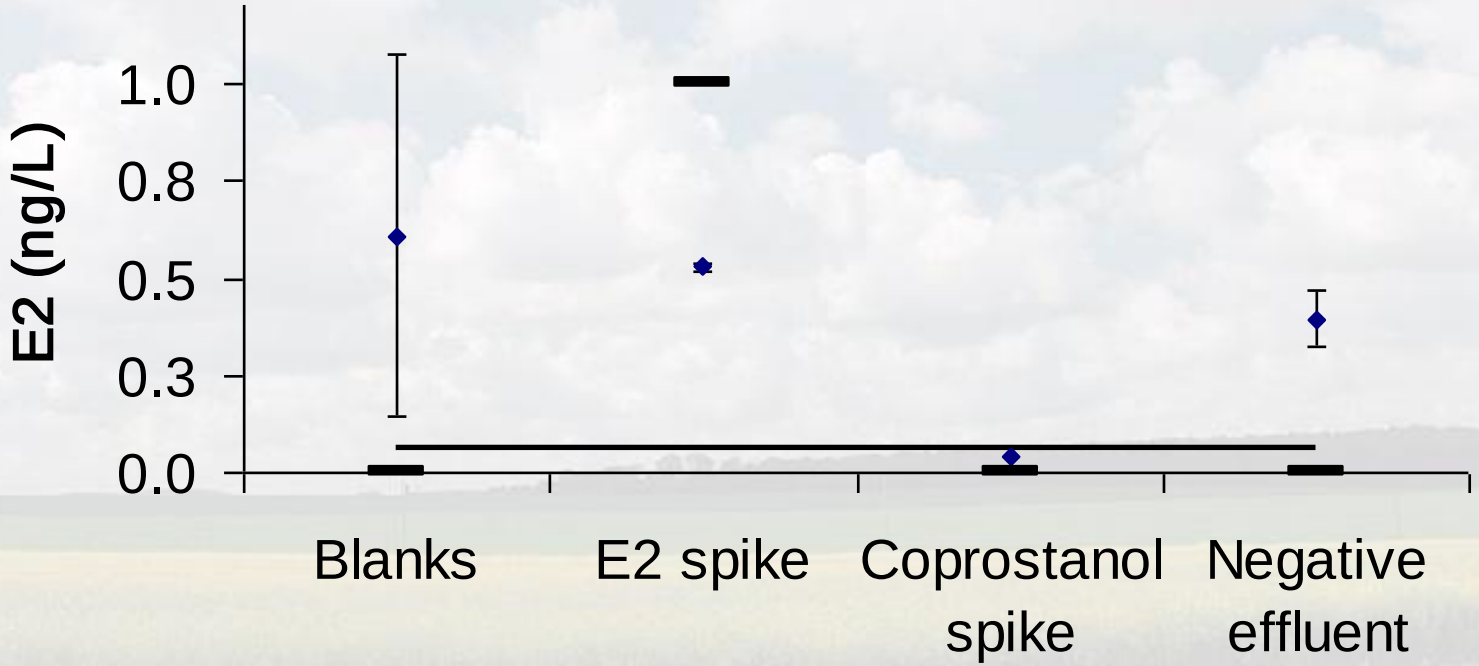
Stage 1 Precision: percentage coefficience of variance in water Type 1 Method (E-screen)



Type 1: Stage 2 Assessment of negative response and selectivity

- UKEA prepared spiked samples of 1 ng E2 and 10 ng Cholesterol. Sent to method laboratory alongside 1 litre volumes of RO (ng/L).
- 6 samples sent of treated sewage effluent (E2 below LOD, negative effluent) and six containing moderate levels of E2 (positive effluent)
- Identify samples spiked with E2 and a negative response for blanks, E1 and negative effluent (below LOD)
- Negative effluent response to be below that of the positive effluent

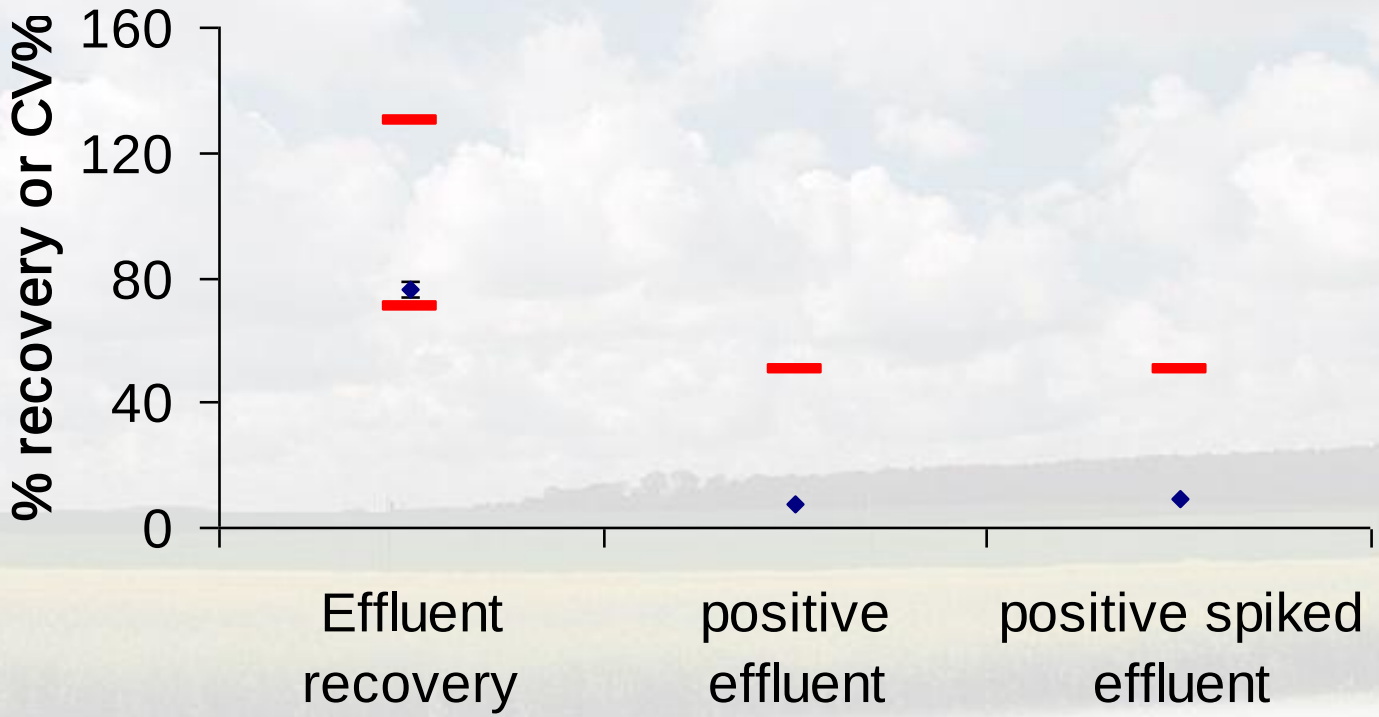
Stage 2: Negative response Type 1 method (E-screen)



Type 1: Stage 3 Assessment of specificity in environmental matrices (E-screen)

- UKEA sent 6 samples of moderately estrogenic effluent (positive effluent) and six samples of positive effluent spiked with 20ng/L E2 (positive spiked effluent).
- Mean recovery: +/- 30%
- CV: <50%

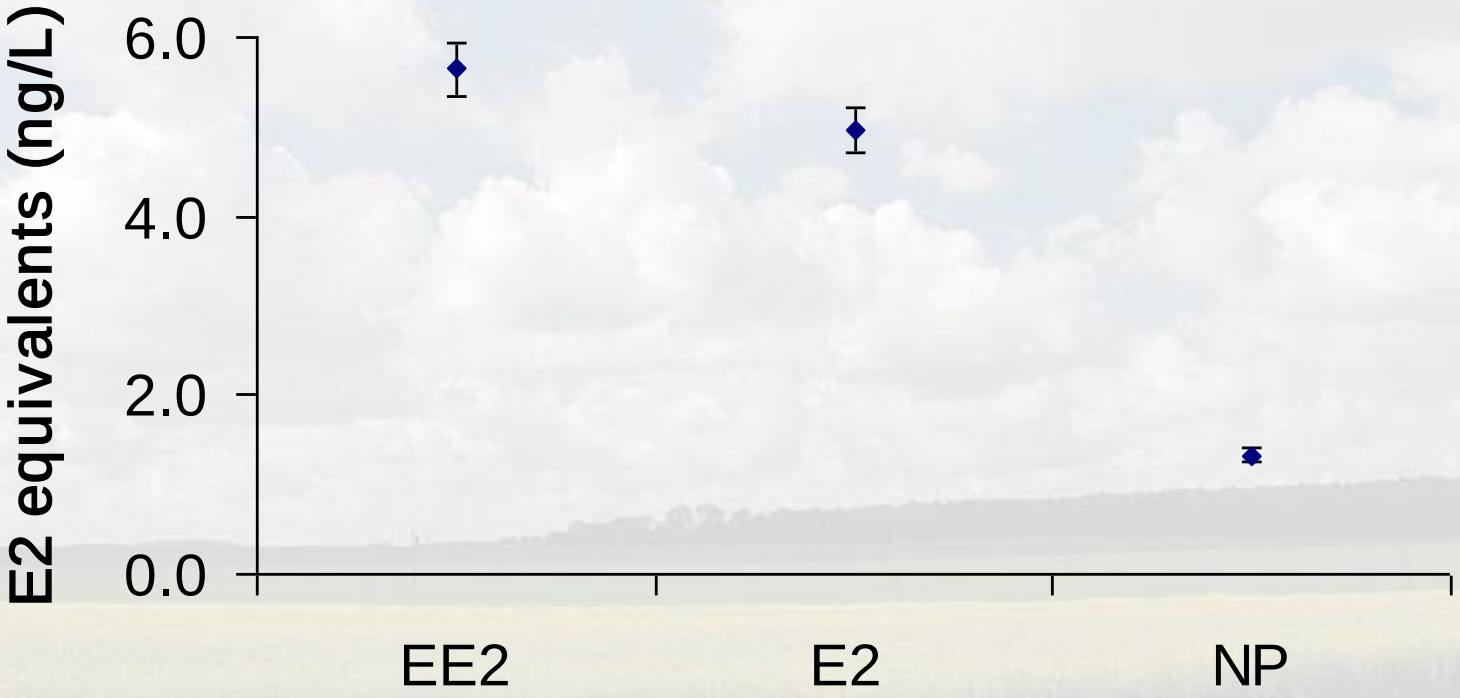
Stage 3 Specificity: percentage recovery and coefficience of variance in water (Type 1 E-screen method)



Type 1: Stage 4 Assessment of Relative Potency of oestrogenic Compounds (E-screen)

- 6 vials spiked with EE2 (10 ng/L) and 6 with 4-NP (10µg/l) alongside 1 litre volumes of RO.
- Potency: EE2>E2>NP

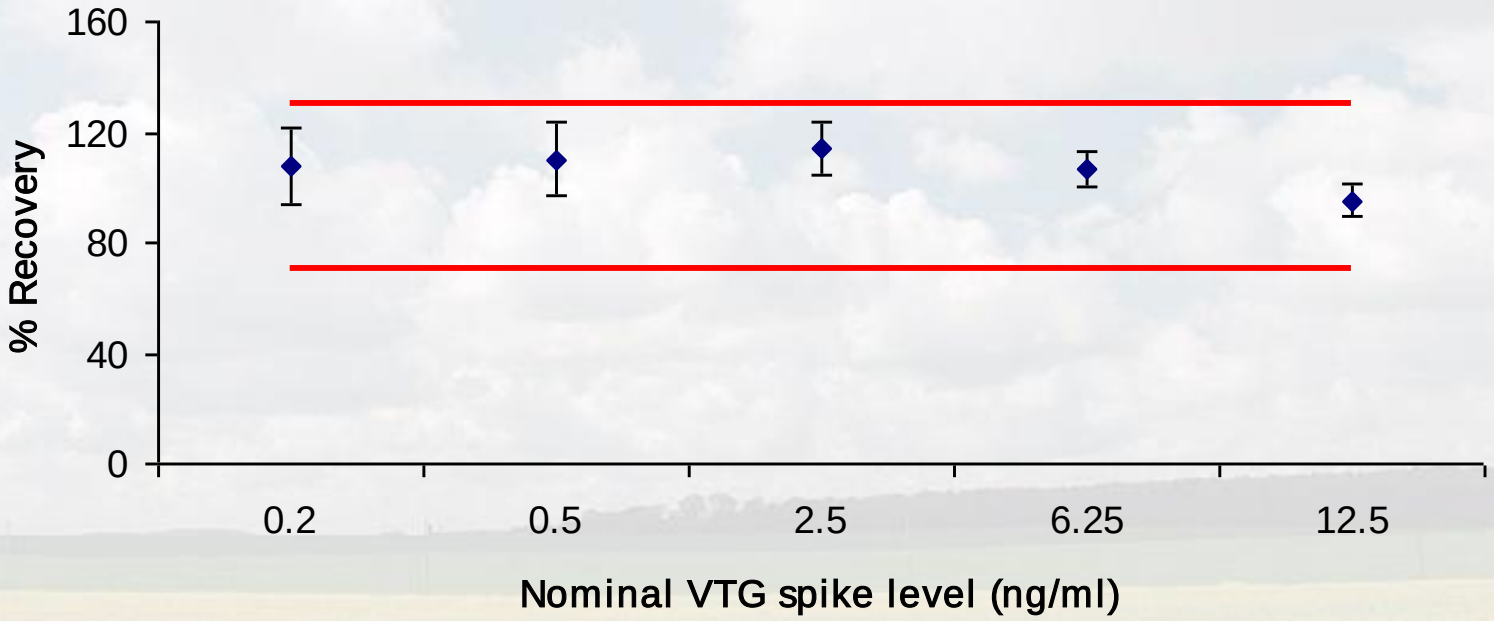
Stage 4: Oestrogenic potency (Type 1 E-screen method)



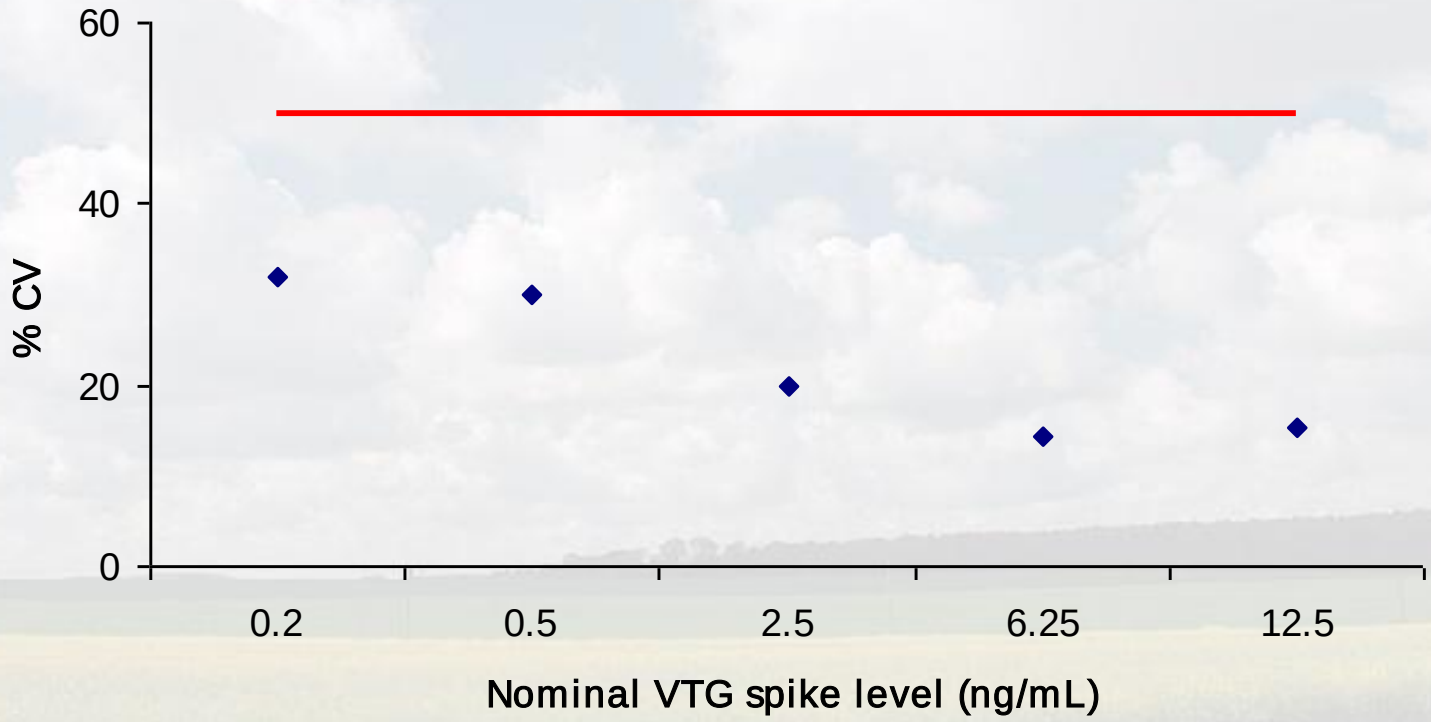
Type 2 method: Stage 1 Assessment of Accuracy, Precision, Linearity and Range

- Low level vitellogenin plasma spiked with a pure lyophilized standard over the assays range (0.2-12.5 ng/ml)
- Assay was conducted on six separate occasions
- Mean recovery: +/- 30%
- CV: <50%
- Calibration curve adhere to a recognised curvilinear model
- Sufficient range and sensitivity

Stage 1 Accuracy: percentage recovery from plasma Type 2 Method (Vtg ELISA)



Stage 1 Precision: percentage coefficience of variance in plasma Type 2 Method (Vtg ELISA)



Type 2 method: Stage 2 Assessment of sensitivity, negative response and selectivity

- Method was altered as the UKEA were unable to provide fish plasma from unexposed males, males contaminated with an unrelated Fathead Minnow blood protein and males with a low level of Vtg
- Only negative response was assessed
- Validation laboratory spiked 6 of 12 unexposed plasma samples with low levels of Vtg (0.01 ng/ml).
- Method assessed by its ability to identify samples spiked with Vtg

Stage 2: Negative response Type 2 method (Vtg ELISA)

Sample no.	Vtg (ng/ml)
1	0.1
2	0.1
3	0.1
4	<0.0123
5	<0.0123
6	<0.0123
7	0.1
8	0.1
9	<0.0123
10	<0.0123
11	0.1
12	<0.0123

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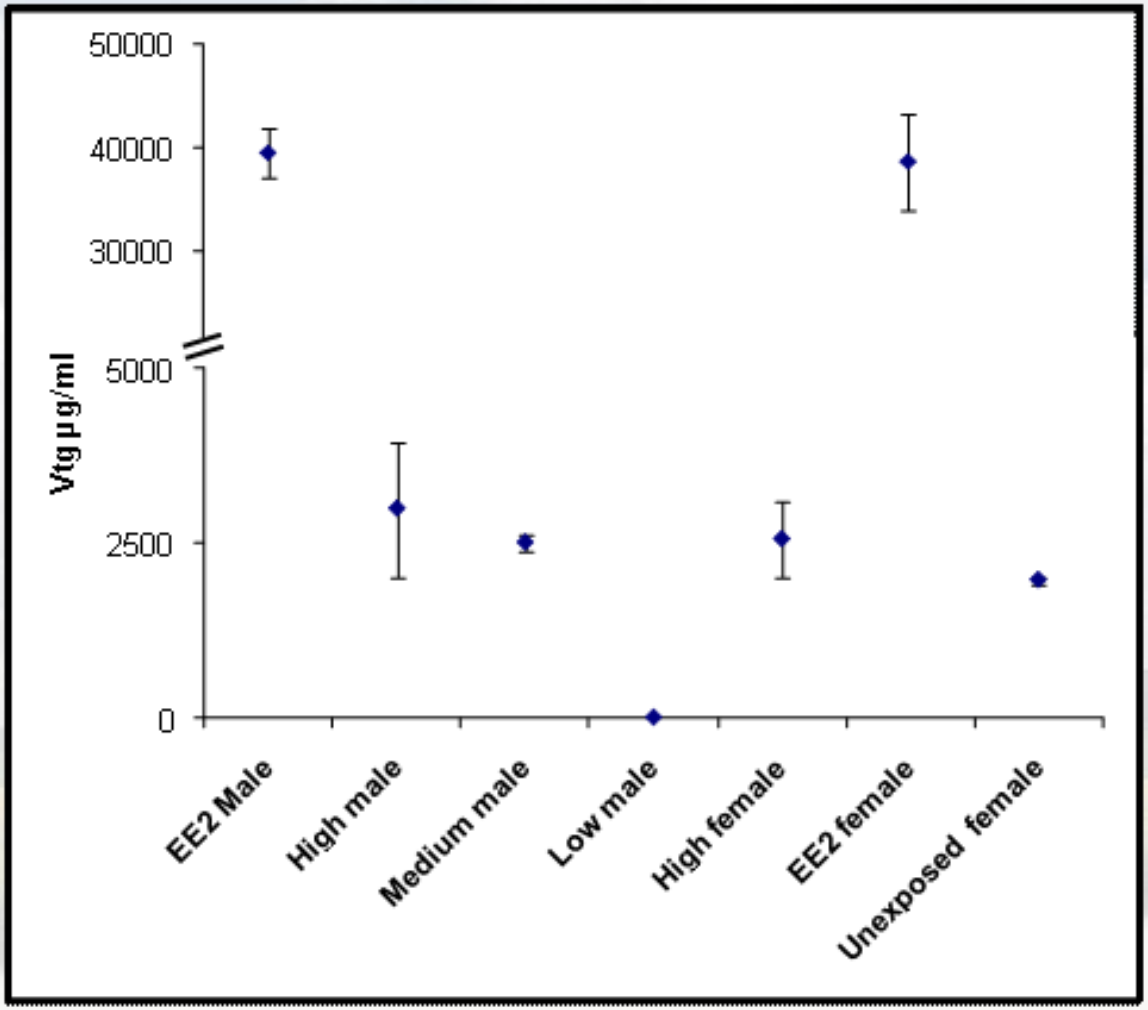
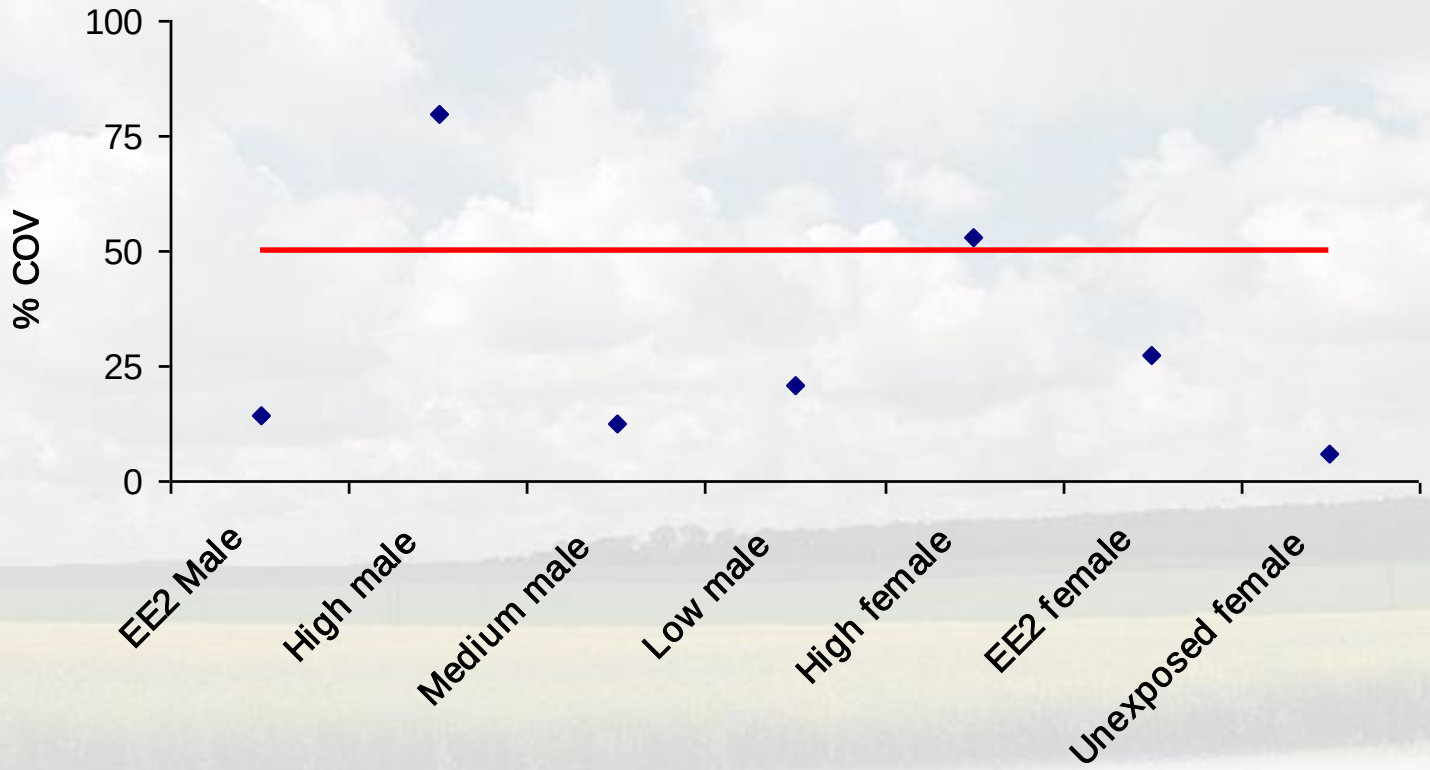
Type 2: Stage 3 Assessment of sensitivity and discriminative ability

- UKEA provided plasma from male and female fish exposed to 18 ng/L EE2 and those exposed to effluents of varying degrees of estrogenicity.
- Method must be able to detect and discriminate between those exposed to estrogens and those not exposed.
- CV: <50%
- Mean Vtg of males exposed to EE2: >750 ng/ml
- Mean Vtg for males exposed to the effluents: high>medium>low
- Mean Vtg for female exposed to EE2 and estrogenic effluents > unexposed

Stage 3 Discriminative ability: Type 2 method (Vtg ELISA)



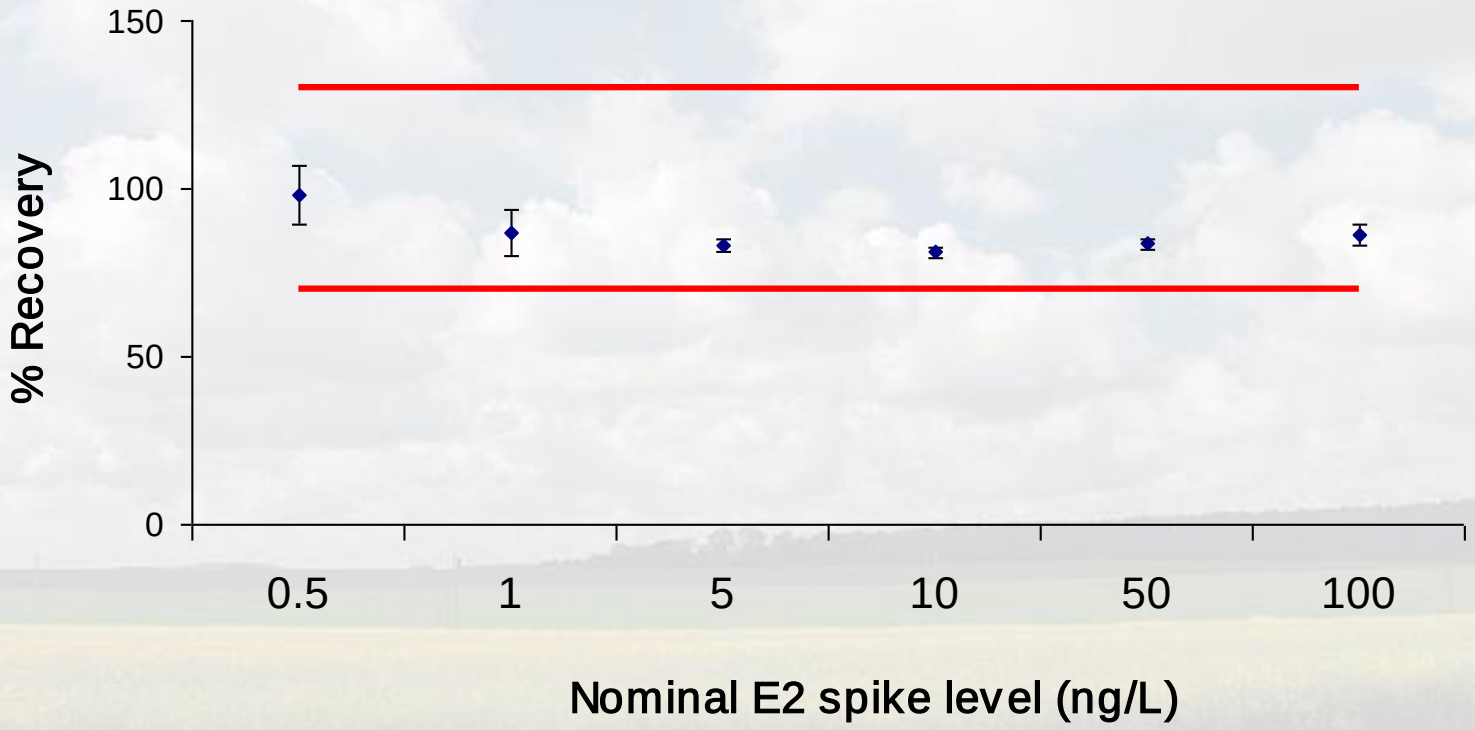
Stage 3 Precision: percentage coefficience of variance in plasma Type 2 method (Vtg ELISA)



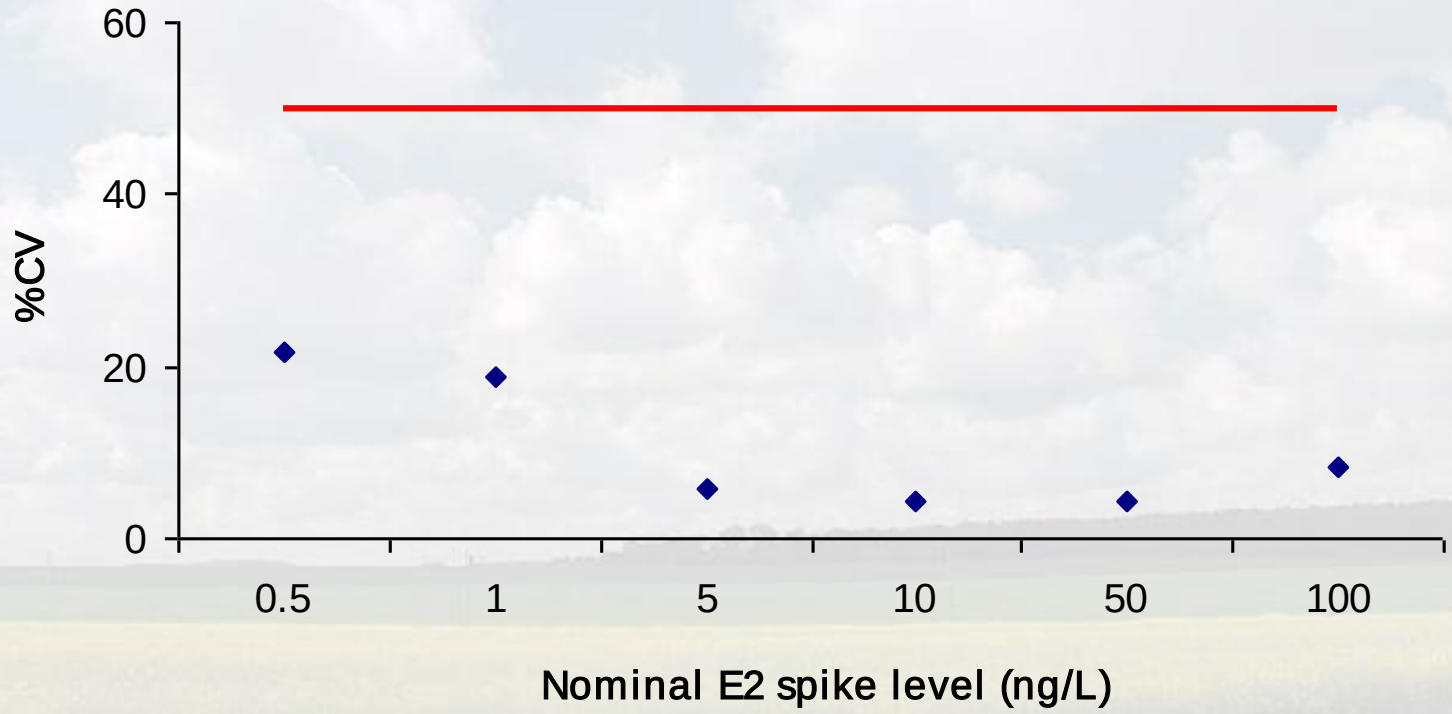
Type 3: Stage 1 Assessment of Accuracy, Precision, Sensitivity, Linearity and Range

- UKEA prepared six independent stocks and from this serial dilutions of 0.5, 1, 5, 10, 50 and 100 ng E2. Sent to method laboratory alongside 500 ml volumes of RO (E2 ng/L).
- Mean recovery: +/- 30%
- CV: <50%
- Calibration curve adhere to a recognised curvilinear model
- Sufficient range and sensitivity

Stage 1 Accuracy: percentage recovery from water Type 3 Method (ELISA)



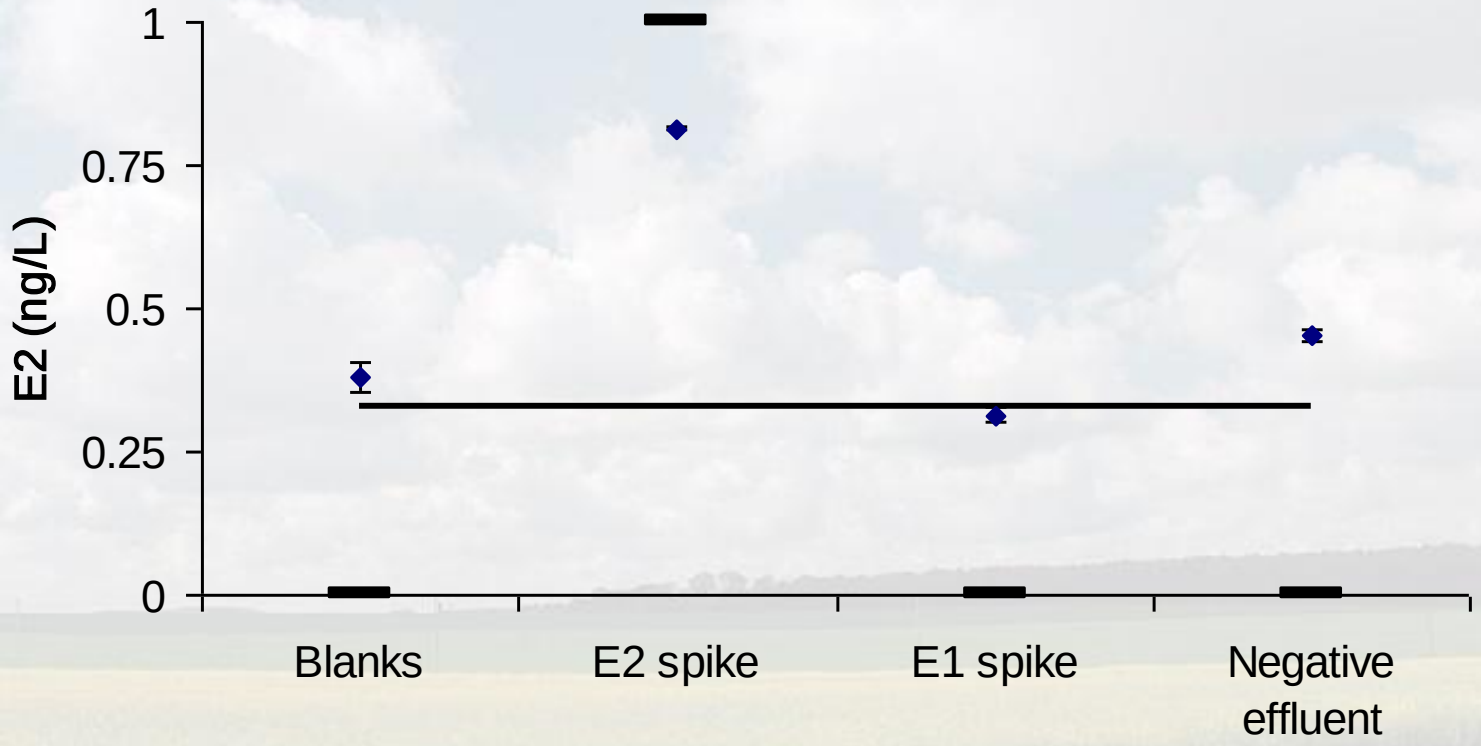
Stage 1 Precision: percentage coefficience of variance in water Type 3 Method (ELISA)



Type 3: Stage 2 Assessment of negative response and selectivity

- UKEA prepared spiked samples of 1 ng E2 and 10 ng E1. Sent to method laboratory alongside 1 litre volumes of RO (ng/L).
- 6 samples sent of treated sewage effluent (E2 below LOD, negative effluent) and six containing moderate levels of E2 (positive effluent)
- Identify samples spiked with E2 and a negative response for blanks, E1 and negative effluent (below LOD)
- Negative effluent response to be below that of the positive effluent

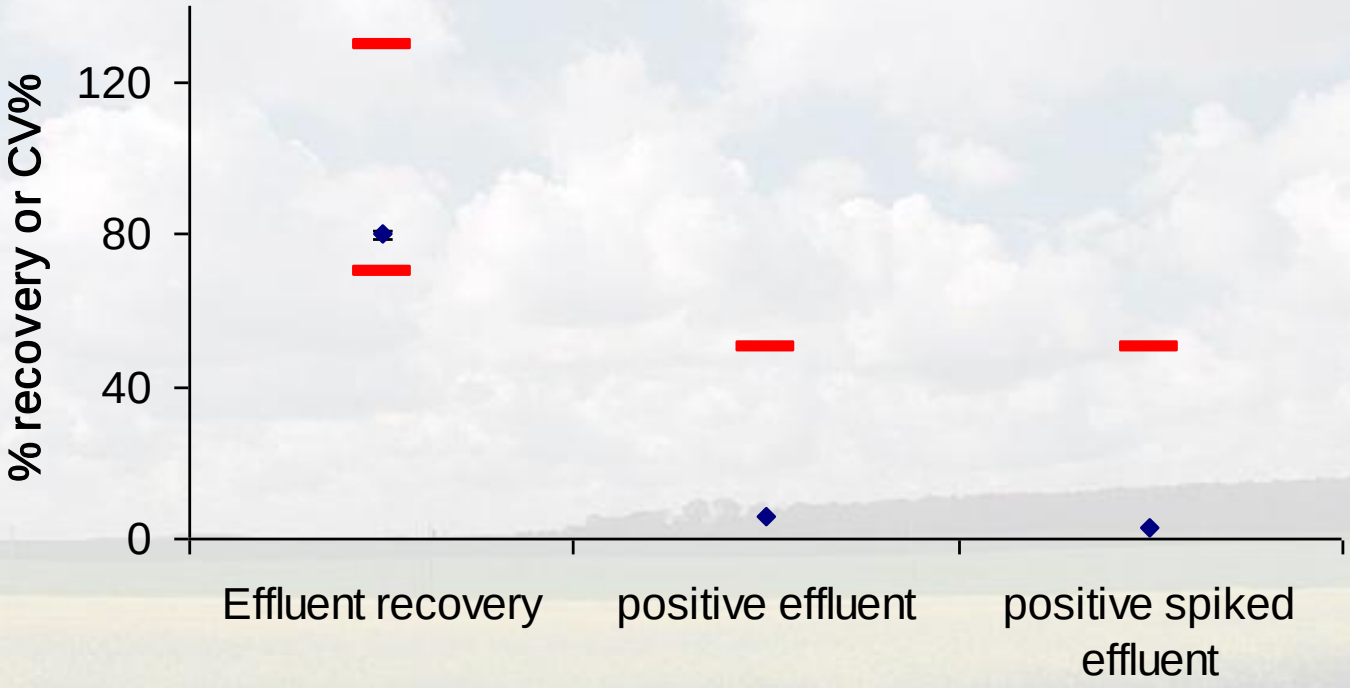
Stage 2: Negative response Type 3 method (ELISA)



Type 3: Stage 3 Assessment of specificity in environmental matrices

- UKEA sent 6 samples of moderately estrogenic effluent (positive effluent) and six samples of positive effluent spiked with 20ng/L E2 (positive spiked effluent).
- Mean recovery: +/- 30%
- CV: <50%

Stage 3 Specificity: percentage recovery and coefficience of variance in water (Type 3 ELISA method)



Summary of the results for type 1-3 methods

Stage	Performance characteristic	Type 1 method (E-Screen)	Type 2 method (Vtg)	Type 3 method (ELISA)
1	Accuracy	<70%	70-130%	70-130%
	Precision	<50%	<50%	<50%
	Linearity	OK	OK	OK
	Sensitivity	OK	N/A	OK
	Range	None	0.2-12.5 ng VTG /ml	0.5-100 ng E2 /L
2	Negative response	Unspiked>LOD	OK	Unspiked>LOD
	Selectivity	OK	Could not be tested	OK
3	Specificity	OK	N/A	OK
	Discriminative ability	N/A	Some difficulties in discrimination between samples	N/A
4	Relative potency	OK	N/A	N/A

Conclusions and recommendations

- Extent to which methods had previously undergone method validation varied greatly
- Blind study invaluable in assessing methods limitations as well as advantages
- Requirement for more assessment during selection process – assay may not be fit for purpose or has specific needs
- Increased amount of chemical analysis to reduce uncertainty
- Recognition for participants