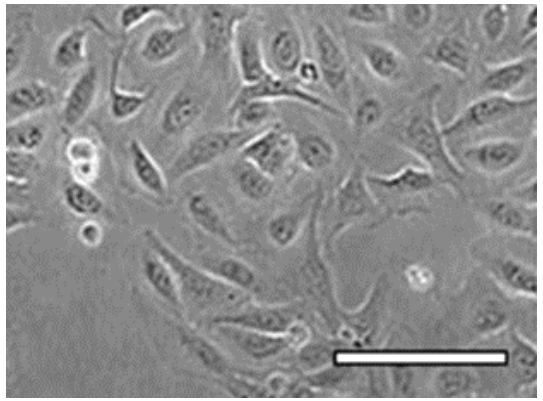
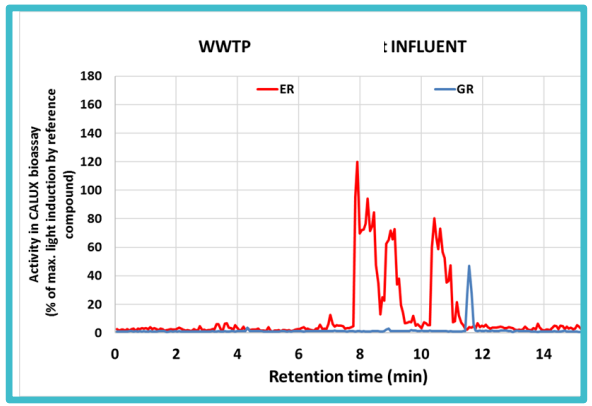
Hormone-like activities in water: which compounds are responsible?

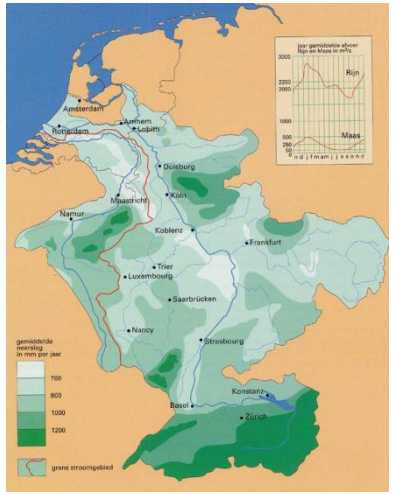




Corine J. Houtman, Rob ten Broek, Dennis Kloes, Yvonne van Oorschot, Martine Rosielle, Bas Spanhaak, Marja H. Lamoree

HET WATERLABORATORIUM

Introduction – The Water Laboratory



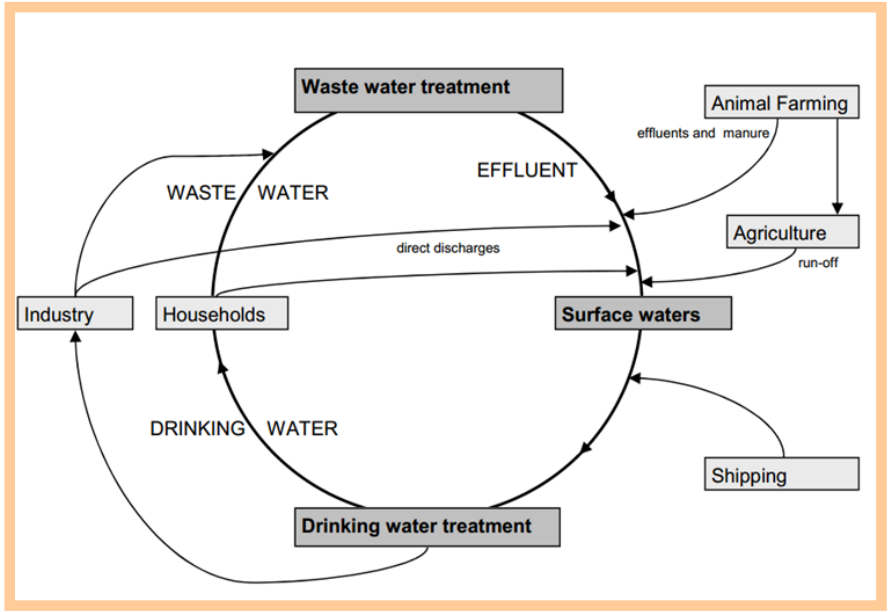
- Central laboratory for 3 Dutch Drinking Water companies
- Use surface water from rivers Rhine, Meuse and Lake Yssel and dune infiltration to prepare drinking water

- Monitoring and Research on presence and toxicology of chemical contaminants in the watercycle

- Using:
  - Chemical target compound analyses
  - Bioassays
  - Screening
  - Combinations thereof

- European and Dutch Legislation
- ***Risk-based approach*** (adopted in EU proposal for a Directive dw, Feb 2018)

  => more room for screening and bioassays in legislative monitoring.

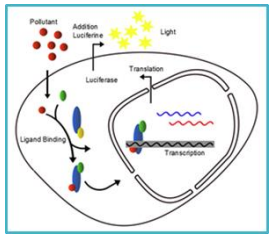


Introduction

- Endocrine disruption is one of the toxicological endpoints relevant for drinking water quality….
- **Former study:**
  - Do known steroids (endogenous, pharmaceuticals) explain steroid hormone activities in WWTPs?

Target analysis

$$\text{Activity}_i = \text{Conc}_i * \text{Relative potency} * \text{Mw}_{ref.comp}/\text{Mw}_i$$

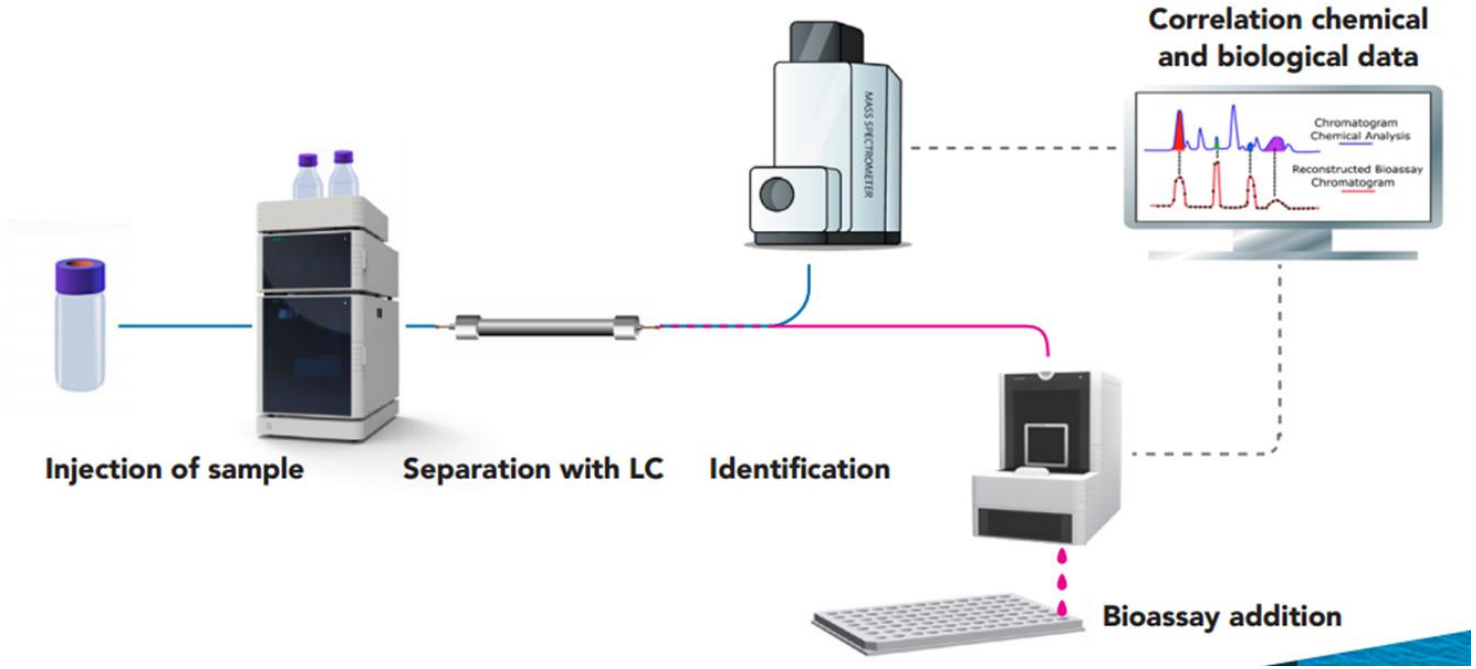


CALUX reporter gene bioassays

- **Conclusion:**
  - 13 steroids detected
  - Fair part of activity explained;
  - Also unexplained activity: e.g. glucocorticoids and anti-androgens in effluent
  - Approach works well if you know which compounds might be involved
  - Houtman et al., BioDetectors 2017 and STOTEN 2018

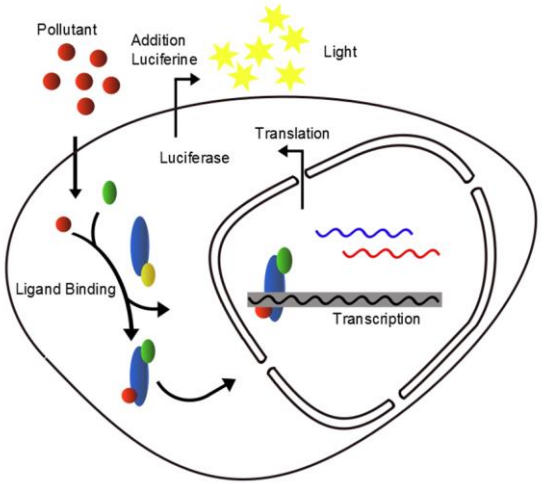
Novel EDA-platform

(VU University Amsterdam, Nick Zwart and Willem Jonker)



- Spot small fractions to bioassay in 384 well format
- “bioassay chromatogram”
- High resolution of fractionation, 1: 1 identification
- Spotter on sale: FractioMate

Steps in Effect-directed Analysis

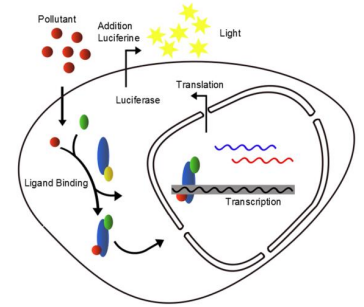


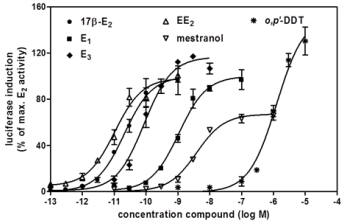
a) Bioassay non-fractionated sample

- CALUX reporter gene assays for **AR**, **anti-AR**, **ER** and **GR** activity

- Androgenic => dihydrotestosterone (DHT)
- Anti-androgenic => flutamide (AR CALUX with addition of DHT on ~EC40 level)
- Estrogenic (ERα) => 17β-estradiol (17β-E2)
- Glucocorticoid => dexamethasone (Dex)

Steps in Effect-directed Analysis





a) **Bioassay non-fractionated sample**
   - CALUX reporter gene assays for **AR**, **anti-AR**, **ER** and **GR** activity

b) **Bioassaychromatograms**
   - Separation on UPLC: 288 fractions
   - CALUX reporter gene assays for **AR**, **anti-AR**, **ER** and **GR** activity
   - Bruker QToF MS

c) **Identification QToF-MS**
   - Bruker software
   - Software and databases on internet

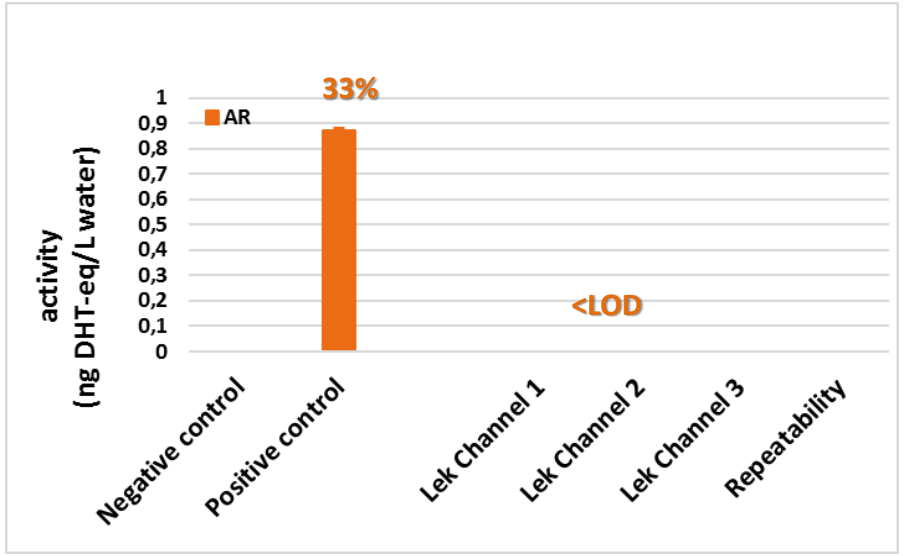
d) **Confirmation with analytical standard**
   - Tret on UPLC
   - Activity in CALUX bioassay

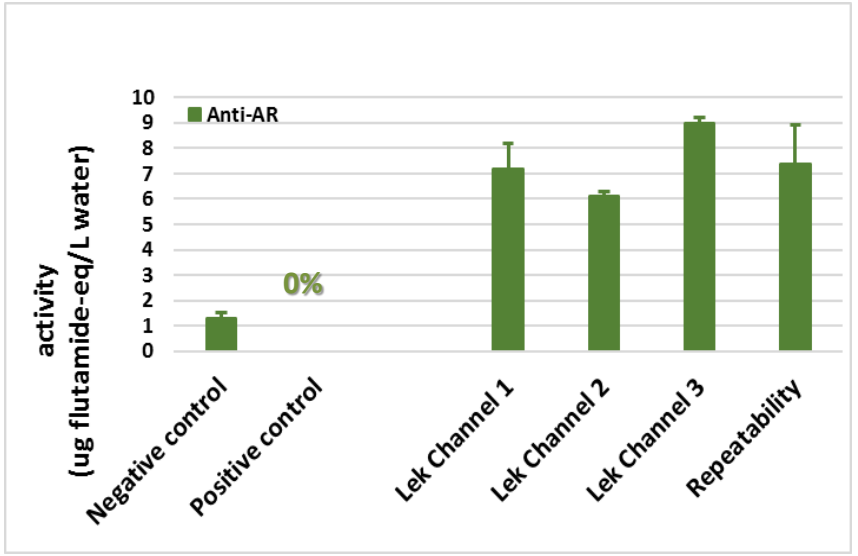
Samples

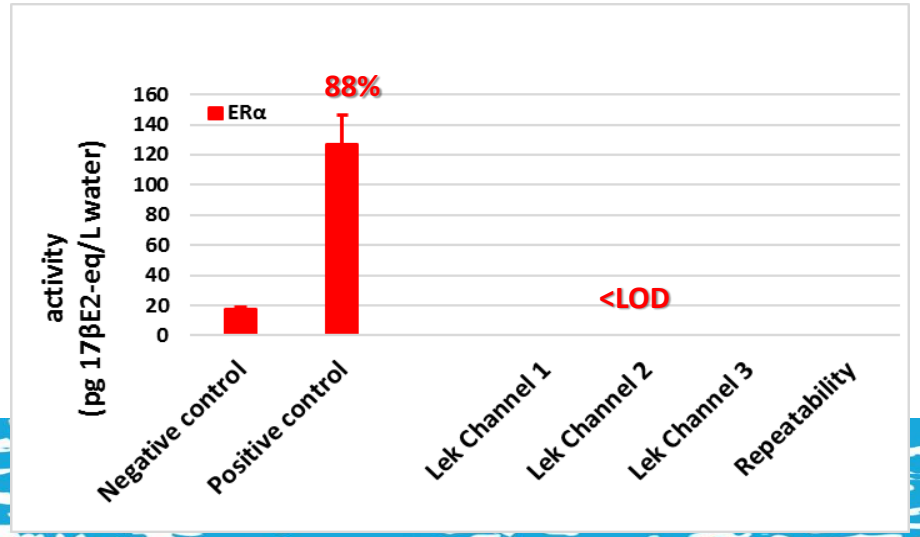
- **Control samples**
  - Negative controls
  - Positive controls (spiked with reference compounds bioassays)
  - Repeatability

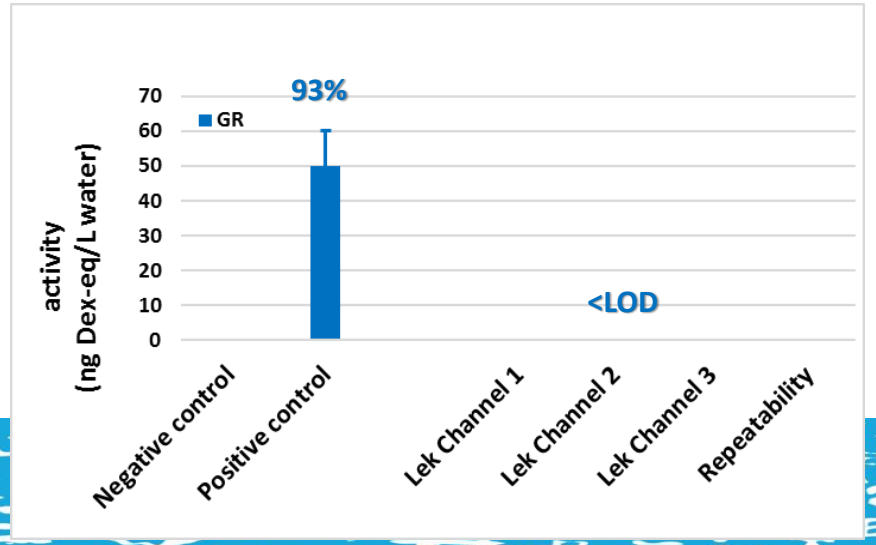
- **Real samples**
  - Surface water
  - Effluent WWTP plant

a. Control samples non-fractionated



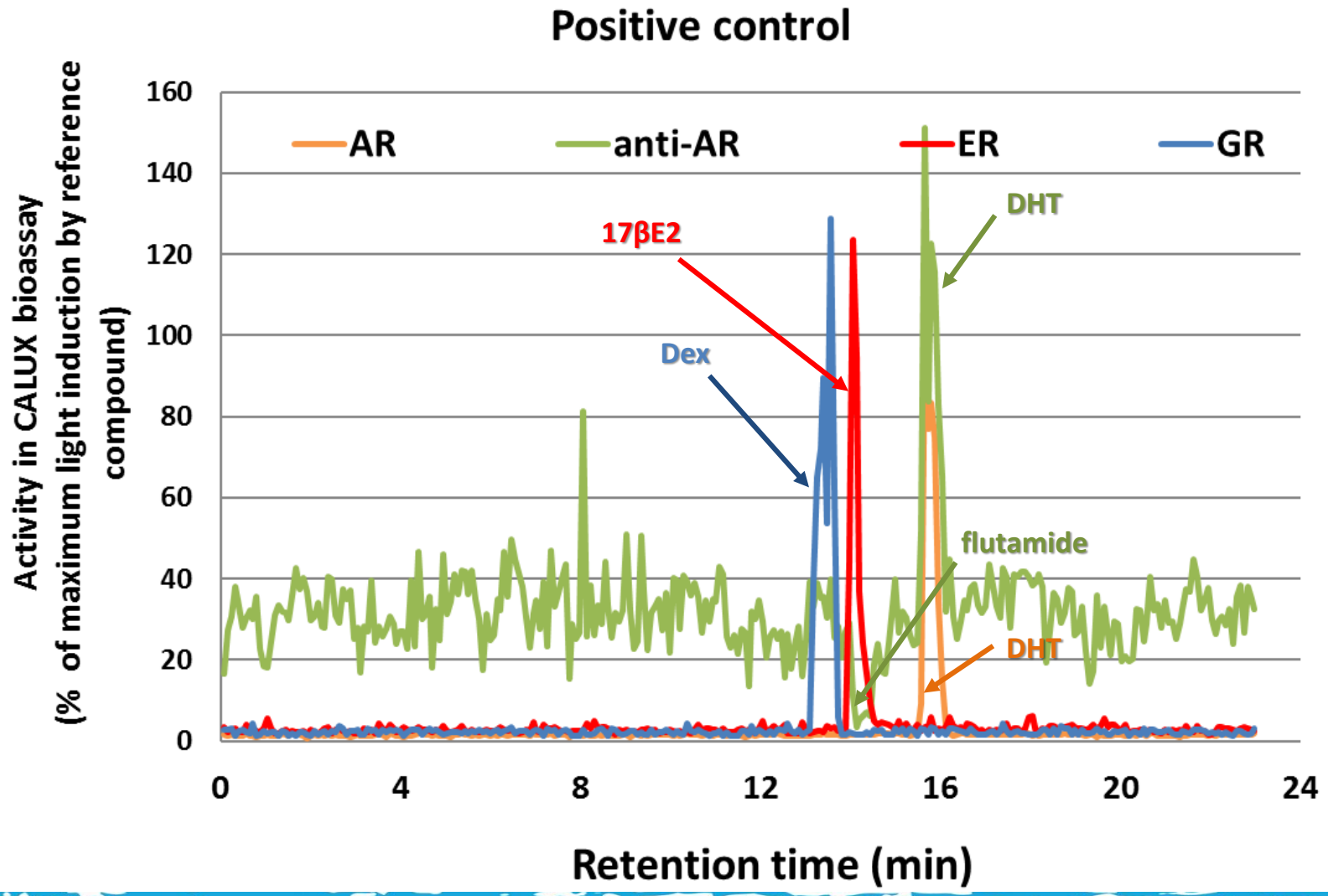






- Negative controls: OK
- Positive controls: OK
- Positive controls **AR** and **anti-AR**: low responses due to mutual masking of activity
- Repeatability: OK: variation <20%
- Repeatability: “bad luck”: only **anti-AR** activity in this sample

b. Control samples: bioassaychromatograms



- Matching with Tret on UPLC-QToF MS

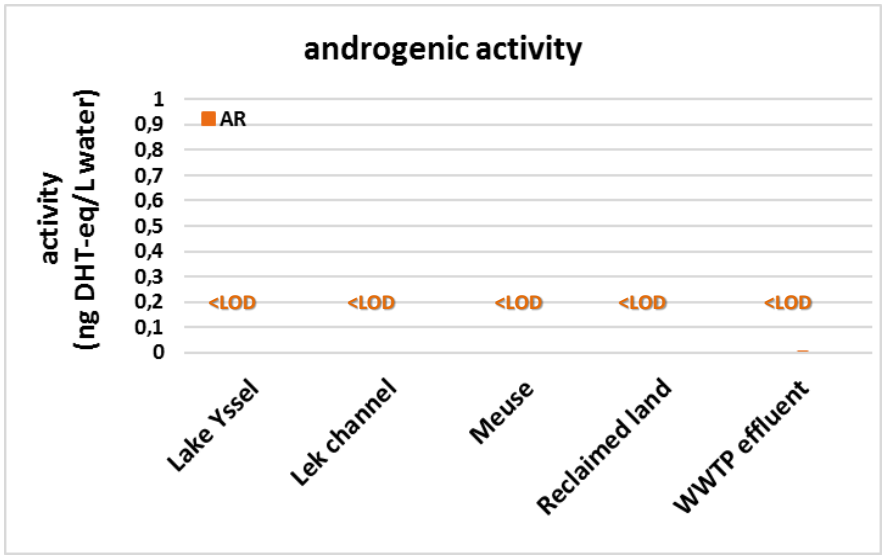
Collection real samples

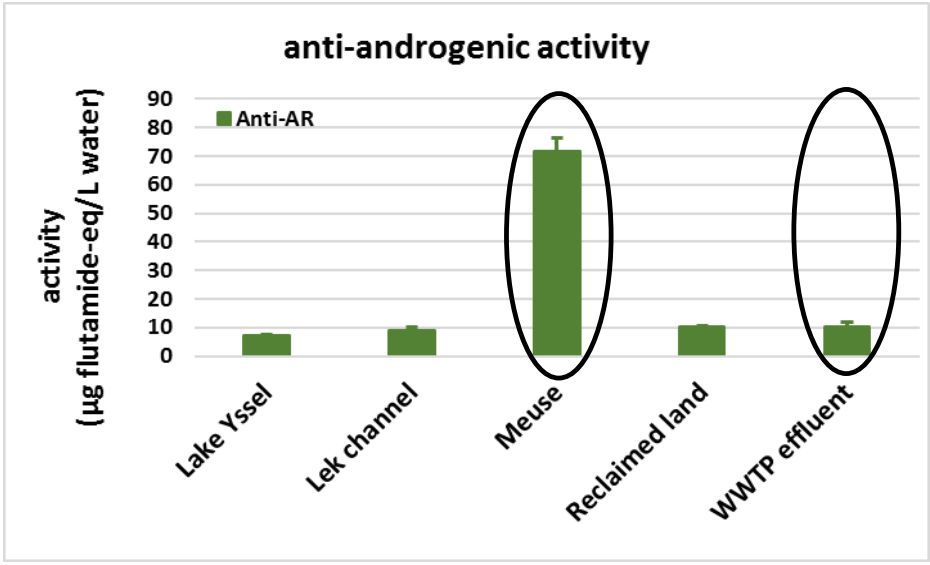
Intake points surface water for dw production

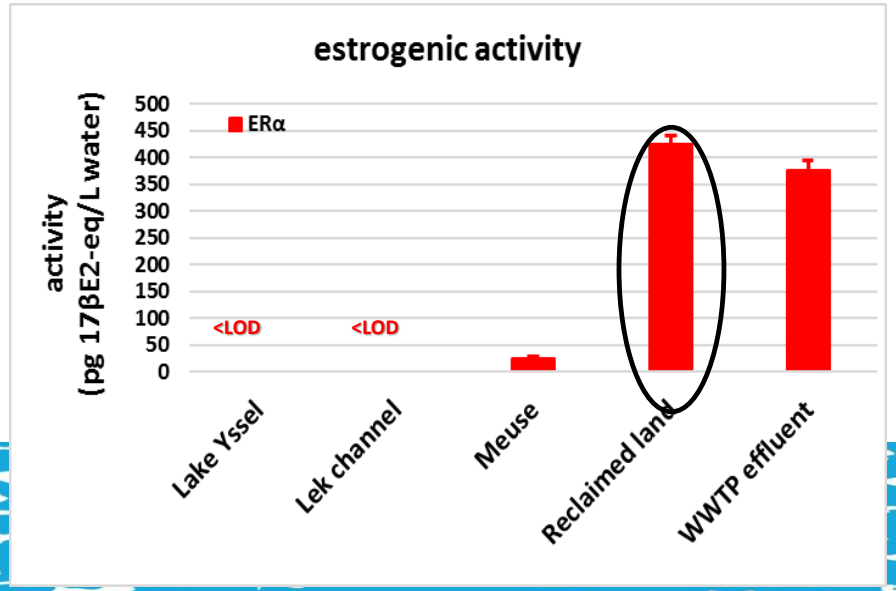
- Lake Yssel
- Lek channel
- Meuse
- Reclaimed land Bethunepolder

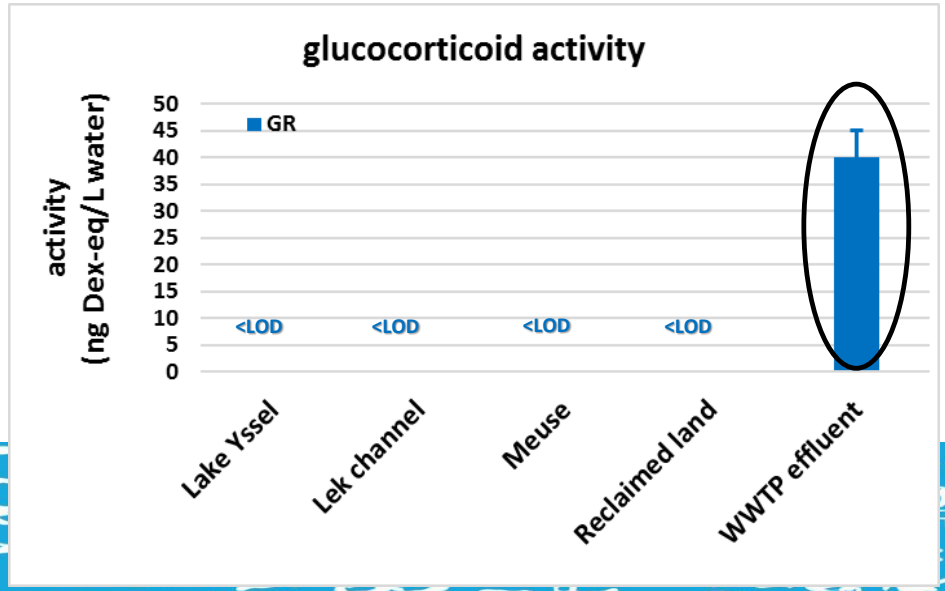
WWTP effluent

a. Activity in non-fractionated samples

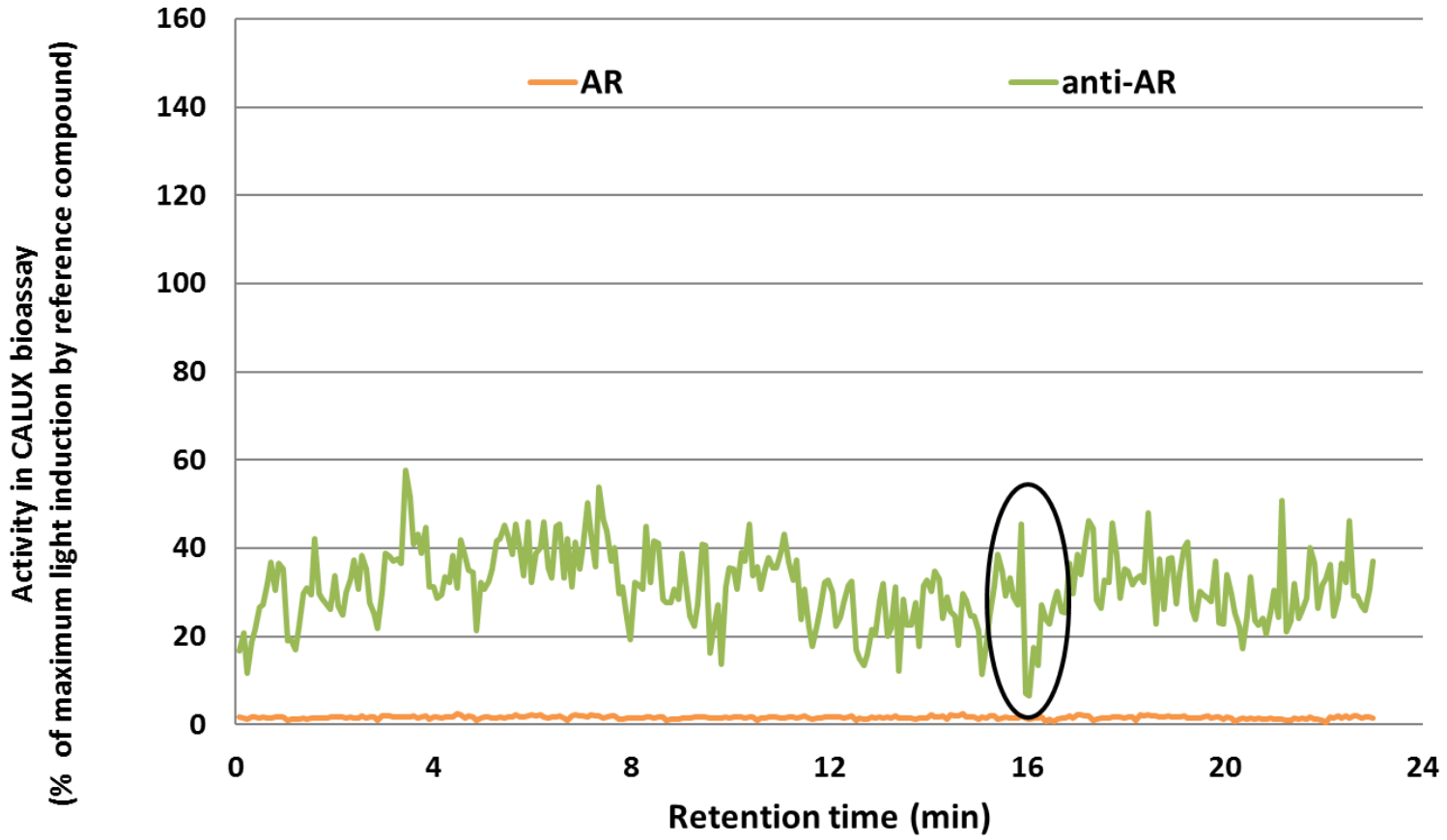






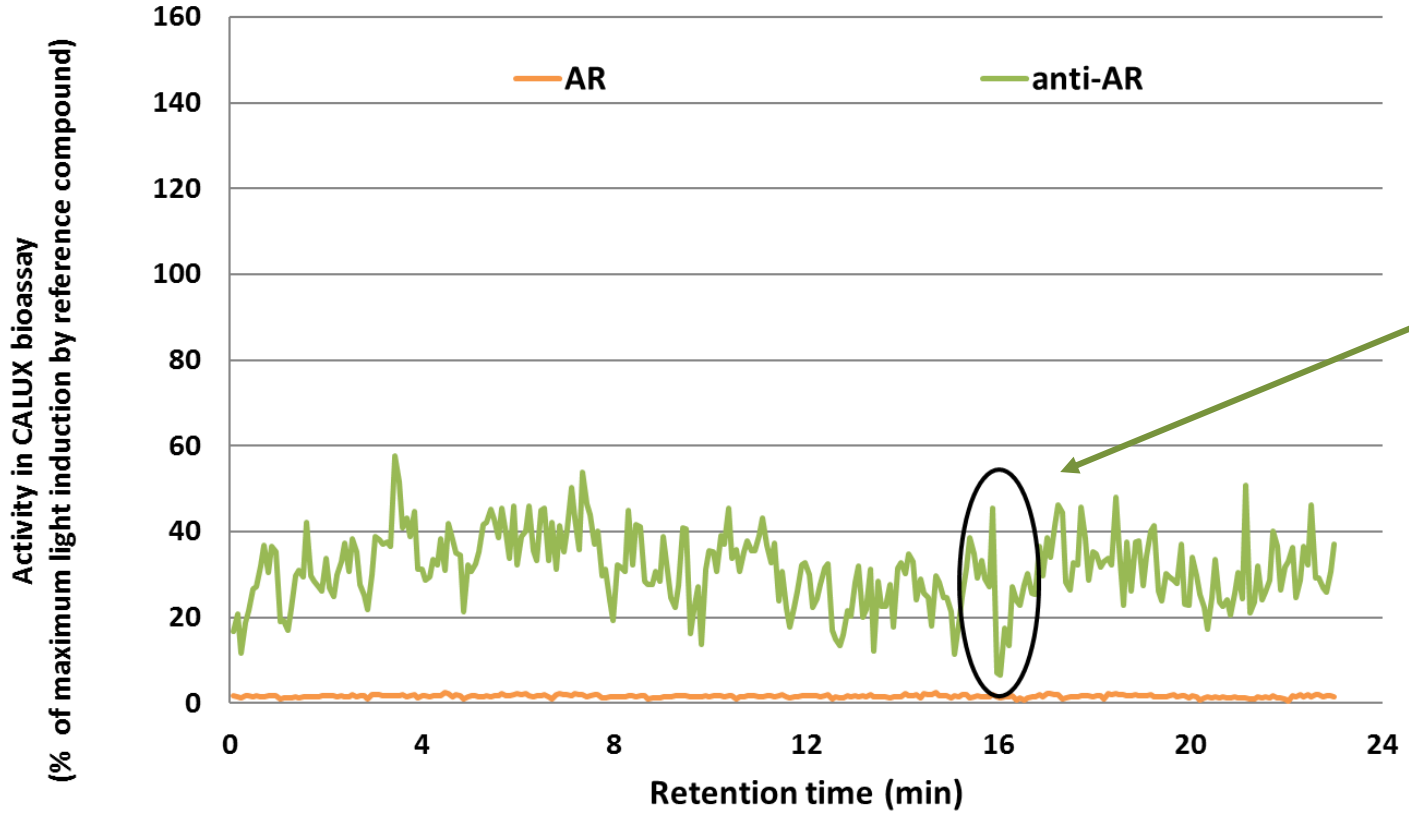


b. Bioassaychromatogram Meuse: activity in anti-AR CALUX



- Indeed negative peak of anti-androgenic activity
- No peak in AR => no mutual masking of AR and anti-AR compounds in this sample

c. Identification by UPLC-QToF-MS

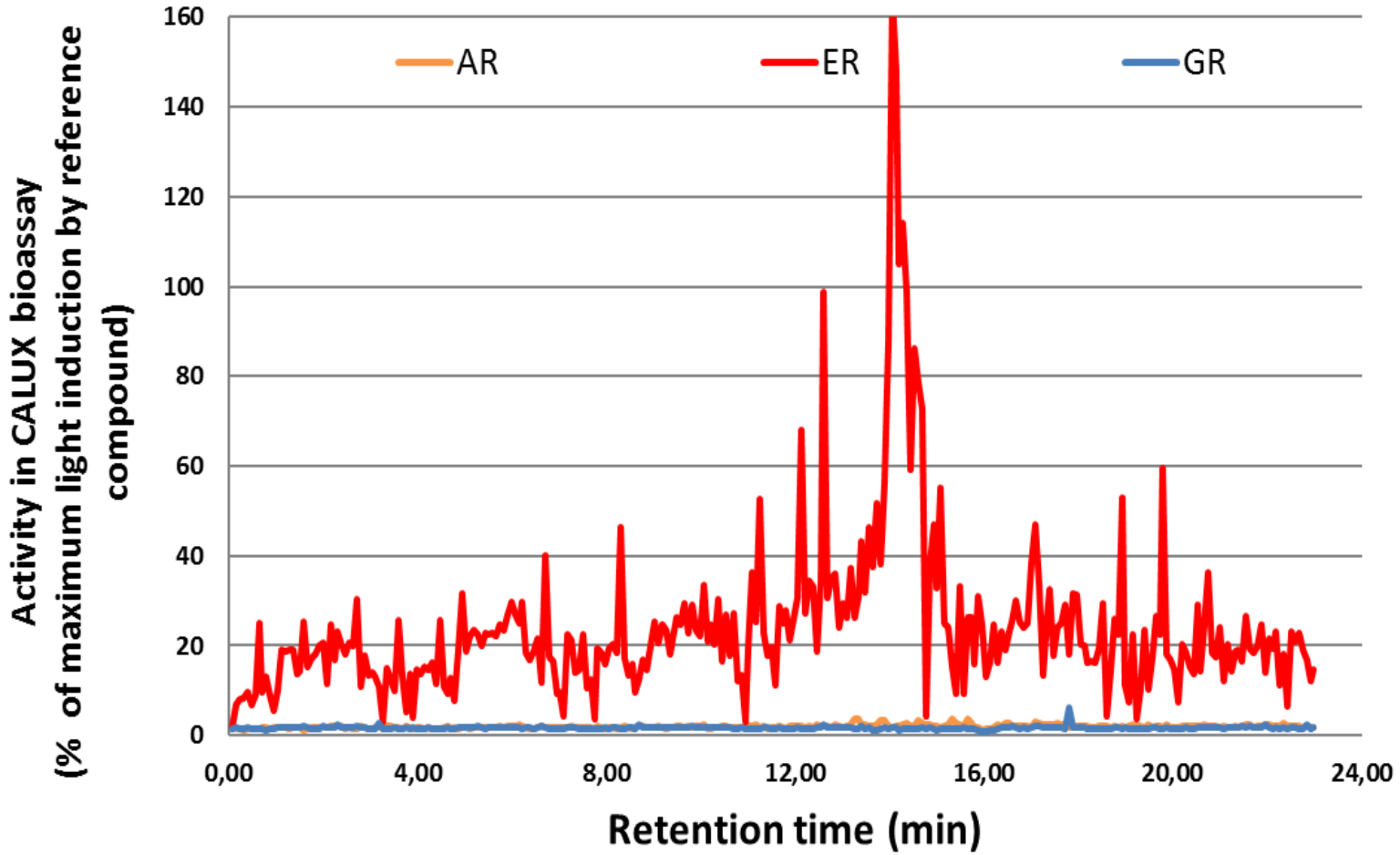


Identified compounds a.o.:

- Fatty acids
- Sulfonic acids
- **Capsaïcin**
  - Chilli pepper
  - (AR)↓ (Zheng et al, 2015)
  - Candidate therapy prostate cancer

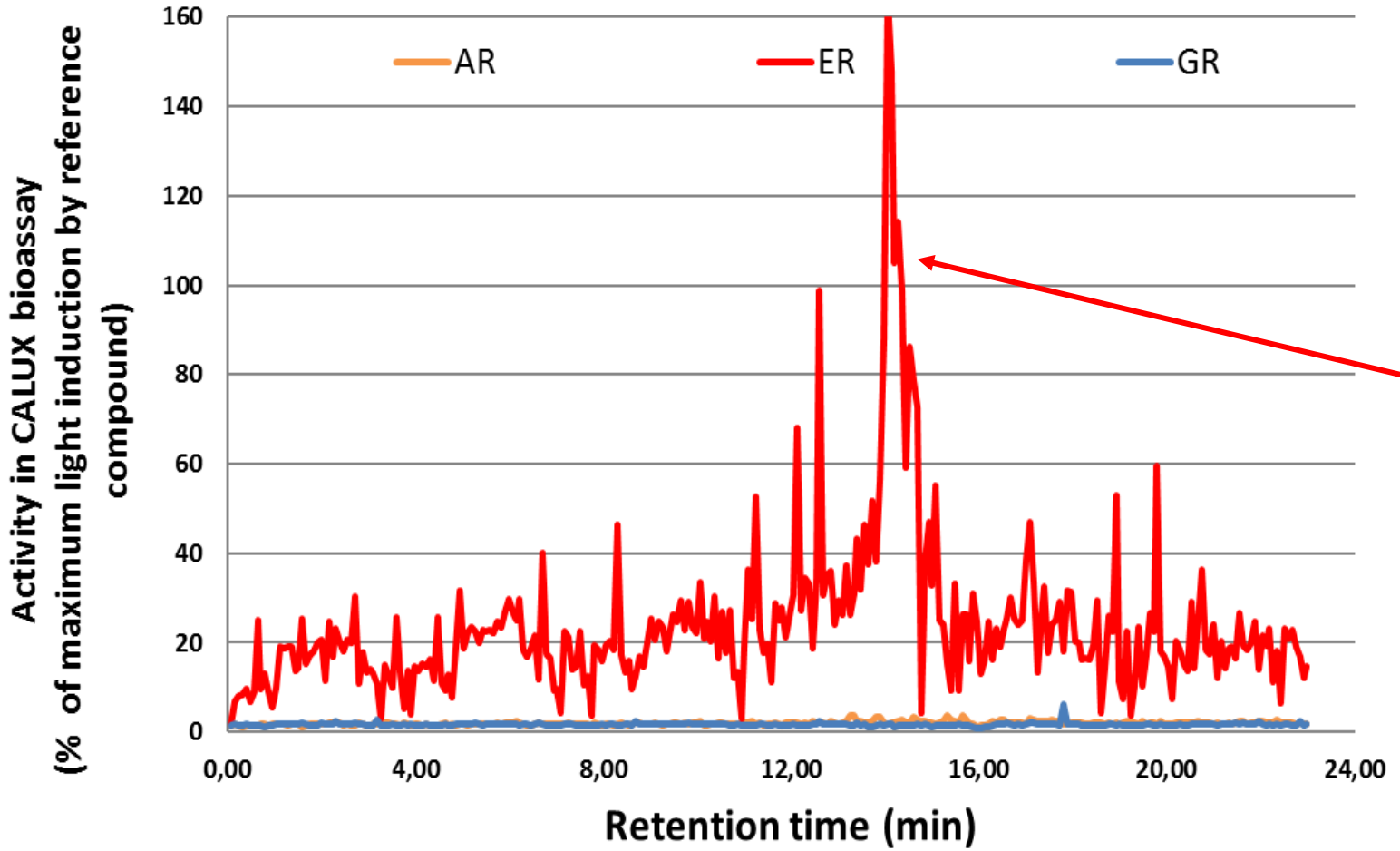
d. Confirmation with analytical standard will follow…

b. Bioassaychromatogram Reclaimed land: activity in ER CALUX



- Large peak(s) around Tret = 14.05 min.
- Tret = 14.05 min is Tret of 17β-E2 (reference compound).

b. Bioassaychromatogram Reclaimed land: activity in ER CALUX



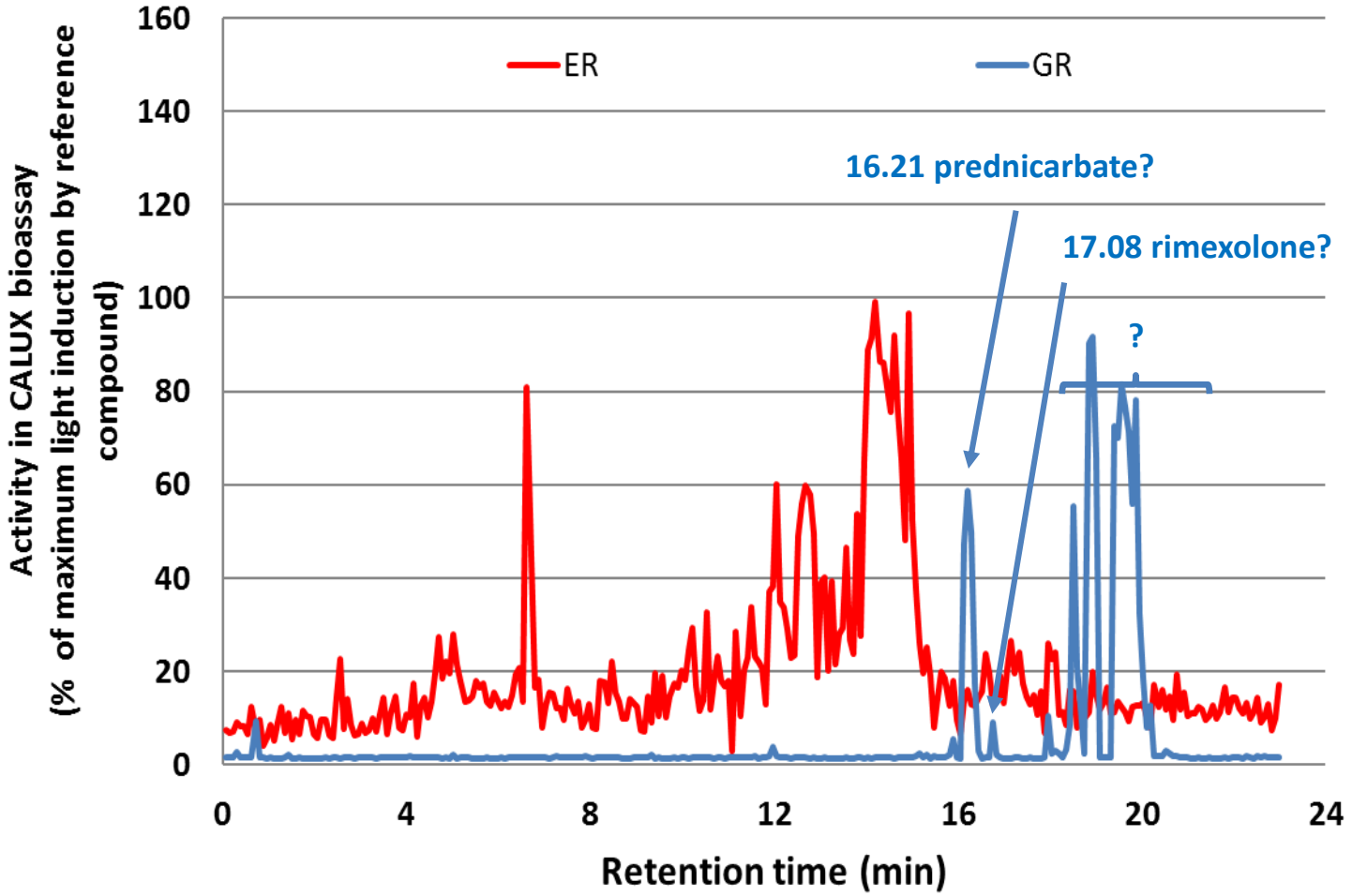
- Large peak(s) around Tret = 14.05 min.
- Tret = 14.05 min is Tret of 17β-E2 (reference compound).

- **estrogens**
  - 17β-E2 potent estrogen in aquatic environment (Lange et al, 2002, Houtman et al, 2011)
  - Probably related estrogens present as well:
    EE2: Tret 14.40
    αE2: 14.57
    E1: 14.40
  - No peak at Tret of E3 (10.70)

d. Confirmation

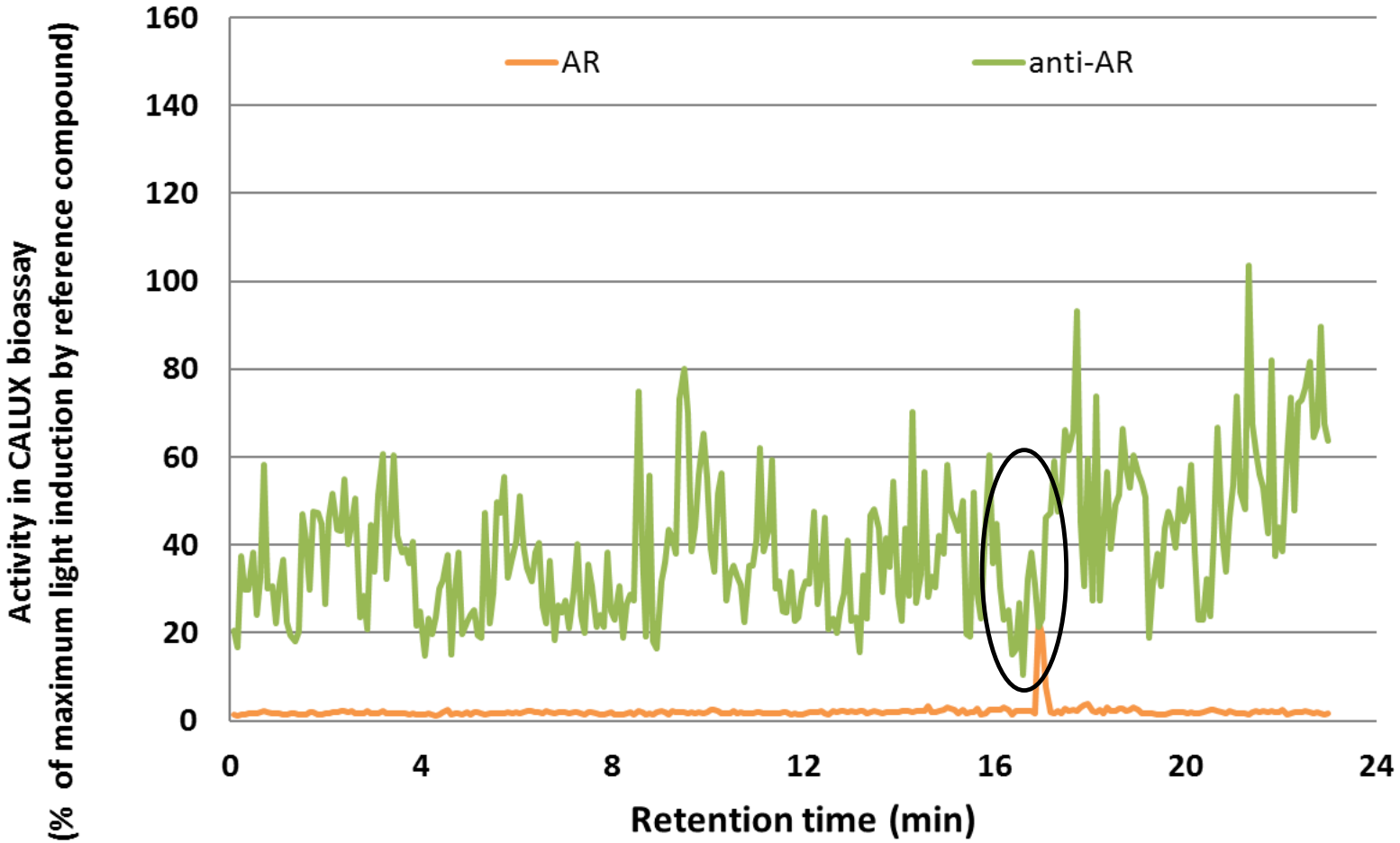
Concentration very low to be visible with MS (0.4 ng/L)

b. Bioassaychromatogram WWTP: activity in GR CALUX



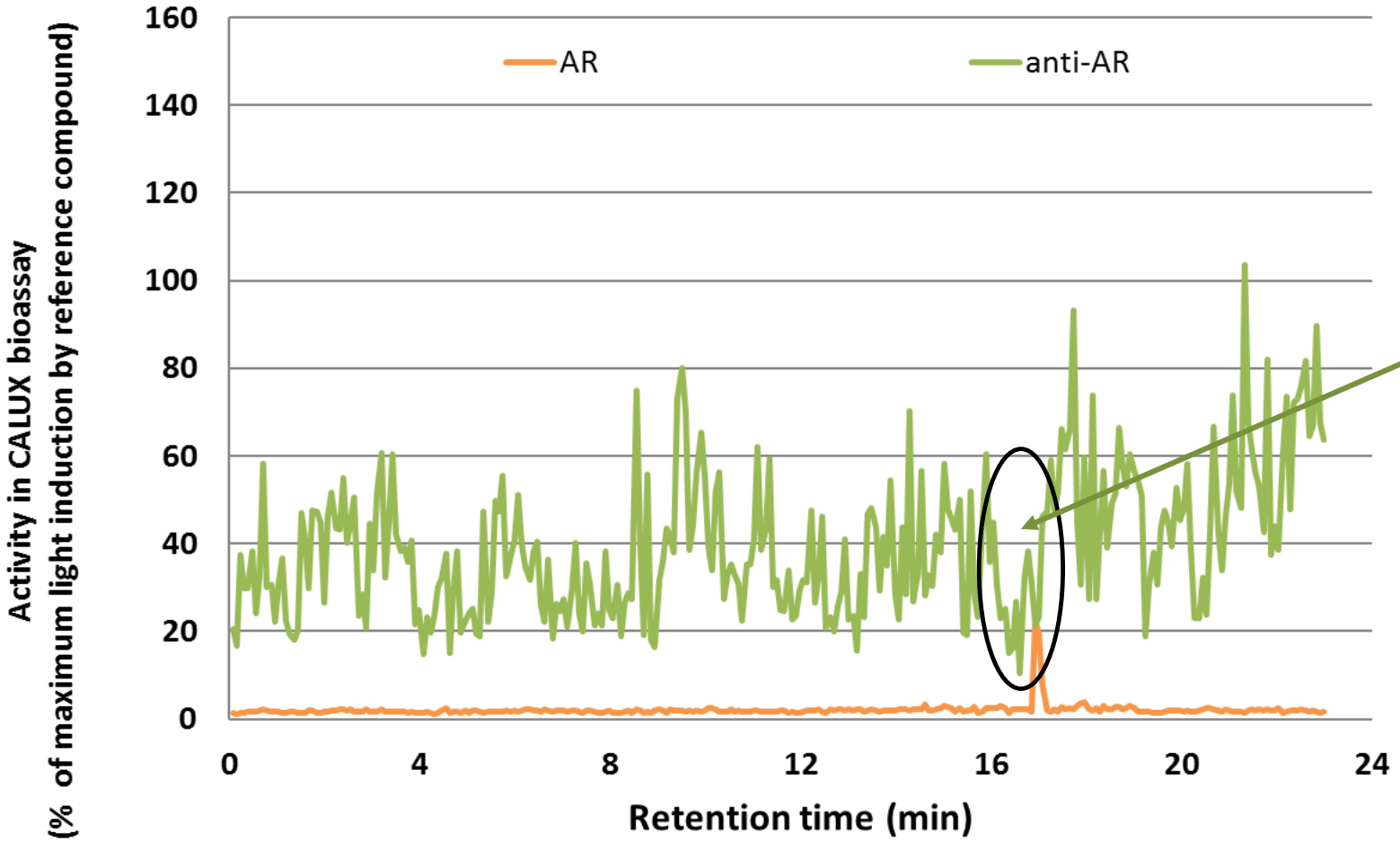
- **GR**: 6 peaks => ≥ 6 compounds
- NO peak at Tret = 13.4 min => dexamethasone not involved
- Peaks later in chromatogram: no masses could be identified => poorly ionisable less polar metabolites of glucocorticoids?
- 2 peaks with matching Tret of standards of synthetic glucocorticoids prednicarbate and rimexolone

b. Bioassaychromatogram WWTP: activity in anti-AR CALUX



- **Anti-AR**: negative peak at 16.4 and 16.6 min
- Probably split up by masked **AR** activity in same sample

c. Identification by UPLC-QToF-MS

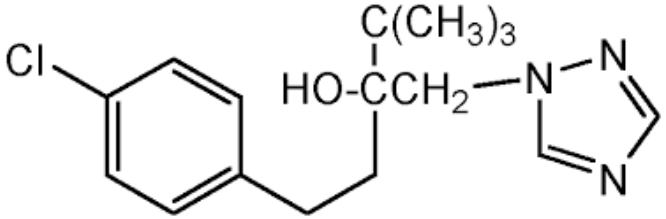


- **Anti-AR**: negative peak at 16.4 and 16.6 min
- Probably split up by masked **AR** activity in same sample

Identified compound:

- **Tebuconazole**

d. Confirmation tebuconazole



Analytical standard on UPLC-QToF-MS:

- Mw 308.1527
- Tret 16.3 min => ≡ Tret bioassaychromatogram

Literature:

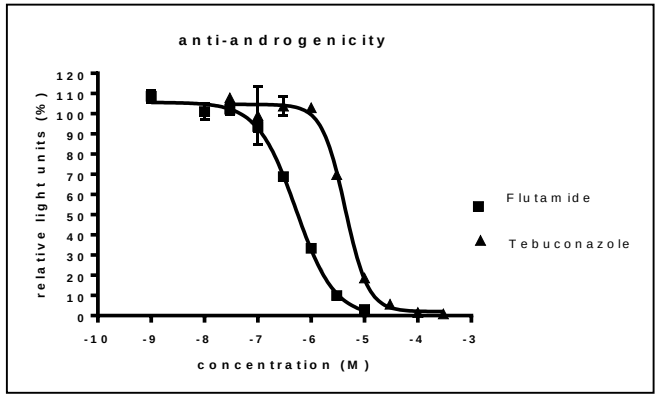
- Fungicide
- Anti-androgenic in Mda-kb2 cells (Christen et al., 2014)

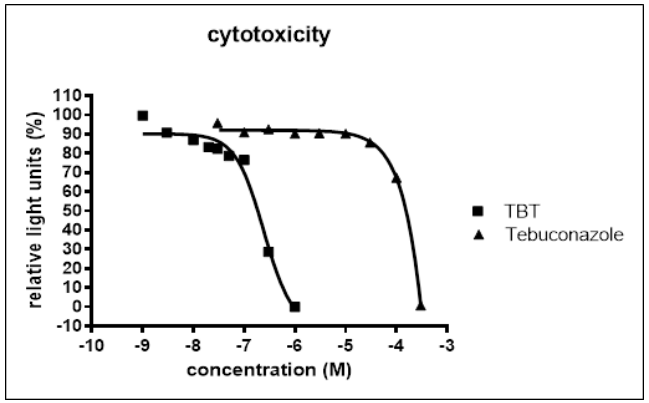
Anti-AR CALUX:

- Anti-androgenic, EC50 4.3 µM
- REP = 0.13 compared with flutamide

Cytotox CALUX:

- Cytotoxic at higher concentrations than anti-androgenicity
- Anti-androgenic effect is real !



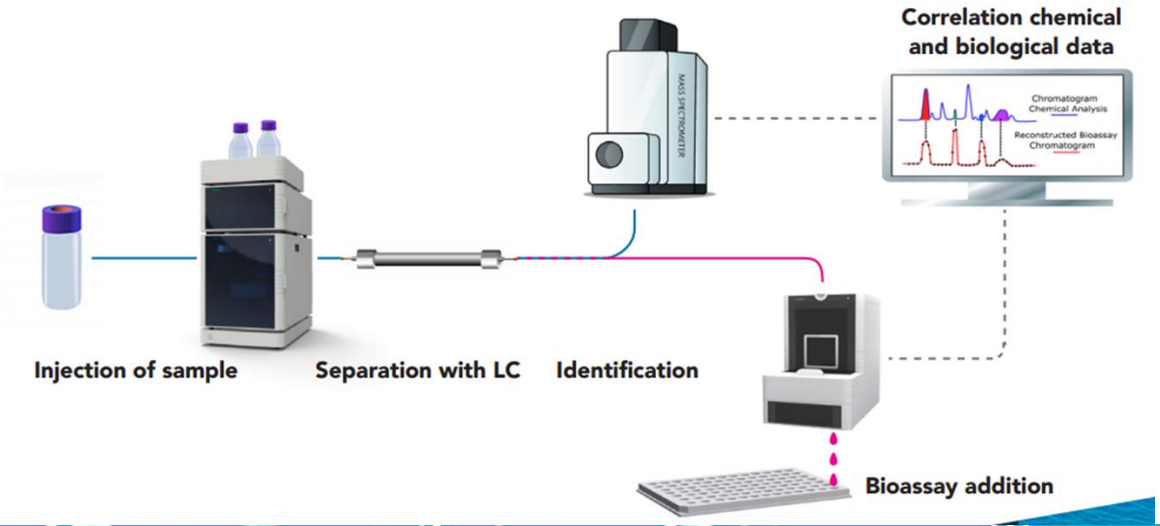


Summary

- EDA-platform:
  - Successful identifications
  - Identification remains bottle neck; large differences between sensitivity of MS and of bioassays between compounds
    - Hormones: Sensitivity bioassays >> MS
    - Anti-androgens, genotoxic compounds: Sensitivity bioassay << MS

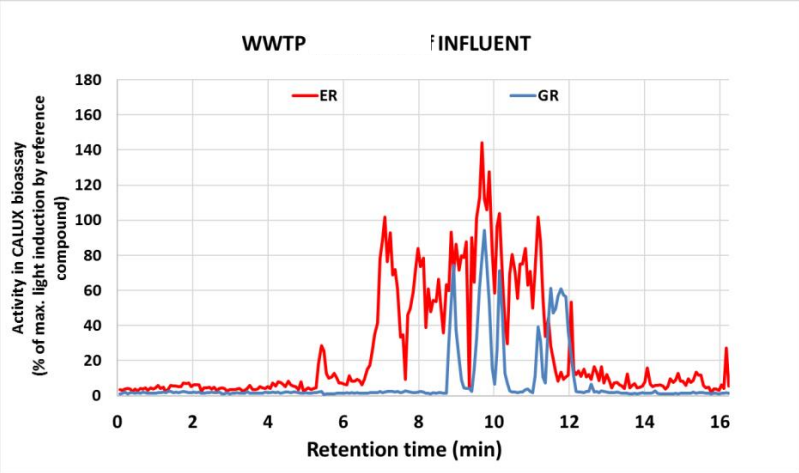
    => Play with split ratio LC to MS or spotter
  - Bioassay chromatogram already contains a lot of information…..

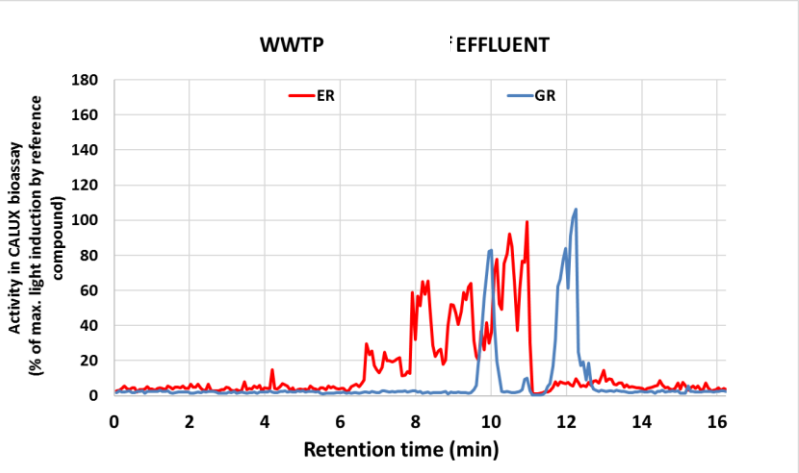
    …also demonstrated in follow-up

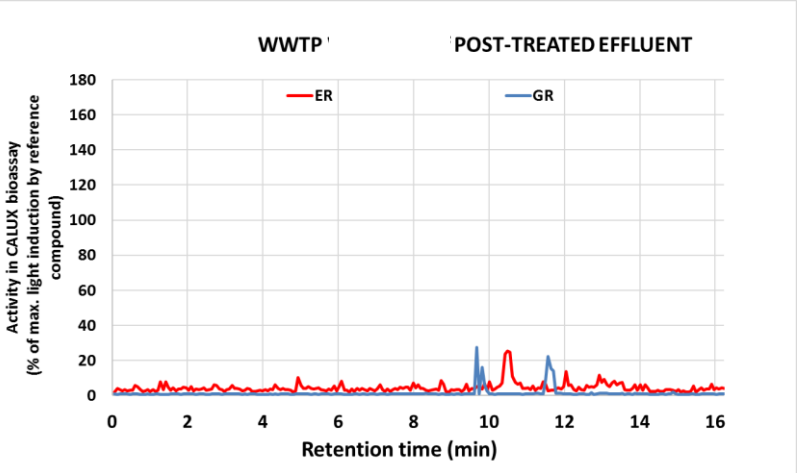


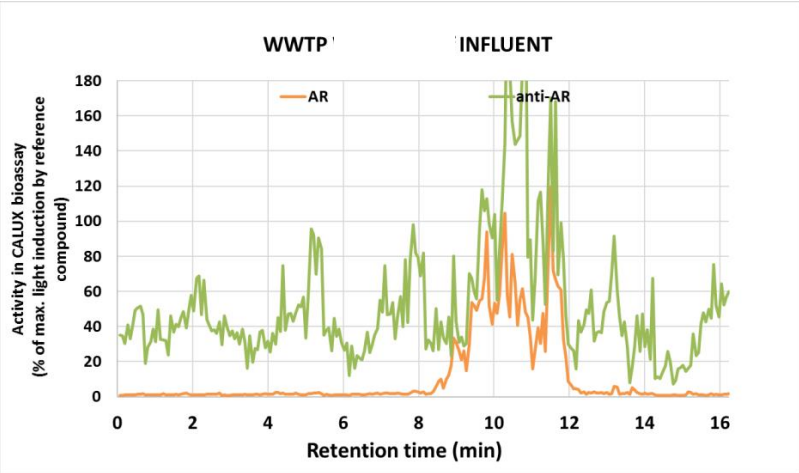
Follow-up study

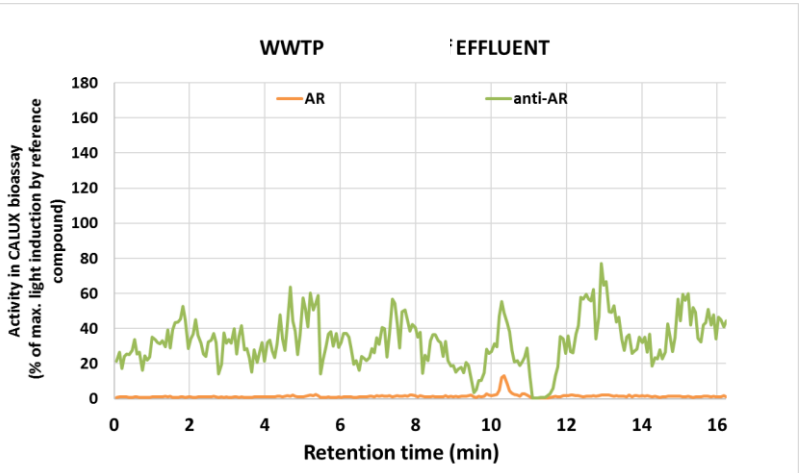
Influent (0.04L) → **Effluent (0.19L)** → **Post-treated effluent (0.19L)**

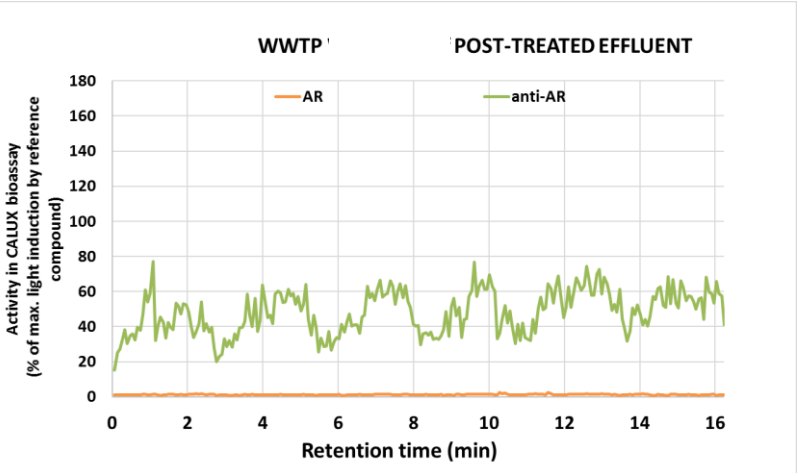








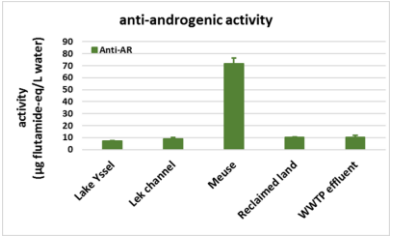




Outlook: how to use the HT-EDA platform

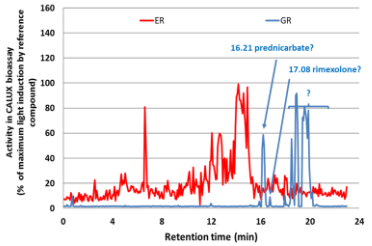
- Tiered approach possible => Not always necessary to do all steps:

  1. Activity measurement in non-fractionated extract
  - Activity detected?
  - *or:* Exceeding trigger value?

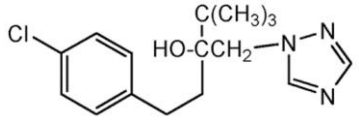


  2. Bioassay chromatogram
  - How many peaks?
  - Tret of known compounds?
  - Comparison of activity patterns between samples (influent ↔ effluent; samples in time)

  ⇒ e.g. record locations twice a year: same peak pattern = same water quality



  3. Identification of active compounds with recorded MS-data & confirmation with pure standard
  - Only if confirmed identity of responsible compounds is highly desired.



- Integral part of risk-based monitoring

Thanks for your attention!

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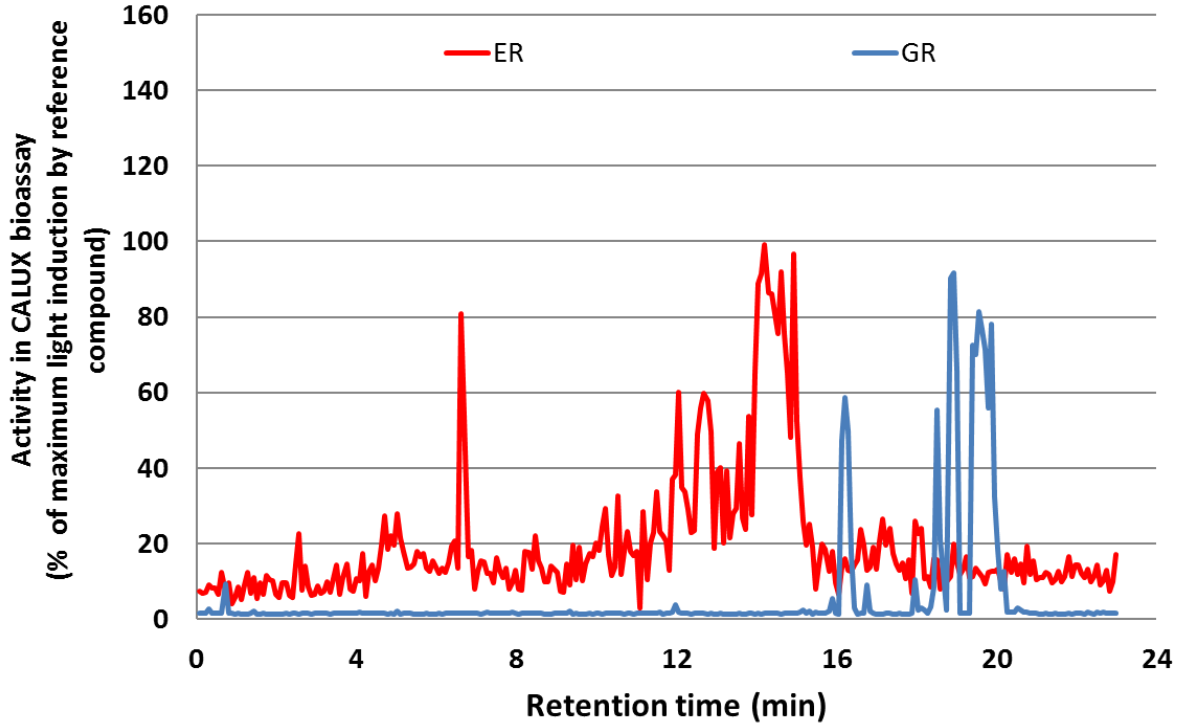
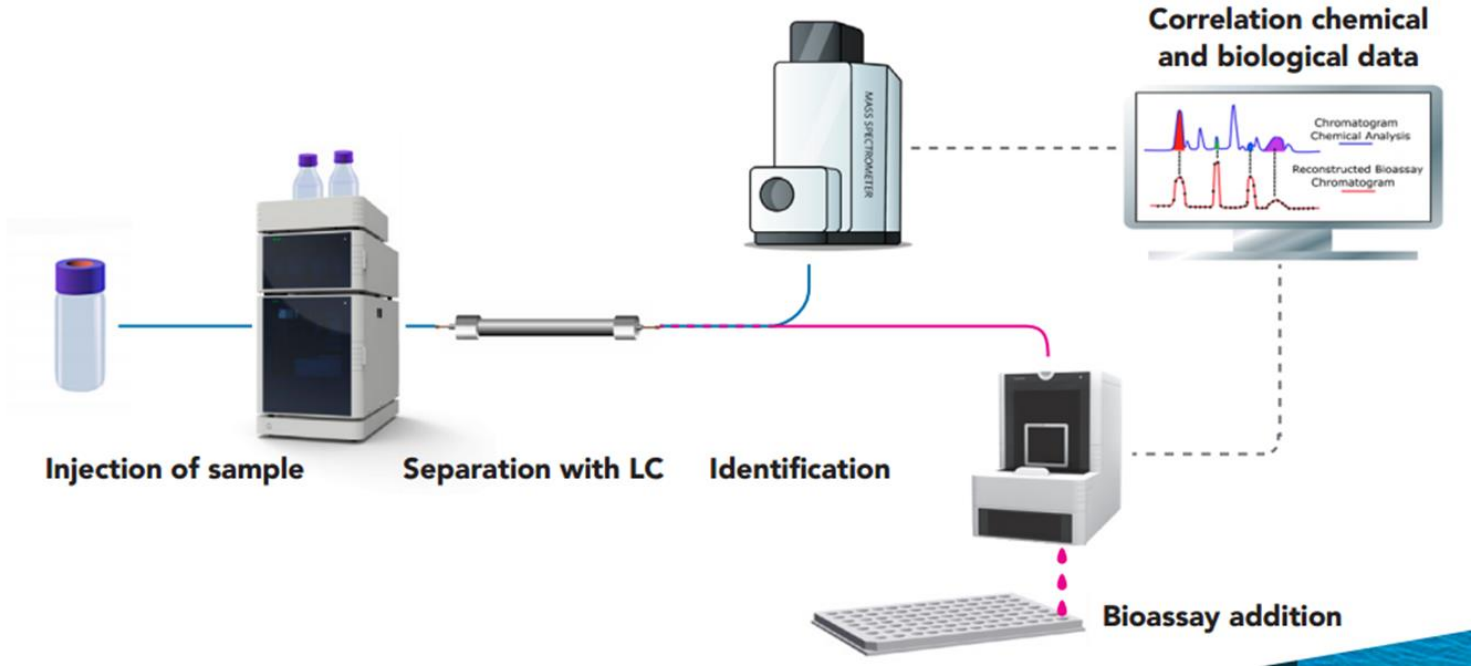
b. Bioassaychromatogram WWTP: activity in ER CALUX



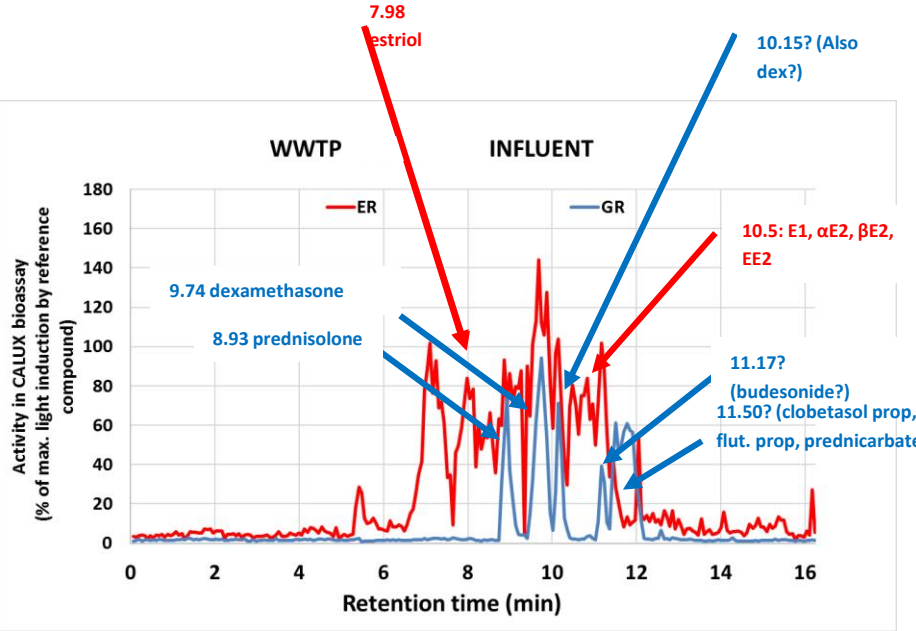
- **ER**: Large peak(s) around Tret = 14.05 min. => estradiol and related hormones E1, E3, 17αE2, EE2,…
- **ER**: spike (one well) at Tret =6.6 min : experimental artifact?

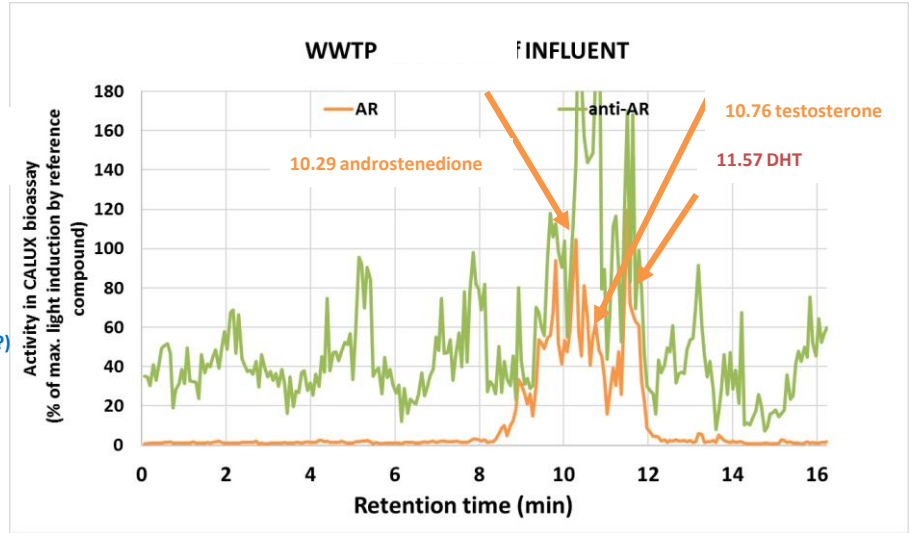
Novel EDA-platform



- Spot small fractions to bioassay in 384 well format
- “bioassay chromatogram”
- High resolution of fractionation, 1: 1 identification
- Spotter on sale: FractioMate

3a. WWTP Wervershoof INFLUENT (0.04 L)

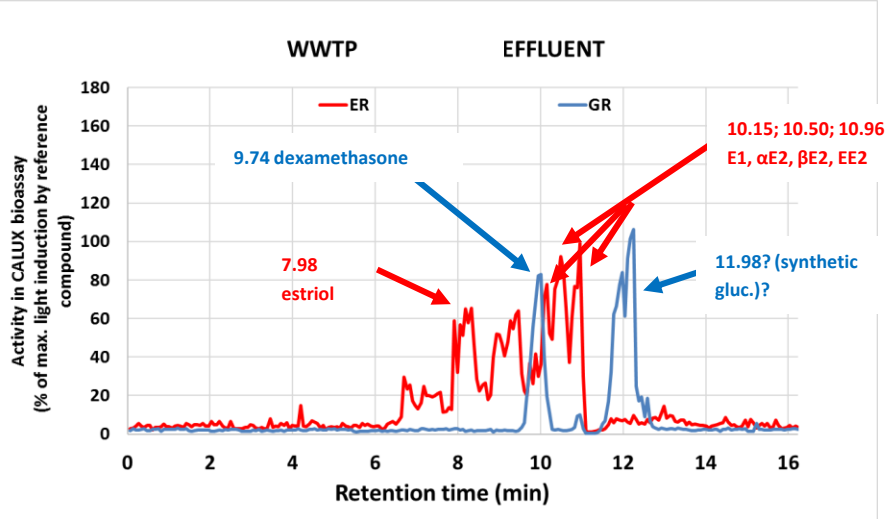


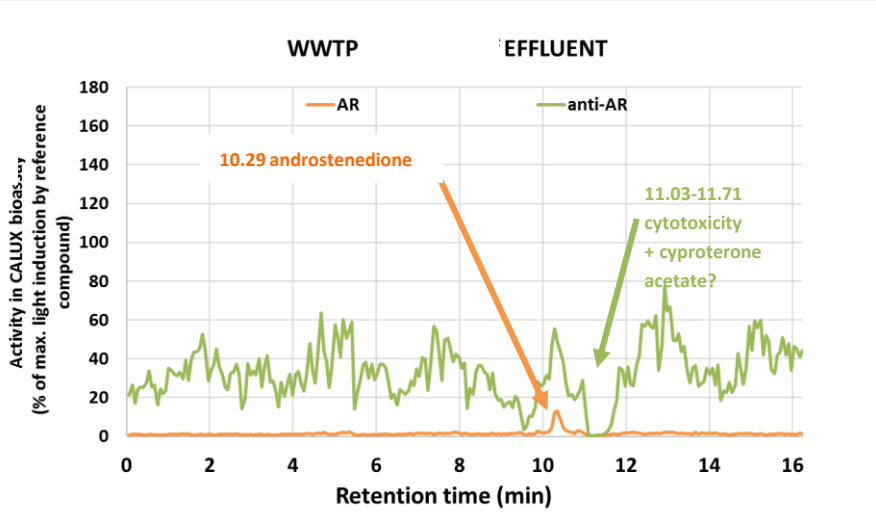


Tentatively identified based on RT:

- ER: E3 and sum of E1, αE2, βE2, EE2 + 4 peaks of unknown estrogens
- GR: prednisolone, dexamethasone, budesonide + 2 unknown peaks (one possibly by synthetic gluco's (fluticasone propionate, clobetasol propionate, prednicarbate)
- AR: androstenedione, testosterone, DHT + 4 peaks of unknown androgens.
- aAR: no compounds, one peak of an unknown anti-androgen.

3b. WWTP Wervershoof EFFLUENT (0.19 L)



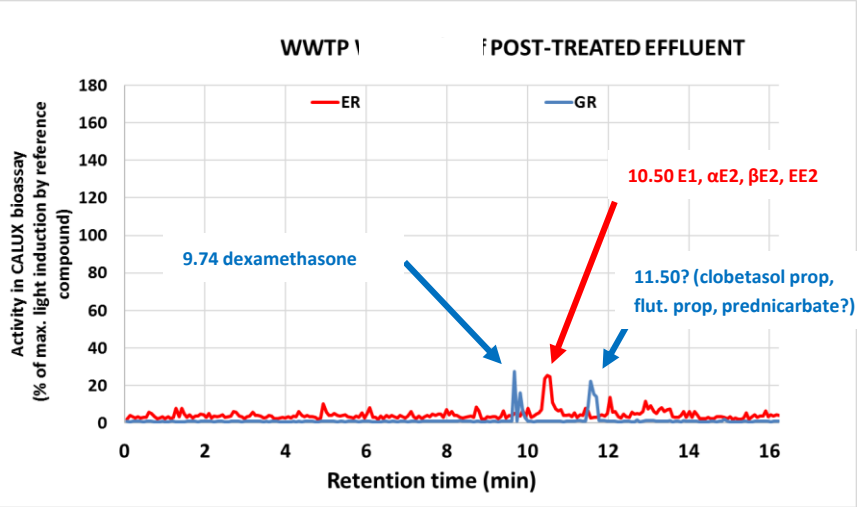


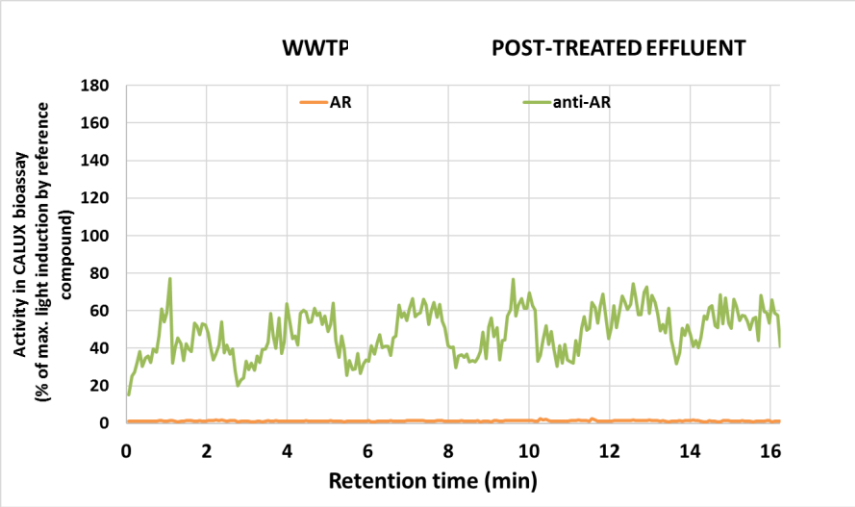
Tentatively identified based on RT:

- ER: E3 and sum of E1, αE2, βE2, EE2 + 2 peaks of unknown estrogens
- GR: dexamethasone + 2 unknown peaks (one possibly by synthetic gluco's (fluticasone propionate, clobetasol propionate, prednicarbate, amcinonide) and/or metabolites
- AR: androstenedione.
- aAR: no compounds, one peak of an unknown anti-androgen.

NB: cytotoxixity at RT 11.1-11.7 in this sample in all hormone assays.

3c. WWTP Wervershoof POST-TREATED EFFLUENT (0.19 L)





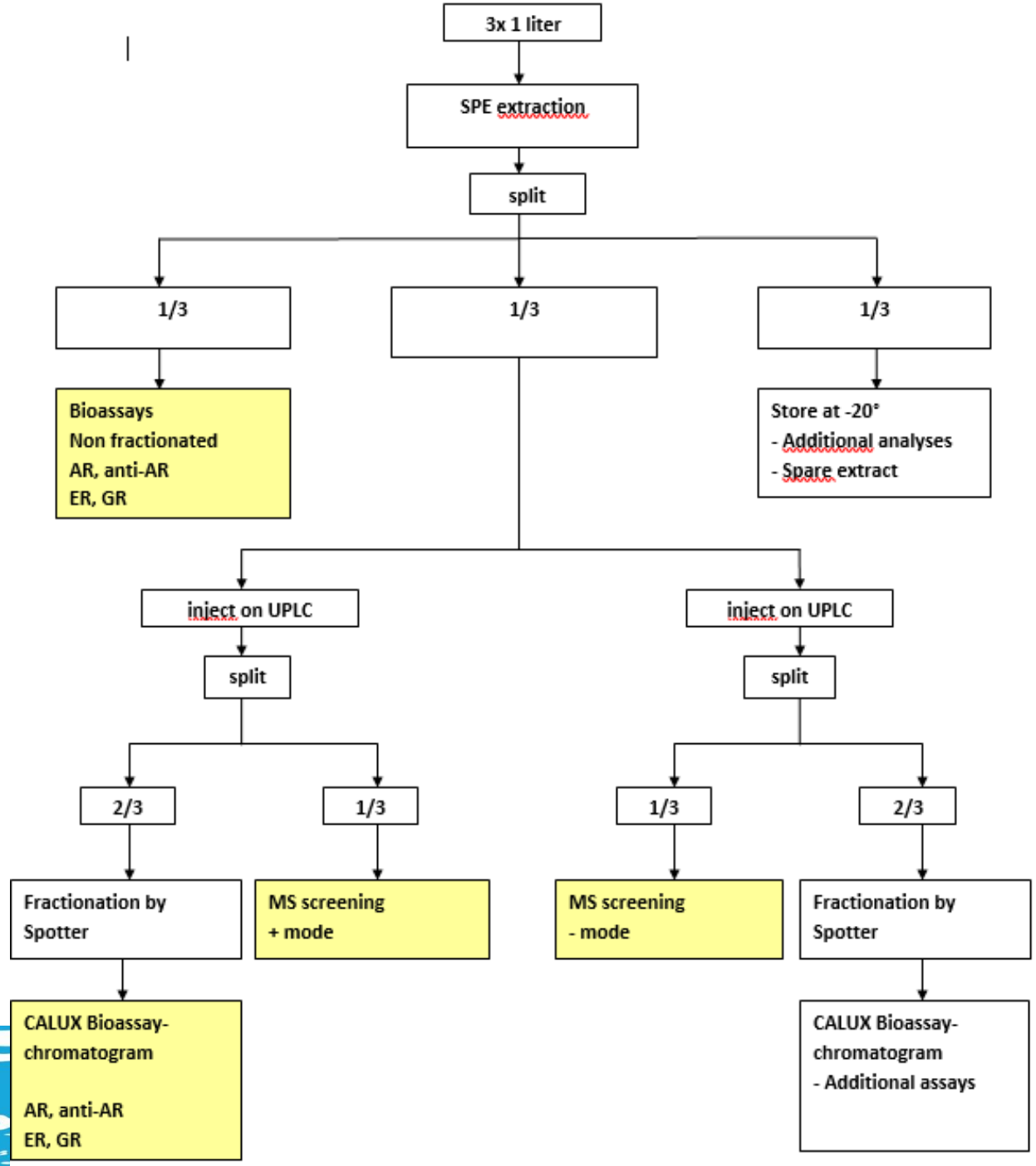
Tentatively identified based on RT:

- ER: sum of E1, αE2, βE2, EE2
- GR: dexamethasone, synthetic glucocorticoids (fluticasone propionate, clobetasol propionate, prednicarbate) and/or metabolites?
- AR: no peaks
- aAR: no peaks

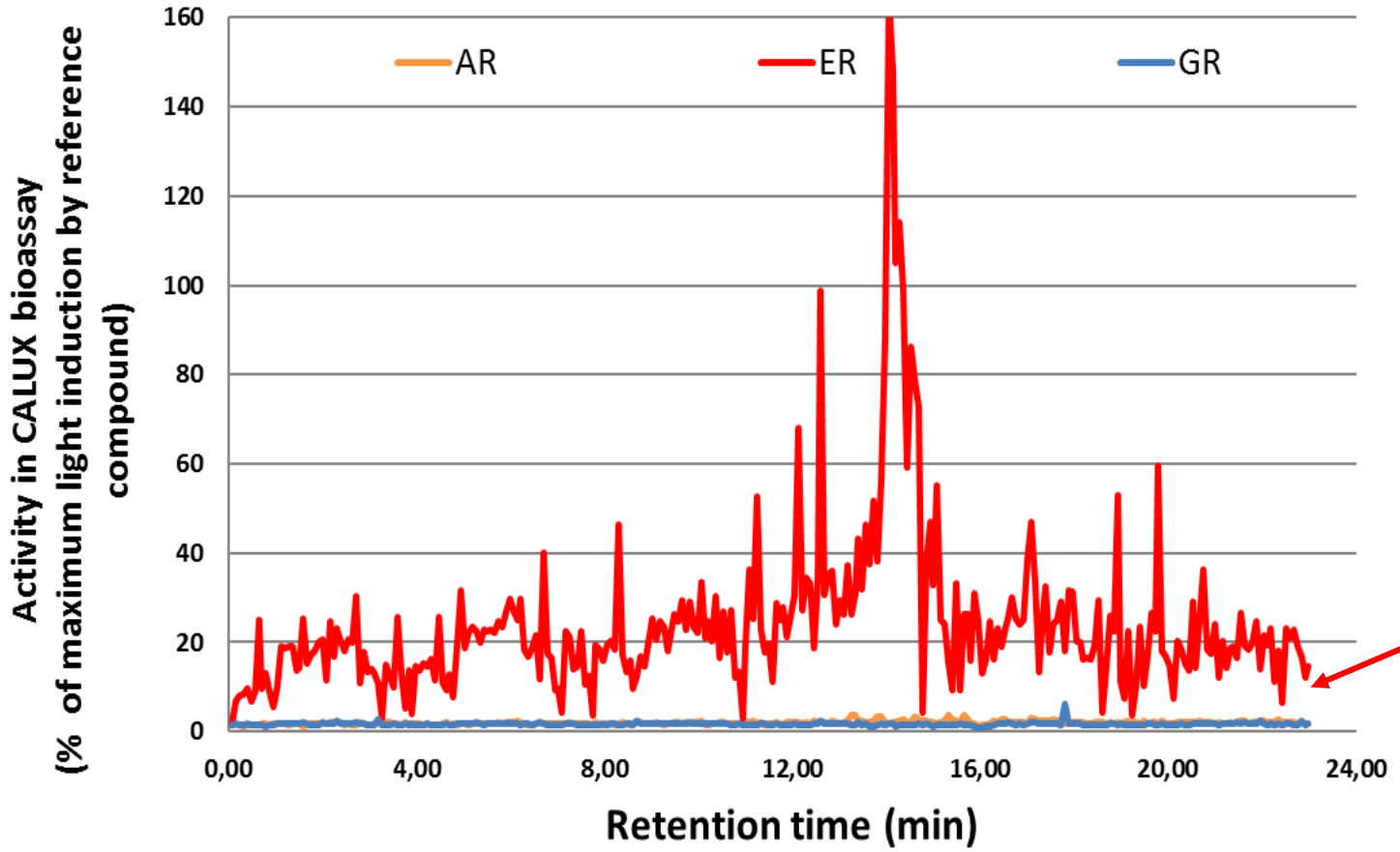
HET WATERLABORATORIUM

Steps

a) Bioassay non-fractionated sample
b) Bioassaychromatogram
c) Identification QToF-MS
   - FMF: monoisotopic mass
   - SmartFormula: Bruto formula
   - Compound Crawler: possible chemical structures
   - MetFrag: to which chemical structure do the obtained fragments match?
   - m/z cloud: compare spectrum with spectra previously uploaded by others
   - Compass isotope pattern: check theoretical isotopic pattern of the candidtae with the one obtained
d) Confirmation with analytical standard
   - Tret on UPLC
   - Activity in CALUX bioassay



c. Tentative identification by Tret



- Large peak(s) Tret 14 – 15 min.
- Tret = 14.20 min is Tret of 17β-E2 (reference compound).

- **estrogens**
  - 17β-E2 potent estrogen in aquatic environment (Lange et al, 2002, Houtman et al, 2011)
  - Probably related estrogens present as well:
    EE2: Tret 14.40
    αE2: 14.57
    E1: 14.40
  - No peak at Tret of E3 (10.70)

d. Confirmation

Concentration very low to be visible with MS (0.4 ng/L)

Use of analytical approaches in Risk-based monitoring

Desk Studies

Lab Research

Risk Analysis

Inventarisation emerging compounds
- Sources dw production
- Treatment
- Distribution
- In house tubing

Unknown compounds/ risks
- Non-target screening
- Suspect screening
- Bioassays
- HT-EDA

Signalling

Data evaluation

Prioritisation

Identification

Prioritisation (e.g. PRIO-model)

Method development

Pilot measurements

Risk-based Monitoring Program

Known compounds / risks
- Target compound analyses
- Bioassays (e.g. PAK-Calux)

Evaluation of monitoring program