Assessment of the suitability and performance of *in vitro* bioassays for analyzing mixture toxicity and ecotoxicity effects of androgenic, thyroid, glucocorticoid and progesterone hormonal activity in environmental and drinking waters

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Introduction

Recent work confirmed that environmental micropollutants may also affect other primary endocrine pathways (than estrogenic) which play a crucial role in the maintenance of homeostasis, sexual development, metabolism, growth and behavior (1,2).

To date, there are only a handful of studies on (anti)progestagenic, (anti)glucocorticoid or (anti)thyroid activity. If chemical analyses alone provide very little information on the biological effects and do not take into account interactions among individual chemicals in mixtures, *in vitro* bioassays are finding increasing added value as screening tools particularly for detecting Endocrine Active Compounds.

The aim of this project was to determine the suitability and relevance of available bioassay tools to quantify endocrine activity in environmental waters, to better assess potential endocrine effects in wildlife and humans.

Objectives

WP1: Review *in-vitro* methods available for non-estrogenic endocrine activity (particularly androgenic, progestagenic, glucocorticoid and thyroid activity) & **identify** the suitable ones for environmental and drinking water samples monitoring;

WP2: Develop & validate methods to extract a large variety of endocrine active compounds from water;

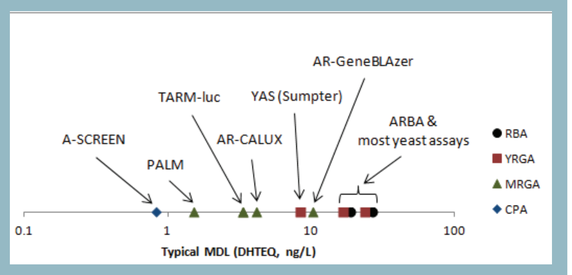
WP3: **Compare** various *in vitro* and one *in vivo* assays to detect thyroid active compounds in water samples;

WP4: Apply a battery of *in vitro* bioassays to measure 7 endocrine agonist and antagonist activities (estrogenic, androgenic, progestagenic, glucocorticoid, thyroid, mineralcorticoid and retinoid) on 3 water matrices (treated wastewater, surface water, drinking water) from 6 countries (France, Germany, the Netherlands, Spain, South Africa & Australia) to determine the levels of endocrine activity in the water cycle.

Methods & Results

WP1 : Literature review of bioassay performance

- Exhaustive literature meta-analysis from 35 assays targeting endocrine pathways .
- Comparison of the overall method sensitivity for each assay and evaluation of its suitability to detect endocrine activity in environmental water samples assessed by comparing Method Detection Limits (MDLs) (see example below).



- **MDL** were calculated by integrating assay sensitivity (based on the EC_{10} value from a reference standard concentration effect curve), the minimum assay dilution (determined by the solvent tolerance of the assay) and a typical solid-phase enrichment factor (of 1,000×).

➢ **Most sensitive methods** are confirmed to be based on Mammalian reporter gene assays and cell proliferation assays.

➢ **Significant sample enrichment** is necessary to detect endocrine activity (other than estrogenic activity) in water samples.

➢ **Selection of a list of 11 *in vitro* bioassays covering 7 endocrine disruptive pathways :**

- ✓ ER-GeneBLazer (estrogenic)
- ✓ AR-GeneBlazer, MDA-kb2 (androgen)
- ✓ PR GeneBlazer, PR-CALUX (progestagen)
- ✓ GR-GeneBlazer, GR-CALUX (glucocorticoid),
- ✓ GH3-Luc (thyroid)
- ✓ RXR-CALUX, HELN-RARα (retinoid acid)
- ✓ HG5LN-MR (mineralcorticoid)

WP2: Extraction methods validation

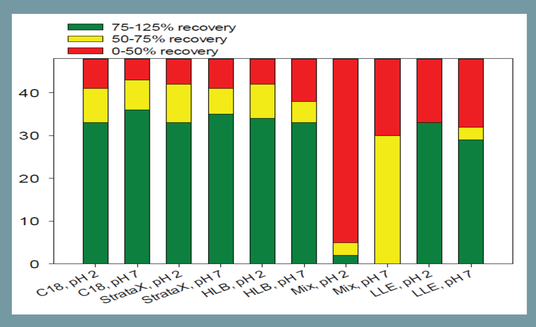
- Based on a prioritization framework and expert opinion, 51 micropollutants under 9 chemical classes were selected for method development.

Androgens (3)	Phytoestrogen (1)	Industrial compounds (6)
Estrogens (4)	Progestogen (1)	Personal care products (3)
Pesticides (13)	Thyroid hormones (2)	Pharmaceuticals (18)

- Four different SPE sorbent materials as well as Liquid-Liquid extraction tested at pH 2 & 7 for the extraction of target analytes from aqueous samples .

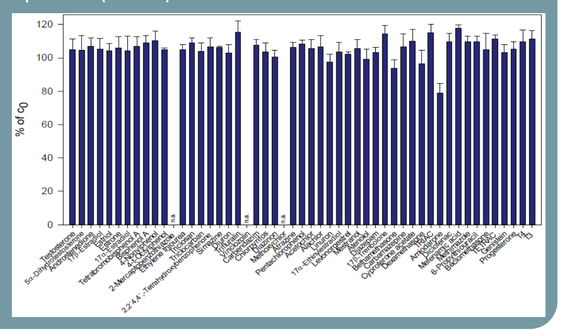
4 different SPE sorbent materials	C18	HLB
	StrataX	Mix

➢ Most SPE methods allowed good recoveries for most compound (see below).



➢ Most efficient extraction method is **SPE with StrataX cartridge (Phenomenex) at pH 2**

➢ **Recoveries rate ranged from 80% to 95%** for the majority of the 51 compounds (see below) & **stability of SPE extract** (methanol) provided excellent recoveries after 3 months storage period (>80%).



WP3 : Thyroid inter-assay comparison

- Comparison of the performance of **7 *in-vitro* bioassays and 1 *in-vivo* bioassay covering crucial Thyroid signaling endpoints** such as:

Biosystem	Endpoints	Assays
in-vitro	Thyroid Hormone **synthesis**	TPO inhibition assay
	Thyroid Hormone **transport**	TTR displacement assay (fluo probe) TTR displacement assay (ANSA) TTR displacement assay (luc based)
	Thyroid Receptor mediated action	TR-β CALUX TRβ-GeneBLAzer GH3-TRE-Luc
in-vitro 	**Amphibian metamorphosis** assay	WatchFrog transgenic amphibian metamorphosis assay

- **Stage 1 on Model compounds**:
  - ✓ 9 reference compounds (T3, T4, TRIAC, TETRAC, amiodarone, PCP, ETU, BP2 and methimazole)
  - ✓ analyzed in all 8 bioassays
- **Stage 2 on Environmental samples:**
  - ✓ 3 environmental waters (treated sewage effluent, surface and drinking) and an ultrapure blank were sampled
  - ✓ extracted by the SPE with **StrataX cartridge pH2**

Preliminary results

➢ Most bioassays can detect correctly the thyroid active model compounds / stage 1.

➢ None of the environmental extracts were active on the thyroid Hormone Receptor (THR) / stage 2.

➢ The most responsive & pronounced effect is the **disruption of the Thyroid Hormone Biosynthesis** as detected in the TPO inhibition assay.

➢ Wastewater was almost completely able to suppress TPO activity at a Relative Enrichment Factor (REF) of 10. The analysis of other results is currently in progress.

Fig. 2.. Results of the TPO inhibition assays with drinking water (DW), waste water (WW) and surface water (SW). The figures on the horizontal axis represent the respective Relative Enrichment Factor (REF).

WP4 : Application of *in vitro* bioassay battery to environmental water samples

- **18 environmental water samples** extracted by the SPE with StrataX cartridge pH2
  - ✓ treated wastewater (WW)
  - ✓ surface water (SW)
  - ✓ drinking water (DW)

  Collected from 6 countries
  - ✓ France, Germany, Netherlands, Spain,
  - ✓ South Africa and Australia
- **Screening of 7 endocrine endpoints /** WP1 review

Endpoint	Assay
Estrogenic	**ER-GeneBLAzer**
Androgenic	**AR-GeneBLAzer**
	AR-CALUX
	MDA-kb2
Glucocorticoid	**GR-GeneBLAzer**
	GR-CALUX

Endpoint	Assay
Progestagenic	**PR-GeneBLAzer**
	PR-CALUX
Thyroid	**GH3-TRE-Luc**
Mineralcorticoid	**HG5LN-MR**
Retinoid X receptor	**HELN-RARa**
	RXR-CALUX

- **Chemical analysis** under 3 different methods

Analytical methods	Chemical compounds
LC-MS target analysis (quantitative result)	***51 compounds***
LC MS "suspect screening" (qualitative result)	***112 compounds used for prioritization***
SPE-silylation GC/MS method	***5 hormones:*** cortisone, cortisol, norethisterone epitestosterone, androsterone **9 further analytes:** benzothiazole, tert-butylphenol, galaxolide, tonalide, dibutylphtalate, pentachlorophenol, triclosan, DEHP, bisphenol A

Preliminary results

- Good qualitative agreement between chemical analysis and bioassay screening
- Common detection in environmental water samples from all countries of low concentrations, indicating global traces contamination of:
  - ✓ Pharmaceuticals (carbamazepine, diclofenac, atenolol)
  - ✓ Herbicides (simazine, diuron)
  - ✓ Personal care products (triclosan)
- **Undetectable endocrine activity in most water samples**, with the exception of:
  - ✓ some low ER activity (2WW and 1 SW)
  - ✓ low GR activity (1 WW and 1 SW)
  - ✓ anti-MR activity in all 6 surface water samples.
- The analysis of other results is currently in progress.

Conclusions

The main conclusions from each of the four work packages (WP) of this study were:

❶ Detection of (non-estrogenic) endocrine activity in water samples will likely require significant sample enrichment (>10,000×) despite the exquisite sensitivity of *in vitro* bioassays.

❷ A simple solid phase extraction method using StrataX SPE cartridges and adjustment to pH 2 yields very good recoveries of a wide range of endocrine active analytes from water.

❸ While there are a variety of *in vitro* thyroid receptor reported gene assays, it appears that environmental water samples do not contain significant concentration of TR active compounds. However, **they do contain compounds that interfere with TPO synthesis.**

❹ **Endocrine activity in various water samples from various countries around the globe was very low, suggesting that water may not be a significant source of exposure to Endocrine Actives Compounds.**

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