Looking beyond estrogenicity: Application of *in vitro* bioassays to measure endocrine activity in environmental waters

Frederic D.L. Leusch[1], Natalie Aneck-Hahn[2], Charlotte Arnal[3], Patrick Balaguer[4], Auguste Bruchet[5], David Du Pasquier[6], Beate I. Escher[7], Mar Esperanza[5], Armelle Hebert[3], Marina Grimaldi[4], Gaela Leroy[3], Peta A. Neale[1], Frank Sacher[8], Marco Scheurer[8], Rita Schlichting[7] and Merijn Schriks[9]

[1]Smart Water Research Centre, Australian Rivers Institute, Griffith School of Environment, Griffith University, Gold Coast, Australia; [2]Environmental Chemical Pollution & Health Research Unit, Faculty of Health Sciences, University of Pretoria, South Africa; [3]Veolia Recherche & Innovation, Département Environnement & Santé, France; [4]IRCM (Institut de Recherche en Cancérologie de Montpellier), Inserm U1194, France; [5]Cirsee - SUEZ Environnement, Pole Analyse Capteurs & Applications, France; [6]WatchFrog, Evry, France; [7]Department Cell Toxicology, Helmholtz Centre for Environmental Research – UFZ, Germany; [8]TZW DVGW Technologiezentrum Wasser, Germany; [9]KWR, Watercycle Research Institute, Nieuwegein, The Netherlands

Introduction

- There is increasing concern about the presence of endocrine active chemicals (EACs) in the aquatic environment due to their potential effects on both human and ecosystem health.
- Most studies focus on estrogenic activity, but other hormones, including androgens, progestagens, glucocorticoids and thyroid hormones, also play a crucial role in the maintenance of homeostasis, sexual development, metabolism, growth and behaviour.[1,2]
- Some work has been conducted on (anti)androgenic activity, but there are very few studies focusing on (anti)progestagenic, (anti)glucocorticoid or (anti)thyroid activity.
- **The current study aimed to determine the suitability and relevance of available bioassays to quantify endocrine activity in environmental waters in order to better assess potential endocrine effects in wildlife and humans.**

Objectives

❶ **Review *in vitro* assays** available for androgenic, progestagenic, glucocorticoid and thyroid activity and **identify** the suitable assays for monitoring environmental waters.

❷ **Develop and validate** methods to extract a large variety of EACs from water.

❸ **Compare** various *in vitro* and one *in vivo* assays to detect thyroid active compounds in water samples.

❹ **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 and Australia) to determine the levels of endocrine activity in the water cycle.

❶ Literature review of bioassay performance

- Thirty five *in vitro* bioassays indicative of (anti)androgenic, (anti)progestagenic, (anti)glucocorticoid, (anti)thyroid and (anti)estrogenic activity were reviewed.
- Assay method detection limit (MDL) was calculated (Fig 1) and compared with hormonal activity in water.
- Current assays sufficiently sensitive to detect (anti)androgenic and estrogenic activity in environmental waters after typical sample enrichment (1000×).
- Reporter gene assays able to detect (anti)progestagenic and glucocorticoid activity in treated wastewater after standard enrichment.
- Current assays unlikely to detect (anti)thyroid activity.

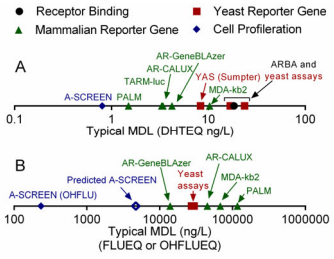

Fig 1: Typically method detection limits for A) androgenic and B) anti-androgenic assays.

❷ Extraction method validation

- Recovery of 51 EACs, including hormones, pesticides and pharmaceuticals, evaluated using solid phase extraction (SPE) and liquid-liquid extraction at pH 2 and 7.
- All commercial SPE sorbent materials gave comparable results (Fig 2), with comparable recoveries at both pH 2 and 7.
- StrataX SPE cartridges, with extraction at pH 2 and elution using methanol, acetonitrile and acetone, were applied in objectives 3 and 4.

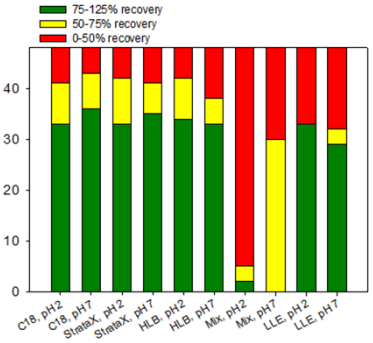

Fig 2: Compound recovery by SPE with different sorbent materials and liquid-liquid extraction at pH 2 and 7.

❸ Thyroid inter-assay comparison

- Thyroid active chemicals and environmental water samples were analysed in a suite of *in vitro* assays indicative of thyroid hormone synthesis, thyroid hormone transport and thyroid receptor mediated activity and an *in vivo* amphibian reporter gene assay (WatchFrog).
- The majority of *in vitro* assays were able to detect known thyroid agonists and antagonists (Fig 3 A), but the environmental samples were not active in the assays.
- In contrast, the SW and WW samples were active in the WatchFrog assay (Fig 3 B).
- The discrepancy between the *in vitro* and *in vivo* results may be due to 1) lack of metabolic activity in the *in vitro* assays or 2) another mechanism of thyroid activity not covered by the *in vitro* assays.

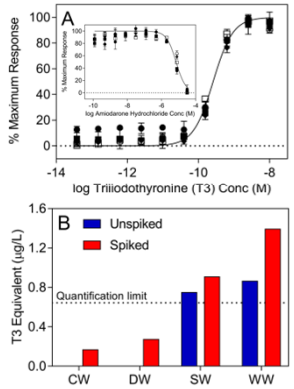

Fig 3: A) Concentration-effect curves for agonist T3 and antagonist amiodarone hydrochloride in TRβ-GeneBLAzer and B) T3 equivalents for spiked and unspiked water samples in the WatchFrog assay

❹ Application to environmental water samples

- *In vitro* assays indicative of estrogenic, androgenic, progestagenic, glucocorticoid, thyroid, mineralcorticoid and retinoid activity *in vitro* assays were applied to wastewater (WW), drinking water (DW), surface water (SW) and control water (CW) from 6 countries, with both agonist (+) and antagonist (-) activity measured.
- Most samples were inactive in the studied assays (Table 1).
- Low ER and GR activity in some WW and SW samples.
- Anti-mineralcorticoid (MR) activity in WW and SW samples.
- General agreement between chemical analysis and bioanalysis observed.

		ER-GeneBLAzer (+)	ER-GeneBLAzer (-)	AR-GeneBLAzer (+)	AR-GeneBLAzer (-)	GR-GeneBLAzer (+)	GR-CALUX (+)	PR-GeneBLAzer (+)	PR-GeneBLAzer (-)	PR-CALUX (+)	GRE-Luc (+)	RXR-CALUX (+)	HG5LN-hRIP (+)	HG5LN-hMR (+)	HG5LN-hMR (-)	HG5LN-hMR (+)
CW	Germany	<0.1	<830	13	<10	<23	<12	<500	<0.5	<0.4	<40	<5	<400	<400	<200	<120	<620
	Australia	<0.1	<830	<2	<20	<23	<12	<500	<0.5	<0.4	<40	<5	<400	<400	<200	<120	<620
	France	<0.1	<830	<2	<20	<23	<12	<500	<0.5	<0.4	<40	<5	<400	<400	<200	<120	<620
	South Africa	<0.1	<830	<2	<20	<23	<12	<500	<0.5	<0.4	<40	<5	<400	<400	<200	<120	<620
	Netherlands	<0.1	<830	<2	<20	<23	<12	<500	<0.5	<0.4	<40	<5	<400	<400	<200	<120	<620
DW	Spain	<0.1	<830	<2	<20	<23	<12	<500	<0.5	<0.4	<40	<5	<400	<400	<200	<120	<620
	Germany	<0.63	<210	21	<6	<5.8	<3	<120	<0.1	<0.1	<10	<1.3	<100	<50	<50	<30	<160
	Australia	<0.63	<210	<0.5	<40	<5.8	<3	<120	<0.1	<0.1	<10	<1.3	<100	<50	<50	<30	<160
	France	<0.63	<210	<0.5	<6	<5.8	<3	<120	<0.1	<0.1	<10	<1.3	<100	<50	<50	<30	<160
	South Africa	<0.63	<210	<0.5	<6	<5.8	<3	<120	<0.1	<0.1	<10	<1.3	<100	<50	<50	<30	<160
	Netherlands	<0.63	<210	<0.5	<6	<5.8	<3	<120	<0.1	<0.1	<10	<1.3	<100	<50	<50	<30	<160
	Spain	<0.63	<210	<2	<20	<5.8	<3	<120	<0.1	<0.1	<10	<1.3	<100	<50	<50	<30	<160
SW	Germany	<0.1	<830	23	<30	<23	<12	<500	<0.5	<0.4	<40	<20	<400	<400	<200	<120	<620 *
	Australia	<0.1	<830	<5	<40	<23	<12	<500	<0.5	<0.4	<40	<20	<400	<400	<200	<120	<620
	France	<0.1	<830	<4	<50	<23	<12	<500	<0.5	<0.4	<40	<20	<400	<400	<200	<120	<620
	South Africa	<0.1	<830	<4	<50	<23	<12	<500	<0.5	<0.4	<40	<20	<400	<400	<200	<120	<620
	Netherlands	<0.1	<830	<3	<40	<23	<12	<500	<0.5	<0.4	<40	<20	<400	<400	<200	<120	<620
	Spain	<0.1	<830	<4	<80	96	<12	<500	1.1	<0.4	<40	<20	<400	<400	<200	<120	150
WW	Germany	<0.6 *	<4200	<16	<100	<120	<60	<2500	<2.5	<2	<200	<25	<2100	<2000	<1000	<600	<3100 *
	Australia	<0.6	<4200	<10	<200	<120	<60	<2500	<2.5	<2	<200	<25	<2100	<2000	<1000	<600	<3100
	France	1.2	<4200	<20	<100	<120	<60	<2500	<2.5	<2	<200	<25	<2100	<2000	<1000	<600	<3100 *
	South Africa	<0.6	<4200	<20	<100	<120	<60	<2500	<2.5	<2	<200	<25	<2100	<2000	<1000	<600	<3100 *
	Netherlands	<0.6	<4200	<10	<100	<120	<60	<2500	<2.5	<2	<200	<25	<2100	<2000	<1000	<600	<3100
	Spain	<0.6	<4200	<10	<100	<120	<60	<2500	<2.5	<2	<200	<25	<2100	<2000	<1000	<600	<3100

Table 1: Bioanalytical equivalent concentrations (ng/L) for all water samples (red: active; yellow: slightly active; green: inactive; grey: to be confirmed)

Conclusions

The main conclusions from each of the four objectives of this study were:

❶ Current *in vitro* assays sufficiently sensitive to detect (anti)androgenic and estrogenic activity in treated wastewater and surface water with typical enrichment factors (1000×), with reporter gene assays likely to detect (anti)progestagenic and glucocorticoid activity in treated wastewater after standard enrichment. Other endpoints require higher enrichment (>1000x).

❷ A simple SPE method using StrataX SPE cartridges and adjustment to pH 2 yields very good recoveries of a wide range of EACs in water.

❸ While there are a variety of *in vitro* thyroid activity assays, it appears that the studied assays are not yet able to detect chemicals in environmental water samples that cause whole organism thyroid responses (as measured by the WatchFrog assay).

❹ Endocrine activity in environmental water samples collected from various countries was very low, suggesting that water may not be a significant source of EACs. Further research should investigate other sources of exposure (e.g. food).

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