Determination of the *in vitro* oestrogenic activity of sediment samples collected from the river Tyne

Final Report to Posford Haskoning Ltd.

Burnham Laboratory

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Summary

The oestrogen receptor (ER) agonist potency was measured in sediment extracts obtained from cores collected from the Tyne estuary using the *in vitro* yeast oestrogen screen (YES). These data were compared with *in vitro* data previously obtained for a number of UK estuaries as part of the UK EDMAR[1] programme. Extracts obtained from sediment samples collected from the Tyne on this occasion showed activity to be between 0.03 and 1.1 μg E2 Kg^{-1}. This compares with 1.3 - 4μg E2 Kg^{-1} that has previously been determined for surface sediments collected from the Tyne.

Introduction

Male flounder (*Platichthys flesus.*) collected from impacted estuaries in the United Kingdom (UK) exhibit biological responses that are consistent with exposure to oestrogenic substances [1-3]. These responses include elevated blood plasma levels of the yolk precursor protein vitellogenin and the presence of testicular oocytes. The degree of oestrogenic contamination, as measured by male vitellogenesis, is greatest in the estuaries of the rivers Tees, Mersey and Tyne [3]. These estuaries receive high inputs of domestic and industrial treated effluents, whilst also being historically impacted by heavy industry. Characterisation of estuarine discharges identified 17β-oestradiol as the main cause of oestrogenic activity with additional activity from androsterone, nonylphenol and (tentatively) *bis*(2-ethylhexyl)phthalate [4]. However, flounder placed in cages in these effluent streams did not show any of the biological responses consistent with exposure to oestrogenic compounds. Further analysis of samples collected from UK estuaries using the receptor based yeast oestrogen screen (YES) showed that a significant proportion of the *in vitro* oestrogenic activity found in estuaries is associated with sediments (Table 1). However, a clear link between high *in vitro* oestrogenic activity in estuarine sediments and oestrogenic responses in flounder has yet to be established.

In vitro receptor mediated response assays such as the YES assays are very powerful in quantifying mechanism specific agonists in environmental samples. This is particularly important when the individual compounds responsible for this activity are unknown. Here we report the application of the YES assay to quantify the *in vitro*

[1] EDMAR: A multi-agency funded research project into the effects of endocrine disrupting compounds in the marine environment.

oestrogenic activity associated with sediment samples collected from the Tyne estuary.

Table 1. Oestrogenic activity of selected UK estuarine surface sediments

Estuary[†]	*In vitro* oestrogenic activity[*]	
	Pore Water ng E2 l^{-1} (median)	**Sediment solvent extract µg E2 Kg^{-1} (median)**
Tyne	19-61 (24)	1.26-3.95 (1.86)
Tees	7-66 (38)	0.04-13 (1.68)
Mersey	< 2	< 0.2-0.94 (<0.2)
Thames	3-7 (3.5)	0.57-2.39 (1.12)
Southampton Water	<2-68 (4)	0.67-2.15 (0.79)

[*] Oestrogenic activity as determined by the yeast oestrogen screen and expressed as an equivalent concentration of 17β oestradiol that would cause the same response.
[†] n=5/6

Methods

Sample receipt
Fifteen sediment samples packed in a cool box containing freezer packs were received from WRc (Dr Ian Johnson) via courier on the 25th November 2001. Samples were stored at -20°C until the samples were extracted.

Sediment sample extraction
A sediment sample is placed in a Teflon® centrifuge tube and centrifuged at 2000 G for 30 min to separate the sediment pore water from the residual particulate material. The pore water is drawn off using a Pasteur pipette and extracted using octylsilane (C8; 1 g) solid phase extraction (SPE) columns. The extracted compounds are recovered from the SPE columns using methanol and reduced in volume using nitrogen. This extract is then tested on the YES assay. The pelleted residual particulate material is dried at room temperature to constant weight, and ground using a pestle and mortar. The fines are passed through a 2 mm stainless steel sieve. Samples (*ca.* 10 g) are extracted with dichloromethane (10 ml), shaken for 30 min, sonicated for 10 min and centrifuged at 1730 G for 10 min. This process is then repeated twice. The supernatant is removed and the extracts combined. The extracts were concentrated to 5 ml using a TurboVap (Zymark, USA; 37°C).

Sediment extract fractionation

A simple fractionation step is employed to reduce the cytotoxicity of the complex extract obtained. Samples (total extract in hexane, 100 ml) are passed through a silica SPE column and the post column hexane collected. The column is then eluted with hexane (10 ml) which is added to the original post column hexane. The column is then consecutively eluted with 10 ml of dichloromethane (DCM), acetone and methanol and each individual fraction reduced in volume to 5 ml for assay.

Assay for oestrogenic activity
The YES assay detects receptor binding oestrogens (e.g. 17β-oestradiol and oestrone) and xenoestrogens (e.g. alkylphenols and Bisphenol A).

In brief, the human oestrogen receptor (hER-α) has been integrated into the yeast genome, together with expression plasmids carrying oestrogen-responsive elements (ERE's) that control the expression of the reporter gene *Lac-Z*. In the presence of oestrogens or chemicals with oestrogenic activity (which bind to, and activate the receptor), β-galactosidase is synthesised and secreted into the assay medium in which the yeast is grown. The β-galactosidase then breaks down the chromogenic substrate chlorophenol red β-galactopyranoside (CPRG). CPRG is initially yellow but breaks down into a red product, the concentration of which can be measured by absorbance.

Samples extracts in methanol are added to the microtitration test plate at a range of concentrations and then allowed to evaporate to dryness at room temperature. The assay medium, that consists of the chromogenic substrate and a growth medium that has been inoculated with yeast cells, is added to the plate. The plate is then incubated for 3 days at 32°C and shaken vigorously for 2 minutes each day. On the third day any change in the colour of the chromogenic substrate is read colourimetrically using a UV/VIS plate-reader (Bio-Tek instruments, Inc.) at an absorbance of 540nm for colour and 620nm for turbidity. A dilution series of 17β-oestradiol (E2) (used as a positive control and oestrogenic standard), together with a solvent blank is assayed alongside each batch of samples.

Oestrogenic activity for each sample is determined by comparing responses to the E2 standard. All equivalent E2 values of a sample are divided by their relevant concentration factors to produce equivalent E2 values for the original sample. All values falling in the linear range of the sample response curve are averaged to produce the final equivalent E2 value (μg E2 Kg^{-1}) of the sample.

Results and Discussion
All data are summarised in Table 2. The highest activity was measured in sediment BH501C, with similar activity of ~ 1 μg E2 Kg^{-1} determined in BH501A, BH502A, BH502B, and BH503A. Lower activity was measured in samples coded BH503B-BH505C.

A previous study of *in vitro* oestrogenic activity in Tyne surface sediments showed the activity to be between 1.26-3.95 (median 1.86) μg E2 Kg^{-1} (Table 1) which is comparable with the activity of samples BH501A, BH502A, BH502B, and BH503A. The activity of the other samples tested is lower than these previous data. Additionally there appears to be a general trend of decreasing *in vitro* oestrogenic activity from BH501-BH505, as well as from A-C.

Conclusions

- The oestrogen receptor (ER) agonist potency of sediment extracts obtained from cores collected from the Tyne estuary was measured using the *in vitro* yeast oestrogen screen (YES) (0.03-1.1 µg E2 kg^{-1}).

- The activity of extracts isolated from four of these samples was comparable with previous data for the Tyne. The other samples tested showed lower activity.

Table 2. Summary of the *in vitro* oestrogenic activity of Tyne sediments

Sample	*In vitro* oestrogenic activity (µg E2 kg^{-1})
BH501A	1.03
BH501B	0.73
BH501C	1.08
BH502A	0.94
BH502B	0.99
BH502C	0.58
BH503A	1.01
BH503B	0.33
BH503C	0.09
BH504A	0.19
BH504B	0.04
BH504C	0.03
BH505A	0.06
BH505B	0.04
BH505C	0.04

* Oestrogenic activity as determined by the yeast oestrogen screen and expressed as an equivalent concentration of 17β oestradiol that would cause the same response.

References

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2. Allen Y, Scott AP, Matthiessen P, Haworth S, Thain JE, Feist S. 1999a. Survey of oestrogenic activity in United Kingdom estuarine and coastal waters and its effect on gonadal development of the flounder *Platichthys flesus*. *Environ Toxicol Chem* 18:1791-1800.

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samples collected from industrialised UK estuaries. *Environ Toxicol Chem* 20 (10): 2165-2170.

Appendix I. Raw data

Table 3. Raw data.

Sample	Weight (g)	Final volume (F1-F4) ml	Conc. Factor	F1 (Hexane) ng E2/ Kg	F2 (DCM) ng E2/ Kg	F3 (Acetone) ng E2/ Kg	F4 (Methanol) ng E2/ Kg	Total YES (ng E2/ Kg)
BH501A	6.92	3	2.31	<10	940	92	<10	1032
BH501B	6.49	3	2.16	<10	697	34	<10	731
BH501C	6.68	3	2.23	<10	1027	55	<10	1082
BH502A	6.96	3	2.32	<10	941	<10	<10	941
BH502B	6.07	3	2.02	<10	804	187	<10	991
BH502C	6.76	3	2.25	<10	522	56	<10	578
BH503A	6.43	3	2.14	<10	601	411	<10	1012
BH503B	6.02	3	2.01	<10	257	69	<10	326
BH503C	6.76	3	2.25	<10	87	<10	<10	87
BH504A	6.35	3	2.12	<10	168	26	<10	194
BH504B	6.83	3	2.28	<10	41	<10	<10	41
BH504C	6.79	3	2.26	<10	25	<10	<10	25
BH505A	6.64	3	2.21	<10	61	<10	<10	61
BH505B	6.97	3	2.32	<10	35	<10	<10	35
BH505C	6.75	3	2.25	<10	38	<10	<10	38