R&D Project 490

Identification of oestrogenic substances in sewage treatment effluents

MAFF / Brunel University

September 1994

R&D Interim Report 490/2/A

IDENTIFICATION OF OESTROGENIC SUBSTANCES IN SEWAGE TREATMENT EFFLUENTS

M Waldock **MAFF**
J Sumpter **Brunel University**

Research Contractors:

MAFF Laboratory, Burnham-on-Crouch
Brunel University, Uxbridge

National Rivers Authority
Rivers House
Waterside Drive
Aztec West
Bristol
BS12 4UD

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Commissioning Organisation
National Rivers Authority
Rivers House
Waterside Drive
Aztec West
Bristol
BS12 4UD

Tel: 0454 624400
Fax: 0454 624409

Dissemination Status

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Statement of Use

This report is the second output from Project 490. It covers the period April 1994 to September 1994. It is to be used for information as to the progress to date and proposed work programme, to March 1995.

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M Stark, ST

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IDENTIFICATION OF ESTROGENIC SUBSTANCES IN STW EFFLUENTS PROJECT NRD 490

Progress report 490/2/A September 1994

MINISTRY OF AGRICULTURE, FISHERIES AND FOOD - DIRECTORATE OF FISHERIES RESEARCH

Fisheries Laboratory, Remembrance Avenue, Burnham-on-Crouch, Essex, CM0 8HA

Project manager Dr M Waldock (MAFF) Prof J Sumpter (Brunel University)
Project duration 1/12/93 - 31/3/96

1. OBJECTIVES

1.1 Overall project objective:

To identify and quantify the component(s) present in sewage effluents that are responsible for the vitellogenic response in fish

1.2 Specific objectives:

1.2.1 MAFF

- to fractionate raw sewage into chemical fractions
- to provide mixtures for hepatocyte testing
- to undertake further identification of those mixtures found to be vitellogenic in fish
- to apply those substances identified above to trout bioassay
- to produce an R&D Note presenting the project findings and recommendations for NRA business development and future work.

1.2.2 Brunel University

- to undertake screening, by laboratory bioassay, of crude and purified fractions of sewage provided by MAFF
- to provide technical support for work undertaken by MAFF, including other physiological determinations in fish, and the production of reports.

2. TARGETS FOR PERIOD APRIL TO SEPTEMBER

A summary of progress in the initial stages of the programme is provide in Report NRA 490/1/A. This report details further progress in assessment of estrogenic activity of crude fractions of sewage.

2.1 MAFF

- production of crude fraction samples to be tested for estrogenic activity
- investigations of other STWs

2.2 Brunel University

- assess toxicity/estrogenic activity of the fractions produced

3. WORK PROGRAMME UNDERTAKEN, APRIL - SEPTEMBER

During this period more than 600 samples of effluent fractions have been produced by the Burnham laboratory and assayed for estrogenic activity by Brunel University.

3.1 Crude Fractionation of Effluents

3.1.1 Strategy:

An assay for determination of estrogens in sewage effluents using a genetically modified strain of yeast has been refined at Brunel University. Appendix 1 describes the methodology. Estrogenic components in sewage are isolated by testing activity of whole sewage, which is then divided into crude and sucessively finer fractions and re-tested.

3.1.2 Crude Fractionation:

Progress:

After improvements in the sensitivity of the yeast assay, further crude (whole, filtered, purged, filtered + purged), and C18 SPE (solid phase extracted) effluent fractions were produced and submitted for testing at Brunel. SPE works on the same principle as liquid chromatography. Sample components are partitioned out of aqueous solution on to a solid phase, and recovered by elution with suitable solvents. Full procedural blanks were prepared using distilled water and have been run for every stage of the fractionation scheme.

One constraint on using the yeast assay is that it must have sterile conditions to work, and the sewage fractions must be sterilised before testing. Although various chemical methods of sterilisation have been attempted, sterilisation of effluent by 0.2µm filtration is still the most effective method used so far. All samples are filter sterilised before testing for estrogenicity and we are unable at present to measure the estrogenicity of `whole' effluent.

Results:

Filter sterilised (liquid phase only) effluent collected from Southend-on-Sea STW has shown estrogenic activity, equivalent to 50ng/L estradiol. In the context of effects previously seen in fish, the effluent would be placed in the category of an inducer of significant levels of vitellogenin production in male trout.

The effect of purging the sample in reduction of activity is slight if any, suggesting it is unlikely that the estrogenic activity is due to volatile components in the effluent. The result of analysis of the volatile fraction of Southend-on-Sea effluent is listed in Progress Report 490/1/A; of the compounds found only p-toluene has been shown to be weakly estrogenic in previous assays at Brunel.

The particulate fractions were retained for further treatment (see later).

3.1.3 C18 extraction of the liquid phase:

Experimental details

Neutral fractions :- 6 x 200 ml aliquots of effluent from Southend STW were filtered to remove any particulate content which would block the SPE cartridges. 5 ml was removed from each sample and retained. The rest of each sample was then extracted using a C18 SPE cartridge, and 5ml of each extracted sample was retained. The `before' and `after' samples were submitted for testing at Brunel University. Each sample was tested at various dilutions as described in Appendix 1.

Acidic and basic fractions :- In order to investigate the effect of pH on the extraction of estrogens the pH of further samples of effluent was adjusted to pH 2 with conc.HCl, or to pH 12 with NaOH. These samples were also extracted by SPE as above, and `before' and `after' samples were submitted to Brunel University.

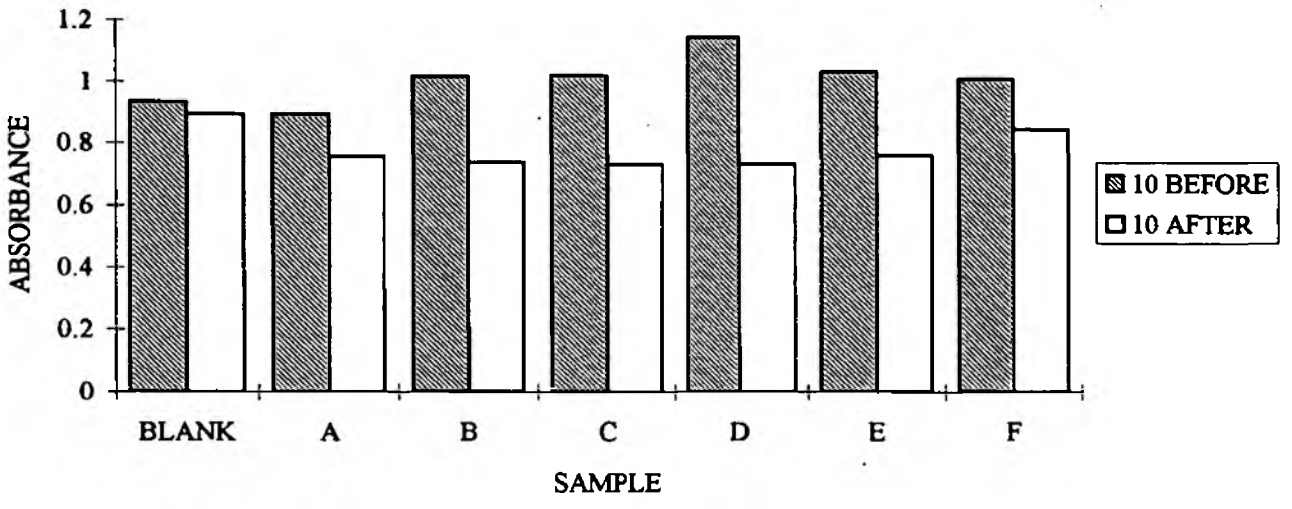


Figure 1. Fractionation of Southend-on-Sea sewage effluent (dissolved phase) using SPE columns. Vertical bars show absorbance in 10µl sample wells before and after passing through the SPE column. Samples A to F are replicates. (Data are not blank corrected).

Results

Passage of the liquid phase through C18 (non-polar) SPE cartridges caused a reduction of estrogenic activity by approximately 80% of the original for filter sterilised sewage effluent (see Fig. 1). This suggests that most of the estrogenic activity contained in the effluent is due to medium to low polarity substances which are trapped on the C18 phase of the SPE cartridge, and is not due to ionic substances, which would not be retained.

3.2 Fine fractionation

In order to begin the finer fractionation of the SPE retained estrogenic samples, a method based on the US EPA toxicity based fractionation procedure was used.

3.2.1 Medium polarity fractions

Method

The C18 cartridges used for the extraction in the previous section were eluted sequentially with a number of water/methanol mixtures ranging in polarity from very polar (0% methanol/100% water) to medium/low polarity (100% methanol). 3ml of each solvent mixture was used, and the resulting fractions submitted for testing.

Results

Estrogenic activity was concentrated in the fractions eluted with 50-85 % methanol (see Fig.2).

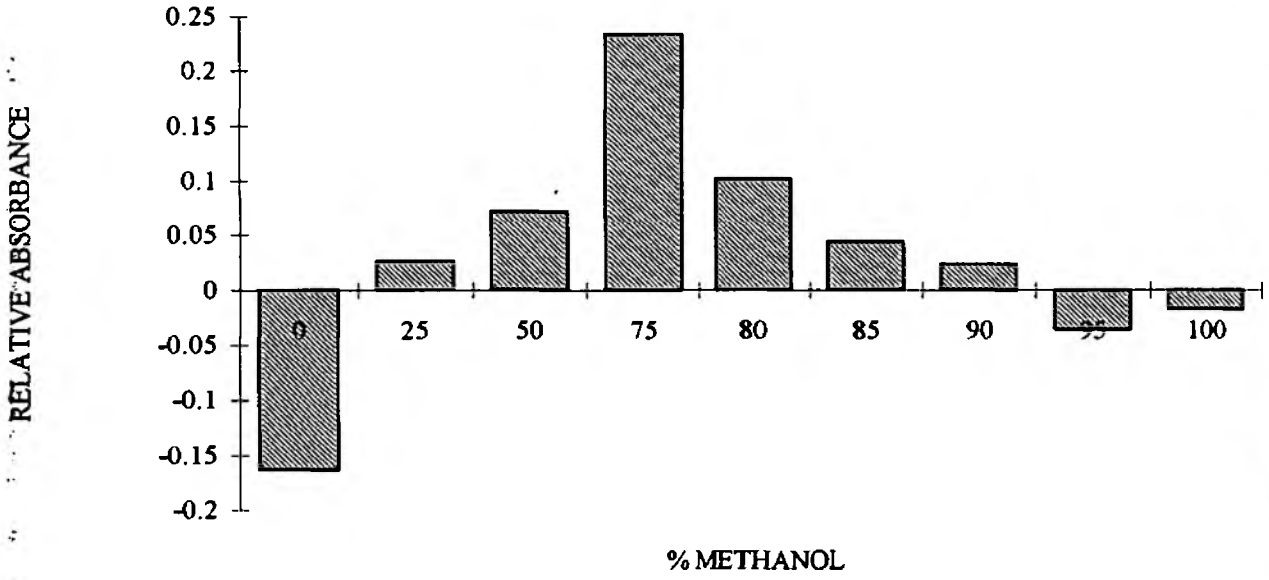


Figure 2. Estrogenicity of samples isolated from C18 SPE columns using increasing methanol to water ratios. Results for 10µl assay samples (Blank corrected values).

3.2.2 Acidified fractions

Method: (as above)

Results:

Acidification of samples before SPE partition had no overall effect on estrogenicity i.e. there was the same reduction of activity after C18 extraction of these samples compared with original filtered effluent. However the concentration of the activity in the 50-85% methanol fractions was greater (see fig.3). Acidification of the sample would have the effect of increasing the elution efficiency of compounds from the C18 SPE cartridge. It is therefore possible that acidification of the effluent leads to more efficient isolation of the components of interest. This will be futher investigated in the near future.

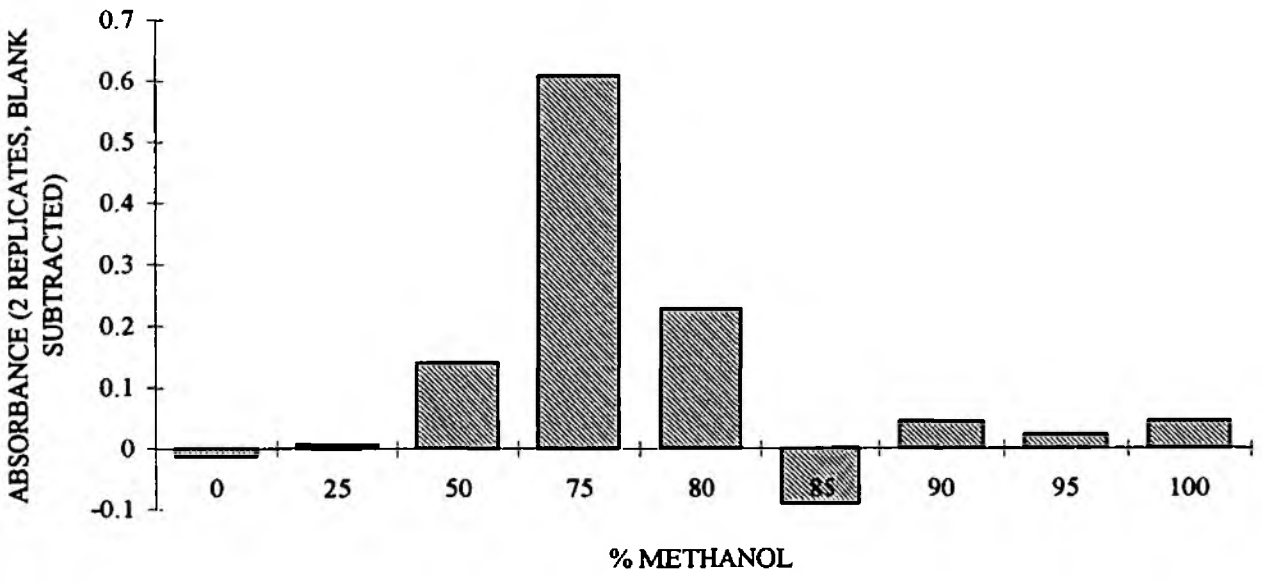


Figure 3. Estrogenicity of acidified samples isolated from C18 SPE columns using increasing percentages of methanol in the elution system. Results for 10µl assay samples. (Note the different scaling to figure 2.)

3.2.3 Low polarity fractions

Method

In order to elute any other compounds which were not removed with 100% methanol, the same SPE cartridges were then sequentially eluted with ether, 50/50 ether/hexane and hexane, in order to collect samples covering the widest possible polarity range. These fractions were then blown down to dryness under nitrogen, made up to 3ml with methanol and submitted for testing at Brunel.

Results

No estrogenic fractions were found.

3. 3 Liquid Chromatography (LC) fractionation

Finer fractionation of the estrogenic samples provided by SPE extraction of filtered effluent was then attempted. The samples of neutral pH SPE effluent containing most

of the estrogenic activity identified in the previous section were recombined and re-extracted using C18 SPE. The cartridge was then eluted with 100% methanol and the resulting concentrated extract separated by reversed phase LC, and individual fractions collected in successive 1.5 ml volumes.

3.3.1 Extraction method

Sample preparation - the most estrogenic fractions (eluted with 50-85% methanol) of 4 (out of 6) of the samples from the previous section were recombined (3ml x 4 x 4 = 48 ml) and diluted with nanopure water to a volume of 500ml (approx. 10 x dilution). This bulked sample was then extracted using two C18 SPE cartridges placed in series to ensure that as much of the analytes of interest were trapped on the second cartridge if the loading capacity of the first cartridge was exceeded. Each cartridge was then eluted with 3 x 2ml of methanol, the eluents combined, and subsequently blown down under nitrogen to a total volume of 500µl. The extraction procedure concentrated the most estrogenic fractions of 800ml of effluent by a factor of 1600 times.

3.3.2 Liquid chromatography

A reversed phase gradient LC method was developed to separate the concentrated fraction, the elution order being polar compounds eluted before non-polar compounds.

Conditions:

Column	25cm x 4.6mm id C18	
Mobile phase	A=water, B=methanol	
Gradient	time	%B
	0	40
	3	40
	30	100
	40	100
	45	40
Detection	UV, 210nm	

In order to introduce a large amount of sample onto the column, a 150µl loop injector was used. Under these conditions, the column was severely overloaded, but the separation remained reproducible during sucessive analytical runs.

Fraction collection

The eluent from the LC column was collected immediately after passing the UV detector in 1.5ml aliquots (giving a total of 30 fractions over 45mins). Each fraction was blown down to approximately 750µl under nitrogen to further increase the final concentration of potential estrogens in each sample. These samples were then submitted to Brunel for testing.

Results

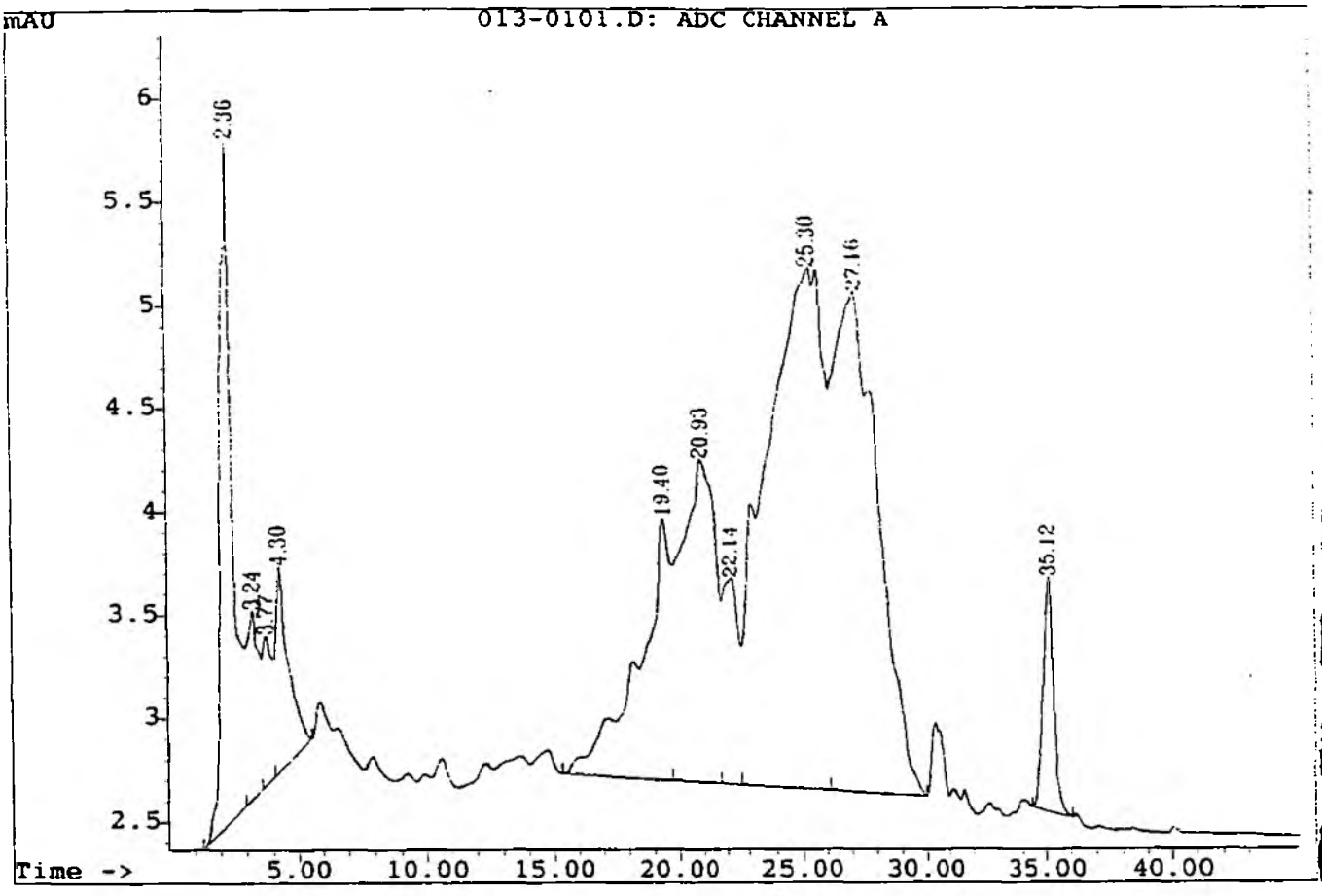
Fractions 9 to 11 (12.0 - 16.5 mins) and 13 to 16 (18.0 - 24.0 mins) were found to be very weakly estrogenic. Fraction 17 (24.0-25.5 mins) was found to be very strongly estrogenic (see fig.4).

3.3.3 Gas Chromatography/Mass Spectrometry (GC-MS) analysis of fraction 17

No significant peaks were found during full scan GC-MS analysis of the fraction showing the most estrogenic activity. This strongly suggests that the estrogenic compound/compounds present in LC fraction 17 are too polar to migrate through GC columns. Alkyl phenol standards have been run under the same conditions, and comparison of the GC/MS chromatograms shows that they are not present in the estrogenic fraction. This finding does not rule out the presence of polar alkyl phenol polyethoxylate surfactants in the sample. Liquid chromatography coupled to mass spectrometry is a more suitable technique for identification of polar compounds and will be used in the future.

3.4 Particulate fraction

Although it has not been possible to measure the estrogenicity of whole effluent, initial investigations have been made into the estrogenicity of the particulate fraction. Glass fibre filter papers used to filter the sewage effluent used in the previous sections were solvent extracted with a series of solvents of increasing polarity (hexane, hexane/ether, ether, methanol, methanol/water, water). The 3 less polar extracts were blown down



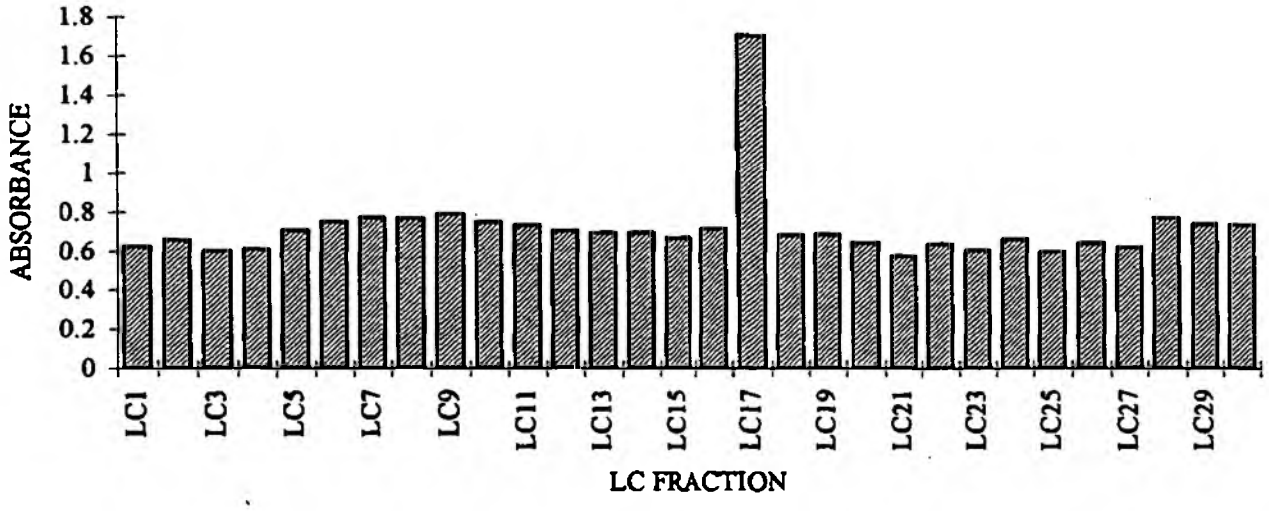


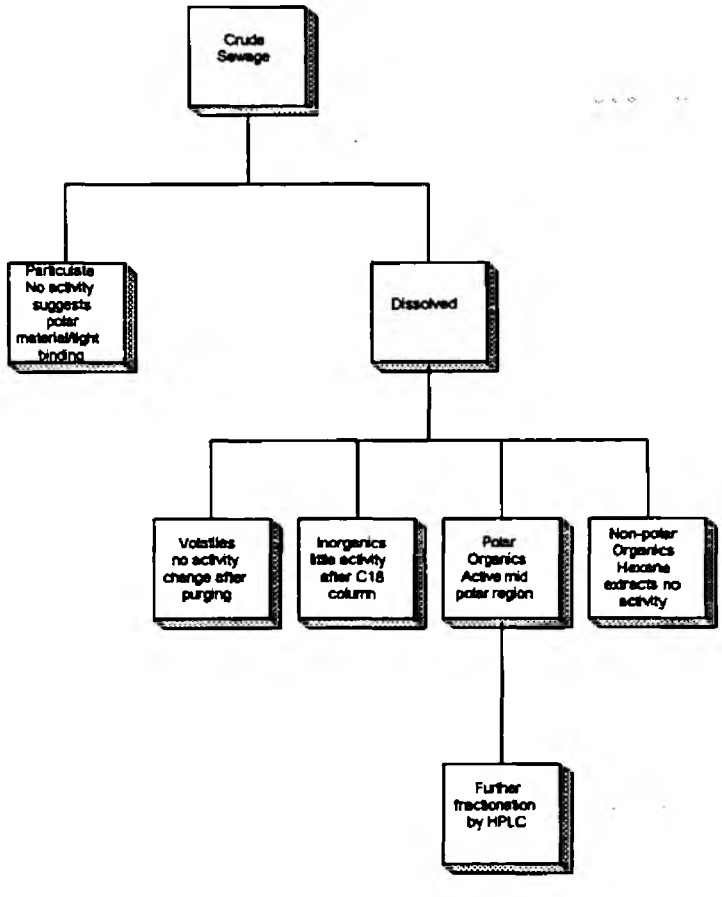
Figure 4a. LC chromatogram of SPE eluant . 4b. Estrogenicity of samples isolated from LC fractions collected in 1.5 ml volumes. (Values are not blank corrected).

to dryness and dissolved in methanol. These were submitted for testing at Brunel. Initial results suggest very little activity is present in these fractions, although this may be due to inefficient extraction procedure or possibly too little sample extracted (4 filter papers extracted, equivalent to passage of about 250ml effluent). Further investigations will proceed in the near future.

3.5 Sewage tracers

Investigations into the use of squalene as a sewage tracer have continued. It is hoped to use this compound as a tracer for effluent in rivers, and to correlate its presence with that of estrogens. A survey of 5 rivers, Wye, G. Ouse, Thames, Lea and Aire for alkyl phenols, coprostanol (a faecal steroid previously used as a sewage tracer), and squalene has been carried out. If squalene presence positively correlates with coprostanol, then squalene will be used as a tracer in its place as the assay developed is very much quicker.

3.2.5 Summary of Progress



These data reflect initial findings for Southend Sewage on one occasion and these preliminary results should be interpreted with care. However the project is now progressing very well and in running to the expected schedule. Time invested in developing the yeast bioassay as an alternative to the hepatocyte assay has paid dividends due to the increased speed at which chemical fractions may be screened for estrogenic activity. A robust methodology for effluent fractionation has been developed and together the two techniques are providing repeatable results. The fact that active fractions have been extracted and remain active on chemical manipulation indicate that the estrogenic component(s) is stable, suggesting that the task of isolation of estrogens should not pose too many difficulties. The methods are presently being used in a semi-quantitative way to target the most estrogenic components within effluents and direct future research, but the system should lend itself to more exact quantification of estrogenicity as the work is developed and repeated.

4. FUTURE WORK

4.1 Further fractionation of the most estrogenic fractions already found will continue, using LC techniques to isolate single compounds for identification by mass spectrometry.

4.2 Investigations of the particulate fractions will be repeated in order to confirm the low estrogenic activity of compounds associated with suspended particulates in sewage.

4.3 Different methods of sterilisation wll be investigated, so that whole effluent can be tested and a scheme for quantification of the activity can be developed..

4.4 STW effluents from Harpenden and E. Hyde, have been subjected to the same fractionation regime in order to confirm that the activity is in the same SPE fractions. These samples have been submitted for estrogen screening.

APPENDIX 1 THE RECOMBINANT YEAST ESTROGEN SCREEN

THE RECOMBINANT YEAST ESTROGEN SCREEN

Introduction

This recombinant yeast strain was constructed in the Genetics Department at Glaxo in order to screen for compounds which act as agonists and antagonists of the human estrogen receptor (hER). As yeast cells do not normally contain an estrogen receptor, the DNA sequence of the hER has been integrated into the yeast, along with a reporter gene (encoding the enzyme β-galactosidase) which is used to measure the receptor's activity. Activated hER (receptor joined to estrogen) causes expression of the reporter gene and the enzyme produced is detected by the inclusion of a chromogenic substrate in the medium. This system provides an excellent method of measuring the activity of the hER in response to estrogens.

The assay involves the overlaying of samples in microtitre plates with media containing the yeast together with the chromogenic substrate Chlorophenol red-beta-D-galactopyranoside (CPRG). In the presence of β-galactosidase, CPRG (normally bright yellow) is metabolised to produce a red colour which can be measured on a plate reader at an absorbance of 540nm. After incubation, control wells will appear light orange in colour (due to background expression of β-galactosidase) and turbid due to growth of the yeast. 'Positive' wells are indicated by either deep red (Agonist) or bright yellow (Antagonist), both with turbid yeast growth.

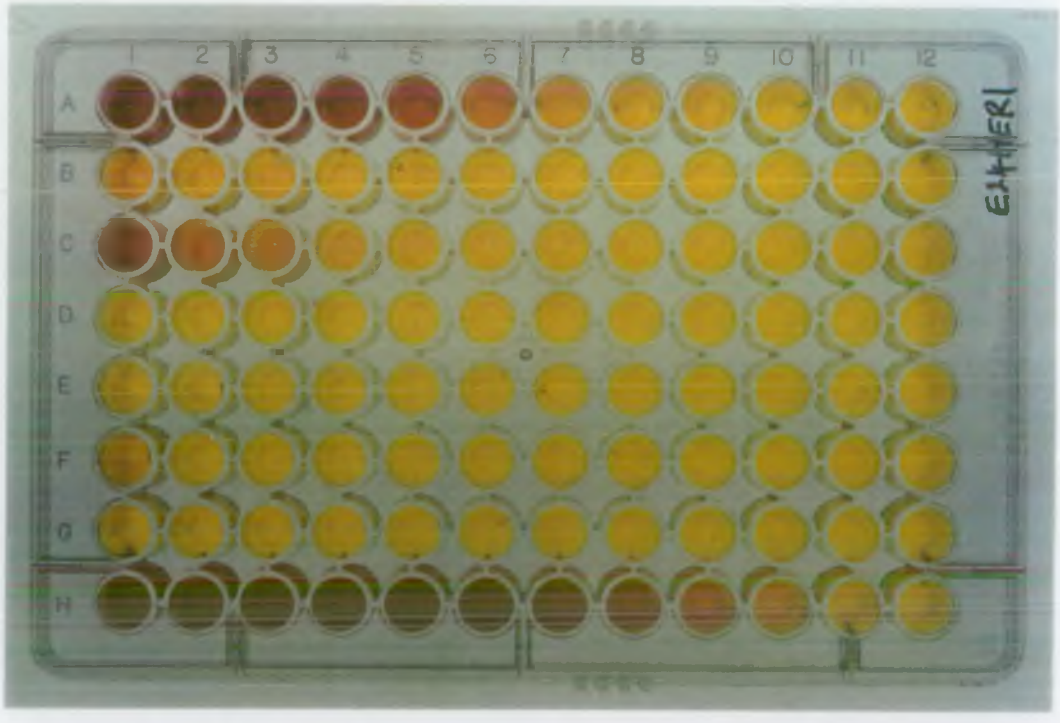


Fig 1. Specificity of the yeast screen. Ten different steroids were tested, each serially diluted from left to right, beginning at 5 x 10 -8 Molar in the left hand well, and ending at 5 x 10-11 Molar in the right hand well. 17β-estradiol (row H) was by far the most active. 17α-estradiol (row A), and 17β-sulphated estradiol (row C) were also estrogenic, albeit much less so. Rows B, D, E and F contained some of the other steroids mentioned in Figure 2, all of which were inactive. Row G is a blank (no samples added).

Specificity of the Yeast Screen

The specificity of the yeast screen was assessed by using a range of steroids and steroid metabolites. 1μMolar solutions of **17β-estradiol** (17b-E2), **17α-estradiol** (17a-E2), **sulphated and glucuronated estradiol** (E2-S04 and E2-Gluc), **testosterone** (Test), **dihydrotestosterone** (DHT), **progesterone** (Prog), **17α-hydroxy-progesterone** (17a-H-P), **4-pregnene-17α20β-diol-3-one** (4-P-17a20b) and **cortisol** (Cort) were made up in ethanol. A dilution curve was made for each steroid by twelve 2-fold serial dilution steps along the plate. A final volume of 10μl of hormone solution was added to the microtitre plate wells. The plate after incubation is shown in Figure 1.
The plate can then be read on the plate reader to produce dose response curves using absorbance as a measure of the response against concentration, as shown in Figure 2. below.

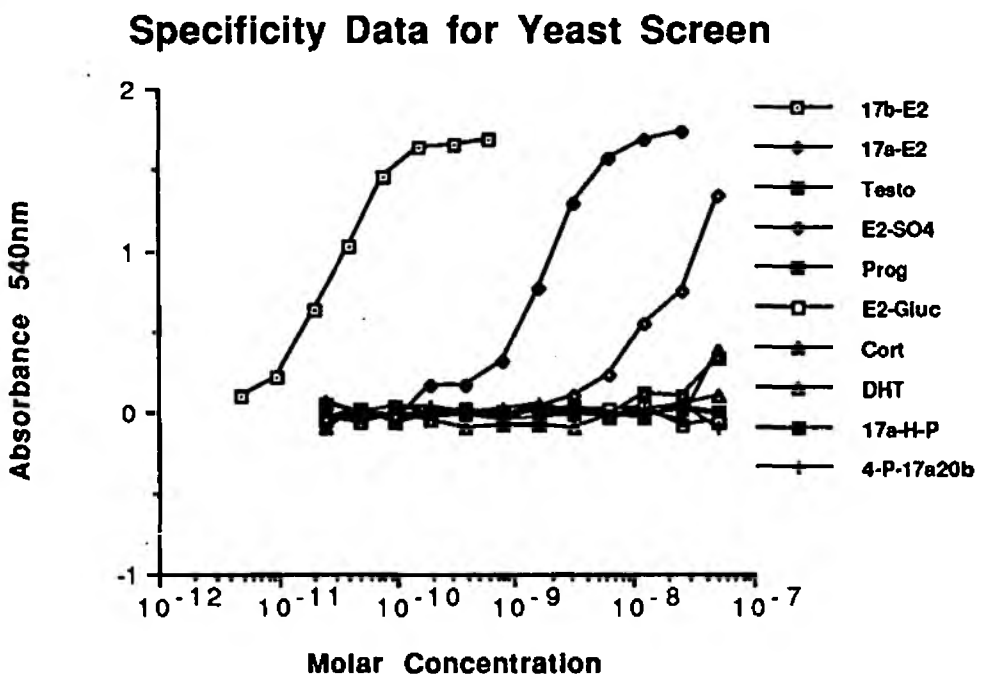


Figure 2. Specificity of the yeast screen to a range of steroids and steroid metabolites.

The data presented in Figure 2. shows that the strongest response was seen in the wells containing the natural estrogen 17β-estradiol. As expected, 17α-estradiol and then the sulphated estradiol metabolite were the next most potent steroids. The other steroids showed no response except testosterone and cortisol which showed a very small response at the highest concentration (three orders of magnitude greater than the concentration of 17β-estradiol required to produce the same response). This was possibly due to a small amount of interaction with the hER at levels well in excess of physiological concentrations . The results show that the screen is highly specific to estrogenic steroids and that their relative order of potency is as expected.

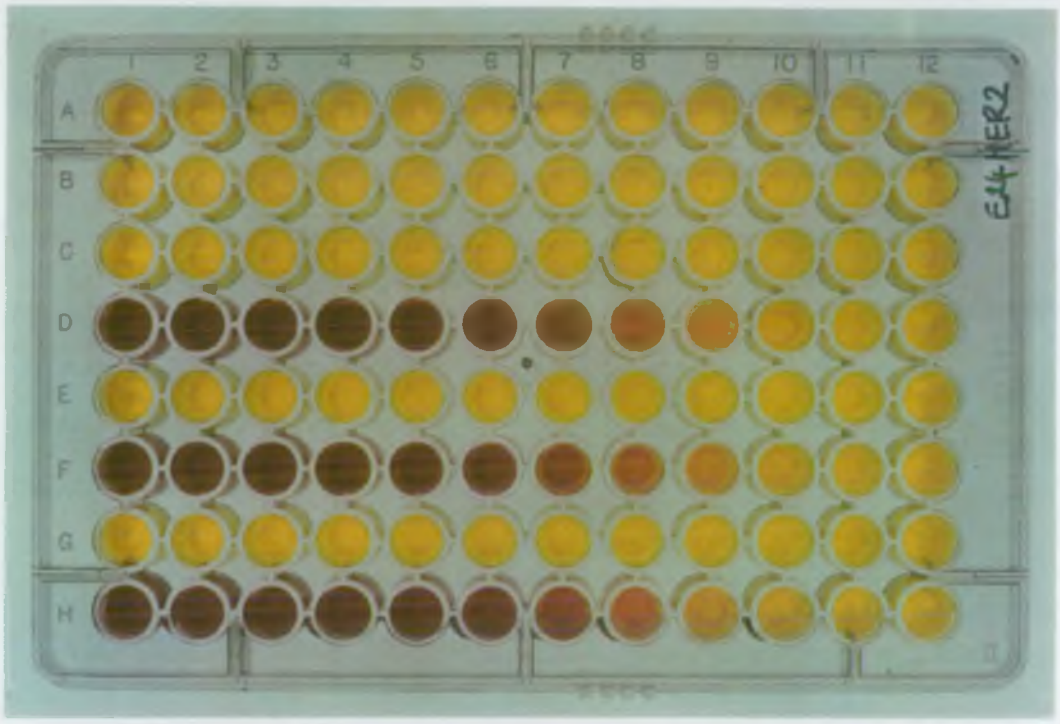


Fig 3. Rows D, F and H are three identical standard curves of 17β-estradiol starting at 3000 ng / L in the left hand well, and diluted down to 1.5 ng / L in the right hand well. The standard curves are separated by blanks (rows E and G). The plate shows that there is no difference between replicate rows, indicating that the screen is highly reproducible.

Sensitivity and Reproducibility of the Screen

The sensitivity and reproducibility of the estrogen screen was assessed by creating three identical standard curves using 17β–estradiol, starting at a concentration of 3000 ng/L and diluting this down to 1.5 ng/L. The standard curves were separated by rows of blanks (no samples added to these wells). The plate after incubation is shown in Figure 3. This plate was then read on the plate reader to produce the graph shown in Figure 4.

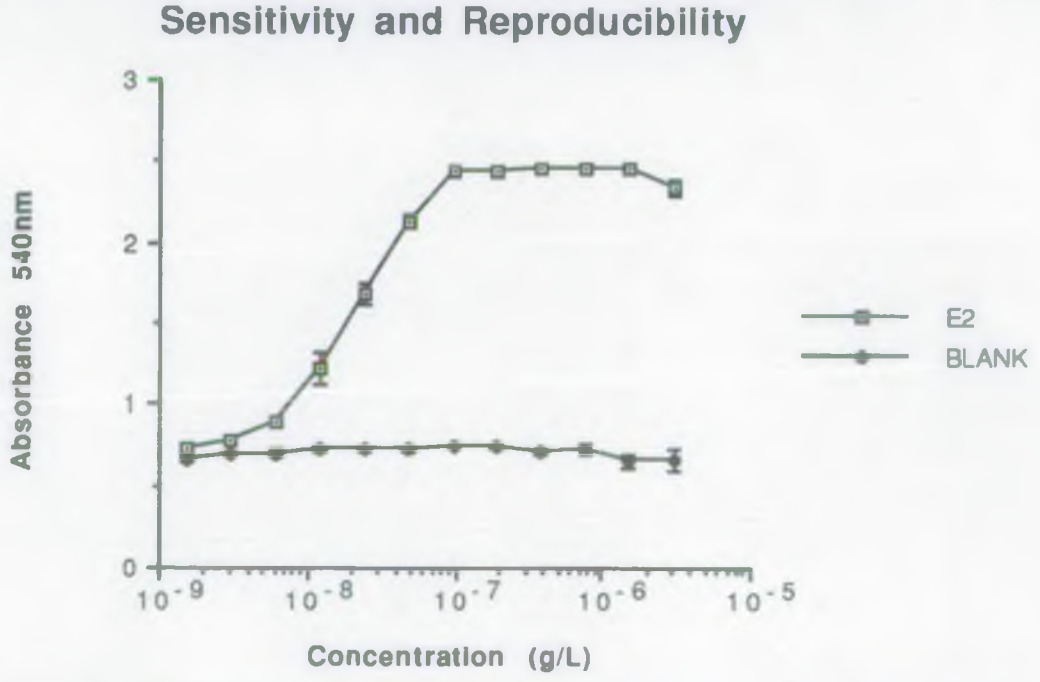


Figure 4. Sensitivity and reproducibility of the yeast screen to 17β-estradiol standard curves.

Figures 3 and 4 show that the screen is highly sensitive. In fact a concentration as low as 3 ng/L of 17β-estradiol can be detected. The tight error bars indicate that the screen is also highly reproducible.

Screening of Effluent Fractions

The recombinant yeast screen is being used at Brunei University to screen effluent fractions produced at MAFF (Burnham-on-Crouch) as part of an NRA contract. The aim of this contract is to discover exactly what it is in effluent that is estrogenic. Figure 5 below shows a plate containing some of these fractions after LC fractionation. It is obvious from this plate that there are definite 'hot' (estrogenic) fractions in the effluent. These fractions can then be further separated at MAFF for re-screening and eventually identification.

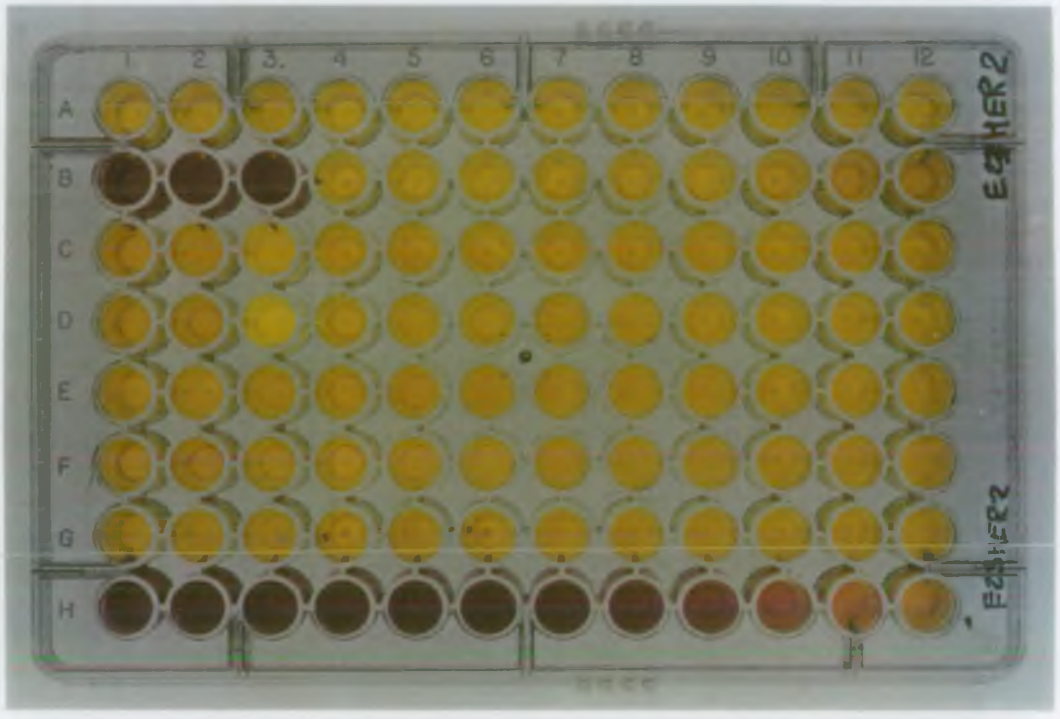


Fig 5. Screening effluent fractions for estrogenic activity. Fractions obtained every 1.5 minutes throughout LC fractionation of effluent were screened in triplicate. Row H corresponds to the 17β-estradiol standard curve and row G is the blank. Wells B1 - B3 show a highly estrogenic sample eluted between 24 and 25.5 minutes from the column.

Conclusion

In view of the specificity, sensitivity and reproducibility of the data presented in this report, it is obvious that this is a excellent screen which has already played a valuable role in this important issue of estrogens in the environment. The short running time of under a week per assay, and the 96- well microtitre plate design, enables us to test a large number of substances (or fractions) cheaply and efficiently.