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***Biodegradation of Estrogenic Compounds and Its Enhancement in a Membrane Bioreactor* – Research Category III, Water Quality**

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Abstract

In the project, we investigated enhancement removal of estrogenic activities in activated sludge. These activities are caused by natural and synthetic substances that mimic the effect of the human hormone estrogen and they potentially can disrupt the endocrine systems of exposed species and the reproductive systems of aquatic fauna. Human and animal wastes are a source of natural and synthetic estrogens to the environment since only a fraction is removed in conventional wastewater treatment. A yeast-based assay developed previously was modified to detect the estrogenic activity in wastewater samples. Using the assay, it was possible to quantify estrogenic activity in range equivalent to between approximately 100ng/L to 100μg/L of the female hormone 17-β estradiol (E2), with sensitivity as low as 0.03ngE2/L. The assay is therefore sensitive to the concentrations of environmental estrogens typically found in wastewater and the new assay may be a useful tool for screening for estrogenic activity. Compared to existing chemical analytical methods, the new test is simpler and covers a wider range of compounds. This is important because by-products of some of the influent estrogens are also active estrogens. For example, E2 is metabolized to estrone and estriol, which are estrogenic. Monitoring the removal of only a few substances may underestimate the estrogenic properties of treatment plant effluents and solids disposed of into the environment. Further experiments were carried out to determine the removal of estrogenic activity from water. Results show that the presence of activated sludge enhances removal of total estrogenic activity by at least 40% within 10-15 days.

Introduction and Problem Statement

Estrogens are substances both natural and synthetic that mimic the effect of the female hormone estrogen in the body and impart estrogenic activity (Arnold *et al*., 1996; Routledge & Sumpter, 1996; Coldham *et al*., 1997). Because of this effect, they potentially can disrupt the endocrine system in the exposed aquatic species.

Since all mammals produce estrogens, human wastes are a major source of estrogens in the environment. These wastes are treated in wastewater treatment plants where some of the estrogens are removed, and the rest is discharged in the effluent or disposed with biosolids. Other

sources of estrogenic compounds include birth control pills (Ternes *et al*., 1999; Johnson *et al*., 2000) and chemicals like detergents. It is important to be able to reduce their concentrations and it would be ideal if this can be achieved using available existing treatment processes.

Objectives

The research objective was to determine if the removal of estrogenic activity was enhanced by the presence of activated sludge solids. A yeast-based assay protocol was developed and used to measure the estrogenic activity.

Experimental Procedures

Method – Yeast Estrogen Assay

The yeast estrogen assay uses a liquid beta-galactosidase test to quantify rapidly estrogenic activity in a sample. The assay methodology is adapted from methods used by Klein, et al. (1994) and Coldham, et al. (1997). It provides means to measure colorimetrically the estrogenic activity in a sample. The method uses genetically modified yeast cells (*Saccharomyces cerevisiae*) which contain both the human estrogen receptor and the *lacZ* gene. The yeast was generously provided by Professor Didier Picard, University of Geneva. Beta-galactosidase is an enzyme encoded by the *lacZ* gene of the *lac*-operon. The enzyme hydrolyzes lactose into glucose and galactose. When the modified (recombinant) yeast cells are exposed to estrogen, the estrogen binds to the estrogen receptor (Picard, pers. comm.). The binding up-regulates the *lacZ* gene to produce beta-galactosidase. This allows the yeast cells to use lactose as a substrate. In the actual assay, a lactose analog, ortho-nitro-phenyl galactopyranoside (ONPG) is used because when hydrolyzed by beta-galactosidase it forms a yellow color due to production of ortho-nitrophenol. **Fig. 1** is a schematic representation of the assay. The yellow color can be measured with a spectrophotometer. Theoretically, the intensity of the yellow color is proportional to the concentration of ONPG hydrolyzed. It is therefore related to the enzyme activity expressed as a result of yeast exposure to estrogenic activities in the sample. A dose-response curve relating galactosidase enzyme activity to estradiol concentration is then used to give an estradiol equivalent measure of the estrogenic activity in wastewater and environmental samples.

Assay Procedure and Standard Curve Development

A standard dose response curve was generated for E2 concentration range from 0.03ng/L (10^{-13}M) to 30mg/L (10^{-4}M) – See **Fig 2**. To prepare standards, E2 was dissolved in denatured ethanol (EtOH) to give a stock solution with a concentration of 10^{-2}M. This solution was further serially diluted 1:10 in EtOH to give E2 standards ranging from 10^{-3}M to 10^{-11}M. The standards were then diluted again 1:100 in a sterile organic feed solution to give standards from 10^{-4}M to 10^{-13}M. The final dilution was done using an organic feed solution rather than water to provide for the same matrix as the samples taken from activated sludge reactor.

A volume of 250µL of each standard in sludge feed was added to a glass culture tube containing 750µL of selective medium - with 50µM $CuSO_4$ to induce estrogen receptor generation. An

inoculum of200µL yeast cell suspension in the selective medium was then added to give a final volume of 1.2mL (0.2%EtOH) and the mixture incubated at 30°C for 20hr.

After incubation, yeast cells were collected by centrifugation (3600rpm for 10min), the supernatant discarded, and the cells re-suspended in Z-buffer. A volume of 100µL of this cell suspension was added to a glass culture tube with 100µL ONPG in Z-buffer, 40µL chloroform, and 40µL SDS in a 30°C water bath and incubated for 6min. The reaction was stopped with 1mL Na_2CO_3, the cell debris removed by centrifugation, and the yellow color absorbance of the supernatant measured at 420nm using a spectrophotometer.

The absorbance readings from the assayed samples were converted into enzyme activities and then E2-equivalent concentrations as follows. The amount of ONP produced before the assay reaction is stopped is measured as yellow color absorbance at 420nm (A_{420}). According to the Lambert-Beer law if the product formation is proportional to measured color development then the measured absorbance is related to the concentration of product by the equation,

$A = \varepsilon.C.L$

where
A = absorbance
ε = molar extinction coefficient
C = concentration of ONP in µmol/L
L = length of light path in cm

Since excess substrate (ONPG) was used in the assay, stopping the assay at T=6min ensured that enzyme activity was maximal and the absorbance was linearly proportional to the concentration of yellow product (ONP). The optimum assay duration was determined in separate experiments.

For ONP, 1µmol ONP has an absorbance of 4.5 at 420nm in a 1cm cuvette (MIC 328, 2001). Then, with some rearranging, the units of beta-galactosidase activity (normalized for cell density of yeast culture) are

$$Activity = \frac{A_{420}\, V_{TOT}}{\varepsilon\, L\, T\, V_{YWS}\, A_{630}} \qquad [\mu mol\, mL^{-1}\, min^{-1}]$$

where for each reaction tube:
A_{420} = absorbance of ONP product measured at 420nm
A_{630} = absorbance (optical density) of yeast cell culture measured at 630nm
V_{TOT} = volume of stopped reaction mixture [mL]
V_{YWS} = volume of yeast cell culture used [mL]
T = time between addition of yeast culture to substrate and stopping with 1M Na_2CO_3 [min]

The activities from an assay of the standards were then plotted against E2 concentration as shown in the standard curve (See **Fig 2**). Activities from sample assays could then be converted into an E2-equivalent concentration by linear interpolation from the standard curve.

Special care must be exercised in preparation of reagents and labware. All glassware used in the experiments was baked at 400°C or greater for 3hours to remove organics. Plastic containers and pipette tips were washed with ethanol before use.

Experimental Setup

Experiment A: Kinetics of Estrogenic Activity Removal

Batch experiments were set up with 100mL activated sludge in 250mL glass flasks. The sludge experiments were spiked with E2 standards to give final E2 concentrations of 20mg/L, 100µg/L and 100ng/L. In one set of flasks the content was mixed continuously (mixed test) while the other was allowed to stand (static test). Controls with no E2 and no sludge were included.

Samples (2mL volume) were collected in 10x75mm baked borosilicate glass culture tubes from each flask over a period of at least 15days. The tubes were then covered with parafilm and frozen so that the entire 15day set could be analyzed at the same time. Before analysis, the samples were defrosted in the refrigerator. Sludge solids were separated out by centrifugation at 3600rpm for 10min, and only supernatant was used in the assay.

An aliquot of 250µL from each sample was incubated with 200µL yeast cells suspension and 750µL selective medium with 50µm $CuSO_4$ for 20hr at 30°C and then assayed in the same way as the standards.

Experiment B: Estrogenic Activity Removal by Acclimated Biomass

Since it is possible that microorganisms may develop higher transformation activities after exposure to E2, the removal of estrogenic activities by the biomass previously exposed to E2 was also evaluated. The sludge from Experiment A was re-spiked with the same concentration of E2 and estrogenic activity remaining determined over an additional 15day period following the same protocol.

Experiment C: Estrogenic Activity Associated with Activated Sludge Biosolids

Since estrogenic compounds show high affinity for solids (as evidenced by their high octanol-water partition coefficient), a set of experiments was devised to evaluate the kinetics of estrogenic activities associated with liquid and solid phases in activated sludge. In batch experiments, biomass from an activated sludge reactor was spiked with different concentrations of E2, similarly to Experiment A. However, sampling was more frequent and samples were separated into solid and liquid phases. Samples with volume of 2mL were collected before adding E2, within 60 seconds after adding E2, then every 5-10min for 1 hour. Immediately after collection, the samples were then centrifuged (3600 rpm for 10min) to separate the solid and aqueous phases. The aqueous phase was decanted into another tube and estrogenic activity evaluated like in the samples from Experiment A. The remaining solid phase was treated with 200µL of chloroform – both to inactivate the microorganisms and to dissolve and concentrate preferentially E2 from the solids (x10). An aliquot of 100µL of the chloroform-dissolved E2 was transferred to a clean culture tube and the chloroform evaporated at 40°C. The E2 coating the tubes was re-dissolved in 250µL of selective medium for analysis in the assay as described previously.

Sludge characteristics

The sludge used for the experiments was obtained from laboratory bench-scale conventional activated sludge system. The sludge had volatile suspended solids concentrations of 0.4-0.7g/L with more than 70% VSS.

Results

Experiments A and B: Kinetics of Estrogenic Activity Removal

Continuously mixed experiments spiked with 20mgE2/L (Fig. 3) showed little removal of estrogenic activity during the duration of the experiments with or without sludge. In the static experiment, an E2-equivalent concentration of less than 1mgE2/L observed in the sludge supernatant and the majority of E2 was probably associated with the sludge solids.

Experiments spiked with 100µgE2/L (Fig. 4) showed between 40 and 90% removal of estrogenic activity within 10 to 15 days after an initial lag of up to 10days. The time lag suggested the possible need for acclimatization of the sludge to E2. This was checked by re-spiking the sludge with an additional 100µg/L after the first set of experiments in Experiment B. When re-spiked, there was a shorter time lag and the activity leveled after about 10days corresponding to an E2-equivalent concentration below 0.2µg/L.

Experiment C: Estrogenic Activity Associated with Activated Sludge Biosolids

Further tests (Experiments C) estimated that within the first hour after E2 addition, up to two-thirds of the estrogenic activity was associated with the sludge solids for E2 concentrations 100 µg/L and higher. The activity associated with solids showed at least 20% decrease within 60minutes, while the activity in the liquid phase changed little. This further emphasizes the enhancement of estrogenic activity by sludge solids.

Background estrogenic activity

The background activity of the sludge used for the experiments is an important factor, especially at low concentrations because the yeast assay is quite sensitive. Controls without sludge did not show much change in activity during the experiment period. For example, in the 100µg/L trials the activity in controls without sludge was 0.27±0.12 – an equivalent of $9.8\pm3.2\times10^{-4}$ µgE2/L. With sludge, the background activity was lower at 0.20±0.13 or an equivalent of $0.05\pm6\times10^{-4}$ µgE2/L. The sludge used in the second trial had a higher initial background activity of 0.39 (0.24µgE2/L) and may explain the longer time taken to reduce the activity to below background levels.

Conclusions

The yeast estrogen assay can be used to evaluate estrogenic activities in activated sludge process. The assay showed a quantitative response for concentrations of 17-β estradiol (E2) from100ng/L to 100µg/L, with sensitivity as low as 0.03ngE2/L. Further enhancements should include preconcentration of the sample. In activated sludge, the experiments showed a measurable removal of total estrogenic activity within the test period of several days. Biomass acclimation is an important factor for faster removal of estrogenic activities. E2 strongly partitions into the biosolids in short time (minutes) thus contributing to fast removal from activated sludge effluent. Continuing work is looking at lower estrogen doses (100ngE2/L) and higher sludge biological solids concentrations (4 to 8g/L). It is expected that since estrogen is removed both by sorption to solids and microbial metabolism, increasing the solids concentrations should be able to remove the estrogenic activity more readily and more effectively in a shorter time frame.

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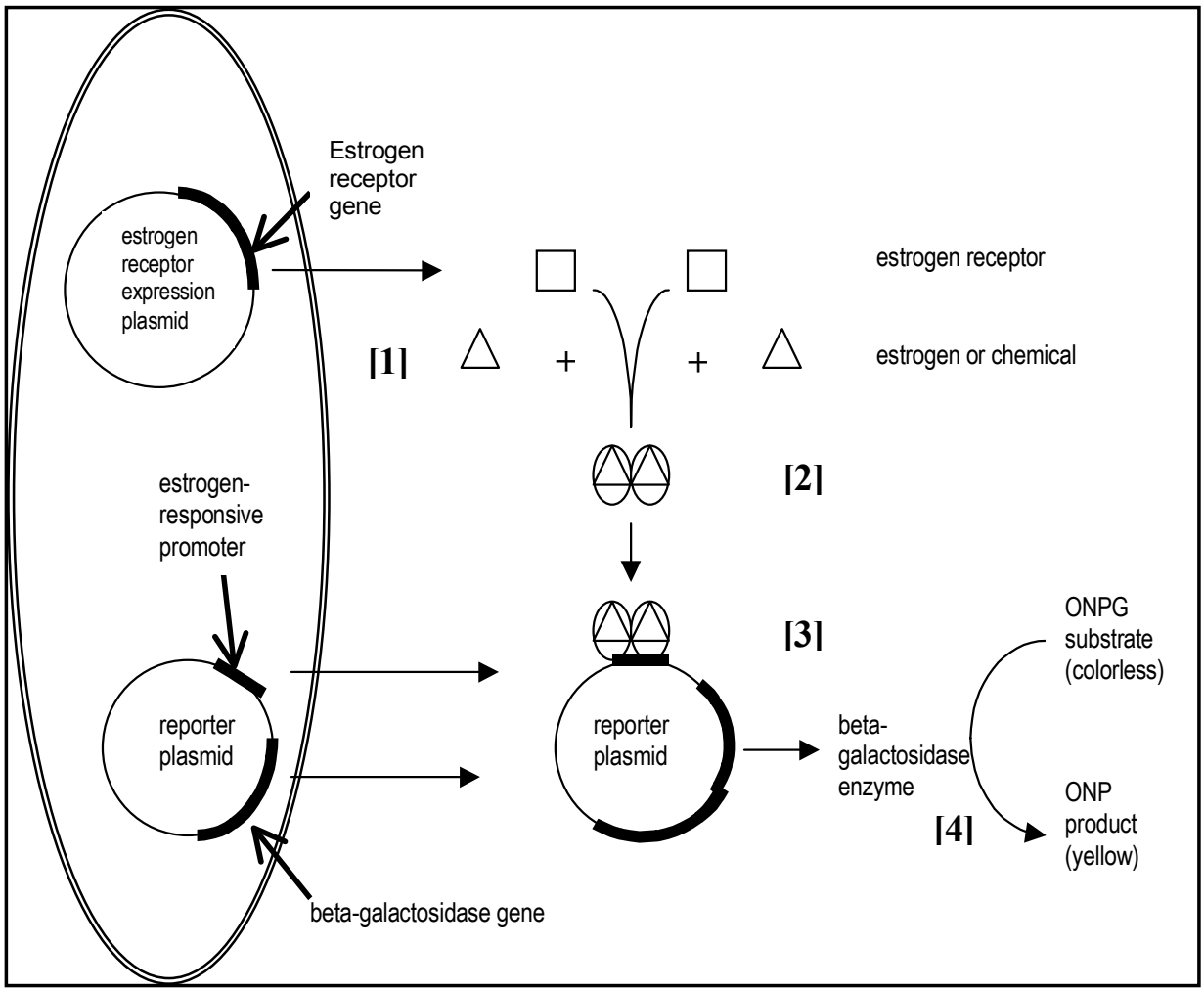

Fig. 1: Yeast-based estrogen receptor assay (Adapted from Activities Feb 1997)
The human estrogen receptor is expressed from its expression plasmid in an inactive form **[1]**. Binding of estrogen or chemical ligand to the estrogen receptor results in dimerization and activation of the receptor to a form capable of binding to estrogen responsive elements on the promoter of the reporter plasmid **[2]**, resulting in the production of the enzyme beta-galactosidase **[3]**. Beta-galactosidase converts the chromogenic substrate ortho-nitrophenyl beta-D- galactopyranoside (ONPG) into a yellow product, ortho-nitrophenol (ONP), which can be measured by absorbance **[4]**.

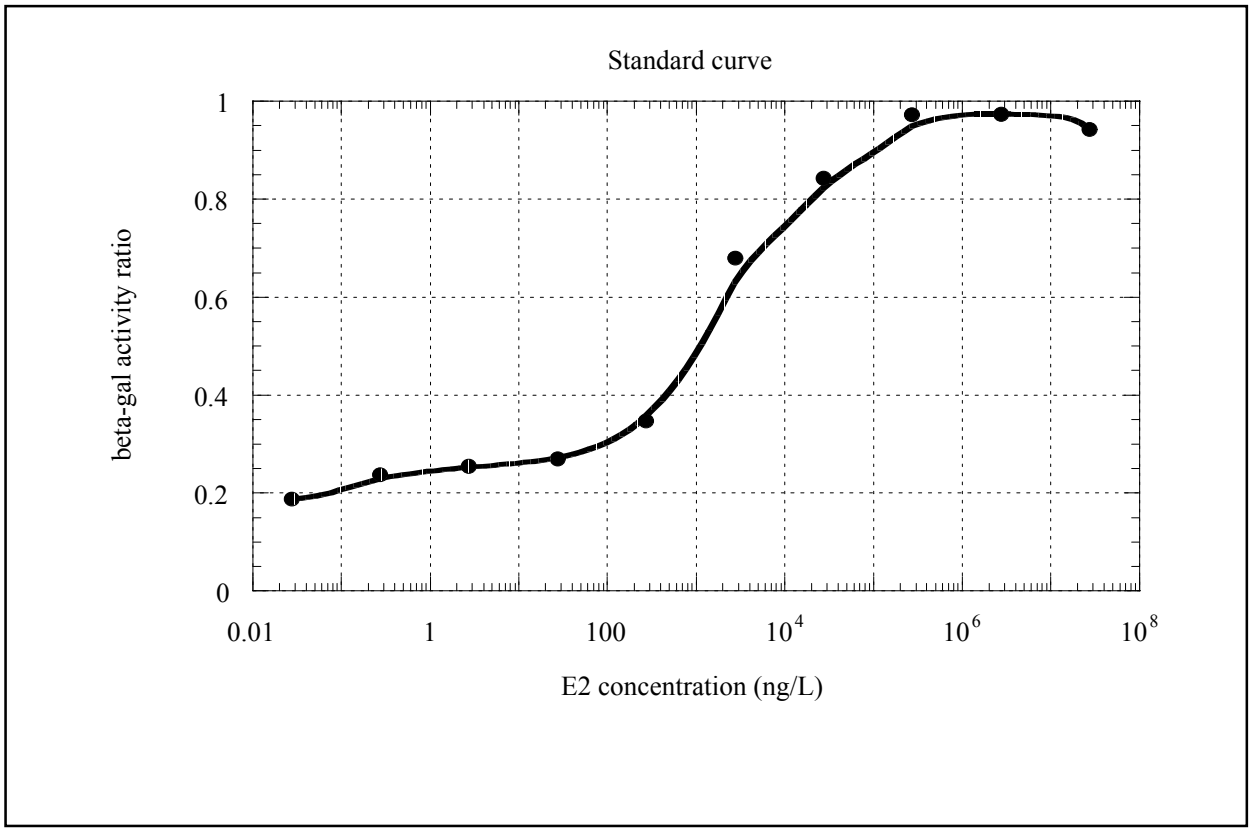

Fig. 2: Standard dose-response curve.
The beta–galactosidase enzyme activity ratio is defined as,
Activity Ratio = [measured activity at given concentration]:[maximum activity measured in assay of standards]. Maximum activity was 0.1336±0.0308μmolONP/min-mLYWS.

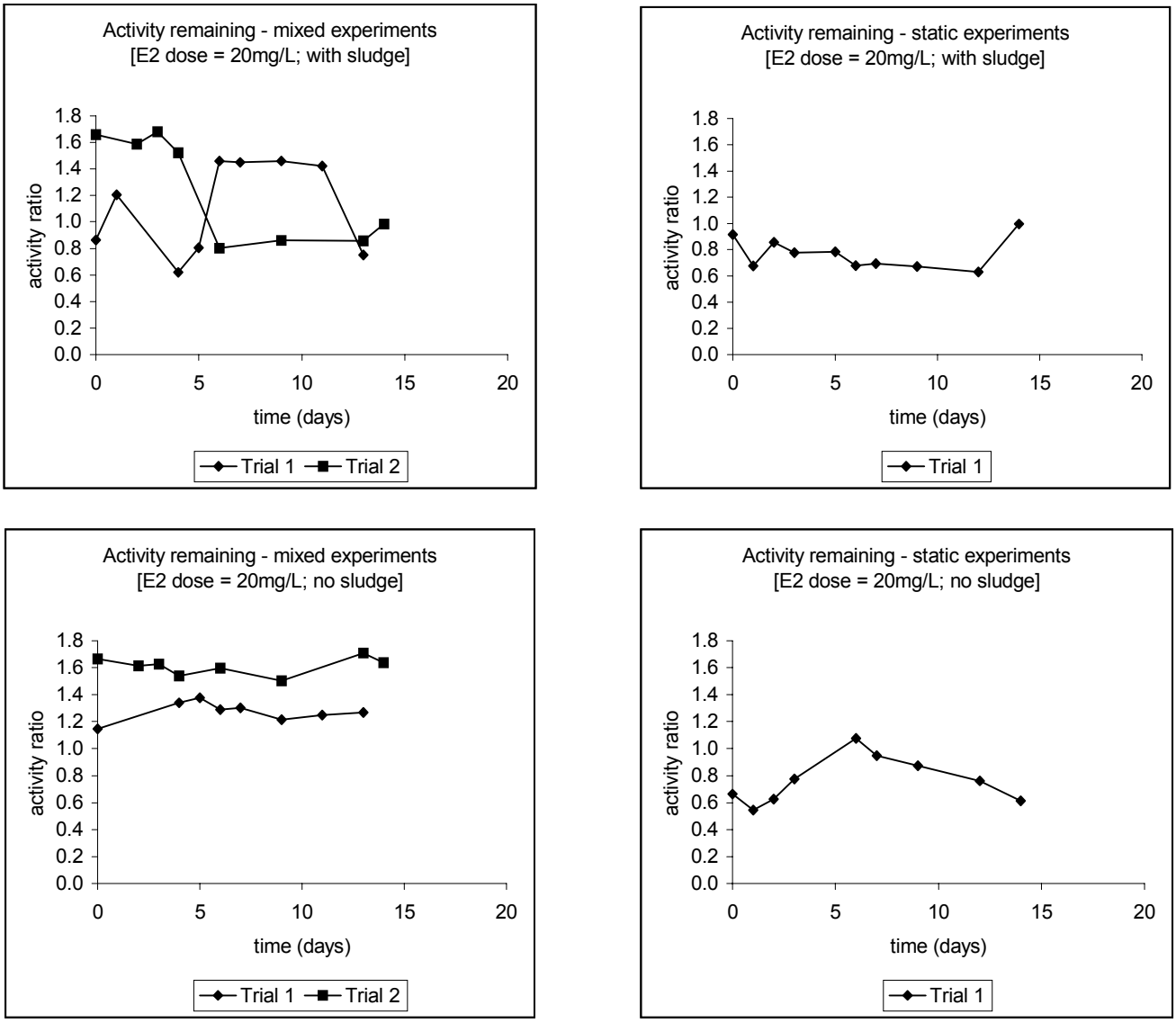

Fig. 3: Removal of total estrogenic activity over time with a 20mgE2/L dose.
Note that samples with activated sludge (AS) showed some reduction of measured activity with time after 7-8 days although measured activity remains high.

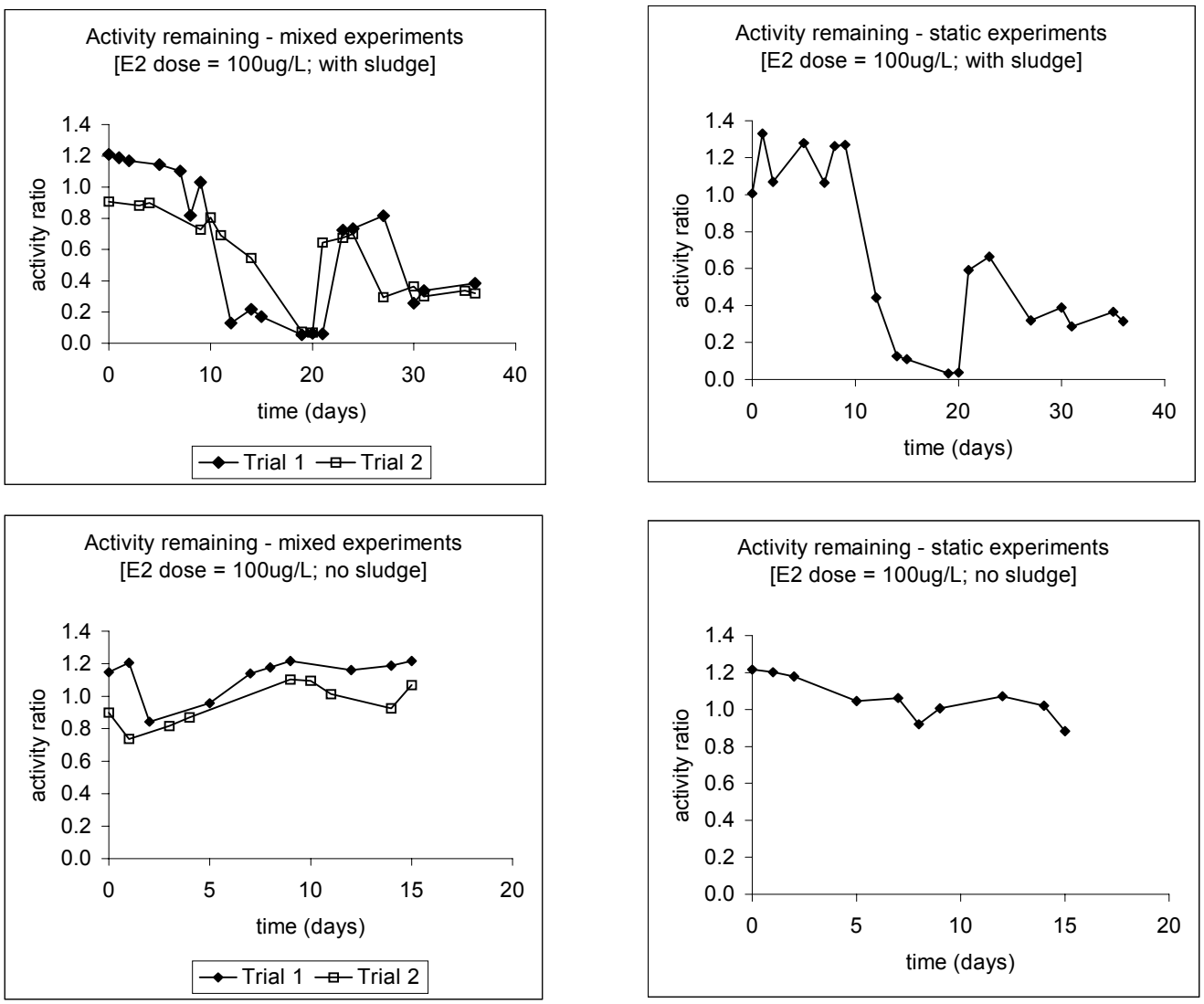

Fig. 4: Removal of total estrogenic activity over time with a 100µgE2/L (0.1mg/L) dose.
Note that samples with activated sludge (AS) showed some reduction of measured activity with time after 8-10days, while experiments with no sludge showed little removal over time. Re-spiked sludge did not show maximum activity, implying more ready removal.

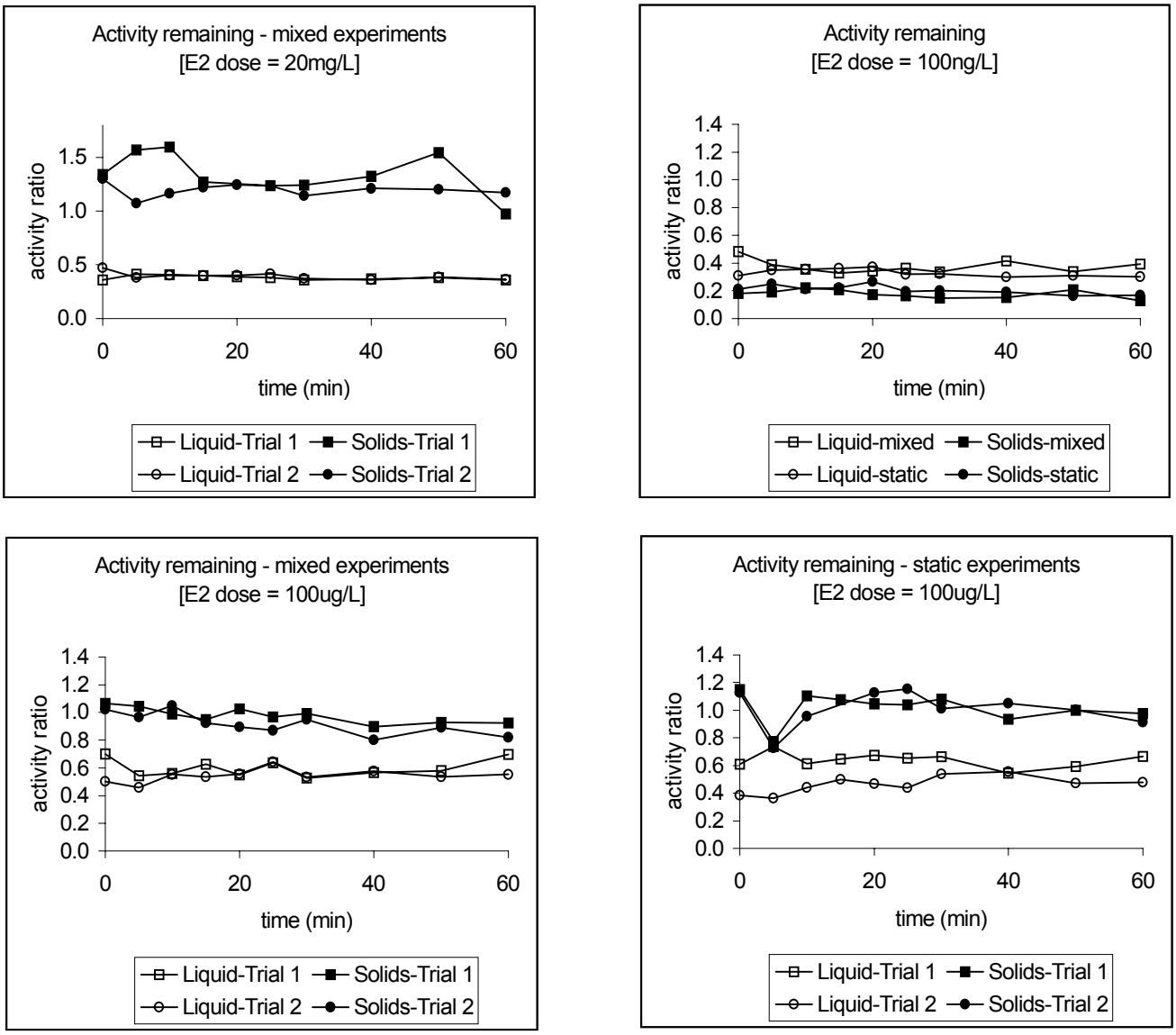

Fig. 5: Estrogenic activity associated with activated sludge solids.
The estrogenic activity associated with solids is estimated to be at least twice that in the liquid phase for 100ug/L concentrations and greater. At 100ng/L it appears that most of the measured activity is in the liquid phase. Further tests and quantitation are proposed to verify these findings.