



Centre for
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Identifying the risk to the aquatic environment of endocrine disruptors derived from agriculture (CT 20071)



**Revised final report to the UK Department for
Environment, Food and Rural Affairs (Defra)**

Project title: **Identifying the risk to the aquatic environment of endocrine disrupters derived from agriculture (CT 20071)**

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FOREWORD

This project was led by the NERC Centre for Ecology and Hydrology, but Cambridge Environmental Consultants (CEA) were sub-contracted to identify suitable farms for study and CEA's ADAS colleagues assisted with some of the field deployments. We would particularly like to thank Robin Hodgkinson and Peter Burt of ADAS for their hard work and for sharing their knowledge of farming practices, and we also thank Dave Wilson and Dave Abel of CEH Lancaster for assisting with various parts of the project. We are also grateful to the farmers (who are to remain anonymous) who permitted us to work on their land and provided information about their farming operations. Finally, we are grateful to the Defra Chemicals and GM Policy Division (Dr Mike Roberts) for funding the main study and for providing constructive comments on the draft report, and to the Environment Agency (Dr Claire Wells; Dr Mark Wilkinson) for conducting oestrogen analyses of the sample extracts. Particular thanks are due to Dr Wells for spotting a calculational error in the original version of the final report.

EXECUTIVE SUMMARY

1. The purpose of this project was to review possible inputs to UK headwater streams of steroid hormones originating from livestock, to investigate hormone contamination in some streams in which concentrations were expected to be maximal, and to draw conclusions about possible risks that these hormones may pose to aquatic organisms.
2. The review concluded that although livestock in the UK excretes more steroid sex hormones (oestradiol and testosterone) than the human population, almost all of the material deposited on soil by livestock and by manure/slurry spreading is likely to be adsorbed and/or degraded in soil before reaching surface waters. Concentrations of oestrogens in field drains are unlikely to exceed 1 ng/l (expressed as 17 β -oestradiol equivalents), a concentration that is probably harmless. However, it is possible that direct excretion by livestock into unfenced streams, and direct run-off to surface waters from slurry stores and hard-standing in livestock farms, may contribute higher concentrations. In other words, poor farming practice may lead to significant steroid hormone pollution.
3. The review also concluded that surface waters in some other countries are contaminated with oestrogens at potentially active concentrations, so it was considered that a survey of UK headwater streams for hormonal activity was justified. The literature search clearly showed that pregnant cattle are the single most important source of natural oestrogens on livestock farms.
4. The chosen sampling strategy was to focus on a limited number of predominantly dairy farms that were considered to represent worst-case conditions for hormone translocation to small headwater streams. Criteria that contributed towards the choice of field sites included stocking type and density, soil type and slope, access of livestock to the stream, application of manure or slurry to the land, possible direct drainage to the stream of waste from leaking slurry stores and hard-standing areas used by livestock, and access permission from the land-owner. Confounding factors such as upstream inputs of hormonally active material from sewage treatment works, septic tank

soak-aways, and industrial discharges, were excluded as far as possible from the study.

5. In order to obtain semi-quantitative, time-integrated samples of hormones in water, locations up- and downstream of livestock activity were sampled on 10 farms using a passive, solid-phase device known as a Polar Organic Chemical Integrative Sampler (POCIS). These were deployed between November 2004 and January 2005 for 3 to 10 weeks (mean = 39 days). At an eleventh site, a field drain issuing from an experimental plot of cracking clay soil treated solely with dairy cow slurry was also sampled with POCIS. At one site, an automatic flow-driven water sampler was deployed alongside the POCIS to capture water soon after heavy rainfall.
6. POCIS and water extracts were assayed for oestrogenic and androgenic activity using the *in vitro* yeast estrogen screen (YES) and yeast androgen screen (YAS), respectively. As part of a separate project, POCIS extracts were also analysed chemically for oestrone (E1), 17 β -oestradiol (E2) and 17 α -ethinylestradiol (EE2) by the Environment Agency.
7. The flow from only one rainfall event was captured in its entirety by the autosampler, but this revealed a background concentration (E2 equivalents) of 0-0.3 ng/l, rising to a transient peak of 9.4 ng/l. Average E2 activity at this site as determined from the POCIS samplers was 1.8-2.7 ng E2 equiv./litre, which provides confidence that the POCIS results are reliable.
8. Estimated oestrogenic activity across all sites (with one exception) lay in the range zero-26.5 ng E2 equiv./litre (mean = 2.0 ng/l; standard deviation = 5.1), based on the POCIS samples. The outlier was 292 ng/l, but this could not be specifically linked with intensive livestock rearing. 92% of monitoring stations (at least one on each farm) contained some oestrogenic activity.
9. In 5 of 9 livestock farms where upstream/downstream comparisons were possible, the downstream oestrogenic activity was higher than upstream, implying inputs from the farms under study. There was one case (Farm 3) where there were no known confounding factors whatever, with very little upstream contamination, and the farm increased activity by a factor of 7.
10. However, upstream activity was sometimes higher than downstream, suggesting possible inputs from phyto-oestrogens and scattered septic tank overflows, and in-stream adsorption and/or degradation. There was a low background level of oestrogenic activity in all but two locations.
11. The data did not generally permit discrimination between different potential sources on the farms, but it seems likely that the observed oestrogenic activity was mainly caused by a combination of slurry spreading and farmyard runoff, with direct excretion to pasture by livestock probably contributing less. In one case (Farm 7 slurry application experiment), activity in the field drain was directly attributable to dairy slurry alone.
12. On 8 of the 11 surveyed farms, oestrogenic activity in the stream (or field drain in one case) exceeded the Predicted-No-Effect-Concentration for E2 of 1 ng/l. In two cases, activity was probably high enough to damage reproduction in fish, although in neither case was livestock itself likely to have been the primary cause.
13. Although no EE2 was detected analytically in any stream, E1 and E2 were ubiquitous, with E2 equivalents ranging from 0.04 to 3.62 ng/l across all but two sites. Furthermore, concentrations downstream of livestock were generally higher than upstream, more markedly so than for the YES data. The oestrogen

concentrations agree well with the YES data and these observations suggest that most of the detected activity was attributable to E1 and E2 derived from livestock. However, the low levels of oestrogenic activity detected by the YES upstream at several stations, and the much higher upstream levels at Farms 11 and 13, could not be explained by E1 or E2, and it is postulated that phyto-oestrogens may have contributed to this signal.

14. Although all streams were assayed with the YAS for androgenic activity, this was only detectable in two cases, and at levels which are unlikely to pose a threat to fish. However, it should be noted that uptake of testosterone by the POCIS has not yet been calibrated.
15. On the basis of this survey, the possibility that natural oestrogens (from both livestock and other sources) in headwater streams are causing adverse effects in fish cannot be excluded.
16. Recommendations are made for further research to discriminate between sources, and to evaluate the risks to fish.

Introduction

Background

Since the late-1980s, much research has been conducted into the phenomenon of endocrine disruption in wildlife (Matthiessen, 2003a). In the United Kingdom, the two most well-studied examples of this concern the masculinisation of female molluscs by tributyltin-based antifoulants (Matthiessen and Gibbs, 1998), and the feminisation of marine and freshwater fish by oestrogenic hormones and their mimics discharged in sewage and industrial effluents (Matthiessen, 2003b; Jobling and Tyler, 2003). In the latter case, it is known that treated sewage discharges contain biologically significant amounts of 17 α -ethinylestradiol (EE2) and 17 β -oestradiol (E2), as well as other oestrogens, and oestrogen mimics such as nonylphenol. These substances are all able to interact with the cytosolic oestrogen receptor in developing and adult fish. This has caused a range of abnormalities including yolk precursor protein (vitellogenin) production in males and juvenile females, the development of oocytes in testes (ovotestis), and the induction of abnormal secondary sexual characteristics such as external genitalia of sexually intermediate appearance. Although it has not yet been established whether these abnormalities are leading to population-level damage in fish, they are certainly impairing reproduction in some species.

The work of Jobling *et al.* (1998) and Gross-Sorokin *et al.* (2004) has clearly shown that increased incidence of some of these abnormalities in roach *Rutilus rutilus* occurs downstream of sewage treatment works (STW) discharges. On the basis of this evidence, the Environment Agency is taking precautionary action by setting up an Endocrine Disruptor Demonstration Programme to pilot test new technology for removing oestrogens from sewage. However, almost no fish populations appear to be entirely free of such changes, and this has led to the suggestion that other sources of oestrogens may be contributing to a proportion of the observed impacts. The main additional source is potentially the oestrogenic material originating more diffusely from livestock but this has never been investigated in the UK. However, a few studies of waters near intensive livestock-rearing areas have been conducted elsewhere.

Early work by Shore *et al.* (1995) showed that chicken manure used as fertilizer in Israel led to oestrogen concentrations in small streams of up to 6 ng/l, while testosterone concentrations reached 28 ng/l. A more recent study of steroid hormones in river water in a predominantly agricultural catchment in the Middle East found peaks of 5-6 ng/l for testosterone (T) and oestrogens respectively following rainfall events (Shore *et al.*, 2004). Kolodziej *et al.* (2004) studied a dairy farming region of California and measured up to 1.9, 17.0 and 0.7 ng/l of testosterone, oestrone (E1) and E2, respectively, in irrigation canals, and slightly lower levels in streams. Soto *et al.* (2004) used the *in vitro* A- and E-screens to test water for hormonal activity close to a North American cattle feedlot over 3 years. Both oestrogenic (0.2-0.5 ng/l E2 equiv.) and androgenic activity were observed, more or less proportional with distance from the feedlot. Orlando *et al.* (2004) in association with Soto *et al.* (2004) found both demasculinized males, and some defeminized female fathead minnows *Pimephales promelas* in proximity to the cattle feedlot areas. This case may be somewhat specialised in representing a potential continual exposure scenario for adjacent wildlife. Finally, Irwin *et al.* (2001) studied ponds on pastures used by beef cattle in

the USA and found up to 1.8 ng/l E2. Female (but not male) turtles *Chrysemys picta* in these ponds had elevated plasma vitellogenin.

Very recently, as yet unpublished research in Ireland (Tarrant *et al.*, 2005) has shown that oestrogenic activity measured by the yeast estrogen screen (YES) in the receiving waters upstream of STWs is present in the range 0.9-2.9 ng E2 equiv./litre. In all cases, no STW or industrial discharges were known to be present upstream of the sampling points, and two sites were specially chosen for their supposedly pristine character. The authors suggest that the oestrogenic activity may be derived from intensive livestock rearing. Further unpublished work from Denmark (Stuer-Lauridsen *et al.*, 2005) confirms that oestrogenic activity up to about 10 ng/l E2 equivalents can be found in so-called 'reference' streams and lakes, and low activity is also present in field drains issuing from manure-treated fields in Denmark.

In summary, there is limited evidence from North America, Israel and Ireland that intensive livestock rearing can produce concentrations of natural steroids in surface waters that are in the biologically active range. The question this project sought to answer was whether UK livestock are also contributing significant amounts of androgenic and oestrogenic hormonal activity to surface waters.

The sampling and assay methods were such that the activity of synthetic androgen and oestrogen agonists would also be potentially detectable. These could include oestrogenic or androgenic veterinary medicines applied to livestock, and plant protection products (PPPs) or their adjuvants, although PPPs were unlikely to be used on livestock farms.

Aims and objectives

- To conduct a literature review of natural E2 and T excretion by livestock, and to estimate possible translocation of livestock-derived hormones to surface waters. - ACHIEVED
- To identify approximately 10 'worst-case' UK headwater streams (in terms of their potential for hormone contamination by livestock) to which translocation of E2 and T are expected to be maximal - ACHIEVED
- To conduct sampling of hormones in the streams using static, solid-phase disc samplers (POCIS), backed up with automatic event-driven water sampling at one site – MAINLY ACHIEVED, ALTHOUGH ONLY PARTIAL WATER SAMPLES WERE OBTAINED DUE TO SAMPLER MALFUNCTION
- To assay POCIS sampler and water extracts for hormonal activity using the yeast oestrogen screen (YES) and yeast androgen screen (YAS). – ACHIEVED for YES and YAS
- To assess whether any hormonal activity found in headwater streams is likely to present a risk to fish. - ACHIEVED

Scope of the study

The scope of the study was considered to encompass a national investigation of streams on livestock farms, given that no relevant investigations on this subject had previously been made in the UK. However, due to resource constraints, it was decided

that there could be no attempt to seek a balanced picture of the risks which hormone excretion by livestock might be posing to freshwater life. In other words, the guiding philosophy was not to use expensive stratified random sampling, or even quantitative chemical analysis, but instead to survey locations, using semi-quantitative *in vitro* bioassays, which might reasonably be considered as worst cases for livestock-derived steroid inputs. The choice of study sites was to be made on the basis of information derived from the literature survey, and on expert knowledge about farming practices and pollutant translocation from farmland to streams. For this to be convincing, it was important to find sites which were traversed by small headwater streams, and where confounding factors (especially STW inputs upstream) were at a minimum. The logic of this approach was that if hormonal activity were to be found at some of the study sites, it would then be necessary to extend the project, not only to gain a more balanced national picture of steroid hormone contamination from livestock, but to assess whether the activity found posed a real risk to aquatic life.

Overall approach

Given that available resources only permitted about 10 farms to be surveyed, it was realised that a clear-cut discrimination of the effects of hormone sources (i.e. direct excretion to farmland; direct excretion to streams; slurry and manure applications to farmland; run-off from hard-standing and leaking slurry stores) and main livestock types (i.e. dairy cattle; beef cattle; pigs; sheep; laying chickens; broiler chickens) could not be achieved. In any case, all four of these sources and at least two of the livestock types probably exist on most pasture-based farms simultaneously. The pragmatic solution was therefore to choose sites where as many risk factors as possible were thought to be present, thus maximising the chance of observing measurable levels of hormonal activity. As well as targeting the sources described above, particularly those involving direct inputs to water rather than via soil, the literature review also suggested that the major focus should be on dairy cattle which tend to contribute far more oestrogen per unit area than other livestock. Some weight, but not over-riding priority, was also given to farms where slopes were steep enough to promote overland flow, or where soils (e.g. cracking clays) were likely to maximise translocation to streams.

The nature of the streams themselves was crucial. In order for the available dilution to be restricted to a minimum, only sites with similar small streams (0.5-2.3 m width) were picked for study. Flows were also similar, although these were not specifically measured except at two stations. Sites were chosen on which the streams arose within the farm itself, or where only a few additional farms were present in the upper catchment, with two exceptions where hamlets or small villages were present upstream. The intention was to sample both downstream of major livestock operations, and upstream in more pristine reference areas, although the latter could not always be found. However, sites downstream of STWs, sewage sludge disposal areas, known septic tank soakaways, or industrial discharges were generally excluded from the study, and there was little arable land which could act as a source of plant protection products within the study catchments.

A practical consideration was that permission of farmers or land-owners had to be obtained before samplers could be deployed. This was partly a matter of courtesy and legality, and partly due to the need to obtain information on local farming practices. It

could be argued that this might have excluded some of the very worst cases of steroid hormone run-off which could result from bad farming practice (e.g. careless storage or disposal of slurry), and this should be borne in mind when assessing the results.

Another major consideration was the time of year at which to conduct the sampling campaign. Although dilution in streams would be expected to be at its lowest in mid-summer, translocation of substances to streams would be most likely to occur in the autumn and early winter when rainfall is higher but before the soil is fully saturated. A further consideration was that cattle tend to be withdrawn from the land during October and November onwards, and although it was not considered essential for livestock still to be present on the fields when sampling took place, it was clear that sampling had to occur in late autumn before excreted hormones had degraded to negligible levels. The aim was therefore to deploy samplers during October 2004, although unavoidable delays in identifying suitable farms delayed these deployments until November 2004 (and hence delayed sampler retrieval until December 2004 or January 2005).

As a result of the literature review, the approach taken to sampling was to focus on oestrogens, but to include a simultaneous search at the same sites for androgenic activity. The constraints on resources prevented the use of automatic flow-driven water samplers as the main sampling tool, and it was felt that random hand-samples would not be able to give a reliable time-weighted average without an excessive number of site visits. It was therefore decided to use some new sampling technology (Polar Organic Chemical Integrative Samplers – POCIS) which is designed to passively accumulate relatively polar substances such as steroids over periods of at least 4 weeks. This would integrate the fluctuating concentrations of steroids during the sampling period and provide a semi-quantitative indication of average exposures. A flow-driven water sampler was, however, installed in parallel with POCIS at one site in an attempt to provide actual water samples from just after a significant rainfall event. The peak hormonal activity in these samples could then be compared with the time-weighted averages in the POCIS.

Studies Conducted

Milestones met

M1: Start-up meeting – July 2004 Met in full.

M2: Identification of sites and deployment of samplers - October 2004 This milestone was met, but delays in the identification of suitable field sites meant that a complete set was not established until early November 2004. Samplers were deployed at all but one site between 9/11/04 and 25/11/04. The final site was the experimental farm on which slurry application experiments were being conducted, and where sampler deployment was delayed until 16/12/04 when the slurry was spread on the experimental plots.

M3: Completion of brief literature review – October 2004 Met in full and submitted to Defra.

M4: Successful interception of run-off events and recovery of samples – December 2004 This milestone was met, but due to late deployment of samplers, they were not all retrieved until the end of January 2005. Late deployment was not necessarily a bad thing because November and December were generally dry months with reduced run-off.

M5: Screening of samples for ED activity – February 2005 Completed in full, but all samples not analysed until the end of April.

M6: Wrap-up meeting – mid-April 2005 Meeting held in London, 22 April 2005

M7: Submission of draft final report – end April 2005 Draft final report submitted early May 2005

M8: Submission of final report – end May 2005

Milestones not met

None

Literature review: ‘The potential steroid hormone contribution of farm animals to United Kingdom freshwaters’

The literature review was provided to Defra in October 2004, but it has since been enlarged and refined, notably with the inclusion of some run-off modelling. The review has now been written up for publication, and the final version is attached at Annex 1. The abstract of the literature review is reproduced in the box below.

The major conclusion from the review is that UK livestock overall excrete more oestrogen than the human population, and in theory could be contributing as much as 15% of the total oestrogen load to UK surface waters if one assumes soil runoff characteristics similar to some pesticides (up to 1% reaching surface waters). For various run-off scenarios following deposits to farmland, this could lead to oestrogen concentrations in shallow streams and ditches in the range 11-413 ng/l E2 equiv. However, the likelihood based on more advanced modelling with the MACRO¹ pesticide translocation model is that the majority of soil-deposited oestrogenic material (>99.999%) will not be translocated to water, with maximum concentrations in field drains failing to reach biologically active levels (<1 ng/l E2 equiv.). This is primarily because of adsorption and degradation processes in the soil. This conclusion of course needs to be tested, which is part of the reason for this project. Furthermore, it should not be forgotten that steroidal material which is not applied to the soil, such as that in drainage from hard-standing and leaking slurry stores, and that directly excreted by livestock into unfenced streams, could be contributing additional but hard-to-quantify inputs.

These conclusions suggest that livestock excretion onto pasture, or slurry-spreading, may cause relatively little steroidal contamination of surface waters, whereas lack of adherence to good farming practice (e.g. allowing livestock to enter streams; allowing drainage from slurry stores and hard-standing to enter streams rather than sewers or septic tanks) may be more important. What is clear is that the main focus in the present study had to be on dairy farms because pregnant cattle excrete far more oestrogen than other types of livestock. The review’s findings also shaped the information which was collected about the field survey sites. In particular, a record

¹ The MACRO model was used because it is a standard exposure prediction procedure used for pesticides in Europe, and permits modelling of the worst-case situation in which substances applied to soil are translocated more or less directly to surface waters via soil macropores and field drains.

was kept of any drainage from areas of hard-standing into the streams, and of whether the livestock had direct access to unfenced surface waters.

Abstract of literature review

The combined farm animal population is considerably larger than the human one in the United Kingdom, implying a possibly important contribution to the environmental load of steroid hormones entering water. To make comparisons on the amount of steroid hormone produced by the different livestock, information was gathered on the structure of the UK farm animal populations and the amount of hormones excreted by animals at each of their life stages. An individual normalised dairy cow excretes two orders of magnitude more, and a normalised pig excretes more than one order of magnitude more steroid oestrogens than a normalised human. In terms of excretion, the combined farm animal population (including sheep and poultry) probably generates around four times more oestrogens than the human population in the UK. The biggest contributor on the animal side is the relatively small dairy cow population. If steroid oestrogens behave like herbicides, in which a worst case loss to surface waters is around 1%, then it could be argued that farm animals are responsible for 15% of all the oestrogens in UK waters. When simulations were made with the MACRO pesticide leaching model, predicted concentrations for field drains failed to exceed 1 ng/L. The rapid biodegradation rates, and high sorption rates taken from the literature and used in the model suggested less than 0.001% of oestrogens would reach the field drains. This survey suggests that direct excretion of steroid hormones by animals into water courses, or discharges from farmyard drains, are likely to be more important sources of contamination rather than via normal agricultural scenarios.

Methods

Site selection

ADAS provided invaluable advice on the basis of their knowledge of farms and farming conditions in England and Wales. As explained above, the aim when choosing sites was to identify 'worst-case' situations in which small streams uncontaminated with non-farm wastes flowed through intensive livestock farms, mainly dairy operations. The criteria used to choose 10 sites for study included high stocking density, soil type favourable to translocation of substances, steep land slope, range of UK regions, access of animals to the stream, relatively clean upstream areas for comparison with potentially contaminated downstream areas, manure or slurry-spreading on the farm, and potential for direct runoff of contaminated water from the farmyard area to the stream. It was impractical to satisfy all criteria at all locations, but an over-riding imperative was to find farms where the landowner was prepared to allow access for field workers and to provide information on farming practices. An eleventh site on an experimental farm was also chosen, specifically to look at translocation of hormones from dairy cow slurry which was applied to an experimental plot from which field drainage could be collected.

The chosen sites and summary descriptions are listed in Table 1a, and possible confounding sources of oestrogens in the study catchments are listed in Table 1b. Data on the identity of the farms are held in confidence by CEH, in order to preserve the anonymity of the farmers. A good geographical spread was obtained, from the Scottish borders of Northumberland, to Devon in the southwest of England. All sites were traversed by small streams or flowing ditches with cross-sectional dimensions of approximately 0.5-2.3 m width by 0.05-0.6 m depth. Land slopes ranged from <1% to

20%, and soils ranged from silts and loams through to cracking clays. Seven of the sites were dairy farms (with pregnant sheep also present in most cases), one had a herd of pregnant beef cattle, one had beef steers, and one was a pig farm. Nine farms were also receiving slurry to varying extents, and seven had the potential for runoff to the stream from areas of hard-standing in the farmyard. In eight cases, livestock had free access to at least some stretches of stream, but on seven farms they had been withdrawn from the fields into sheds during the 2-5 weeks prior to the start of sampling. Figure 1 shows a typical example of the streams which were sampled by the project (Farm 13), and Figure 2 shows the downstream POCIS sampling point on Farm 3 including the auto-sampler tube.

It should be noted that rainfall was generally very low in the November/December 2004 period, with regional mean rainfall figures in the range 37-91% of the 1961-1990 long-term average. The weather was wetter in January 2005 (45-117% of long-term average). This implies that runoff of hormones during the main study period may have been less than one might expect in an average year.

Calculation of steroid loads from livestock

These assessments rely on the predicted faecal and urinary excretion of sex hormones based on the literature review, and from measured values reported in the scientific literature for oestrogens in farm waste. We cannot make predictions for androgens in farm waste products since there are no measurements available in the literature. The hormone assessments can tell us something about the likely input loads at the different sites. Rainfall data and the ditch dimensions can also help give a qualitative indication of likely steroid hormone contamination. However, following the MACRO modelling exercise carried out for the literature review, it is clear we must be cautious when converting these values and assumptions into predicted ditch concentrations. In essence, the MACRO exercise suggested that given the sorptive and relatively biodegradable nature of the steroid hormones only a negligible amount would reach drains and ditches. Thus, the unpredictable, accidental events of direct excretion/waste application in, or near streams and ditches are believed to be crucial.

Farm 1

This farm has 100 sows and so would be expected to represent the upper end of the pig spectrum in terms of oestrogen production. It would appear that pig slurry (yard washings) on this farm is disposed of to some fields near the test stream. Clearly, the oestrogen content of the slurry would depend on the amount of rainwater dilution that had occurred in the collecting pit. Raman *et al* (2004), examined a number of different pig waste collection systems in North American pig farms and these may provide a guide to potential slurry oestrogen concentrations. A slurry pit associated with farrowing sows was reported as having 4 µg/L E2 and 6 µg/L E1, giving us a potential slurry load of 6 µg/L E2 equiv from this source.

Farm 2

This farm has 200 dairy cows but most were transferred to sheds while the samplers were *in situ* and so were contributing mainly a slurry load. Examination of Farm 2 indicated a possibility of slurry contamination of the adjacent stream. The Raman *et*

al (2004) study of different farm waste collection systems in North America measured mean slurry pit concentrations 1.5 µg/L E2 and 4.5 µg/L E1 (3 µg/L E2 equiv) for dairy farms. This value may serve as a guide to UK dairy farm slurry pit concentrations and for our case a maximum of 75 mg E2 equiv/ha could be expected. Only 12-18 cows were actually grazing the 5 ha catchment during the sampling period which would give us 2.4 mg/d E2/ha. If we were to assume that the 200 store lambs in the fields had the same oestrogen excretion as non-pregnant sheep/rams then they would generate 0.4 mg/d E2.

Farm 3

The 110 dairy cows were present on the fields during the first two weeks of sampling and would be predicted to generate 73 mg/d E2 equiv during this period. The slurry application from this herd, given the reported application rate, would have delivered a maximum of 67 mg E2 equiv/ha. The flock of pregnant ewes would be predicted to deposit only 0.7 mg/d E2 equiv. This site appears to have the greatest slope (20%) from the fields to the water course of any of the test sites. Also notable here is a potential upstream point source from a farmyard drain.

Farm 4

The dairy cattle on Farm 4 were solely a source of slurry during the sampling period, and in this case, given the reported application rate, a maximum of 84 mg E2 equiv/ha could have been delivered. The flock of pregnant ewes would be predicted to deposit 1.6 mg/d E2 equiv. Site visits have confirmed that there is a possibility of direct access of the grazing animals to the stream in certain fields.

Farm 6

This farm is mixed with both sheep and beef cattle. The cattle were in the sheds and so were not generating excreta directly to the fields. There are have been few studies to date on oestrogen excretion from ewes (Bamberg *et al.*, 1986; Lange *et al.*, 2002), therefore it is not possible to predict with confidence what their outputs might be. From the literature review we predict an individual pregnant ewe is excreting 5.3 µg E2 equiv/d thus a 2,900 flock would generate 15.4 E2 mg equiv/d, but most of these were outside the micro-catchment of interest. There is direct access for the grazing animals to the water course. We are unaware of any literature on oestrogen values for beef cattle and slurry. In consequence we have used the oestrogen values for dairy cow slurry and farm yard manure derived from Raman *et al* (2004) which are likely to result in an overestimate.

Farm 7

This is an experiment on an experimental husbandry farm where slurry has been applied to a mole-drained plot. The application rate of dairy cow slurry would be predicted to give an oestrogen load of 120 E2 equiv/ha.

Farm 8

Although this farm has a large dairy cow herd, as this has been over-wintered in farm sheds during the sampling period, and no farm yard waste/slurry has been applied to the fields there is no apparent route from which oestrogen stream contamination could occur from this source, other than from excreta deposited on the fields prior to the sampling period. A calculation has been made, however, for the 300 ewe flock on the same basis as that described above indicating 1.6 mg E2 equiv/d could be produced. There would not appear to be a high contamination risk unless some very close, or direct access to the water course has occurred for the grazing sheep.

Farm 9

A calculation for the small ewe flock has been made as described in the discussion of Farm 6. This site is notable for the application of farm yard manure. From the research of Raman *et al* (2004) we would predict dairy cow manure would contain 39 (SD 29) µg/kg E1 and 18.4 (SD 9) µg/kg E2 which we can convert to 31 µg/kg (31.4 mg/tonne) E2 equiv. It is not known what the application rate was in this case.

Farm 11

The most likely source of any oestrogen contamination to the adjacent stream here is from a slurry application applied one week previous to the deployment of the passive sampler. The Raman *et al.* (2004) slurry values (3 µg/L E2 equiv) would deliver 150 mg E2 equiv/ha given the reported application rate. However, the 240 pregnant ewes would be delivering 1.3 mg/d E2 equiv wherever they were grazing.

Farm 13

As in Farm 11, a dairy cattle slurry application has occurred, albeit at a slightly lower application rate giving an expected 120 mg E2 equiv/ha at the reported application rate. The 300 grazing ewe input would be expected to be 1.6 mg/d E2 equiv.

Farm 14

There is a small beef cattle herd with direct stream access. Unfortunately the oestrogen excretion cannot easily be predicted since no literature on beef cattle oestrogen excretion appears to exist. However, to start out one could consider that the beef steer is similar to a cycling or pre-oestrous female cow. A cycling dairy cow is reported to excrete around 320 µg/d total oestrogens in urine (Monk *et al.*, 1974). From the data of Hoffmann *et al.* (1997) E1-glucuronides and E2-glucuronides (the conjugates most likely to be transformed back to free hormones) represent 5% and 1.5% respectively of the total steroid excretion in dairy cows. This would indicate that the cycling dairy cow is excreting 16 µg/d of potentially available E1, and 5 µg/d of potentially available E2. Desaulniers *et al* (1989) examined the total oestrogen presence in faeces of cycling cows, which was around 256 µg/d. Of these total oestrogens if we use the different proportions indicated by the Hoffman *et al.* (1997) paper (11% E1 and 32% E2), then the cycling dairy cow is excreting 28 µg/d of available E1, and 82 µg/d of potentially available E2. This suggests a combined

output of 44 $\mu\text{g/d}$ of available E1, and 87 $\mu\text{g/d}$ of available E2 from a cycling dairy cow. In humans the male excretes only about half the amount of oestrogens of a cycling female (Johnson and Williams, 2004), so we might speculate the same might be appropriate for cattle, giving us now 22 $\mu\text{g/d}$ of available E1, and 43 $\mu\text{g/d}$ of available E2 (50 $\mu\text{g/d}$ E2 equiv.) which would generate 1.1 mg/d E2 equiv for the herd. The literature review predicted an individual beef steer could generate 300 $\mu\text{g/d}$ testosterone, thus, the herd in this case would be generating 6.6 mg/d testosterone.

Figure 1. View of the downstream POCIS deployment site on Farm 13.



Figure 2. Downstream sampling point at Farm 3, showing POCIS cylinder staked to streambed and tube to autosampler.



Sampling

The main sampling tool was the Polar Organic Chemical Integrative Sampler (POCIS), which is described in detail by Alvarez *et al.* (2004), Jones-Lepp *et al.* (2004), and Petty *et al.* (2004) and was supplied by Exposmeter SA, Sweden. It has already been used by Petty *et al.* (2004) for exactly the present application i.e. sampling of steroids from water and measurement of hormone activity using the yeast oestrogen screen (YES). In essence, the POCIS consists of solvent-washed solid-phase adsorption medium (trade name 'Oasis') which is able to sequester hydrophilic molecules including steroids. The adsorption medium is sandwiched between two disc-shaped semi-permeable plastic membranes held in place by two metal compression rings which are in turn mounted inside a protective perforated stainless steel cylinder (Figs 3 and 4).

Figure 3. POCIS discs mounted between metal compression rings



Figure 4. POCIS deployed in a stream (Farm 8 downstream) inside its perforated stainless steel cylinder.



Alvarez *et al.* (2004) have shown that over periods of a few weeks, POCIS discs essentially act like an infinite sink for polar molecules. Each substance will have a characteristic uptake rate which can be measured in the laboratory, allowing a semi-quantitative estimate of the average exposure concentration (see POCIS calibration below).

At each farm, a site was chosen upstream and downstream, respectively, of areas where inputs from livestock were expected, although in a few cases it was impossible to find an upstream location. Distances between the up- and downstream sites ranged from 100 to 1300 m (Table 2). In the case of Farm 7 (slurry application to a 0.17 ha experimental plot), the sampling site was in the field drain issuing from the plot. At each of these sites, between November 2004 and January 2005 inclusive, two newly-unwrapped POCIS discs (one plus a spare in case of damage) were deployed in a perforated stainless steel cylinder which was staked to the stream bed with its long

axis parallel to the current (Figs 2 and 4). In all cases, the cylinders remained submerged for a deployment period of 3 to 10 weeks (mean = 39 days) (Table 2), and all discs were recovered intact. In a few cases, POCIS were used in two sequential deployments. POCIS discs were wrapped in methanol-washed aluminium foil, labelled with location and date, and stored at -20°C to await extraction. The spares from each site were sent to the Environment Agency (Leeds and Nottingham) for oestrogenic hormone analysis by LC-MS/MS (see details below).

At one location (Farm 3), an automatic water sampler was also installed at the downstream site, programmed to take hourly samples once the stream level had risen in response to a significant rainstorm. Unfortunately, the autosampler's inlet tube became blocked with debris, so only the initial 6 hourly samples were obtained from the first rainfall event on 22/12/04. The next event (8/1/05) was very small and was missed due to a software problem, so a full set of samples (12 h) was only obtained from a somewhat larger event on 22/1/05.

Daily flow data were recorded at Farms 3 and 7. On Farm 3, flow peaked at 241 l/sec on 22/12/04 during a 15 mm rainfall event (which came after a week of steady rain), and at 105 l/sec on 22/1/05 during a 16 mm event. Note that the rainfall data are for Farm 2 nearby. At Farm 7, the mean daily flow in the field drain was equivalent to 1.2 mm rainfall (range 0.3-6.5 mm), with the main flows occurring in response to a 21 mm rainfall event just after the POCIS was deployed on 16/12/04.

At each site, fine surficial sediment (to 5 mm depth) was sampled at the same time as the POCIS and stored at -20°C. This was done as 'insurance' in the event that none of the other samples gave positive results, in which case it might be expected that steroidal material could be associated with the sediment.

Sample extraction for bioassay

POCIS discs

One of each pair of discs from each site was processed as follows. Discs were removed from the freezer and allowed to equilibrate to room temperature. The foil was carefully removed, the disc was rinsed with tap water to remove adherent sediment and detritus, the identity of the sample was recorded, and the bolts holding the compression discs together were loosened. The disc assembly was placed in a vacuum oven at 40°C and 500 mBars partial vacuum for 30 mins in order to dry the adsorbent. During this period, glass extraction columns were set up in the fume cupboard and rinsed with 10 ml methanol. After removal from the oven, the disc array was disassembled, the membranes were carefully detached from the stainless steel collars and the adsorbent powder carefully scraped into a funnel placed in the neck of the extraction column. The adsorbent was eluted with 50 ml of analytical grade extraction solvent (toluene : methanol : dichloromethane; 1:1:8). The eluate was collected into labelled 100 ml quickfit flasks with glass stoppers and stored at -20°C until required. Samples were subsequently reduced in volume to approximately 5 ml by rotary evaporation. The remaining 5 ml was then dried under a stream of N₂ in a heating block at 40°C and redissolved in 0.5 ml of absolute ethanol. This final aliquot was stored in a capped glass vial at -20°C to await testing for oestrogenicity and androgenicity.

Water samples from auto-sampler

Water samples collected from the in situ auto-sampler device were received at CEH Lancaster and stored at 4°C. At time of retrieval from the sampler by ADAS personnel, 100 ml of analytical grade dichloromethane was added to each litre sample of water, and mixed. This served the purpose of partitioning any chemicals of interest present within the organic phase and reducing the likelihood of degradation due to bacterial or other agents within the aqueous phase. In the laboratory, the bottles were shaken thoroughly and both the organic and aqueous phases of each water sample were transferred to 1.0 litre separating funnels, held in stands and clamps. The funnels were capped and shaken thoroughly. They were then placed in the stands to allow settling and separation of the two phases. The organic phase was collected via the tap in the base of the separating funnel in a labelled 200 ml quickfit flask with a glass stopper. When necessary, anhydrous sodium sulphate was added to samples to remove any aqueous contamination. The stoppered flasks were stored at -20°C until being reduced in volume by rotary evaporation, drying under N₂ and redissolving in 500 ml absolute ethanol, exactly as described for the POCIS extracts.

POCIS calibration

Prior to deployment of the POCIS discs in the field some laboratory studies were carried out in order to provide information on the recoveries of material likely to be achieved from the POCIS discs and also to provide data from which some estimation of the clearance efficiency of the discs could be made. This information is necessary to allow any oestrogenicity (or androgenicity) detected in the POCIS extracts to be used to estimate likely water-borne concentrations of oestrogenic substances (as E2 equivalents) present during the deployment of the discs. The scope of these studies was limited because most of the available discs were required for deployment in the field.

Calibration method

Five beakers were set up, four of which contained 1000 ml distilled water with 17β-oestradiol (E2; 0.001, 0.01, 0.1, 1.0 mg; Sigma-Aldrich) and ³H-17β-oestradiol (approx 10⁶ dpm; Amersham International), the fifth contained ³H-E2 only. The solutions were held at approximately 20°C and were stirred continuously. A POCIS disc was suspended in each of the four beakers containing unlabelled and ³H-E2. The fifth beaker acted as a control to estimate adsorption of E2 to the internal surfaces and so contained a POCIS disc holder only, with no membrane or adsorbent. At intervals 1.0 ml aliquots of water were collected from each beaker. Each aliquot was added to a 5 ml scintillation vial together with 4.0 ml scintillation fluid (Ecoscint A, National Diagnostics). Because of practical constraints imposed by the use of radiolabelled substances, it was possible to run only 2 beakers at a lower temperature. These were set up as described above, containing 0.1 mg l⁻¹ of E2, and held at 10°C.

Due to shortage of time and resource, no calibration was performed with testosterone. However, it is expected to have a very similar uptake rate to E2 as the molecules are of a very similar size and shape.

Calibration results

Uptake of E2 from solution was independent of concentration. There was no difference in the rate of uptake, or total uptake, between solutions containing from 0.001 mg l⁻¹ to 1.0 mg l⁻¹ E2. Uptake approximated a linear profile with some deviation during the first phase of uptake. We attribute this to the adsorption of E2 by the glass surfaces of the beaker. (Note: Inconsistent results were obtained from control beakers and therefore the precise magnitude of loss arising from non-specific adsorption to the beaker cannot be calculated from these data. The following estimates of clearance are approximations which to some extent overestimate clearance). At 20°C, between 14h and 86h during which period uptake was linear, 39% of E2 in solution was adsorbed. This represents clearance of 390 ml of solution over a period of 72 h which equates to 0.129 litres/day. At 10°C uptake was slightly slower, between 18h and 112h, 35.4% of the total was lost from solution. This represents clearance of 354 ml of solution over a period of 94h which equates to 0.09 litres/day. These clearance figures closely resemble those quoted by Alvarez *et al.* (2004) for the uptake of a range of organic chemicals in a turbulent (stirred) system (0.03 – 0.12 litres/day). Overall recoveries of E2 from the discs, derived from the measured radioactivity in the reconstituted extract of the disc adsorbent, ranged between 33 – 55% of the starting total. No directly equivalent figures are available for comparison although Alvarez *et al.* (2004) quote higher recoveries (>80%) for a range of other analytes under controlled conditions (not waterborne exposures). There is no obvious explanation for the relatively low recoveries, so future research should investigate this issue.

Because of the limited number of discs available for evaluation, these data should be considered preliminary and will be improved upon in future studies.

YES and YAS assays

The assays were performed on POCIS and water extracts by means of recombinant reporter gene assays known as the Yeast Estrogen Screen (YES) and Yeast Androgen Screen (YAS). These cell lines contain the human oestrogen and androgen receptor genes linked to a reporter gene coding for β-galactosidase. The production of this enzyme is indicative of oestrogen or androgen exposure, and leads to a colour change in the test medium in 96-well plates which is detected spectrophotometrically on a plate reader. Full details of the methodology were described by Routledge and Sumpter (1996). In this project, the YES and YAS assays were calibrated against 17β-oestradiol and testosterone standards, respectively.

Oestrogen analysis

POCIS extracts were conducted at the Leeds office of the Environment Agency's National Laboratory Service (NLS). POCIS contents were extracted in a glass column with 40 ml methanol which was reduced to 1 ml by evaporation in a Turbovap system, and made up to 2 ml with methanol, which was finally split into 2 equal aliquots, each representing 100 mg of POCIS sorbent. One aliquot was then sent to the Nottingham office of NLS for analysis.

Following addition of internal standards, the extracts were concentrated under a nitrogen stream to facilitate a solvent exchange prior to fractionation using size-

exclusion chromatography (gel permeation). The fraction containing the oestrogens was collected. This fraction was then concentrated prior to another solvent exchange to facilitate an aminopropyl cartridge cleanup step.

The resultant extract was then taken to dryness and immediately a buffer solution added, followed by a dansyl chloride solution. This mixture was heated briefly to aid the reaction, cooled, and transferred to a vial for analysis. Analysis was carried out using LC-MS/MS with photoionisation interface. Quantification of the oestrone (E1), 17 β -oestradiol (E2), and 17 α -ethinyl oestradiol (EE2) was achieved using an internal standard method with calibration against absolute standard solutions. Calibration showed that total error was less than 50% for each compound of interest. The reporting limit based on previous work was set at 0.1 ng/l for EE2 and 0.15 ng/l for E1 and E2.

Table 1a. Summary of conditions on the surveyed farms, and predicted steroid load.

Farm number	Environment Agency region	Livestock	Livestock access to stream?	Slurry/manure spreading?	Potential for farmyard runoff?	November-January 2004/2005 rainfall for the region (as a % of the 1961-1990 average)	Soil type	Slope of land	Stream size (width x approx. depth) m	Estimated oestrogen load (E2 β equiv.)
1	Anglian	Sows (in sheds)	No	+	+++	49-74%	Clay	3-7%	0.6m wide 0.1m deep	Small – 6 μ g/l expected in slurry
2	Southwest	Dairy cattle (some on fields, some in sheds); Store lambs (on fields)	Yes	+++	+	47-80%	Clay	5-10%	1.5m wide 0.08m deep	2.8mg/d from livestock; 75 mg/ha from slurry
3	Southwest	Dairy cattle (on fields for first 2 weeks); Ewes (on fields)	Yes	++	+++	47-80%	Silt-clay loam	10-20%	1.1m wide 0.06m deep	73.7 mg/d from livestock; 67 mg/ha from slurry
4	Northwest	Dairy cattle (in sheds); Ewes (on fields)	Yes	+++	+	61-115%	Clay	1-3%	0.6m wide 0.1m deep	1.6 mg/d from livestock; 84 mg/ha from slurry

Farm number	Environment Agency region	Livestock	Livestock access to stream?	Slurry/manure spreading?	Potential for farmyard runoff?	November-January 2004/2005 rainfall for the region (as a % of the 1961-1990 average)	Soil type	Slope of land	Stream size (width x approx. depth) m	Estimated oestrogen load (E2 β equiv.)
6	Northeast	Pregnant beef cattle (in sheds)	Yes	++	+	37-117%	Silt	<1%	1m wide 0.15m deep	15 mg/d from livestock; 27 mg/d from slurry plus up to 314 mg/ha from fym
7	Thames	None (dairy cow slurry applied experimentally)	No	+++	-	45-66%	Cracking clay	<1%	Field drain issuing from 0.17 ha experimental plot	120 mg/ha from slurry
8	Northwest	Dairy cattle (in sheds); Ewes (on fields)	Yes	-	-	61-115%	Fine/coarse loam	3%	0.54m wide 0.08m deep	1.6 mg/d from livestock
9	Northwest	Dairy cattle (in sheds); Ewes (on fields)	Yes	+	-	61-115%	Fine loam	2%	0.88m wide 0.12m deep	0.9 mg/d from livestock; (plus 31.4 mg/tonne from fym)
11	Northwest	Dairy cattle (in sheds)	No	+ (just before sampling began)	+	61-115%	Fine/coarse loam	2%	1.64m wide 0.10m deep	1.3 mg/d from livestock; 150 mg/ha from slurry
13	Northwest	Dairy cattle (in sheds); Ewes (on fields)	Yes	++ (just before sampling)	+	61-115%	Fine loam	<1%	1.72m wide 0.43m deep	1.6 mg/d from livestock;

Farm number	Environment Agency region	Livestock	Livestock access to stream?	Slurry/manure spreading?	Potential for farmyard runoff?	November-January 2004/2005 rainfall for the region (as a % of the 1961-1990 average)	Soil type	Slope of land	Stream size (width x approx. depth) m	Estimated oestrogen load (E2 β equiv.)
				began)						120 mg/ha from slurry
14	Wales	Beef steers (on fields)	Yes	-	-	65-90%	Sandy silt loam overlying sandy loam	1-2%	2.32m wide 0.06m deep	1.1 mg/d from livestock; plus 6.6 mg/d of testosterone

Table 1b. Possible confounding sources of oestrogenic activity in the study catchments.

Farm number	Possible confounding sources of oestrogens	
	Upstream of the upper sampling point	Between sampling points
1	A few pasture fields and woods only	One possible domestic septic tank soakaway
2	A few pasture fields and woods only	None
3	A few pasture fields and a minor road	None
4	No upstream sampling point	One possible domestic septic tank soakaway in entire catchment
6	No upstream sampling point. However, the river 'control' in fact drains a large area of land which includes many farms, an army camp, a small sewage treatment works (consented discharge = 1 m ³ /day), and a small trade discharge from a water treatment works (consented discharge = 8 m ³ /day)	None in entire catchment
7	No upstream sampling point	None – experimental plot only.
8	Several pasture fields and a minor road	Two possible septic tank soakaways
9	Several pasture fields and ~3 possible septic tank soakaways	None
11	A few pasture fields and woods only	3 possible septic tank soakaways
13	Three small livestock farms plus a hamlet with approximately 10 septic tanks	One livestock farm in addition to the study farm
14	Several farms and two villages with probable septic tanks, although the majority of the upstream stretch is a winterbourne in which little water flowed during the survey period.	One septic tank soakaway

Table 2. POCIS disc exposure and extraction record for YES/YAS.

Farm number	Date of sample collection	Duration of disc deployment (days)	Disc no.	Extract no.	Comments	Approx. distance between up-and down-stream sites (m)
1	24.12.04	33	1	E22	UPSTREAM	1000
	24.12.04	33	2	E23	DOWN-STREAM	
2	25.1.05	45	1A	E2	UPSTREAM	900
	25.1.05	45	2A	E3	DOWN-STREAM	
2	11.12.04	31	1B	E8	UPSTREAM	900
	11.12.04	31	2B	E9	DOWN-STREAM	
3*	25.1.05	45	1A	E4	UPSTREAM	700
	25.1.05	45	2A	E5	DOWN-STREAM	
3*	11.12.04	31	1B	E20	UPSTREAM	700
	11.12.04	31	2B	E21	DOWN-STREAM	
4	17.12.04	32	1	E24	DOWN-STREAM	-
			Downstream only			-
6	21.12.04	39	1	E18	DOWN-STREAM	-
	21.12.04	39	2	E19	River 'control'	-
7	14.1.05	29	-	E25	Disc 2, weir 18 Field-drain	-
8	21.12.04	42	1	E16	DOWN-STREAM	500
	21.12.04	42	2	E17	UPSTREAM	

Farm number	Date of sample collection	Duration of disc deployment (days)	Disc no.	Extract no.	Comments	Approx. distance between up-and down-stream sites (m)
9	22.12.04	43	1	E14	UPSTREAM **	700
	22.12.04	43	2	E15	DOWN-STREAM**	
11	22.12.04	43	1	E12	UPSTREAM	1300
	22.12.04	43	2	E13	DOWN-STREAM	
13	25.1.05	73	1A	E6	UPSTREAM	600
	22.12.04	40	2A	E7	DOWN-STREAM	
14	6.1.05	42	1	E10	UPSTREAM	100
	6.1.05	42	2	E11	DOWN-STREAM	
14	24.1.05	18	1	E26	DOWN-STREAM	

* An automatic, flow-driven water sampler was also located in the downstream position at Site 3.

** The discs from Site 9 were originally mis-labelled but quality control procedures detected the error. Those marked 'upstream' were in fact 'downstream', and *vice versa*. This table shows the correct locations.

Results

Oestrogenic activity

A summary of the Farm 3 autosampler results for oestrogenic activity in streamwater is shown in Table 3 and Figure 5, and a summary of the POCIS data for estimated average oestrogen activity at all sites is shown in Table 4 and Figure 6. The data presented have not been adjusted for recovery, which was approximately 50%. Plots of extract concentration-response curves are shown in comparison with one of the oestradiol calibration curves in Figures 7-10. For some samples, the measured absorbance at higher volumes of extract was less than the absorbance at smaller volumes. We interpret this to indicate that these extracts contained substances that were cytotoxic. This interpretation is supported by the reduced turbidity (= less cells) measured in these wells (data not shown).

Because the POCIS discs were deployed for varying periods, the data in Table 3 have been normalised to a 30 day uptake period, assuming that uptake was linear during the whole deployment. Rainfall events were small and sparse during the study period, so this procedure probably did not introduce significant bias. The estimated average concentrations in the original streamwater were then calculated using the laboratory-measured clearance rate at 10°C of 0.09 litres of streamwater per day.

It is apparent from Figure 5 that the autosampler failed on 22 December 2004 before capturing the activity peak one might expect to be associated with the peak of the hydrograph. However, the 22 January data reveal such a peak, and show that it exceeded 9 ng E2 equiv./litre (EEQ/l). It should be noted that this was approximately 2 months after cattle were withdrawn into sheds, and that the baseline activity (0-0.3 ng EEQ /l) was lower than that observed one month after the cattle were withdrawn (0.2-0.9 ng EEQ/l).

Because of the assumptions and uncertainties involved in calculating average concentrations based on the activity in the POCIS discs, the comparison between the actual activity in the autosamples at the downstream Farm 3 site and the calculated average activity for the same site is important. The calculated average activity (Table 4) for the month preceding 11 December was 2.7 ng EEQ/l, while the average value for the succeeding period to 25 January was 1.8 ng EEQ/l. These values lie between the baseline and peak activity in the autosamples, thus providing confidence that the predicted average values derived from the POCIS samples are of the correct order of magnitude.

Taking the POCIS data as a whole (Table 4), it is clear that oestrogenic activity was detectable at most sites, and that all but one of the E2-equivalent concentrations lay between zero and 26.5 ng/l (mean = 2.0 ng/l; s.d. = 5.1), with one outlier of 292 ng EEQ/l. In 5 of the 9 cases where it is possible to make a direct comparison between the upstream and downstream values, the downstream activity was higher than upstream, indicating that livestock farming activities were probably contributing oestrogens to the stream. In these cases, the streamwater activity increased by a factor of 2-27.

Table 3. Oestrogenic activity measured in the streamwater samples taken by autosampler from the downstream site at Farm 3 during two rainfall events. ND - not detectable - no discernible signal on the assay plate with the volume of extract employed.

Sample	Oestradiol equivalents in 500 µl extract (ng)	Volume of original sample (ml)	Oestradiol equivalents in stream water (ng/l)
Sample 1; 23.12.04	0.16	250	0.63
Sample 2; 23.12.04	0.06	250	0.25
Sample 3; 23.12.04	0.10	250	0.40
Sample 4; 23.12.04	0.07	250	0.27
Sample 5; 23.12.04	0.22	250	0.90
Sample 6; 23.12.04	0.08	250	0.32
Sample 1; 24.1.05	0.32	1000	0.32
Sample 2; 24.1.05	0.11	1000	0.11
Sample 3; 24.1.05	ND	1000	ND
Sample 4; 24.1.05	0.06	1000	0.06
Sample 5; 24.1.05	9.43	1000	9.43
Sample 6; 24.1.05	1.88	1000	1.88
Sample 7; 24.1.05	0.17	1000	0.17
Sample 8; 24.1.05	1.62	1000	1.62
Sample 9; 24.1.05	ND	1000	ND
Sample 10; 24.1.05	0.05	1000	0.05
Sample 11; 24.1.05	0.03	1000	0.03
Sample 12; 24.1.05	0.03	1000	0.03

Table 4. Estimated average oestrogenic activity in streamwater at all sites sampled with POCIS. ND - not detectable - no discernible signal on the assay plate with the volume of extract employed.

Site number	date of collection	Upstream / downstream	Oestradiol equivalents in 500 µl of extract (ng)	Oestradiol equivalents in 500 µl of extract normalised to 30 days exposure (ng)	Estimated average oestradiol equivalents in stream water (ng/l)
1	24.12.04	UPSTREAM	4.31	3.92	1.4
1	24.12.04	DOWNSTREAM	9.66	8.78	3.2
2	25.1.05	UPSTREAM	2.12	1.41	0.5
2	25.1.05	DOWNSTREAM	1.79	1.19	0.4
2	11.12.04	UPSTREAM	3.01	2.91	1.1
2	11.12.04	DOWNSTREAM	1.45	1.40	0.5
3	25.1.05	UPSTREAM	0.69	0.46	0.2
3	25.1.05	DOWNSTREAM	7.25	4.83	1.8
3	11.12.04	UPSTREAM	1.09	1.05	0.4
3	11.12.04	DOWNSTREAM	7.41	7.18	2.7
4	17.12.04	DOWNSTREAM	9.62	9.02	3.3
6	21.12.04	DOWNSTREAM	0.21	0.16	0.06
6	21.12.04	River 'control'	0.81	0.62	0.2
7	14.1.05	Field-drain	3.12	3.22	1.2
7	14.1.05	Field-drain	4.32	4.46	1.6
8	21.12.04	UPSTREAM	ND	ND	ND
8	21.12.04	DOWNSTREAM	1.44	1.03	0.4
9	22.12.04	UPSTREAM	0.48	0.34	0.1
9	22.12.05	DOWNSTREAM	0.15	0.10	0.04
11	22.12.05	UPSTREAM	1130.03	788.39	292.0

Site number	date of collection	Upstream / downstream	Oestradiol equivalents in 500 µl of extract (ng)	Oestradiol equivalents in 500 µl of extract normalised to 30 days exposure (ng)	Estimated average oestradiol equivalents in stream water (ng/l)
11	22.12.04	DOWNSTREAM	ND	ND	ND
13	25.1.05	UPSTREAM	174.34	71.65	26.5
13	22.12.04	DOWNSTREAM	1.42	1.07	0.4
14	6.1.05	UPSTREAM	0.29	0.21	0.08
14	6.1.05	DOWNSTREAM	8.45	6.04	2.2
14	24.1.05	DOWNSTREAM	2.67	4.44	1.6

Figure 5. Oestrogenic activity in streamwater from the downstream site at Farm 3, plotted for two rainfall events captured by autosampler.

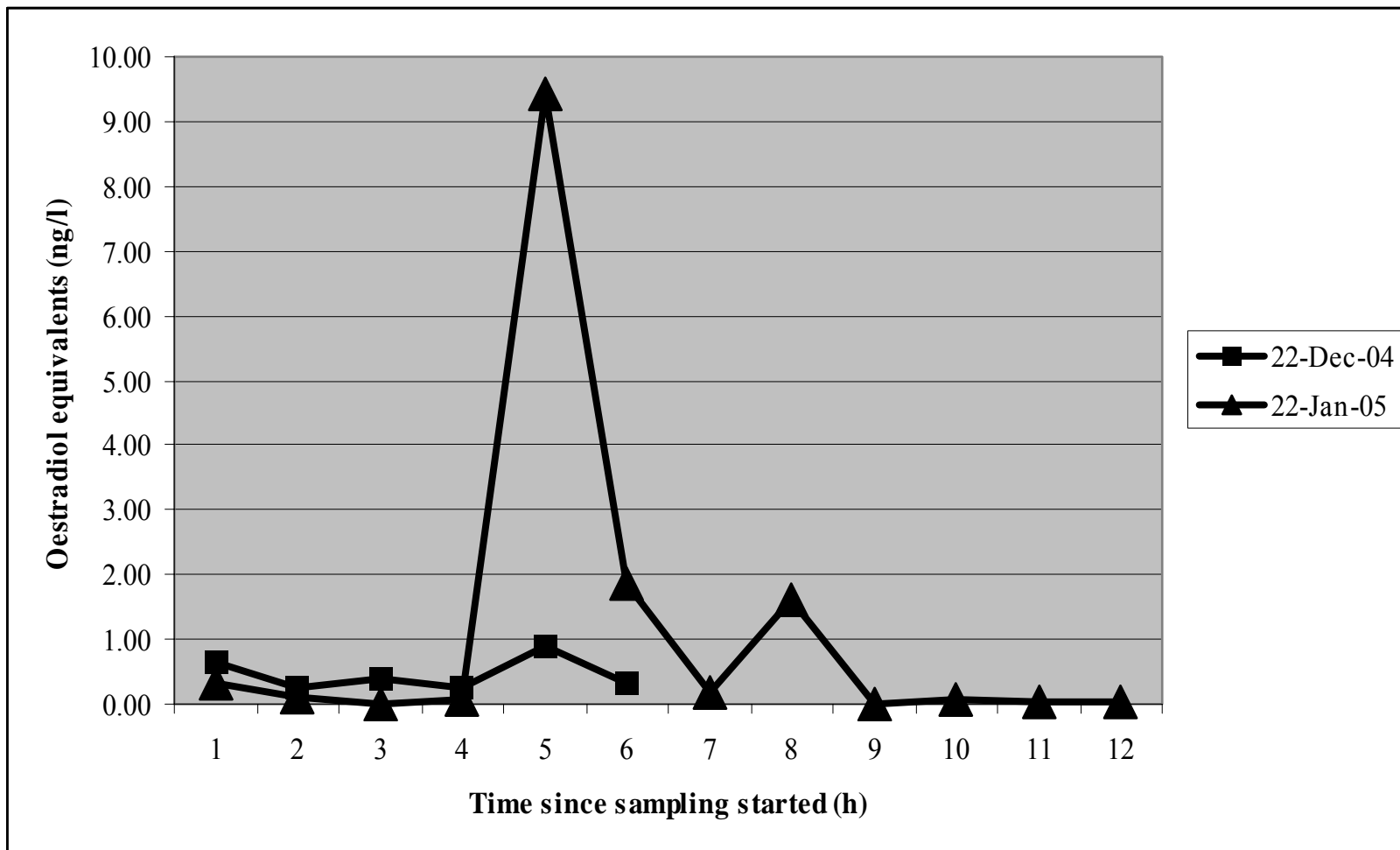
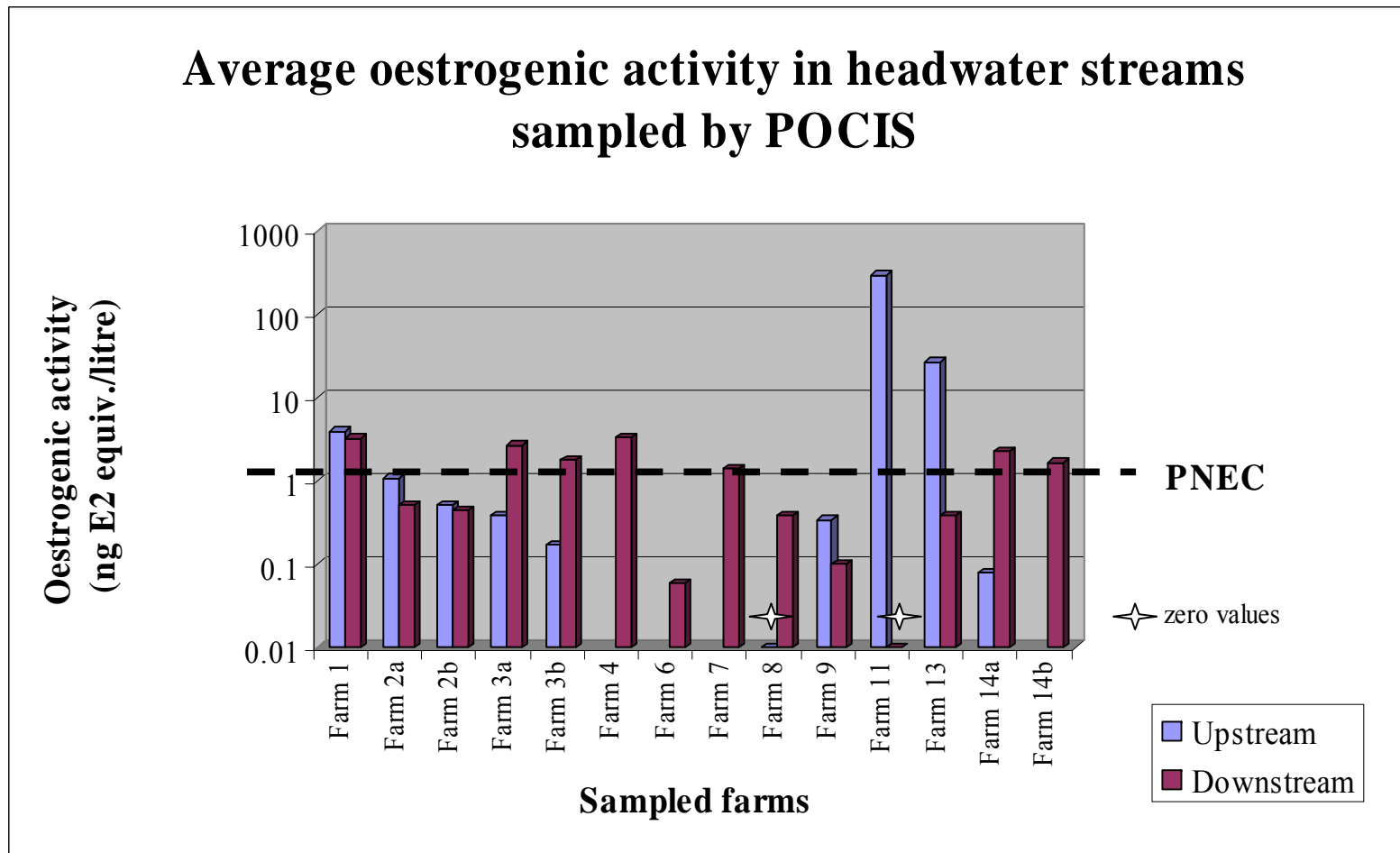


Figure 6. Average oestrogenic activity in streamwater from all sites, as determined by YES assay of POCIS extracts. The Predicted No Effect Concentration (PNEC) for oestradiol is shown as a horizontal dashed line.



However, in the remaining 4 cases, there was no change or even a loss of activity as the stream flowed through the farm. Furthermore, it is clear that only one of the upstream sites (Farm 8) was completely free of oestrogenic activity. This suggests that livestock farming may only have been contributing a proportion of the observed activity, and Table 1b identifies some possible sources. Each farm is different, so the data are discussed farm-by-farm below in comparison with Table 1b.

Farm 1. The only known upstream source of activity (1.4 ng EEQ/l) was a few pasture fields from which livestock had been withdrawn earlier, suggesting that livestock may have caused this signal. The disposal of dirty water from the pig pens appears to have been responsible for the increase downstream (3.2 ng EEQ/l), although a contribution from one possible septic tank cannot be ruled out.

Farm 2. In both sampler runs, upstream activity (0.5-1.1 ng EEQ/l) was higher than downstream (0.4-0.5 ng EEQ/l). The only known upstream source was a few pasture fields containing store lambs.

Farm 3. In both sampler runs, the farm clearly contributed to the oestrogenic activity in the stream (upstream: 0.2-0.4 ng EEQ/l; downstream: 1.8-2.7 ng EEQ/l). There are no known confounding factors, so this appears to be a clear example of the influence of livestock farming. It should be noted that this farm was very much a worst case, with a combination of pregnant cattle and sheep in the fields, direct access of livestock to the stream, slurry applications, and probable direct runoff from the farmyard to the stream.

Farm 4. There was no upstream sampling site on this farm, but the downstream activity (3.3 ng EEQ/l) probably arose from a combination of earlier grazing livestock and slurry applications. At most, there is one septic tank in the whole catchment.

Farm 6. It is no surprise to find some activity (0.2 ng EEQ/l) in the river 'control' due to the large number of sources upstream, including a small STW. The river does not influence the downstream site (a tributary ditch), and the weak activity at this point (0.06 ng EEQ/l) is presumably caused solely by the pregnant beef cattle on the catchment.

Farm 7. This is the slurry application experiment, and there are no confounding factors. The implication of this is that the dairy slurry application (40 m³/ha) was the sole cause of the 1.2-1.6 ng EEQ/l activity in the field drain. Note that the two POCIS measurements were for duplicate samplers deployed side-by-side at the same time.

Farm 8. In this case, the upstream catchment contributed no oestrogenic activity and contained no likely sources except some pasture with pregnant ewes. The downstream activity (0.4 ng EEQ/l) was therefore likely to have been caused by livestock farming, although two possible septic tanks in the catchment may have contributed.

Farm 9. The farm contributed nothing to the oestrogenic activity downstream (0.04 ng EEQ/l), but the upstream activity (0.1 ng EEQ/l) was presumably related both to the pasture fields above the farm, and to 3 possible septic tanks.

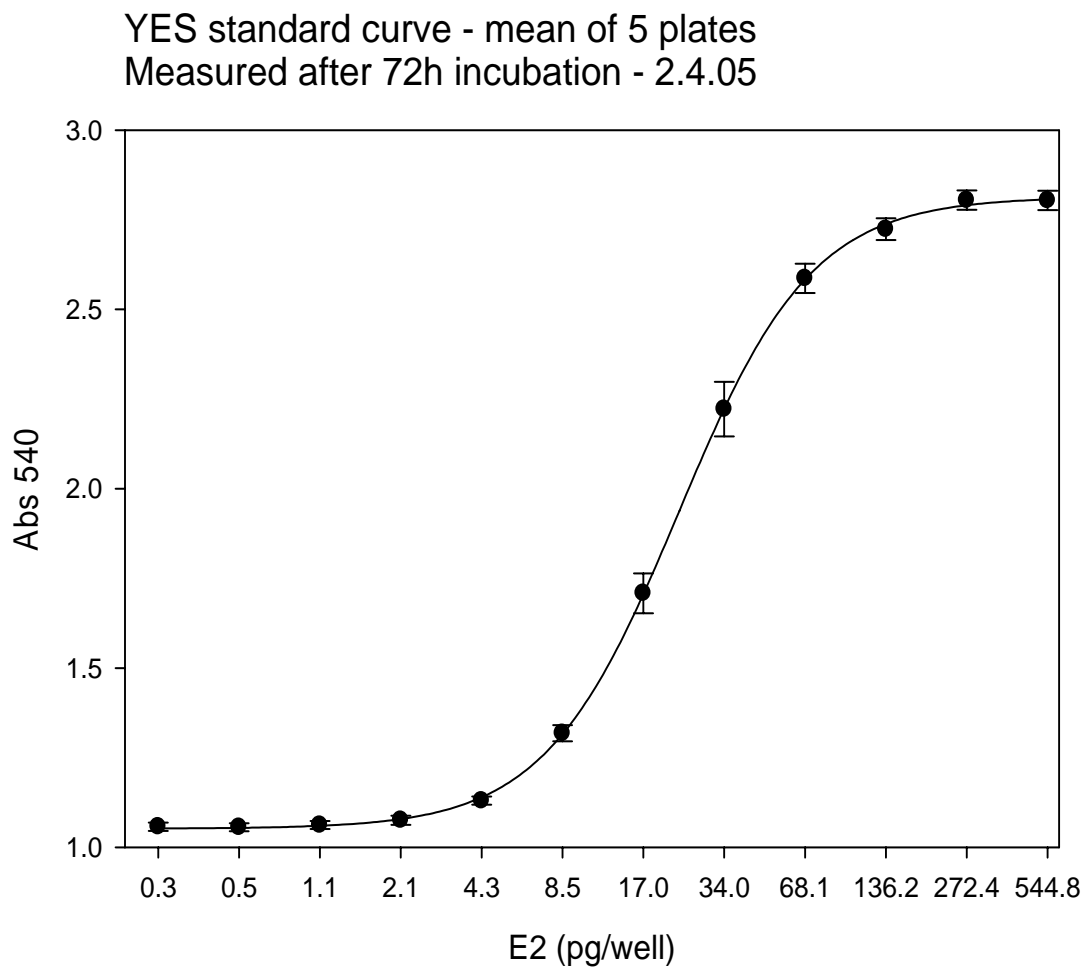
Farm 11. The results from this farm are an apparent anomaly (292 ng EEQ/l upstream; 0 ng EEQ/l downstream), given that there are no known upstream sources of oestrogenic activity other than a few empty pasture fields. Possible downstream sources apart from recently vacated pasture included slurry spreading and several suspected septic tanks. The farmer has been contacted in order to double-check the possibility of upstream sources but no response has been received. Sediment samples have been retained, and it is possible that their future analysis will shed light on these observations.

Farm 13. The substantial upstream activity (26.5 ng EEQ/l) is almost certainly related to the fact that the stream flows through a hamlet with approximately 10 septic tanks and three small livestock farms. The downstream activity was apparently much less (0.4 ng EEQ/l), although it should be noted that the downstream sampler was not left in place for as long as the upstream one (40 d compared with 73d). Operations on the farm do not appear to have added much activity to the stream.

Farm 14. Although there are several villages and farms above the upstream sampling point, the incoming oestrogenic activity was low (0.08 ng EEQ/l), probably because the upstream stretches were not flowing significantly during the study period. The study farm contributed some activity (1.6-2.2 ng EEQ/l), but as the grazing cattle were all steers and there was no slurry spreading from the dairy cattle kept under cover, it seems likely that the single septic tank in the lower catchment was also contributing.

Although it appears that there is no correlation between the predicted oestrogen load from livestock (direct excretion to farmland, plus slurry) and measured oestrogenic activity downstream, this is probably misleading. Such a correlation takes no account of possible inputs from livestock excreting directly into streams, or from farmyard runoff. Nevertheless, the low correlation coefficient (0.26) suggests that other oestrogen sources (e.g. septic tanks) are almost certainly contributing to the total observed activity.

Figure 7. Standard curve for 17 β -oestradiol in the YES assay.



Key to figures 8-10 symbols: The graphs show the absorbance (540 nm) for a range of volumes of the sample extracts from each site.

Site 1

Open circles: downstream
Solid circles: upstream

Site 2

Open circles: downstream B
Solid circles: upstream B
Open triangles: downstream A
Solid triangles: upstream A

Site 3

Open circles: downstream B
Solid circles: upstream B
Open triangles: downstream A
Solid triangles: upstream A

Site 4

Open circles: downstream

Site 6

Open circles: downstream
Solid circles: river "control"

Site 7

Open circles: field drain
Solid circles: field drain

Site 8

Open circles: downstream
Solid circles: upstream

Site 9

Open circles: downstream
Solid circles: upstream

Site 11

Open circles: downstream
Solid circles: upstream

Site 13

Open circles: downstream
Solid circles: upstream

Site 14

Open circles: downstream A
Solid circles: upstream A
Open triangles: downstream B

Figure 8. YES response curves for POCIS extracts from Farms 1-4.

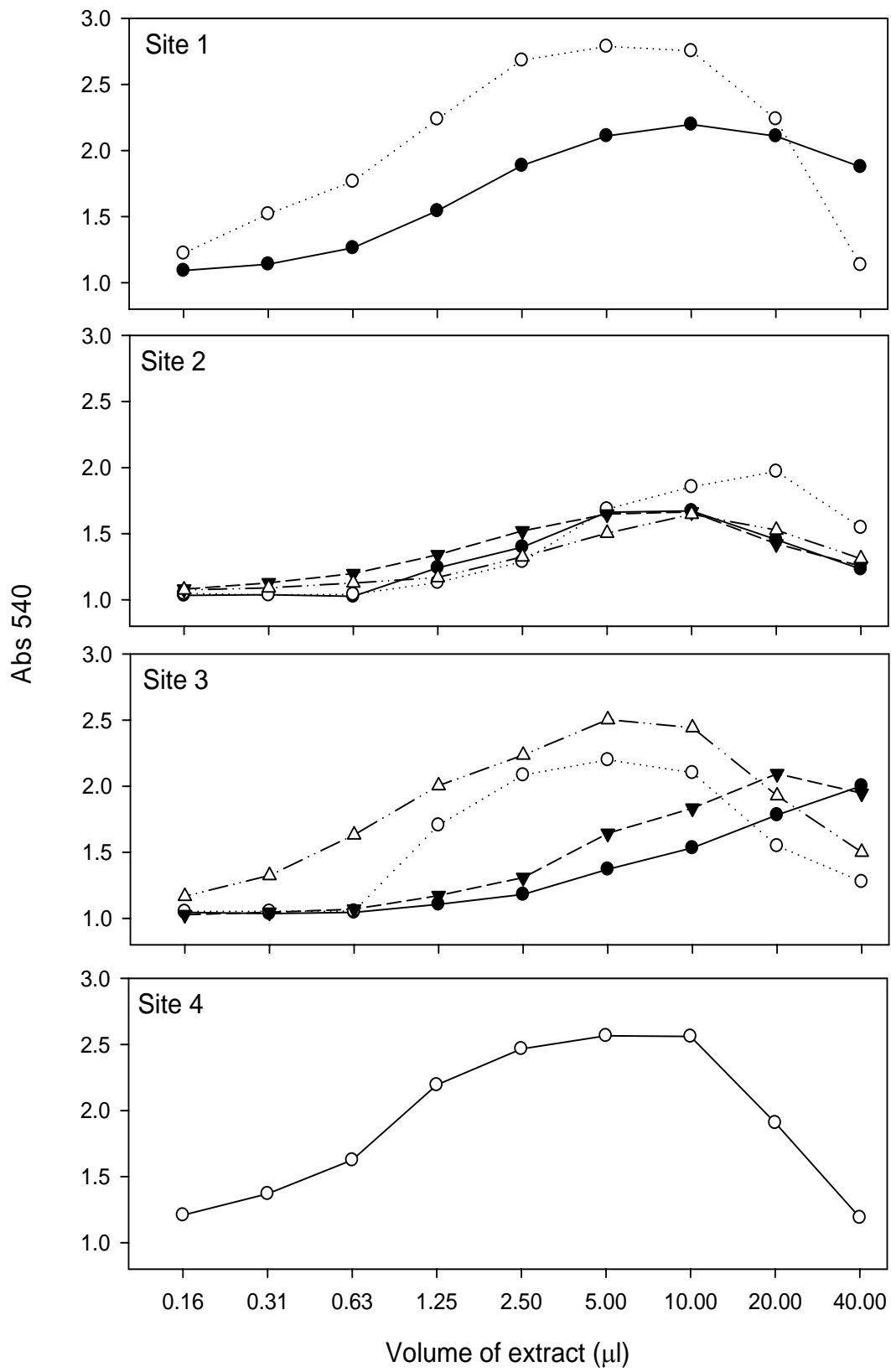


Figure 9. YES response curves for POCIS extracts from Farms 6-9.

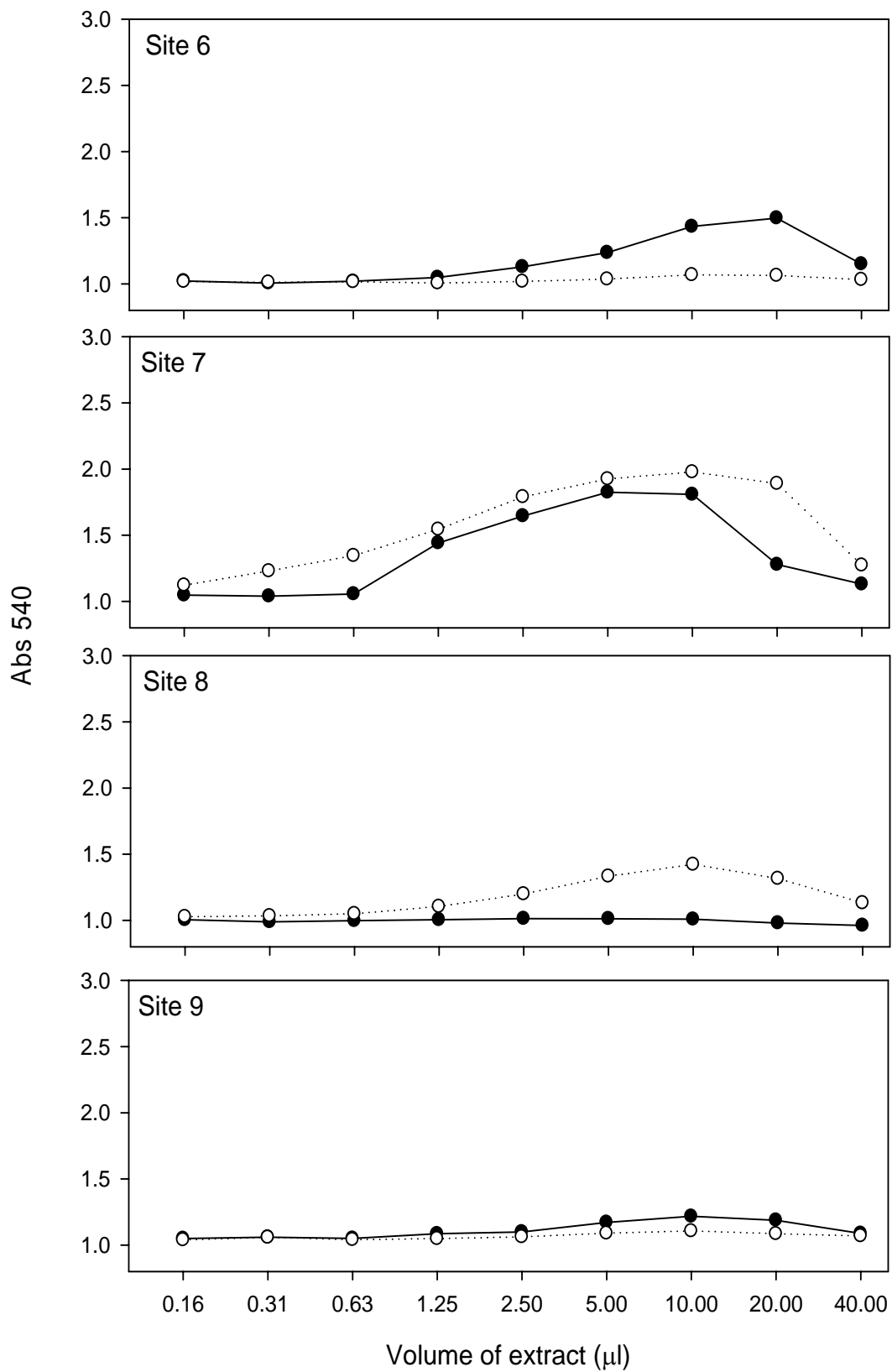
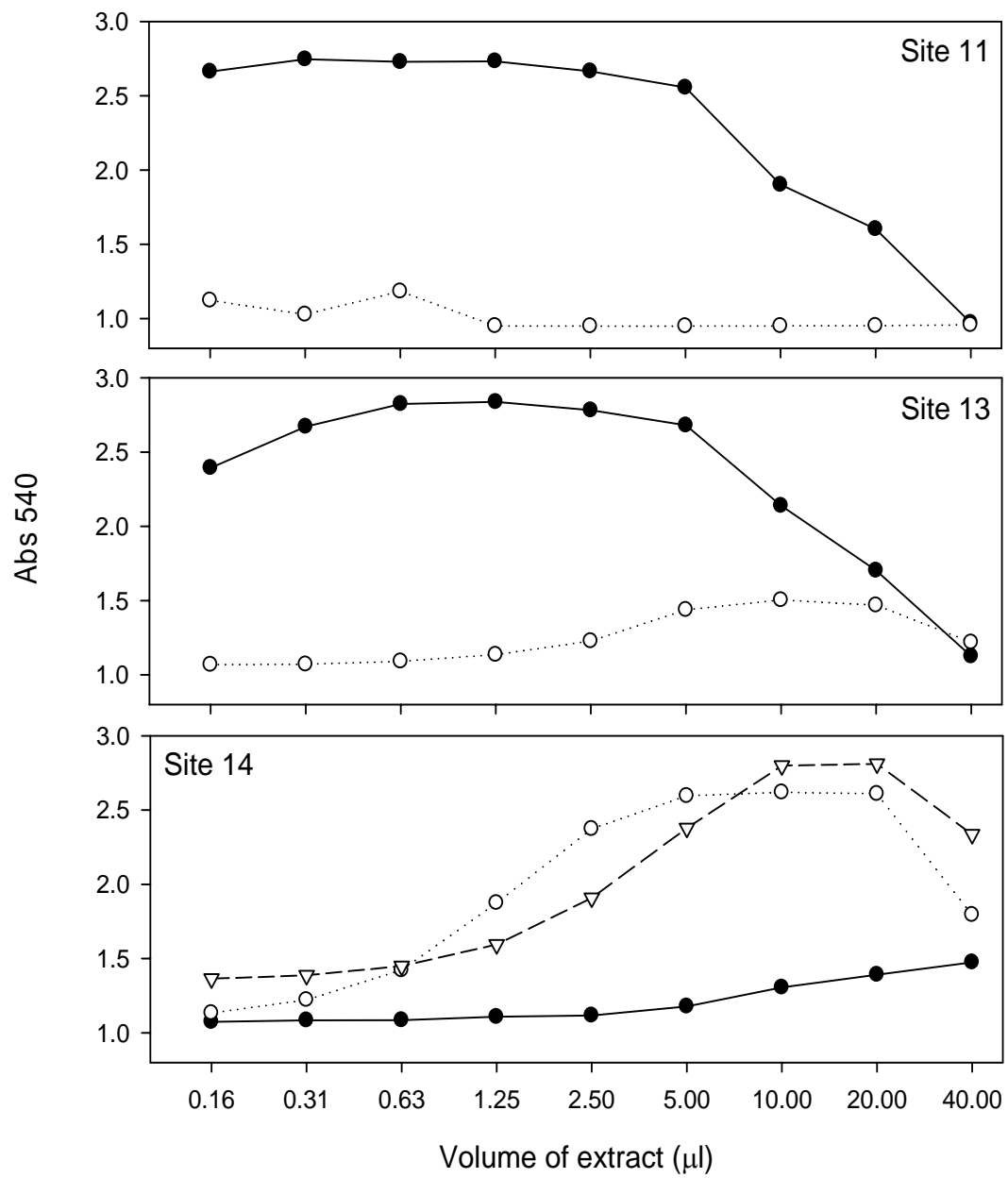


Figure 10. YES response curves for POCIS extracts from Farms 11, 13 and 14.



Analysis of oestrogenic hormones (E1, E2, EE2)

The analytical data are shown in Table 5, and the E2-equivalent values are plotted by site in Figure 11. They have been related back to average concentrations in the streams in the same way as for the YES data.

It is clear that EE2 was absent from all samples, which thus provides no support for the hypothesis that seepage from septic tanks was contributing to the upstream activity seen by YES at some sites (although such seepage is not ruled out because the pharmaceutical use of EE2 is far from universal). Furthermore, E1 was consistently present at higher concentrations than E2, which is to be expected given that dairy cattle excrete over twice as much E1 as E2 (see review). Overall, concentrations of E2-equivalents ranged from 0.04 to 3.62 ng/l which appears to agree well with the levels of activity seen by the YES (0 to 3.3 ng/l) at all but two sites. Furthermore, the difference between the upstream and downstream signals from E1 and E2 was much more marked than for the YES at sites 1-4, revealing clearly the contribution to oestrogenic activity in streams from the presence of livestock on some farms. The remaining farms appeared to contribute little E1 or E2 to the streams. On average, the downstream E2-equivalent concentration was a factor of 16 times higher than upstream (range, downstream/upstream: 0.5 – 60.9).

A regression analysis of the two measures of activity (i.e. normalised E1/E2 and YES) at individual sites did not provide a best-fit line with a slope significantly different from zero ($P = 0.1$) and the analytical data could explain only 12% of the variability in the YES data ($r^2 = 0.12$). However, log transformation of both data sets improved the amount of variation in the YES data accounted for by the analytical data ($r^2 = 0.24$) and a significant deviation from zero in the gradient of the slope was also evident ($P = 0.02$; Figure 12). Although the reason for the improved fit of the data when log-transformed is not immediately clear, the latter analysis would seem to confirm that the measured water-borne steroids account for some of the oestrogenicity detected in the YES assay. Nonetheless, a considerable portion of the oestrogenicity cannot be attributed to E1 or E2. This is emphasised by inspection of the data for the upstream stations at Farms 11 and 13, which had very high activity in the YES (292 and 26.5 ng/l respectively), but much lower levels of E1/E2 (2.26 and 0.28 ng E2 equiv./l, respectively). As described above, there are no known oestrogen sources in the upper catchment of Farm 11, while the stream on Farm 13 flows through a small hamlet with septic tanks. In addition, several other catchments (i.e. 1, 2, 3, and 14) also showed higher YES activity than normalised E1/E2 concentrations above the farm.

Table 5. Chemical analytical data for oestrogens extracted from the duplicate POCIS discs – estimated average concentrations in stream water (ng/l). The righthand column shows the calculated E2-equivalent values.

Site number	Date of sample collection	Comments	E1	E2	EE2	Calculated E2 equivalent*
1	24.12.04	UPSTREAM	0.13	0.00	0	0.04
1	24.12.04	DOWNSTREAM	3.02	0.34	0	1.34
2	25.1.05	UPSTREAM	0.15	0.00	0	0.05
2	25.1.05	DOWNSTREAM	1.46	0.20	0	0.69
2	11.12.04	UPSTREAM	0.23	0.00	0	0.08
2	11.12.04	DOWNSTREAM	2.62	0.34	0	1.21
3	25.1.05	UPSTREAM	0.11	0.00	0	0.04
3	25.1.05	DOWNSTREAM	4.83	0.56	0	2.17
3	11.12.04	UPSTREAM	0.21	0.00	0	0.07
3	11.12.04	DOWNSTREAM	4.67	0.53	0	2.09
4	17.12.04	DOWNSTREAM	9.31	0.52	0	3.62
6	21.12.04	DOWNSTREAM	0.10	0.00	0	0.03
6	21.12.04	River 'control'	0.32	0.00	0	0.11
7	14.1.05	Field-drain	No data	No data	No data	No data
7	14.1.05	Field-drain	No data	No data	No data	No data
8	21.12.04	UPSTREAM	0.61	0.00	0	0.20
8	21.12.04	DOWNSTREAM	0.19	0.00	0	0.06
9	22.12.04	UPSTREAM	1.27	0.11	0	0.53
9	22.12.05	DOWNSTREAM	0.88	0.00	0	0.29
11	22.12.05	UPSTREAM	4.11	0.89	0	2.26

11	22.12.04	DOWNSTREAM	2.59	0.23	0	1.10
13	25.1.05	UPSTREAM	0.59	0.08	0	0.28
13	22.12.04	DOWNSTREAM	0.40	0.09	0	0.22
14	6.1.05	UPSTREAM	0.28	0.00	0	0.09
14	6.1.05	DOWNSTREAM	0.45	0.00	0	0.15
14	24.1.05	DOWNSTREAM	0.31	0.00	0	0.10

*Assuming that $E1 \cdot 0.333 = E2$ equivalent; Thorpe *et al.*, 2003.

Figure 11. Upstream/downstream comparison of measured hormone residues (E2 equivalents) in streamwater. Asterisks denote missing samples.

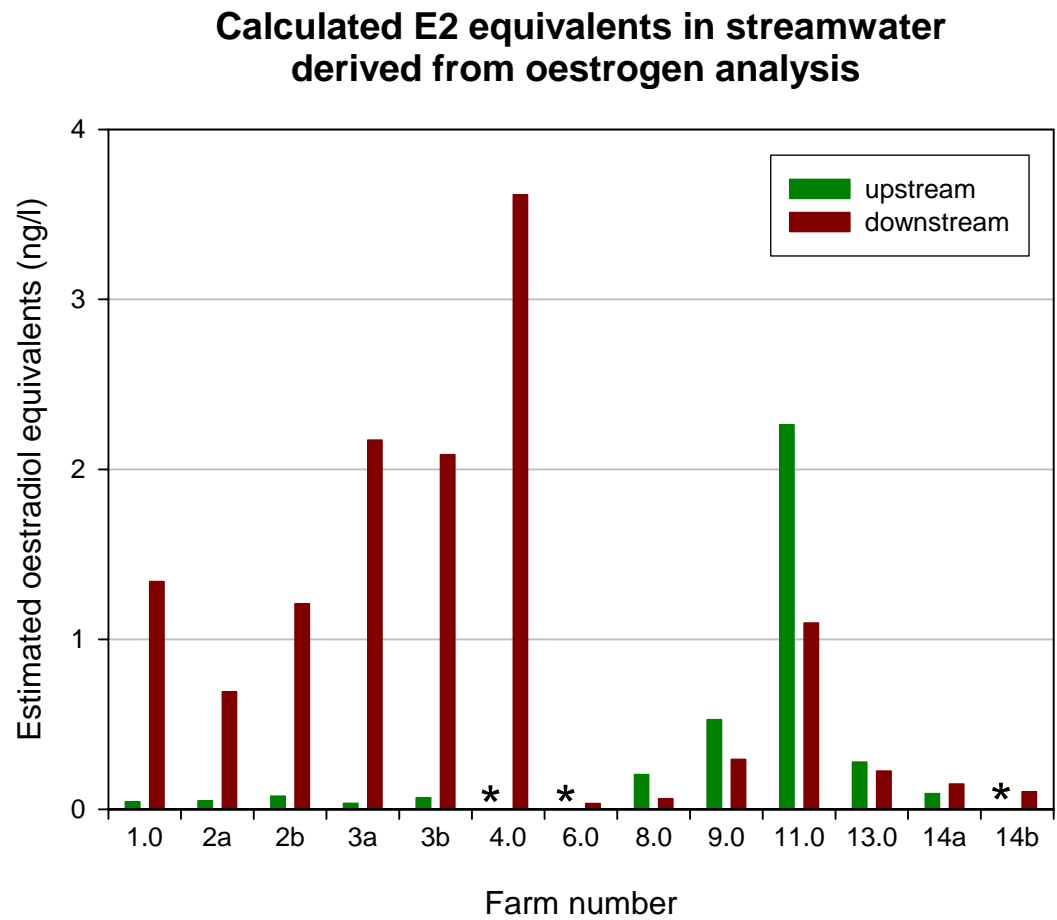
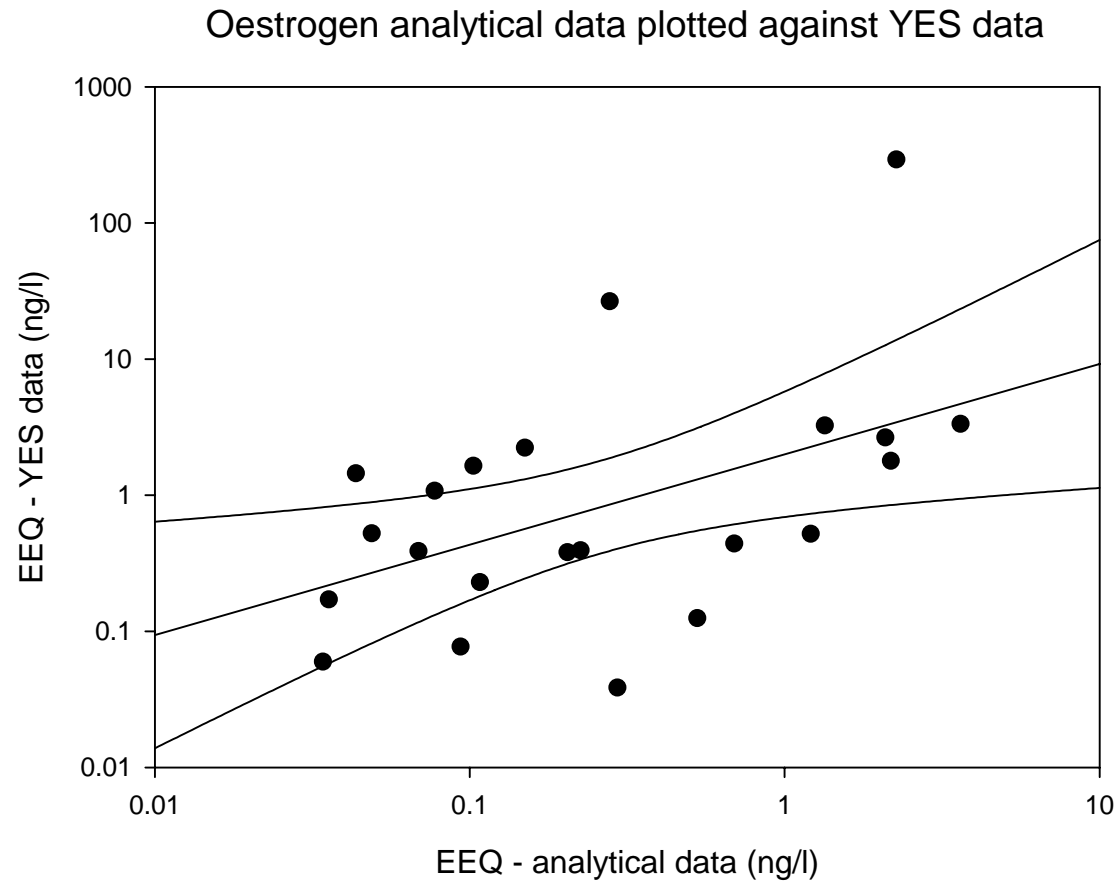


Figure 12. Comparison of YES data and E2 equivalent analytical data. The linear regression line and 95% confidence intervals are shown ($r^2 = 0.242$, $P = 0.02$).



Androgenic activity

The YAS standard curve for testosterone is shown in Figure 13, and the YAS response curves obtained from the extracts of POCIS discs deployed at the various sites are shown in Figures 14-16. Finally, the YAS response curves from the Farm 3 downstream autosample extracts are shown in Figure 17. The calculated testosterone (T) equivalent concentrations in the autosamples are listed in Table 6. As with the oestrogen activity data, results have not been adjusted for recovery, and the data have been normalised to a 30 day exposure period. Measurements in the laboratory of testosterone uptake rate by the POCIS discs were not made, but were assumed to be similar to the rate for oestradiol (0.09 l/day) due to the great similarity in the characteristics of the two molecules.

Reference to Figure 17 shows that high concentrations of water extract were able to produce cytotoxicity in the YAS in the same way as in the YES. However, androgenic activity generally (in both water and POCIS extracts) was low or absent. Table 6 demonstrates that during the rainfall event of 22/12/04, concentrations of androgenic activity in streamwater rose from zero to a maximum of 4715 ng T equiv./l, returning to near baseline after 5 hours. However, no androgenic activity at all was detected during the later rainfall event of 22/1/05, even though oestrogenic activity was still present at that time. In other words, what little androgenic activity was being mobilised in the Farm 3 catchment declined rapidly after the dairy herd was withdrawn to sheds in late November 2004 (although the two events may not be related).

Turning to the YAS assays of POCIS extracts (data not tabulated), all but one demonstrated no androgenic activity whatever, including the sample from downstream on Farm 3 where transient activity was detected by the autosampler in late November. This suggests that background levels of androgenic activity were generally negligible, and that the brief spike detected by the Farm 3 autosampler on 22/11/04 contributed little to the long-term average level of activity.

The exception was the POCIS sample from the upstream site on Farm 11, from which it was estimated that the average concentration of androgenic activity in the stream at that point was 18.3 ng T equiv./l. It will be recalled that the POCIS at this site also accumulated a very high level of oestrogenic activity, and it therefore seems possible that the two observations are linked to the same (unknown) source. Although it would be unwise to place great reliance on these data as the testosterone uptake and recovery rates were not calibrated, note that the simultaneous presence of plant-derived oestrogenic and androgenic activity is not beyond the bounds of possibility.

Unfortunately, while the Environment Agency were kindly able to analyse POCIS extracts for oestrogenic hormones, this service did not extend to androgens, so the identity of the activity at Farms 3 and 11 is not known.

Figure 13. Testosterone standard curve obtained with Yeast Androgen Screen.

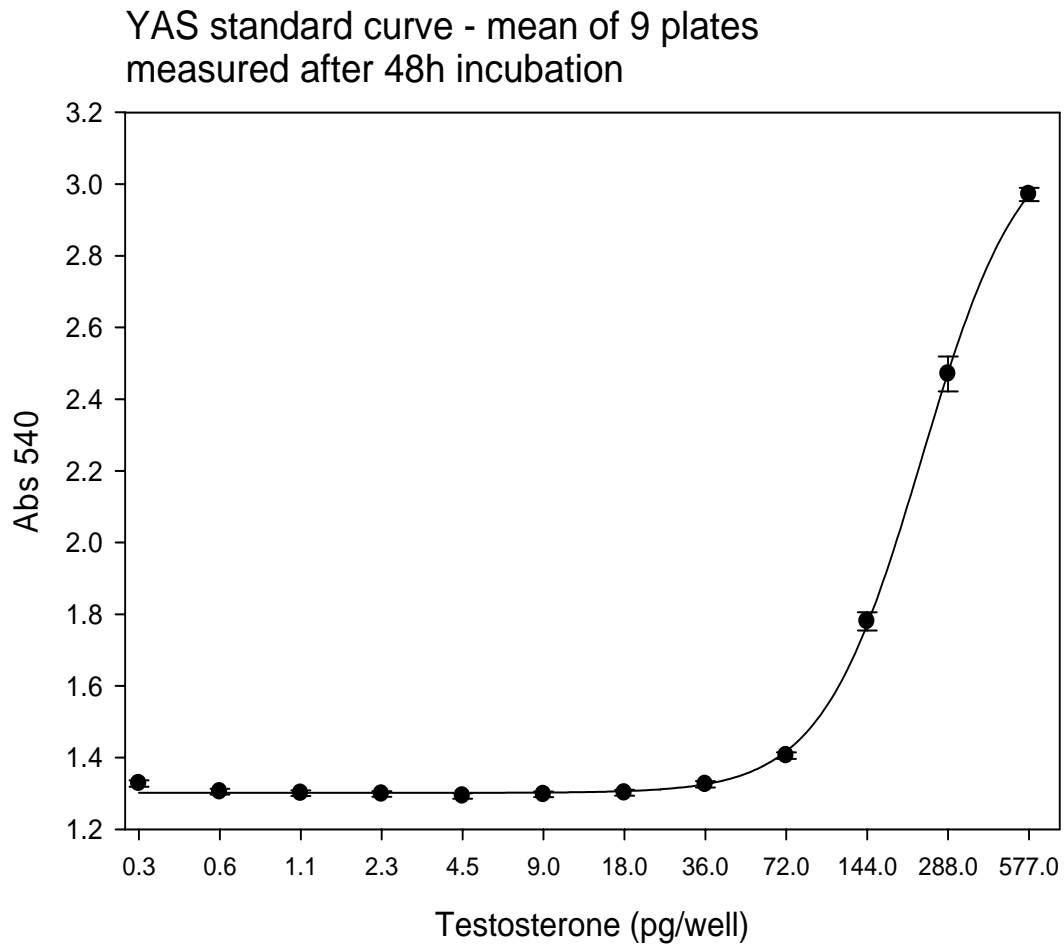


Figure 14. YAS response curves for Farms 1-4.

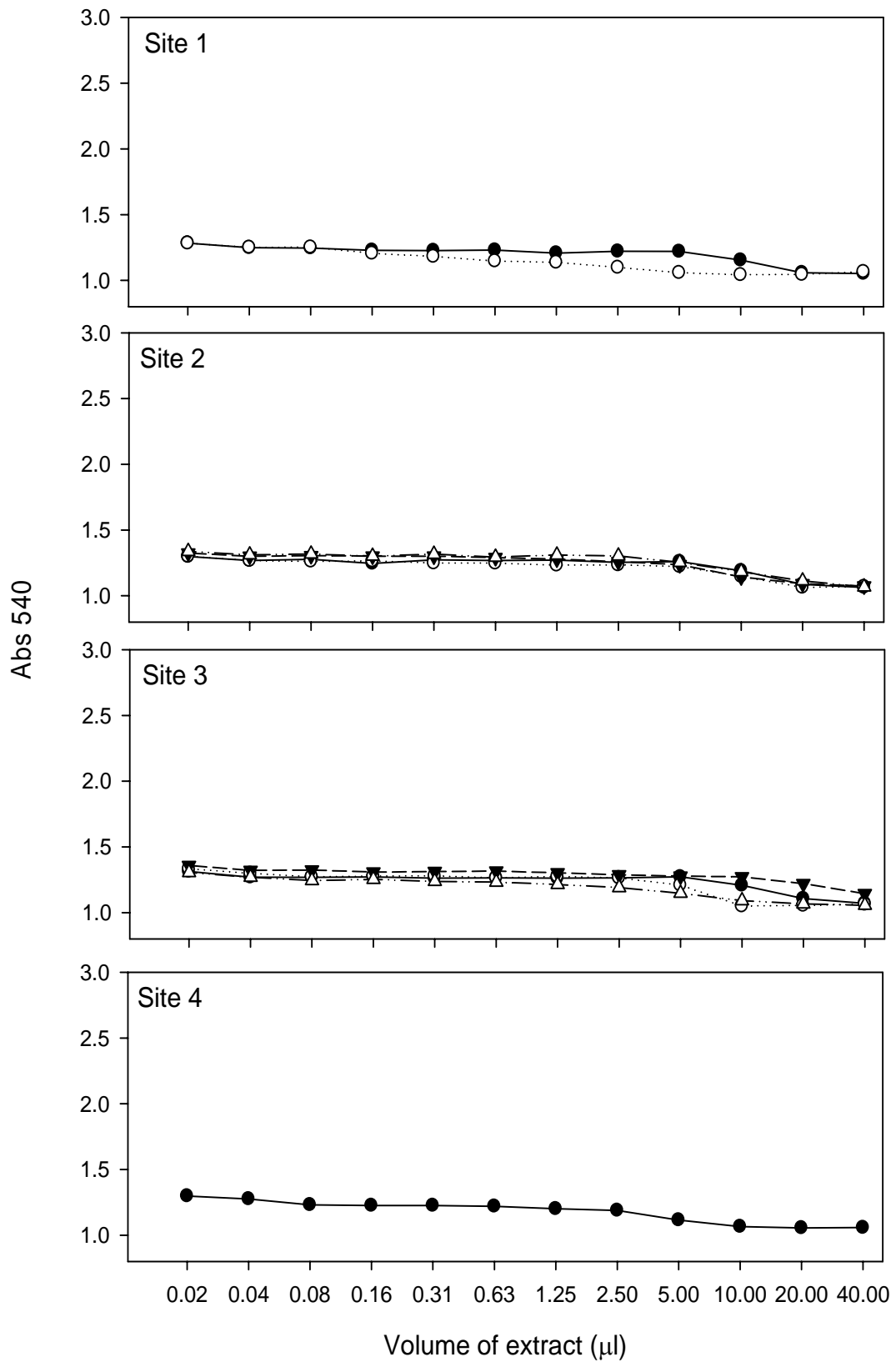


Figure 15. YAS response curves for Farms 6-9.

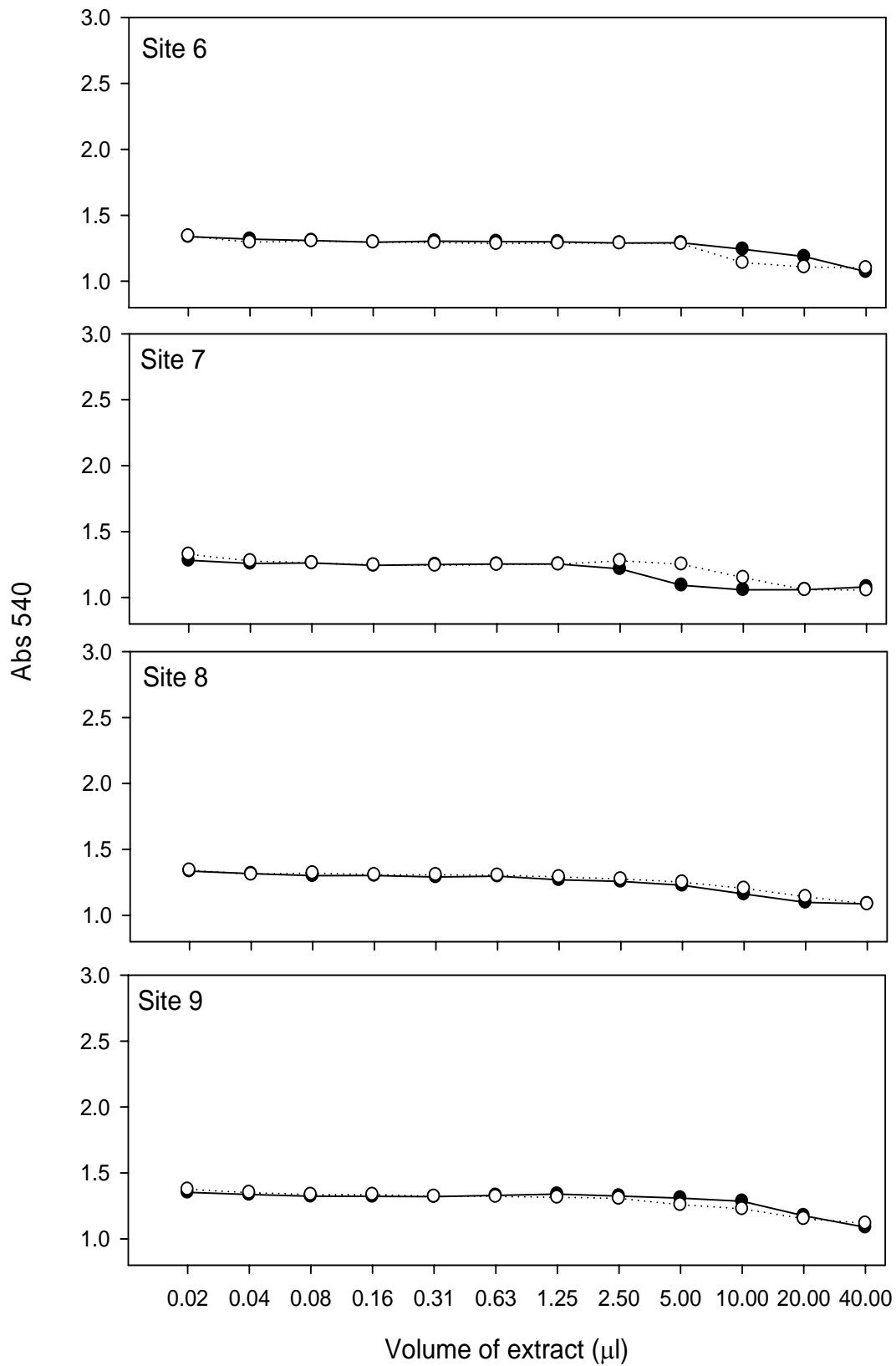


Figure 16. YAS response curves for Farms 11, 13 and 14.

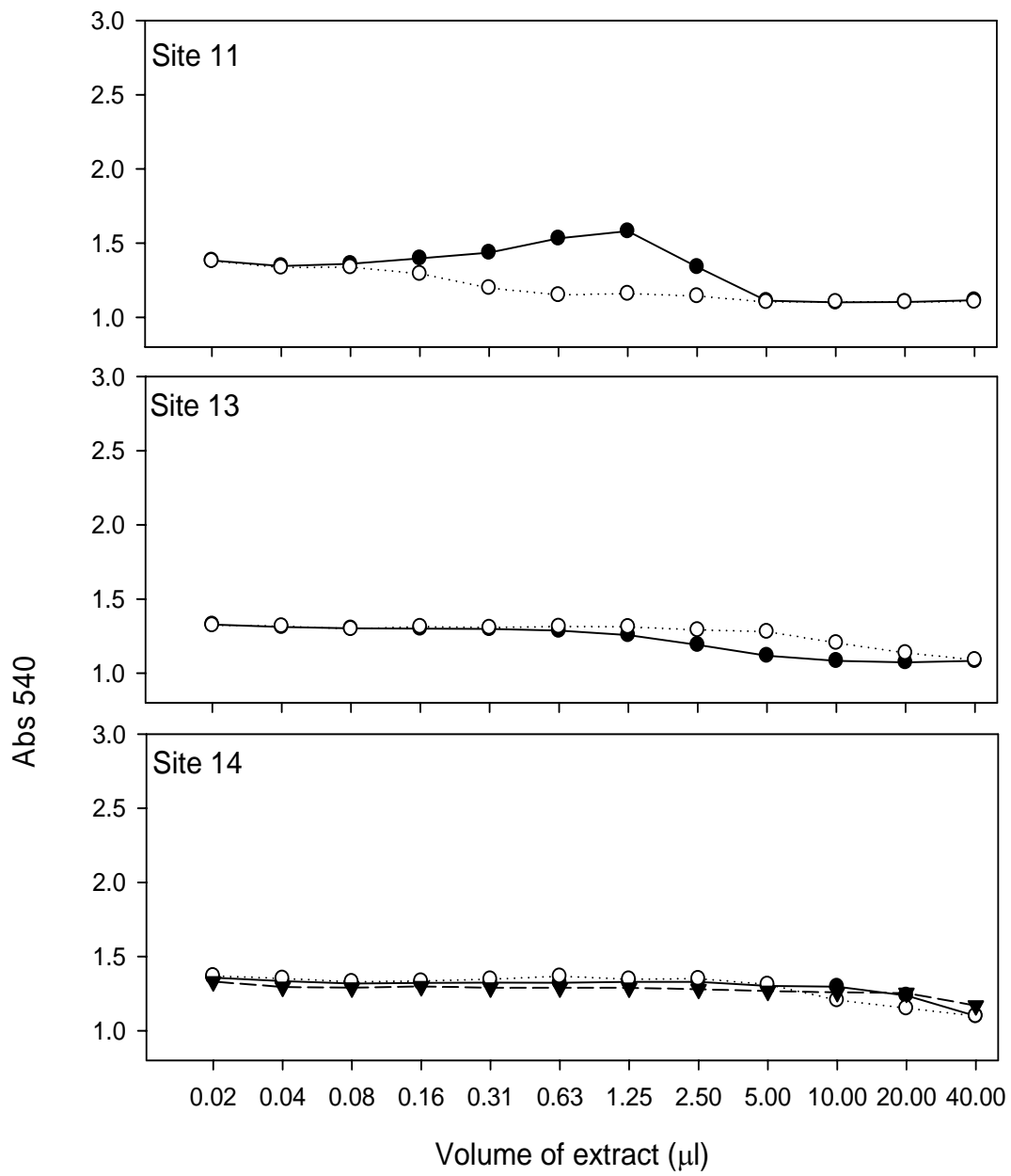


Figure 17. YAS response curves for autosamples collected at Farm 3.

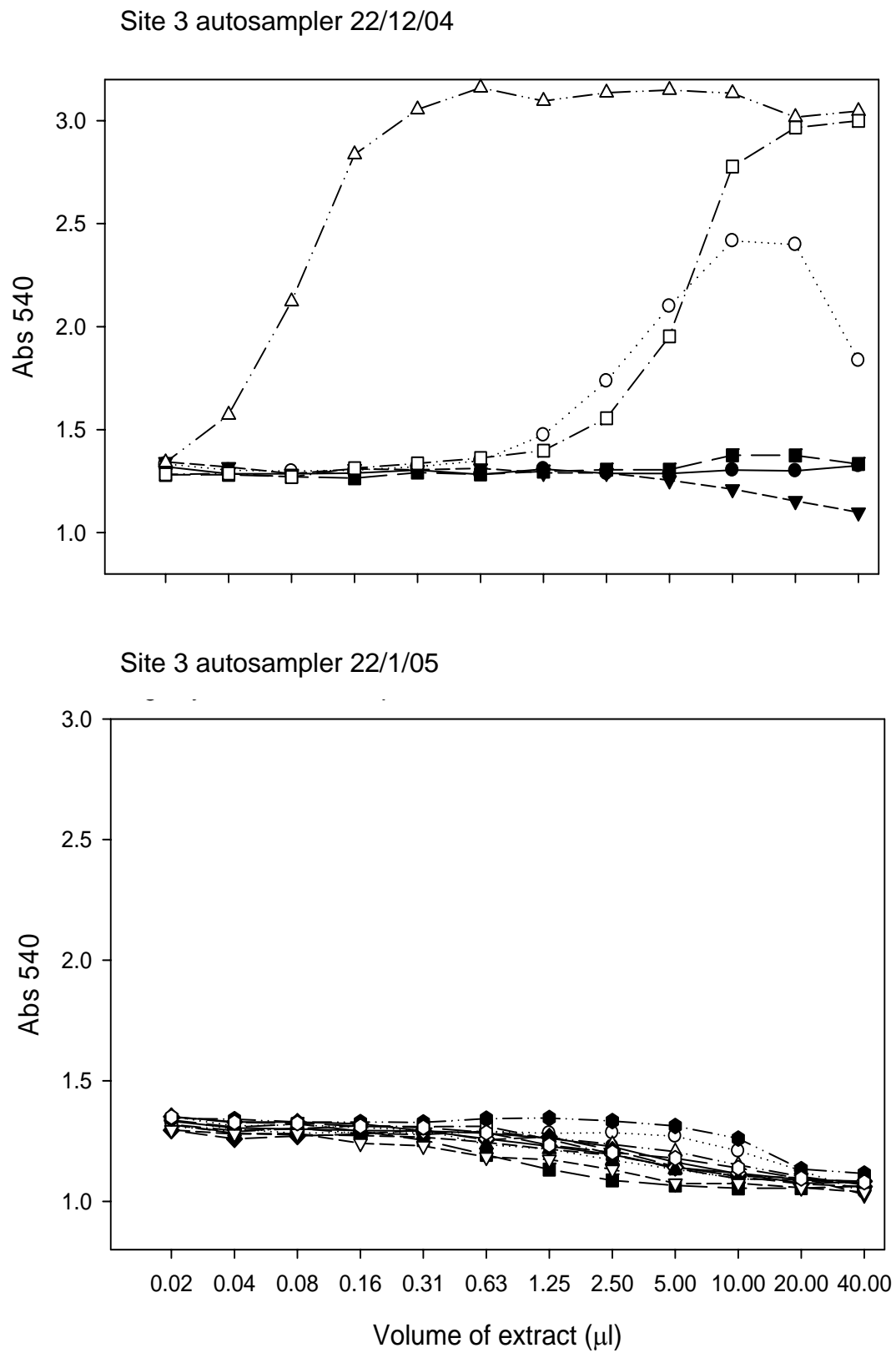


Table 6. YAS data for Farm 3 autosamples expressed as testosterone equivalents (TEQ)

Sample	Date	TEQ (pg in 500 ul extract)	Volume original sample (ml)	TEQ (pg/l)	TEQ (ng/l)
1	22.12.04	ND	250	0	0
2	22.12.04	19394	250	77576.2	77.6
3	22.12.04	ND	250	0	0
4	22.12.04	1178876	250	4715503.2	4715.5
5	22.12.04	3202	250	12807.2	12.8
6	22.12.04	16209	250	64837.2	64.8
1	22.1.05	ND	1000	0	0
2	22.1.05	ND	1000	0	0
3	22.1.05	ND	1000	0	0
4	22.1.05	ND	1000	0	0
5	22.1.05	ND	1000	0	0
6	22.1.05	ND	1000	0	0
7	22.1.05	ND	1000	0	0
8	22.1.05	ND	1000	0	0
9	22.1.05	ND	1000	0	0
10	22.1.05	ND	1000	0	0
11	22.1.05	ND	1000	0	0
12	22.1.05	ND	1000	0	0

ND= not detectable. Abs540 less than or equal to blank

Discussion

The results of these studies suggest that oestrogenic contamination of headwater streams in livestock farming areas is widespread in England and Wales, at least on farms considered to be ‘worst-case’ oestrogen sources. Origins of this activity are not solely attributable to livestock, and it is likely that phyto-estrogens derived from plants, and even oestrogenic material in rainwater, may be ‘topping up’ the livestock signal, although we have no direct evidence for this at present. Septic tank overflows and cess pits were also possibly contributing activity at some sites, although the absence of EE2 does not support this view. The levels of oestrogenic activity found lie in the same range as, or occasionally higher than, those reported for some agricultural surface waters in Israel (Shore *et al.*, 1995; 2004), North America (Irwin *et al.*, 2001; Kolodziej *et al.*, 2004; Soto *et al.*, 2004), Ireland (Tarrant *et al.*, 2005) and Denmark (Stuer-Lauridsen *et al.*, 2005). In these cases from other countries, levels of oestrogenic activity in rural streams and lakes range from zero to about 10 ng E2 equiv./ litre.

It could be argued that the farms monitored during this study were not indeed ‘worst-cases’ because sampling generally began soon after cattle had been withdrawn to sheds for the winter. However, the downstream oestrogenic activity at Farm 3 in Nov/Dec 2004 (2.7 ng EEQ/l) where the whole dairy herd was on the pasture for the first half of the POCIS-deployment period was not higher than on several other farms where the animals were under cover throughout. This observation cannot be explained by differentially low rainfall in the region of Farm 3. It would be desirable in future to conduct studies slightly earlier in the season (October), but this information suggests that grazing animals contribute relatively little to the overall oestrogenic contamination of streams – slurry application and farmyard runoff may be the main sources. This is supported by the modelling conducted for the literature review.

Routes of this oestrogenic activity to headwater streams are probably various. However, the appearance of a brief peak in oestrogenic activity in the 24 January autosamples echoes similar peaks in water-soluble herbicides which occur after rainfall in many headwater streams draining arable catchments (e.g. Matthiessen *et al.*, 1992). This implies that at least some of the measured contamination reaches streams via seepage and drainflow during rainstorms, because the 24 January event was probably not intense enough to have caused overland flow (rain data are not available for Farm 3, but at the nearby Farm 2 this event was recorded as 21.2 mm over the previous 3 days). More data are needed on this point, but it seems that stream organisms are being chronically exposed to time-averaged levels of oestrogenic activity up to about 3 ng EEQ/l, supplemented by brief spikes of activity reaching 10 ng EEQ/l or more after rainstorms.

These transient spikes may have little biological relevance. Although recent unpublished data (Maunder *et al.*, 2005) show that sticklebacks can bioaccumulate E2 in the blood by up to 50-fold within 6 hours of exposure via the ambient water, this can be rapidly lost again when external concentrations decrease. It is probably more appropriate to consider the POCIS discs as surrogate organisms for which the time-averaged exposure concentration is of greatest significance.

A considerable amount of data on the impacts of oestrogens on aquatic life has been published, and fish appear to be most at risk, although relatively little is yet known about the susceptibility of invertebrates. On the basis of a thorough literature review, Young *et al.* (2002) proposed a long-term Predicted-No-Effect-Concentration (PNEC) for aquatic life of 1.0 ng/l for E2. A critical study is that of Metcalfe *et al.* (2001) who exposed Japanese medaka fish to E2 for 100 days from hatching to sexual maturity and measured *inter alia* the induction of male intersex individuals with oocytes in their testes. For this, the most sensitive endpoint, the Lowest-Observed-Effect-Concentration (LOEC) was 10 ng/l, and the No-Observed-Effect-Concentration (NOEC) was 1 ng/l. However, it should be noted that data for E2 based on a fish full life cycle test are not available, and it is possible that these would be more sensitive than the medaka partial life cycle test. The proposed PNEC of 1 ng/l may therefore not protect fish populations against all adverse effects.

The implication of this published information is that average long-term E2-equivalent concentrations in excess of 1 ng/l, if bioavailable, are likely to cause ovotestis and other oestrogen-induced intersexual abnormalities (e.g. vitellogenin induction) in some fish. In fact, due to the nature of the POCIS sampling method, it is likely that the levels of oestrogenic activity and oestrogen residues were indeed fully bioavailable to fish and other aquatic organisms. 46% of the POCIS measurements were above 1 ng EEQ/l, representing 8 of the 11 surveyed farms. If the PNEC were to become an Environmental Quality Standard (EQS), several farms would potentially be in breach. However, probably in only two cases (Farms 11 and 13) did measured activity reach levels (292 and 26 ng EEQ/l, respectively) that might be considered a significant threat to fish reproduction, and in neither of these cases did the activity appear to be primarily related to livestock rearing.

Turning to the analytical data on oestrogenic hormones sampled by the duplicate POCIS disks, it is clear that most oestrogenic activity could be attributed to E1 and E2. However, the high upstream activity at sites 11 and 13, and the lower upstream activity at several other sites was attributable to neither E1/E2 nor EE2. Although it is possible that the upstream activity on Farm 13 (and possibly Farm 14) relates to an unknown synthetic oestrogen or oestrogens (e.g. alkylphenols) derived from septic tanks known to be present in these catchments, the absence of EE2 diminishes this possibility, and it seems more likely that most upstream activity is related to farming operations. The only veterinary medicines detectable by the YES which were known to have been used on cattle were oestradiol-containing intra-vaginal devices (PRIDs – see below), but the residue from these would also have been picked up by the chemical analyses.

One is therefore forced to the tentative conclusion that much of the oestrogenic activity in apparently pristine upper catchments is produced by the cutting or other processing of plant matter – i.e. by the release of phyto-oestrogens. In the areas under study, a possible candidate may be grass silage. Although no evidence is currently available to support this hypothesis, it is known that grass silage contains high concentrations of free oestrogenic activity, particularly attributable to daidzein and biochanin A (Khodabandehlou *et al.*, 1997). The relative potencies of daidzein and biochanin A compared with E2 β in the YES are only 0.001 and 0.009, respectively (Coldham *et al.*, 1997), so if the observed activity was indeed due to these phytoestrogens, their average concentrations in the stream must have been in the $\mu\text{g/l}$

range. It is known that approximately 1000 tonnes of grass silage on Farm 11 had 'spoiled' during the period of study and it is possible that some of this material had found its way into the upper stream. This theory can only be examined through chemical analysis of further samples, and it would be worth analysing the sediment samples from Farm 11.

The conclusions about relatively low risks to fish at most of the surveyed sites should be regarded as tentative until fish from headwater streams of this type have been investigated for oestrogenic effects. Routine fish population data are not gathered by the Environment Agency for streams of this size, but they are known to provide a habitat for small species such as stickleback and minnow, and some are breeding sites for migratory salmonids. The levels of oestrogenic activity are close (within a factor of 10) to those which would indeed cause reproductive effects in some fish species, and the uncertainties involved in the survey approach could easily have led to some under-estimation of activity. For example, recoveries from the POCIS samplers were in the region of 50%, implying that true concentrations may have been double those reported. Furthermore, the winter of 2004/05 was exceptionally dry, so it is to be expected that mobilisation of steroid residues into the streams would have been lower than in wet years.

The data obtained with the YAS suggest that androgenic activity was generally absent from the streams we studied, although it appeared briefly in late November 2004 at the downstream site on Farm 3, and at a more sustained average level at the upstream site on Farm 11. Beef cattle excrete approximately 300 µg T/day/head (see review), but pregnant females presumably excrete far less. Furthermore, T has a greater potential to be leached out of soil than E1 or E2 (Das *et al.*, 2004), and removal half-lives of only 1.0-1.7 h have been measured in soil columns (Casey *et al.*, 2004). It is therefore unsurprising that androgenic activity in the monitored streams was the exception rather than the norm, and that the activity in the Farm 3 autosampler was only present while cattle were still on the land. The elevated levels of activity at the upstream site on Farm 11 are inexplicable without supporting analytical data, but there may be some link with the similarly high levels of oestrogenic activity at that site. It has been suggested (Svenson and Allard, 2004) that decaying wood is a source of androgenic activity in Swedish pulpmills, and the upstream catchment at Farm 11 did contain significant areas of forest, but this can only be investigated by targeted analytical work.

Relatively little is known about the biological effects of androgenic activity on aquatic life. However, Katsiadaki *et al.* (2002) have shown that the male glue protein spiggin can be induced in female 3-spined sticklebacks (*Gasterosteus aculeatus*) by 5 α -dihydrotestosterone, with a LOEC of 2000 ng/l after 5 weeks exposure. In the same system, the LOEC for 17 α -methyltestosterone after 3 weeks was only 100 ng/l. Later work (Hahlbeck *et al.*, 2004 a&b) showed that 17 α -methyltestosterone at 1000 ng/l produced both kidney hypertrophy and spiggin induction in juvenile sticklebacks, and interfered with sexual differentiation. There are no published data for the effects of testosterone itself on fish, but it is reasonable to suppose that it has a similar order of potency. Although the available data are very sparse, it will be apparent that the average activity seen in the stream at Farm 11 (18.3 ng T equiv./l) was well below that which would be expected to cause androgenic effects in fish. If it had been sustained, the peak level seen in the Farm 3 autosampler (4715 ng T equiv./l) would

probably have been biologically active, but such a transient exposure would be unlikely to cause any problems.

The survey for androgenic activity therefore shows that androgenic activity in the headwater streams under study was generally absent, and when present was unlikely to cause biological effects in fish, but it should be remembered that the androgen assay procedure was not calibrated. However, it is possible that higher levels of activity may be present in areas where beef cattle predominate, especially earlier in the year when animals are still on the pasture.

Conclusions

1. Field drains and headwater streams on many farms in intensive livestock-rearing areas of the UK are likely to contain oestrogens, while androgens only appear sporadically. In the present survey, 92% of the monitoring stations (at least one on each farm) revealed measurable oestrogenic activity, whereas weak androgenic activity was only detected at 2 sites.
2. The oestrogenic activity cannot be attributed solely to livestock, and some probably derives from phyto-oestrogens, and possibly from human-derived hormones in septic tank overflows or cess pits (although human sources seem unlikely due to the absence of EE2). In most cases, however, activity is mainly attributable to E1 and E2 derived from livestock.
3. The data do not allow clear discrimination between different livestock sources, but spreading of cattle-slurry and run-off from farmyards appear to be more important than direct excretion to farmland.
4. These conclusions apply mainly to cattle and sheep farms – intensive pig and chicken rearing were not sufficiently studied.
5. On 8 of the 11 surveyed farms, oestrogenic activity in the stream (or field drain in the case of Farm 7) exceeded the Predicted-No-Effect-Concentration for 17 β -oestradiol in water, and in two cases (not directly attributable to livestock) the activity was probably sufficient to cause reproductive effects in fish.
6. Safe levels of androgens in water have not been firmly established, but there is little doubt that the sporadic appearance of weak androgenic activity in this survey is of minor consequence for fish, and probably also for invertebrates.
7. There are uncertainties and margins of error in the survey process, but it cannot be concluded that the environment in UK headwater streams is safe from oestrogen pollution.
8. Further research is required to establish the true extent, major sources and ecological consequences of oestrogen contamination in headwater streams.

Recommendations

1. A second survey of hormone activity in UK headwater streams is recommended, again using a combination of modelling, fully validated POCIS sampling, YES bioassay methodology, and chemical analysis of oestrogenic molecules (including vertebrate sex hormones, phytoestrogens and hormone mimics).
2. This survey should take a stratified random approach in order to provide a more balanced picture, should be designed to include all major potential livestock sources (species, and routes to water), and should additionally include consideration of potential contamination from septic tanks, phytoestrogens and rainwater. For example, boron could be used as a sensitive tracer of contamination from septic tanks, and full use should be made of analytical techniques to identify unexplained activity (e.g. phyto-oestrogens).
3. The possibility of using more controlled studies on experimental husbandry farms should be considered as a way of obtaining definitive information about the relative importance of different hormone sources.

4. The monitoring of oestrogen activity and concentrations should be accompanied by a survey for vitellogenin (and possibly spiggin) induction in caged fish held in a proportion of the monitored streams, in order to confirm that the *in vitro* activity is indeed bioavailable under natural conditions.
5. Attempts should also be made to sample wild fish (e.g. roach, stickleback) from streams well above sewage treatment works to establish the prevalence of intersex individuals, and to find out whether fish from pristine areas with no hormonal inputs are free of this condition.
6. On the basis of this report, it is predicted that caged male fish in many headwater streams will show some vitellogenin induction, but that the ovotestis condition in wild fish will only occur sporadically, and not at all in truly pristine streams.

Contacts with Other Organisations

The main external contact has been with the Environment Agency (contact: Dr Claire Wells) who expressed an interest in the hormonally-based veterinary medicines which may have been given to livestock, and which might appear in surface waters alongside natural hormones – see Table 7 below.

Table 7. Hormonally-based veterinary products approved for use in cattle in the UK

Product	Active	Hormone type	Also indicated for pigs
Chorulon	human chorionic gonadotrophin	gonadotrophin	
Crestar	oestradiol valerate	steroid	
Dalmarelin	lecirelin (synthetic analogue of gonadotropin releasing hormone GnRH)	gonadotrophin releasing hormone	
Eazi Breed Cidr Cattle Device	progesterone	steroid	
Fertagyl	gonadorelin (synthetic GnRH)	gonadotrophin releasing hormone	
Folltropin	FSH	gonadotrophin	Yes
Fostim 6000	gonadotrophin (equine)	gonadotrophin	Yes
Ovagen	FSH	gonadotrophin	
Oxytocin S	oxytocin	anterior pituitary	Yes
Pluset	LH	gonadotrophin	
PMSG	PMSG	gonadotrophin	Yes
Prid	progesterone	steroid	
Prostavet	etiproston (synthetic analogue of PgF2 α)	prostaglandin	
Receptal	buserelin (synthetic analogue of GnRH)	gonadotrophin releasing hormone	
Reprocline	carbetocin (oxytocin analogue)	anterior pituitary	Yes
Super Ov	FSH (pig)	gonadotrophin	
Cycloprost	dinoprost (synthetic analogue of PgF2 α)	prostaglandin	
Dalmazin	cloprostenol (synthetic analogue of PgF2 α)	prostaglandin	
Enzaprost T	dinoprost (synthetic analogue of PgF2 α)	prostaglandin	Yes
Estroplan Injection	cloprostenol (synthetic analogue of PgF2 α)	prostaglandin	
Estrumate	cloprostenol (synthetic analogue of PgF2 α)	prostaglandin	
Gabrostim Injection	alfaprostol (synthetic analogue of PgF2 α)	prostaglandin	Yes
Lutalyse	dinoprost (synthetic analogue of PgF2 α)	prostaglandin	Yes
Noroprost	dinoprost (synthetic analogue of PgF2 α)	prostaglandin	
Prosolvlin	luprostiol (synthetic analogue of PgF2 α)	prostaglandin	Yes

It will be noted that only one of these medicines (Crestar - oestradiol valerate) is likely to produce a positive response in the yeast screens used to assay the sample extracts for oestrogenic or androgenic activity. We have evidence that oestradiol benzoate is also used to treat cattle as a component of Progesterone Releasing Intravaginal Devices (PRIDs) by some veterinary surgeons. **Each of these contains 10 mg of oestradiol benzoate. Only one was used on each of Farms 11 and 13 in the year prior to the survey, and none at all on Farms 1, 2, 3, 4, 6, and 14, but 89 were used on Farm 8 without any obvious effect on the level of oestrogenic activity in the Farm 8 stream (it was one of the least contaminated).** With Defra's approval, it was eventually agreed in a separate contract that the project would

provide the EA with the replicate POCIS sampling discs that had been deployed at each site in case of damage and which were now surplus to requirements. The EA agreed to analyse extracts of these discs for oestrogens and supply the data to the project (in addition to their in-house requirement for analyses of veterinary medicines). The project also agreed to supply the EA with anonymised site data, and with any information the farmers could supply on their use of hormonal veterinary medicines.

Publications

Published/in press papers to date

Johnson, A.C., Williams, R.J., and Matthiessen, P. (2005). The potential steroid hormone contribution of farm animals to United Kingdom freshwaters. In press *Science of the Total Environment*. – see Annex 1

Conference presentations and posters

Matthiessen, P., Johnson, A., Pepper, T. and Pottinger, T. (2005). Endocrine disrupting activity in streams draining intensive livestock farms in the United Kingdom – a pilot study. Oral paper presented to the SETAC-Europe meeting, Lille, May 22-26 2005.

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Annex 1. Pre-publication literature review.

Title: The potential steroid hormone contribution of farm animals to freshwaters: the United Kingdom as a case study.

Authors: A.C. Johnson, R.J. Williams, and P. Matthiessen.

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