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Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms

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Abstract

Most studies of hormonal activity in rivers have focused on inputs from sewage treatment works (STW), and their consequences for endocrine disruption in fish. It is possible that livestock is contributing to this hormonal activity in rivers. This study represents a search for evidence of steroid hormone contamination in streams associated with livestock farms. The majority of the 10 sites selected were streams running through dairy farms, although some examples of beef, sheep and pigs were included. Passive water samplers (Polar Organic Chemical Integrative Samplers – POCIS) were deployed up- (control) and down-stream of the farms for 3 to 10 weeks (mean = 39 days) during the period November 2004 to January 2005. At one site, water samples were also taken automatically during rainfall events. All samples were solvent-extracted. Total oestrogenic activity in concentrates of the extracts was analysed using the Yeast Estrogen Screen (YES) calibrated against 17β-oestradiol (E2), while oestrone (E1), E2 and 17α-ethinylestradiol (EE2) were analysed by liquid chromatography–mass spectrometry (LC–MS/MS). Stream water from the entirety of only one rainfall event was sampled directly, but this revealed background activity (E2 equivalents) of 0–0.3 ng/l, rising to a transient peak of 9.4 ng/l. Average oestrogenic activity at this site as estimated from the POCIS samplers was 1.8–2.7 ng E2 equiv./l. Estimated average oestrogenic activity across all sites (with one exception) lay in the range 0–26.5 ng E2 equiv./l (mean = 2.0 ng/l; S.D. = 5.1), based on the POCIS samples. The outlier was 292 ng/l, and this could not be specifically linked with livestock rearing. 92% of monitoring stations (at least one on each farm) contained some oestrogenic activity, and activity was higher at downstream sites in 50% of cases. Although no EE2 was detected analytically in any stream, E1 and E2 were almost ubiquitous, with E2 equivalents ranging from 0.04 to 3.6 ng/l across all sites. Furthermore, steroid concentrations downstream of livestock were higher than upstream in 60% of cases, more markedly so than for the YES data. In several cases, activity upstream was greater than downstream, and this tended to be associated with higher activity than could be accounted for by the hormone analyses. Both the YES and chemical analytical data suggest that fish in headwater streams on or near some livestock farms may be at risk of endocrine disruption.

Keywords: Oestrogens; Farm animal excretion; Manure; Slurry; Headwater streams; Endocrine disruption

1. Introduction

Since the late 1980s, much research has been conducted into the phenomenon of endocrine disruption

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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 17α-ethinylestradiol (EE2) and 17β-oestradiol (E2), as well as other oestrogens, and oestrogen mimics such as nonylphenol. These substances are all able to activate the intracellular oestrogen receptor in developing and adult fish. Exposure to environmental oestrogens has been linked to 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 (Mills and Chichester, 2005), laboratory studies with feral intersex roach *Rutilus rutilus* indicate that they are experiencing impaired reproduction (e.g. Jobling et al., 2002).

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 occurs downstream of sewage treatment works (STW) discharges. Based on this evidence, the England and Wales Environment Agency is proposing to take 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 oestrogenic changes, and this has led to the suggestion that other sources of oestrogens may be contributing to a proportion of the observed impacts. Possibly the additional oestrogenic contamination originates from that excreted by livestock (e.g. Raman et al., 2004), but so far there has been little evidence to substantiate this hypothesis (Hanselman et al., 2003). Nevertheless, it is certainly possible that oestrogens excreted directly by livestock or applied in slurry could persist long enough in soils to provide a source of contamination for surface waters (Colucci and Topp, 2001, 2002; Colucci et al., 2001; Jacobsen et al., 2005).

A few studies of waters near intensive livestock-rearing areas have been conducted, and these have recently been reviewed by Johnson et al. (in press). They suggest that oestrogenic activity in surface waters near intensive livestock farms may be high enough in some places to cause endocrine disruption in some aquatic organisms (e.g. Soto et al., 2004; Orlando et al., 2004). 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 of 0.9–2.9 ng E2 equiv./l. 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, Ireland and Denmark that intensive livestock rearing is linked to natural oestrogens in surface waters that are in the biologically active concentration range. However, batch, microcosm, column and modelling studies (Colucci and Topp, 2002; Das et al., 2004; Johnson et al., in press) have indicated that steroid hormones are likely to be strongly sorbed and rapidly degraded in soil, leaving little to escape into drain water. This preliminary study therefore attempted to find evidence on the ground for oestrogenic contamination of streams associated with farms containing livestock representative of Northern Europe.

2. Materials and methods

2.1. Site selection

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 (which Johnson et al., in press, had identified as likely to produce the highest amounts of steroidal effluent). In other words, this was not a random survey of streams in general. The main factor used to choose 10 sites for study was a high predicted steroid load (Table 1), based on criteria including high livestock stocking density, soil type favourable to translocation of substances, steep land slope, access of animals to the stream, manure or slurry-spreading on the farm, and potential for direct runoff of contaminated water from the farmyard area to the

Table 1
Summary of conditions on the surveyed farms, and predicted oestrogen load

Farm number	England and Wales region	Livestock	Livestock access to stream?	Slurry/ manure spreading? [a]	Potential for farmyard runoff? [b]	November–January 2004/2005 rainfall for the region (as a % of the 1961–1990 average, and total)	Stream size (width × approx. depth) m	Estimated oestrogen load (E2 equiv.)
1	Southeast England	120 sows (in sheds)	No	+	+++	49–74% 96 mm	0.6 m wide 0.1 m deep	Small – 6 μg/l expected in slurry
2	Southwest England	200 dairy cattle (some on fields, some in sheds); 200 lambs (on fields)	Yes	+++	+	47–80% 261 mm	1.5 m wide 0.08 m deep	2.8 mg/day from livestock; 75 mg/ha from slurry
3	Southwest England	70 dairy cattle (on fields for first 2 weeks); 140 ewes (on fields)	Yes	++	+++	47–80% 261 mm	1.1 m wide 0.06 m deep	73.7 mg/day from livestock; 67 mg/ha from slurry
4	Northwest England	250 dairy cattle (in sheds); 300 ewes (on fields)	Yes	+++	+	61–115% 328 mm	0.6 m wide 0.1 m deep	1.6 mg/day from livestock; 84 mg/ha from slurry
6	Northeast England	27 pregnant beef cattle (in sheds)	Yes	++	+	37–117% 181 mm	1 m wide 0.15 m deep	15 mg/day from livestock; 27 mg/day from slurry plus up to 314 mg/ha from farmyard manure
7	Central Southern England	None (dairy cow slurry applied experimentally)	No	+++	−	45–66% 119 mm	Field drain issuing from 0.17 ha experimental plot	120 mg/ha from slurry
8	Northwest England	450 dairy cattle (in sheds); 300 ewes (on fields)	Yes	−	−	61–115% 328 mm	0.54 m wide 0.08 m deep	1.6 mg/day from livestock
9	Northwest England	110 dairy cattle (in sheds); 170 ewes (on fields)	Yes	+	−	61–115% 328 mm	0.88 m wide 0.12 m deep	0.9 mg/day from livestock; (plus 31.4 mg/tonne from farmyard manure)
11	Northwest England	170 dairy cattle (in sheds)	No	+ (just before sampling began)	+	61–115% 328 mm	1.64 m wide 0.10 m deep	1.3 mg/day from livestock; 150 mg/ha from slurry
13	Northwest England	65 dairy cattle (in sheds); Ewes (on fields – number unknown)	Yes	++ (just before sampling began)	+	61–115% 328 mm	1.72 m wide 0.43 m deep	1.6 mg/day from livestock; 120 mg/ha from slurry
14	South-east Wales	22 beef steers (on fields)	Yes	−	−	65–90% 344 mm	2.32 m wide 0.06 m deep	1.1 mg/day from livestock

[a] Slurry/manure spreading: −: none; +: <25% of farm area or washings only; ++: 25–50% of farm area; +++: >50% of farm area.
[b] Farmyard runoff potential: −: none; +: low potential or none observed; +++: high potential or runoff observed.

stream. Farms were generally chosen where upstream control areas had as little farming and human activity as possible for comparison with potentially contaminated downstream areas,. It was impractical to satisfy all criteria at all locations, and an over-riding imperative was to find farms where the landowner was prepared to allow access for scientists 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 alone which was applied to an experimental plot from which field drainage could be collected.

Details of the farms are given in Table 1, although the anonymity of farmers has been preserved by not publishing names and precise locations. A good geographical spread was obtained, from the Scottish border region of northern England to the southwest of England, with a focus on the areas of most intense dairying. All the farms were traversed by small streams or permanently 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 impervious concrete in the farmyard. In eight cases, livestock had had free access to at least some stretches of stream, but on seven farms this access ceased after they had subsequently been withdrawn from the fields into sheds during the 2–5 weeks prior to the start of sampling.

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). For the whole 3-month period, the cumulative total rainfall for the regions in question ranged from 96 to 344 mm. This implies that runoff of hormones during the main study period may have been less than one might expect in an average year. Sampling points were generally situated up- and down-stream from the farms, with distances between these sites ranging between 100 and 1300 m. There were few known sources of oestrogenic contamination above the upstream sampling sites, with the exception of occasional septic tank soakaways associated with isolated houses. However, in the case of Farms 13 and 14, there were several upstream farms and small villages with septic tanks. In no case were there any sewage treatment works discharges upstream.

Table 1 shows the loads of oestrogens predicted to emanate from each farm, based on the faecal and urinary excretion rates given by Johnson et al. (in press). However, caution must be exercised when extrapolating to possible concentrations that might occur in streams. The MACRO modelling exercise run by Johnson et al. (in press) suggests that events such as direct excretion to streams by wading livestock may be more important than seepage through soil, during which there will be strong sorption and biodegradation.

2.2. 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). Furthermore, recent work has shown that it is a more efficient means of sampling dissolved organics in streams than spot water samples (Alvarez et al., 2005). Briefly, the POCIS consists of solvent-washed solid-phase adsorption medium (trade name 'Oasis') which is able to sequester hydrophilic molecules including steroids. The absorption 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.

Alvarez et al. (2004) have shown that over periods of a few weeks, POCIS discs essentially act like an infinite sink for polar molecules at environmental concentrations. 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 downstream, and in most cases also upstream (as a control), of areas where inputs from livestock were expected. In a few cases, it was impossible to find an upstream location. 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 were deployed in a perforated stainless steel cylinder which was staked to the stream bed with its long axis parallel to the current. In all cases, the discs remained submerged for a deployment period of 3 to 10 weeks (mean=39 days), and all discs were recovered intact. The variation in deployment times was due to occasional logistical problems. In a few cases, POCIS were used in two sequential deployments (a and b). Recovered discs were wrapped in methanol-washed aluminium foil,

labelled with location and date, and stored at −20 °C to await extraction.

At one location (Farm 3), an automatic water sampler (ISCO 3700 – Isco Inc.; 12×950-ml glass sample bottles) 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 not recorded due to a software problem. However, a full set of samples (12 h) was obtained from a somewhat larger event on 22/1/05.

Daily water flow data were recorded at Farms 3 and 7. On Farm 3, estimated flow peaked at 256 l/s on 22/12/04 during the first (incomplete) autosampler run, after a 15-mm rainfall event (which came after a week of steady rain), and at 76 l/s during the second autosampler run on 22/1/05 after 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 of 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.

2.3. Sample extraction for bioassay

2.3.1. 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 mbar partial vacuum for 30 min in order to dry the adsorbent. During this period, glass extraction columns were set up in a fume cupboard and rinsed with 10 ml methanol. After removal from the oven, the disc array was disassembled, the membranes were 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 N_2 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. Blank discs were processed in the same way to check that the POCIS were uncontaminated with oestrogens.

2.3.2. Water samples from autosampler

Water samples collected from the in situ auto-sampler device were received at CEH Lancaster within 2 days of collection and stored for no more than 8 weeks at 4 °C in the dark. At time of retrieval from the sampler, 100 ml of analytical grade dichloromethane had been added to each 900 ml sample of water, and mixed in a clean glass bottle. 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-l 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 the extract was reduced in volume by rotary evaporation, then dried under N_2 and redissolved in 0.5 ml absolute ethanol, exactly as described for the POCIS extracts.

2.4. 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 oestradiol likely to be achieved from the POCIS discs and also to provide data from which an estimation of the clearance efficiency of the discs could be made. This information was necessary to allow any oestrogenicity 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.

Five new glass 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 [1,2,6,7-^3H]17β-oestradiol (^3H-E2; 2.77 TBq/mmol; Amersham International; approx. 10^6 dpm), 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 ^{3}H-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 during a 158-h incubation period, 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 of E2, and held at 10 °C. Due to anomalous results from the control beaker, an additional two control beakers were set up, each containing ^{3}H-E2, one with and one without a disc holder, with continuous stirring at approximately 20 °C. Samples (1.0 ml aliquots) were collected and counted at intervals over a 96-h period.

2.5. Yeast Estrogen Screen (YES) assays

The assays were performed on POCIS and water extracts by means of a recombinant reporter gene assay known as the Yeast Estrogen Screen (YES). This cell line contains the human oestrogen receptor gene linked to a reporter gene coding for β-galactosidase. The production of this enzyme is indicative of oestrogen 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 have been described by Routledge and Sumpter (1996). In this project, the YES assay was calibrated against 17β-oestradiol (E2). The limit of detection in the assay wells lay between 2.1 and 4.26 pg E2, which translates to approximately 50–100 pg/l in the original stream water.

2.6. Oestrogen analysis

The second POCIS discs from each site were extracted and analysed for oestrogens (oestrone – E1, E2 and EE2) by the England and Wales 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 (Caliper Life Sciences), 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 analysed for oestrogens and the other was retained for other purposes.

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 (Isolute NH2, 500 mg/6 ml, Argonaut Technologies Inc).

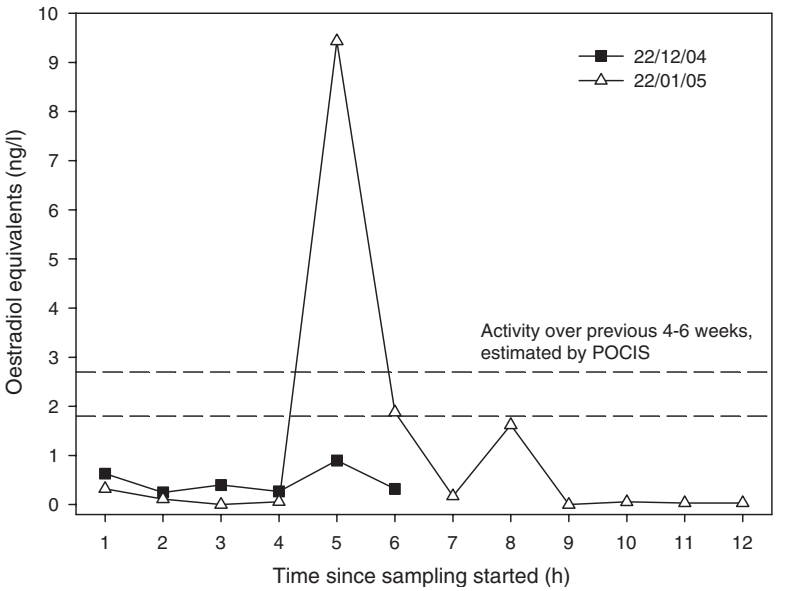

Fig. 1. Oestrogenic activity measured by Yeast Estrogen Screen (YES) in stream-water from the downstream site at Farm 3, plotted for two rainfall events captured by autosampler. The horizontal dashed lines represent the average oestrogenic activity for the 4–6 weeks preceding each autosample run, as estimated from the POCIS samplers (2.7 ng EEQ/l for month to 22/12/04; 1.8 ng EEQ/l for month to 22/1/05).

The resultant extract was taken to dryness and a buffer solution immediately added, followed by derivitisation with a dansyl chloride solution (1 mg/ml). This mixture was heated briefly to aid the reaction, cooled, and transferred to a vial for analysis. Analysis was carried out using liquid chromatography/mass spectrometry (LC–MS/MS) (API3000; Applied Biosystems) with a photoionisation interface. Quantification of the E1, E2, and 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.

3. Results

3.1. POCIS 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 to 1.0 mg/l E2. Uptake approximated a linear profile with some deviation during the first phase of uptake. This deviation may have been due to the adsorption of E2 by the glass surfaces of the beaker. However, no systematic decrease of activity was observed in the control beakers. At 20 °C, between 14 h and 86 h during which period uptake was linear, 39% of E2 in solution was absorbed. This represents complete clearance of 390 ml of solution over a period of 72 h which equates to 0.129 l/day. At 10 °C, uptake was slightly slower – between 18 h and 112 h, 35.4% of the total was lost from solution. This represents a clearance of 354 ml of solution over a period of 94 h which equates to 0.09 l/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 l/day), and provide confidence that the POCIS discs can be used to provide a semi-quantitative estimate of the average oestrogen concentrations in the streams during the period of deployment. Overall recoveries of E2 from the discs, derived from the measured radioactivity in the

Table 2
Estimated average oestrogenic activity in stream-water at all sites sampled with POCIS

Farm number	Duration of POCIS deployment (days)	Date of POCIS collection	Upstream (U)/ downstream (D) of farm	Oestradiol equivalents in 500 µl of extract (ng)	Oestradiol equivalents in 500 µl of extract normalised to 30 days of exposure (ng)	Estimated average oestradiol equivalents in stream water (ng/l)
1	33	24/12/04	U	4.31	3.92	1.4
	33	24/12/04	D	9.66	8.78	3.2
2a	31	11/12/04	U	3.01	2.91	1.1
	31	11/12/04	D	1.45	1.40	0.5
2b	45	25/1/05	U	2.12	1.41	0.5
	45	25/1/05	D	1.79	1.19	0.4
3a	31	11/12/04	U	1.09	1.05	0.4
	31	11/12/04	D	7.41	7.18	2.7
3b	45	25/1/05	U	0.69	0.46	0.2
	45	25/1/05	D	7.25	4.83	1.8
4	32	17/12/04	D	9.62	9.02	3.3
6	39	21/12/04	D	0.21	0.16	0.06
7	29	14/1/05	Field-drain	3.12	3.22	1.2
	29	14/1/05	Field-drain	4.32	4.46	1.6
8	42	21/12/04	U	ND	ND	ND
	42	21/12/04	D	1.44	1.03	0.4
9	43	22/12/04	U	0.48	0.34	0.1
	43	22/12/05	D	0.15	0.10	0.04
11	43	22/12/05	U	1130.03	788.39	292.0
	43	22/12/04	D	ND	ND	ND
13	73	25/1/05	U	174.34	71.65	26.5
14a	40	22/12/04	D	1.42	1.07	0.4
	42	6/1/05	U	0.29	0.21	0.08
14b	42	6/1/05	D	8.45	6.04	2.2
	18	24/1/05	D	2.67	4.44	1.6

a and b designate two sequential deployments of POCIS samplers on the respective farms.
ND – not detectable/no discernible signal on the assay plate with the volume of extract employed.

reconstituted extract of the disc adsorbent, ranged between 33% and 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).

3.2. Oestrogenic activity

A summary of the Farm 3 autosampler results for oestrogenic activity in stream water is shown in Fig. 1, and a summary of the POCIS data for estimated average oestrogen activity at all sites is shown in Table 2. The data presented have not been adjusted for recovery, which was approximately 50%. Plots of some typical extract concentration–response curves are shown in Fig. 2, in comparison with one of the oestradiol calibration curves in Fig. 3. 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 (=fewer cells) measured in these wells (data not shown).

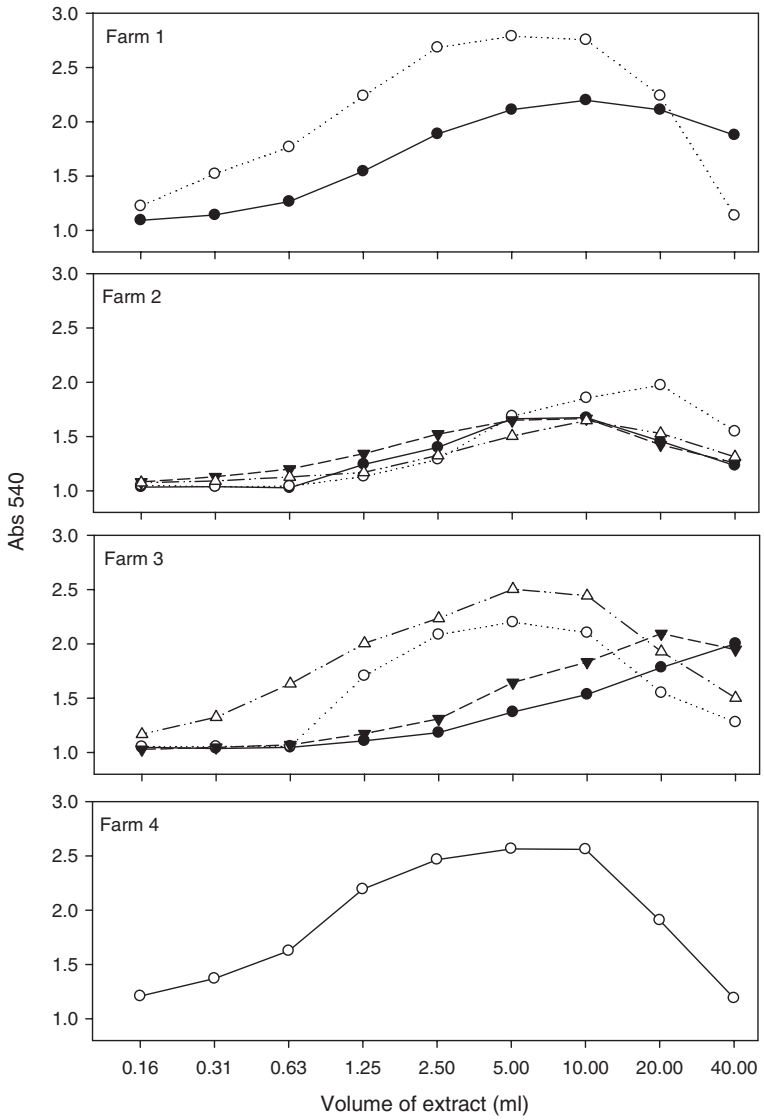

Fig. 2. A selection of dose–response curves (Farms 1–4) for the Yeast Estrogen Screen exposed to a range of dilutions of POCIS extracts. The open symbols represent downstream sites and the solid symbols upstream sites. For Farms 2 and 3, the December sample is represented by triangles, and the January sample by circles.

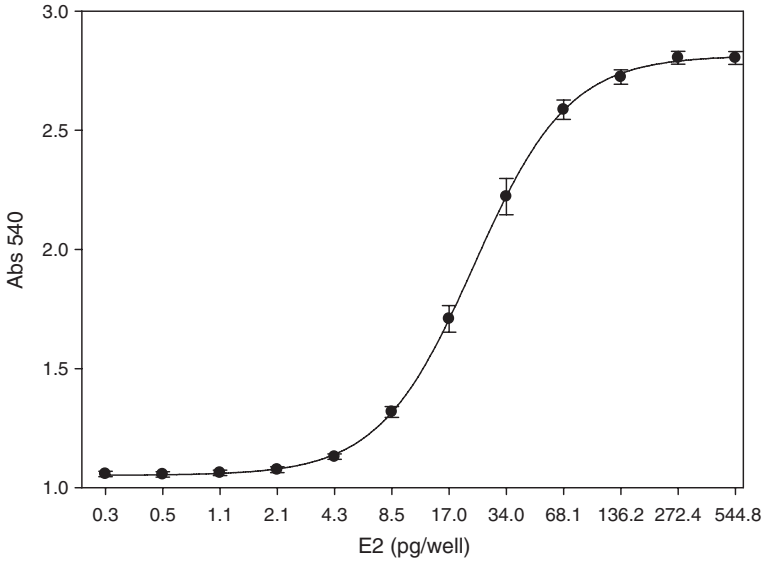

Fig. 3. Oestradiol calibration curve for the Yeast Estrogen Screen (YES), measured after 72 h incubation. Each point represents the mean of 5 plates. Error bars represent the standard error of the mean.

Because the POCIS discs were deployed for varying periods (due to logistical difficulties), the data in Table 2 have been normalised to a 30-day uptake period, assuming on the basis of the calibration experiments 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 stream water were then calculated using the laboratory measured clearance rate at 10 °C of 0.09 l of stream water per day.

It is apparent from Fig. 1 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 (on the basis of other studies with water-soluble herbicides). However, the 22 January data reveal such a peak, and show that it exceeded 9 ng E2 equiv./l (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 1 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 as a means of calibration. The calculated average activity (Table 2) for the month preceding 11 December (sample 3a) was 2.7 ng EEQ/l, while the average value for the succeeding period to 25 January (sample 3b) 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 2), oestrogenic activity was detectable at most sites, and all but one of the E2-equivalent concentrations lay between 0 and 26.5 ng/l (mean = 2.0 ng/l; S.D. = 5.1), with one outlier of 292 ng EEQ/l. 12 of the 25 activity measurements exceeded the proposed predicted-no-effect-concentration (PNEC) for E2 of 1 ng/l (Young et al., 2002). On 4 of the 8 farms 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 stream water activity increased by a factor of 2–27.

However, in the remaining 4 cases, there was a loss of activity as the stream flowed through the farm, although it should be noted that the upstream sampler on Farm 13 was left in situ for much longer than the downstream one (due to access problems). In the other three cases where activity upstream was higher than downstream (Farms 2, 9 and 11), there was negligible past or present deployment of livestock above the upstream sampler, and hardly any (≤ 3) septic tank overflows in the upper catchment. Furthermore, it is clear that only one of the upstream sites (Farm 8) was completely free of oestrogenic activity. This suggests that livestock (and to a much lesser extent, human)

excretion may only have been contributing a proportion of the observed activity. However, the slurry application experiment (Farm 7) revealed oestrogenic activity (1.2–1.6 ng EEQ/l) in the field drain, and this can be firmly attributed to dairy slurry alone.

Overall, there was no significant difference between the means of the upstream (32.2 ng EEQ/l) and downstream (1.3 ng EEQ/l) oestrogen activities (Kruskal–Wallis test, $p=0.78$). Although there only appears to be a weak relationship between the predicted oestrogen load from livestock (mg/day – direct excretion to farmland, plus slurry; Table 1) and measured oestrogenic activity downstream, this is probably misleading. It is likely to reflect the many unique, site-specific factors which override such considerations as herd size and field slope. These local factors might include livestock excreting directly into streams, or farmyard runoff.

3.3. Oestrogenic hormone concentrations (E1, E2, EE2)

The analytical data are shown in Table 3, and the E2-equivalent values are plotted by site in Fig. 4. They have been related back to average hormone concentrations in the streams in the same way as for the YES data. Overall, the proposed E2-equivalent PNEC was exceeded during 7 of the 23 hormone measurement periods.

EE2 was absent from all samples, which lends some weight to the assertion that human sewage may not have been playing a major role at these sites. E1 was generally found at higher concentrations than E2, which is to be expected given that dairy cattle excrete over twice as much E1 as E2 (see Johnson et al., in press), and that E2 degrades to the more stable E1 in water. Overall, concentrations of E2-equivalents ranged from 0.04 to 3.62 ng/l, which appears to agree well with the levels of activity detected 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, which again suggests that, at least on some farms, livestock is contributing to oestrogenic activity in streams. Downstream normalised concentrations of E1/E2 were higher than upstream concentrations in 6 out of the 10 cases where upstream–downstream comparisons were possible. The remaining farms

Table 3
Estimated average steroid oestrogen concentrations in stream water sampled with POCIS discs

Farm number	Date of POCIS collection	Upstream (U)/downstream (D) of farm	E1 (ng/l)	E2 (ng/l)	EE2 (ng/l)	Calculated E2 equivalent (ng/l)*
1	24/12/04	U	0.13	0.00	0	0.04
	24/12/04	D	3.02	0.34	0	1.34
2a	11/12/04	U	0.23	0.00	0	0.08
	11/12/04	D	2.62	0.34	0	1.21
2b	25/1/05	U	0.15	0.00	0	0.05
	25/1/05	D	1.46	0.20	0	0.69
3a	11/12/04	U	0.21	0.00	0	0.07
	11/12/04	D	4.67	0.53	0	2.09
3b	25/1/05	U	0.11	0.00	0	0.04
	25/1/05	D	4.83	0.56	0	2.17
4	17/12/04	D	9.31	0.52	0	3.62
6	21/12/04	D	0.10	0.00	0	0.03
7	14/1/05	Field-drain	No data	No data	No data	No data
	14/1/05	Field-drain	No data	No data	No data	No data
8	21/12/04	U	0.61	0.00	0	0.20
	21/12/04	D	0.19	0.00	0	0.06
9	22/12/04	U	1.27	0.11	0	0.53
	22/12/05	D	0.88	0.00	0	0.29
11	22/12/05	U	4.11	0.89	0	2.26
	22/12/04	D	2.59	0.23	0	1.10
13	25/1/05	U	0.59	0.08	0	0.28
	22/12/04	D	0.40	0.09	0	0.22
14a	6/1/05	U	0.28	0.00	0	0.09
	6/1/05	D	0.45	0.00	0	0.15
14b	24/1/05	D	0.31	0.00	0	0.10

The right-hand column shows the calculated E2-equivalent values.
a and b designate two sequential deployments of POCIS samplers on the respective farms.
* Assuming that E1 * 0.333 = E2 equivalent; Thorpe et al., 2003.

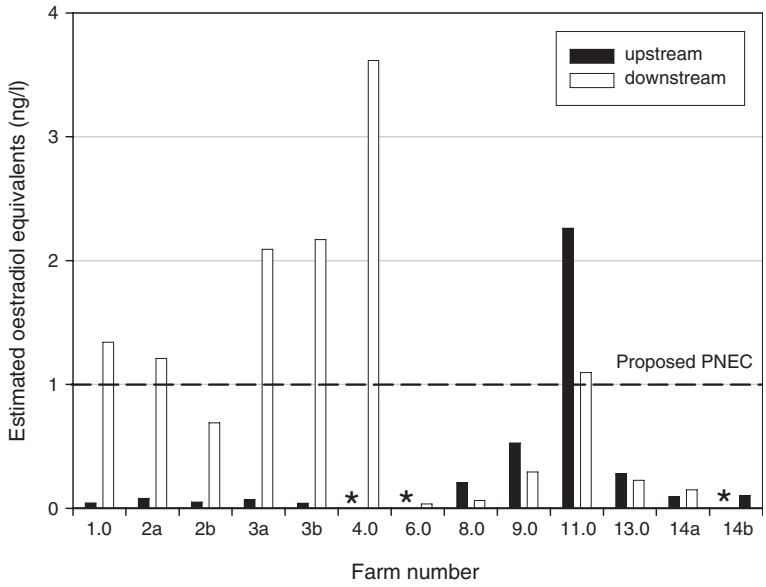

Fig. 4. Chemical analytical data for E1 and E2 from each POCIS sampler, expressed in E2-equivalents (EEQ/l). The proposed PNEC for oestradiol is shown as a horizontal dashed line. Asterisks indicate no data.

appeared to contribute little E1 or E2 to the streams, but on average, the downstream E2-equivalent concentration was a factor of 16 times higher than upstream (range of 0.5–61). Overall, however, the difference between the E2-equivalent sex steroid concentrations at the upstream (mean = 0.36 ng/l) and downstream (mean = 1.01 ng/l) sites was not statistically significant (Kruskal–Wallis test, $p = 0.072$).

A regression analysis of the two measures of activity (i.e. normalised E1/E2 and YES) at individual sites gave a best-fit line with a slope that was not significantly different from 0 ($p = 0.1$) 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

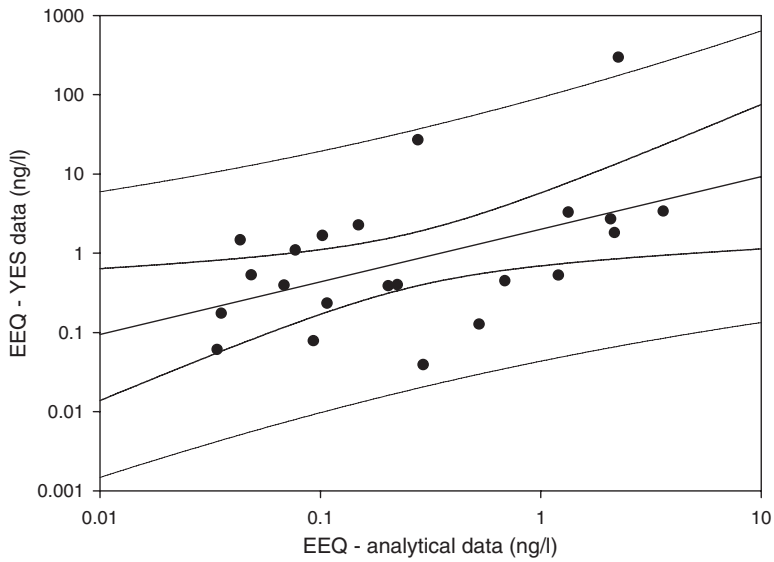

Fig. 5. Comparison of E2-equivalent (EEQ) YES data and EEQ steroid analytical data, both derived from POCIS samples. The linear regression line is shown ($r^2 = 0.242$, $P = 0.02$). The inner curves represent the 95% confidence intervals of the regression line, and the outer curves represent the 95% prediction intervals for new data points.

significant deviation from 0 in the gradient of the slope was also evident ($p=0.02$) (Fig. 5). 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 vertebrate 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.

4. Discussion

4.1. Representativeness of the data

The results of these studies suggest that oestrogenic contamination of headwater streams associated with livestock farms is widespread in England and Wales, although it must be emphasised that we focused on 'worst-case' examples. Origins of this activity are not solely attributable to livestock, however, and it is likely that non animal sources (possibly silage – see below) are 'topping up' the livestock signal, although we have no direct evidence for this at present. Septic tank overflows and cess pits could be contributing activity at some sites, but the absence of EE2 does not support this view (although only about one-fifth of households would be a source of this substance – Johnson and Williams, 2004). 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 0 to about 10 ng E2 equiv./l.

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. Perhaps slurry application and farmyard runoff were the main sources as suggested in the review conducted by Johnson et al. (in press).

4.2. Routes of contamination

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 some of the measured contamination reaches streams via seepage and, in some cases, 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). Soil seepage from slurry application must also have led to the oestrogenic activity observed in the field drain on Farm 7, but more data are needed on this point.

4.3. Sources of contamination

Considering the analytical data on oestrogenic hormones sampled by the duplicate POCIS disks, it is clear that most oestrogenic activity could be attributed to natural E1 and E2 derived from livestock. Enquiries with the farmers revealed minor use of only one veterinary medicine (Progesterone-Releasing Intravaginal Devices, or PRIDs, containing inter alia 10 mg oestradiol) that might have given rise to additional oestrogenic activity, but this would have been detected by the chemical analyses. 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 oestrogen mimic or mimics (e.g. alkylphenols) derived from the few septic tanks known to be present in these catchments, the absence of EE2 diminishes (but does not completely eliminate) this possibility, and it seems more likely that most upstream activity is related to farming operations or natural processes. It is unlikely that the upstream activity was due to oestrogen-mimicking pesticides because the catchments were essentially grass- and wood-land with very low use of plant protection products of any type.

Equally, the concentrations of atmospherically deposited anthropogenic organochlorines and polycyclic aromatic hydrocarbons, which are associated with oestrogenic activity in the sediments of some upland lakes (Garcia-Reyero et al., 2005), would be extremely low in stream waters due to their low solubility.

It is therefore tentatively concluded that much of the oestrogenic activity in relatively pristine upper catchments may be produced by the cutting or other processing of plant matter – i.e. by the release of phytoestrogens. The oestrogenic activity of a river in Japan has already been largely attributed to these substances, particularly genistein (Kawanishi et al., 2004), but the source was unclear. In the areas described in the present study, a possible candidate source 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 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 streams must have been in the μg/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 been disposed of on site and found its way into the upper stream.

4.4. Exposure of, and possible effects on, stream organisms

Data from the present study suggest that stream organisms on many livestock farms are being chronically exposed to time-averaged concentrations 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, although these transient spikes may have little biological relevance. Recent unpublished data (Maunder et al., submitted for publication) show that 3-spined sticklebacks *Gasterosteus aculeatus* can bioaccumulate E2 in the blood by up to 50-fold within 6 h of exposure via the ambient water, but 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. Based on a thorough literature review, Young et al. (2002) proposed a tentative long-term Predicted-No-Effect-Concentration (PNEC) for freshwater life of 1.0 ng/l for E2. A critical study was that of Metcalfe et al. (2001) who exposed Japanese medaka fish *Oryzias latipes* 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. It should be noted that data for E2 based on a fish full life cycle test were not available to Young et al. (2002), but a subsequent life-cycle test (Seki et al., 2005), also using medaka, has given a LOEC of 8.66 ng E2/l and a NOEC of 2.86 ng E2/l for abnormal sexual differentiation and reproductive impairment. The proposed PNEC of 1 ng/l may therefore be sufficiently protective for fish populations.

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 (which only captures molecules from solution), it is likely that the estimated levels of oestrogenic activity and steroid oestrogen residues were indeed maximally bioavailable to fish and other aquatic organisms, and that additional though less bioavailable residues may have been associated with suspended and bed sediments. 48% of the POCIS YES measurements and 30% of the POCIS E1/E2 measurements were above 1 ng EEQ/l, representing 73% and 50% of surveyed farms, respectively. If the PNEC were to become an Environmental Quality Standard (EQS), several farms would therefore 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 major threat to fish reproduction, and in neither of these cases did the activity appear to be directly related to livestock rearing.

4.5. Need for field studies of fish in headwater streams

This conclusion about the existence of probable risks to fish populations at relatively few 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 England and Wales Environment

Agency from streams of this size, but they are known to provide a habitat for small species such as three-spined stickleback (*G. aculeatus*) and minnow (*Phoxinus phoxinus*), and some are breeding and nursery sites for migratory salmonids (e.g. *Salmo salar*). The levels of oestrogenic activity are close to (within a factor of 10), or in two cases exceed, 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. Finally, if more cattle had been in the fields during the survey, it is possible that this would have caused more hormone translocation to streams, although this was not apparent on the one farm where animals were still present.

5. Conclusions

1. Field drains and headwater streams on many farms in intensive livestock rearing areas of the UK are likely to contain oestrogens. In the present survey, 92% of the monitoring stations (at least one on each farm) revealed measurable oestrogenic activity.
2. In most cases, activity appears to be mainly attributable to E1 and E2 derived from livestock. However, oestrogenic activity cannot be attributed solely to this source, and some possibly derives from phytoestrogens, and from human derived hormones in septic tank overflows or cess pits.
3. The data do not allow clear discrimination between different livestock sources, but spreading of cattle-slurry and run-off from farmyards may be more important than direct excretion to farmland or streams.
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 at least once the Predicted-No-Effect-Concentration for 17β-oestradiol in water, and in two cases (not directly attributable to livestock) the activity was probably sufficient to cause reproductive damage in fish.
6. 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.
7. Further research is required to establish the true extent, major sources and ecological consequences of this oestrogen contamination.

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