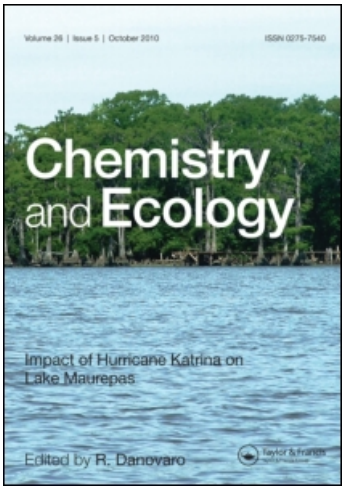

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ESTROGENIC EFFECTS OF EFFLUENTS FROM SEWAGE TREATMENT WORKS

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The occurrence of hermaphrodite fish in the lagoons of sewage treatment works led us to hypothesize that sewage effluent might contain a substance, or substances, estrogenic to fish. To test this hypothesis, we placed cages containing rainbow trout in the effluent from sewage-treatment works, and one to three weeks later measured the vitellogenin concentration in the plasma of the fish. Vitellogenin is a protein synthesized by the liver of oviparous fish in response to estradiol stimulation; it is then conveyed by the blood to the ovary, where it is sequestered by oocytes to form the yolk. Thus, the presence of vitellogenin in the plasma is indicative of estrogenic stimulation of the liver. An initial study, at a sewage-treatment works, showed that plasma vitellogenin concentrations rose rapidly and very markedly (over 1000-fold in three weeks) when trout were maintained in the effluent. An extensive nationwide survey was then conducted. Results were obtained from fifteen sewage-treatment works distributed throughout England. In all cases, exposure of trout to effluent resulted in a very pronounced increase (500 to 100,000-fold, depending on site) in the plasma vitellogenin concentration. Induction of vitellogenesis was also observed in carp, but to a much lesser extent than in trout.

The identity of the estrogenic substance is unknown. It is suggested that the two most likely possibilities are ethynylestradiol, originating from pharmaceutical use, or alkylphenol-ethoxylates (APE), originating from the biodegradation of surfactants and detergents during sewage treatment.

Laboratory studies on the potency of ethynylestradiol demonstrated that levels as low as 1 to 10 ng l^{-1} could generate the response shown by the caged fish and that positive responses may arise at 0.1 to 0.5 ng l^{-1}. Further work is in progress on the potency of APE.

KEY WORDS: sewage effluent, vitellogenesis, estrogenesis, ethynylestradiol, alkylphenol-ethoxylates

INTRODUCTION

Some of the more significant cases of environmental pollution have arisen from the purposeful use of biologically active materials. The classical example is the mid-century impact of organochlorine pesticides such as DDT; a more recent one is the effect on oysters and other molluscs of tributyltin, as used in ship paints to prevent settlement of sessile organisms (Waldock, 1986). An understanding of the biological consequences of such contamination has an *a priori* element which is not always so obvious for pollution which arises from disposal as opposed to use. For example, the adverse biological effects of PCBs and some heavy metals are of concern to us but their modes of action remain conjectural.

Following the widespread adoption of hormonal contraception procedures, general concern has been expressed in the popular media about the potential effects of constituents of contraceptive pills entering waste waters and rivers. Scientific concern has also been expressed, particularly on the possible consequences of contamination by estrogens (Richardson and Bowron, 1985; Aherne *et al.*, 1985).

The present study of estrogenic effects on fish of effluent water from sewage treatment works (STW) began as a consequence of the casual observation by anglers of hermaphrodite fish in STW lagoons and its subsequent confirmation by a follow-up survey. An attempt to assay the effluent by *in vivo* and *in vitro* mammalian methods was inconclusive and virtually uninterpretable, but a more direct and specific fish assay was available. This was based on the measurement of plasma concentrations of vitellogenin (Sumpter, 1985), the yolk precursor which is found in all egg laying animals. Vitellogenin is produced naturally by the liver in females under the control of estrogens secreted by the ovary but it can also be synthesized in both females and males following exposure of fish to exogenous estrogens (Clemens, 1978).

A vitellogenin radioimmunoassay (RIA) was developed for the rainbow trout (*Oncorhynchus mykiss*) and the overall plan for the assessment of STW effluents was to place trout in cages in or near to effluent discharges. To test the hypothesis that the constituents of the contraceptive pill contributed to the estrogenic effect of STW effluents, their potency was assessed under laboratory conditions. In addition, since many of the rivers receiving STW effluents support coarse fish rather than trout, an RIA for carp (*Cyprinus carpio*) vitellogenin was developed (Tyler and Sumpter, 1990) and used in preliminary field and laboratory tests.

MATERIALS AND METHODS

Rainbow trout were purchased from several sources. Ideally they were all-male stock produced by masculinization (Purdom, 1984). However, when it was necessary to buy ostensibly mixed-sex stock there were occasions when all the fish were females due to the adoption of all-female techniques (Bye and Lincoln, 1986) by many UK hatcheries. Fish were immature and had to be killed in order to identify their gender. Immature carp were purchased from a single supplier. All fish were acclimatised (for a few days at least) in tanks provided with mains water, predominantly from a bore-hole source.

In field trials the fish were held in galvanised steel cages and were not fed during the periods under test. Blood was sampled by heparinized syringe from the caudal sinus after anaesthesia with 2-phenoxyethanol. Fish were then killed, measured for length and sexed. Blood was centrifuged and the plasma stored in liquid nitrogen prior to assay. The vitellogenin assay was conducted as described by Sumpter (1985) or Tyler and Sumpter (1990) and was performed blind on coded samples.

Five series of field trials were performed as follows:

i. Trout were held at the main STW at which hermaphrodite fish had been observed, at a nearby but smaller STW with a largely rural catchment zone, at a MAFF Experimental Trout Culture Unit just below a small STW, and at two control sites comprising tap water supply (largely bore-hole in origin) and spring supply (the farm of origin of the fish). This was a preliminary trial and fish were exposed for varying times.

ii. Following positive results from (i), in a further preliminary trial trout were placed in effluent at the main STW for assessment of the time-dependence of the vitellogenic response.
iii. The main trial embraced the study of 30 STWs chosen throughout England and Wales to include several from each Water Authority region; the choices were made on the advice of individual Water Authorities and on separate assessments of the likelihood that trout could survive in the effluent streams.
iv. A series of trials was made using carp at selected sites used for (iii).
v. A series of trials of linked STWs, potable water intake points and intermediate sites was made using trout.

In laboratory tests of the potency of contraceptive pill constituents, trout and carp were held in 1600 l glass aquaria with a flow-through of water into which stock solutions of steroids in distilled water were fed via a peristaltic pump and mixing chamber. In one trial, treatment was *via* intra muscular injection.

The purpose of the field trials was to investigate the occurrence of estrogenic substances in STW effluents and not to quantify the vitellogenic response in relation to content and concentration of substances in the effluent. The extreme variability of effluent composition, flow rate and dilution during the trials made inter-site comparisons invalid and repeat trials under identical conditions impossible. In the data from these trials the individual levels of vitellogenin varied widely. However, the effect at the main STW and results from subsequent studies of other STW effluents were so obvious that tests of statistical significance were inappropriate, but in cases where the picture was less clear, non-parametric tests were applied to aid interpretation of the data (Conover, 1980).

Analysis of the data from the laboratory tests was with the IBM SPSS statistical package. In experiments in which all the fish in a group (usually the controls) had plasma vitellogenin levels below the detection limits of the RIA, the analysis was done on the counts per minute values obtained from the gamma counter in order to introduce variation within the group.

RESULTS

Field trial i.: Winter 1986–1987

The results shown in Table I clearly indicate that enhanced levels of plasma vitellogenin arose in male and female fish held at site A, the main STW, with some indication also of increased levels at the smaller STW (site B) and the Experimental Trout Unit. The levels in males at the control sites were at the limit of the resolving power of the assay. This was therefore the first clear indication of an estrogenic activity in STW effluent.

Field trial ii.: Spring 1988

Male fish were used and exposed to effluent at the main STW for 3 weeks or held as controls in tap water. Ten fish were sampled at the end of weeks 1 to 3 in each group. The results showed a steady climb in mean vitellogenin levels at 33 $\mu g\ ml^{-1}$ for week 1, 192 $\mu g\ ml^{-1}$ for week 2 and 373 $\mu g\ ml^{-1}$ for week 3. Variations were again large and the population standard deviations were 25, 149 and 272, respectively.

Table I Preliminary trials measuring serum vitellogenin levels in rainbow trout at specific sites.

Site	Exposure (days)	Males			Females		
			vitellogenin $\mu g\ ml^{-1}$			vitellogenin $\mu g\ ml^{-1}$	
		n	mean vitellogenin	range	n	mean vitellogenin	range
STW site A*	33	4	20.2	0.1–78.0	14	63.6	1.5–130.0
	67	9	174.2	27.0–400.0	9	407.3	87.0–880.0
STW site B	33	10	0.6	<0.02–4.7	10	1.8	0.1–8.8
Experimental trout	34	10	10.0	0.03–36.5	6	12.5	0.3–42.5
unit below STW	61	13	0.3	<0.02–1.2	10	3.5	0.3–10.0
	91	12	13.5	0.06–71.0	12	20.2	2.7–148.0
Control site	91	14	0.03	<0.02–0.05	17	1.1	0.2–2.5
mains water	Resident	7	0.04	<0.02–0.10	5	2.7	0.7–6.1
Control Farm-spring water	Resident	40	0.03	<0.02–0.15	42	1.3	0.05–6.5

*STW sites are coded to fulfil confidentiality requirements.

At the control site, levels were again around the limits of detection at 0.1, 0.06 and 0.04 $\mu g\ ml^{-1}$, respectively. Three-week periods were adopted for future STW exposures.

Field trial iii.: Summer 1988

This was the main field trial and the results are given in Table II. Despite careful choice, at 12 of the sites the fish were unable to survive and at three others works plant malfunction was judged to be responsible for the death of all fish. At each of the 15 sites where fish survived the 3-week exposure, clear evidence of large increases in plasma vitellogenin was obtained. Even in male fish, where vitellogenin is usually not detectable, the observed levels were in mg ml^{-1} concentrations, falling within the range normally observed in mature females during egg formation. At the control sites, the levels of vitellogenin were generally low except for a very mildly elevated value at site 3. Surviving fish at most sites were in poor condition. As a check for stress-related effects, fish were placed in the influent and effluent channels of a major trout farm as poor water quality of an effluent can stress rainbow trout. No vitellogenin elevation was observed when influent fish (18 females: mean vitellogenin 32.6 $\mu g\ ml^{-1}$)were compared to those in the effluent (13 females, 10.5 $\mu g\ ml^{-1}$).

Field trial iv.: Winter 1988

Immature common carp (*Cyprinus carpio*) were deployed at ten of the STWs previously used successfully for trout. At one, site K, plasma samples were taken weekly for 6 weeks. At the others, samples were taken after three-week exposures. The results are given in Table III. Vitellogenin levels were very low overall compared to those for rainbow trout and amongst the controls a few fish gave surprisingly high values. A non parametric test of significance Kolmogorov-Smirnov (Conover, 1980) indicated that significantly high values were obtained at sites J, L and N and the series at site K did seem to show an increasing trend albeit not of statistical

Table II Results of nationwide survey of estrogenic activity in STW effluents. Means of samples of 8 to 20 surviving fish.

	Site	Vitellogenin levels in $\mu g\ ml^{-1}$ following 3 weeks exposure	
		Male	Female
Control sites	1	–	4.5
(Spring or tap water)	2	0.05	–
	3	1.80	88.3
	4	0.05	23.5
	5	0.22	–
STW sites	A	–	470
(final effluent)	C	23	–
	D	3 100	–
	E	147 000	112 000
	F	–	10 000
	G	–	48 000
	H	7 600	6 000
	I	2 100	3 800
	J	–	13 200
	K	–	10 500
	L	–	3 000
	M	54 000	–
	N	65 000	–
	O	19 200	–
	P	15 600	–
	12 sites	Fish unable to survive	
	3 sites	Works failure	
	1 site	Cage and fish stolen	

Table III Plasma vitellogenin ($\mu g\ ml^{-1}$) levels in common carp after 3 weeks exposure at STW sites. At site K fish were maintained for 6 weeks and bled weekly.

STW site	n	mean	sd	max	min
K week 1	12	0.02	0.02	0.07	0.01
" 2	12	0.04	0.03	0.09	0.01
" 3	20	0.06	0.03	0.11	0.03
" 4	12	0.10	0.00	0.10	0.10
" 5	12	0.20	0.53	1.85	0.20
" 6	12	0.34	0.86	3.00	0.01
E	20	0.64	2.56	11.50	0.01
Q	20	0.17	0.25	1.00	0.01
R	20	1.05	4.46	20.00	0.01
L	20	2.16*	2.50	8.50	0.02
S	19	1.20	2.32	9.50	0.01
A	20	0.43	1.88	8.40	0.01
J	20	15.64*	5.67	20.00	3.15
C	20	0.09	0.09	0.30	0.01
N	20	2.28*	1.96	5.80	0.01
Controls	40	0.55	2.23	13.00	0.002

*Significantly higher than control levels.

significance with the test used. These conclusions must be treated with caution. Due to a delay in the placement of fish and an early onset of cold weather, the water temperatures on sites were around 5°C. It is likely that this is too low for normal vitellogenesis in carp, a species for which a normal English summer is too cold for natural spawning. (Hernandez *et al.*, 1992).

Field trial v.: Summer 1989

This trial was conducted because of concern for public health raised by the earlier work, even though tap water controls had consistently given negative results and a very early assay of rainbow trout held as 'sentinels' in potable water intake protection tanks by Water Authorities had also proved negative. Forty two females and 14 males were sampled from 8 localities and the mean vitellogenin levels found were 0.83 and 0.11 $\mu g\ ml^{-1}$, respectively.

Four rivers were chosen where fish in cages could be deployed at a STW, a downstream abstraction point and at an intermediate position. This requirement meant that previously used STWs were not appropriate. The results are shown in Table IV. Technical problems rendered this trial incomplete, despite an attempt to replicate it. Enhanced vitellogenin levels were observed in male trout at the STWs

Table IV Vitellogenin levels ($\mu g\ ml^{-1}$) in male rainbow trout at STW, abstraction points downstream and intermediate localities. Means and SEM of ten fish per sample.

River	Month	Control	STW	Intermediate	Abstraction
V	May	<0.005	898 ± 233**	0.016 ± 0.01 NS	0.076 ± 0.05*
	August	<0.010	2,154 ± 395**	NO TEST	<0.010 NS
W	May	<0.005	x	0.290 ± 0.21*	0.240 ± 0.16*
	August	<0.010	x	0.110 ± 0.05*	+
X	May	<0.010	2,078 ± 614**	NO TEST	<0.010 NS
T	May	<.005	+	<0.005 NS	<0.005 NS

x Placement of fish precluded by technical detail in Home Office licence
+ Fish died
*P<0.05)
**P<0.001) Statistical significance of response compared to controls.
NS – Not Significant)

but little effect was evident downstream for rivers V and X. For river W there was evidence for a slight increase in vitellogenin levels in downstream abstraction and intermediate points. This is a slow, lowland river whereas river V is a fast-flowing chalk stream. The results suggest that substantial dilution in rivers with strong flows may largely dissipate the estrogenic effect, but this may not be the case in slow flowing rivers. On the fourth river T, fish died at the STW but showed no evidence of enhanced vitellogenin levels at either of the downstream localities.

LABORATORY TRIALS

An *a priori* argument at the outset was that constituents of the contraceptive pill might be implicated in an estrogenic effect of waste waters. The principal estrogen

Table V Plasma vitellogenin levels ($\mu g\ ml^{-1}$) in male rainbow trout injected with estradiol-17β (E) and 17α-ethynylestradiol (**EE**). Means of 4 fish. Temperature 8.5°C

Dose ($\mu g\ kg^{-1}$)	Days after injection					
	1	5	10	15	20	25
1 E	<10	15	18	22	13	10
EE	**<10**	**39**	**87**	**155**	**66**	**88**
100 E	<10	52	61	84	63	75
EE	**<10**	**130**	**172**	**480**	**221**	**184**
500 E	<10	82	92	173	108	126
EE	**<10**	**261**	**3 750**	**15 050**	**13 110**	**90 750**
1 000 E	<10	256	240	5 690	6 950	4 295
EE	**<10**	**158**	**3 360**	**13 250**	**16 000**	**20 300**

Controls for each steroid were < 10 $\mu g\ ml^{-1}$ on each sampling day

in the pill is 17 α–ethynylestradiol but its 2-methyl ester (Mestranol) is sometimes used. The main metabolites of these artificial steroids are the α- and β-glucuronides.

In a preliminary trial to assess whether ethynylestradiol could induce vitellogenesis in trout, male fish were given a single intramuscular injection of varying doses of ethynylestradiol and, for comparison, similar doses of the natural steroid estradiol-17β. Sham injected controls were run for each group of fish and individuals were identified by tagging. The results in Table V show that the artificial steroid 17 α-ethynylestradiol can induce vitellogenesis in trout and that it is very much more potent than estradiol-17β.

In a second trial, male fish were exposed by immersion to ethynylestradiol, Mestranol and the α- and β-glucuronides at a concentration of 25 ng l^{-1}. Blood samples from 10 individually identified fish at each treatment were taken two days after the start of treatment and daily thereafter, up to the sixth day. All steroids other than ethynylestradiol were ineffective by comparison with untreated controls. The effect of ethynylestradiol is shown in Figure 1. A logarithmic increase in serum vitellogenin occurred over the 6 day period to reach levels approaching those commonly found in mature females.

Finally, within this series, male trout and immature carp were exposed by immersion to different concentrations of ethynylestradiol for a preliminary evaluation of dose response. At 9.5°C (Table VI), carp responded much less strongly than trout but significant enhancement was apparent at steroid concentrations at and above 10 ng l^{-1}. In the parallel trout group, the lowest steroid concentration of 1 ng l^{-1} appeared ineffective but the samples used in the vitellogenin assay were over-diluted and below the sensitivity of the assay. In a later series of experiments conducted at a higher temperature (Table VII), significantly enhanced levels of vitellogenin were found with all treatments. The responses at 10 ng l^{-1} in Tables VI and VII are within the range of observations at STWs as shown in Table II.

DISCUSSION

This paper confirms that estrogenic substances are present in the effluent of STWs. Its preliminary nature and the difficulty of conducting repeatable, replicated trials in field conditions precluded the application of full scientific rigour. The resultant data

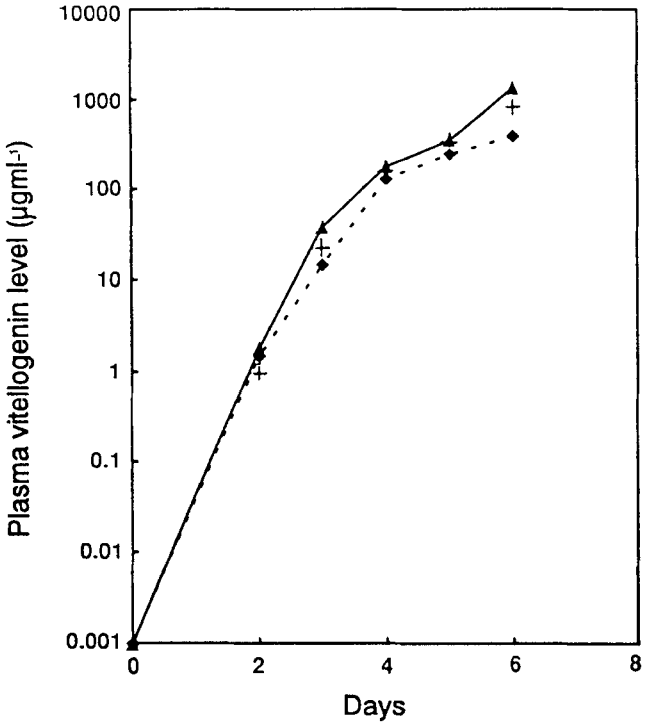

Figure 1 Effect on plasma vitellogenin levels (μg ml^{-1}) of immersion of male trout in 25 ng l^{-1} of ethynylestradiol for 6 days. + mean of 10 fish; ▲ individual fish showing greatest response over 6 days; ◆ individual fish showing least response over 6 days.

Table VI Vitellogenin levels (μg ml^{-1}) in male rainbow trout and immature carp exposed to 17α-ethynylestradiol by immersion for 10 days (temperature 9.5°C). Mean and SEM of 10 fish in each group

Dose (ng l^{-1})	Plasma vitellogenin (μg ml^{-1}) mean ± SEM	
	Carp	Trout
Control	<0.01	<1.0
1	<0.01	<1.0#
10	0.15 ± 0.08*	630 ± 140**
25	0.84 ± 0.26**	4970 ± 73**
50	216 ± 26**	11200 ± 800**

Statistical significance of response compared to controls
*P<0.05
**P<0.001
#Samples overdiluted for assay

set, although imperfect, indisputably demonstrates the nationwide distribution of estrogens in STW effluents; fuller and more detailed studies are currently in progress.

Placing rainbow trout in the effluent of sewage-treatment works caused a rapid and very pronounced increase in their plasma vitellogenin concentrations. It is

Table VII Vitellogenin levels in male trout after 10-day exposure to 17α-ethynylestradiol by immersion (temperature 16.5°C)

Dose (ng l^{-1})	n	Vitellogenin ($\mu g\ ml^{-1}$)	Range ($\mu g\ ml^{-1}$)
Control	10	<0.01	All<0.01
0.1	10	0.06 ± 0	0.02–0.01*
0.5	8	9.71 ± 5.03	0.34–46**
1.0	10	149 ± 112	0.18–1150**
10.0	7	37400 ± 4130	16500–48000**

Statistical significance of response compared to control
*P<0.05
**P<0.001

unlikely that this marked response was non-specific, because no effect was observed in fish placed in the effluent of a major trout farm and, in any case, stress causes a decrease in the plasma vitellogenin concentration (Carragher *et al.*, 1989; Campbell *et al.*, 1993). It is well established that the synthesis of vitellogenin in trout is under the control of estrogens; in female trout, estradiol-17β is the naturally occurring estrogen primarily responsible for stimulating vitellogenesis during sexual maturation (van Bohemen *et al.*, 1982; Scott and Sumpter, 1983). In a comprehensive series of experiments, Bromage and colleagues injected a large number of different steroid hormones into rainbow trout, and assessed their ability to stimulate vitellogenin synthesis (reviewed in Bromage and Cumaranatunga, 1988); they found that estrogens were very much more effective than other steroids. LeGuellec *et al.* (1988) describe primary and secondary stimulation of vitellogenesis in male trout by injection of estradiol-17β. Thus, it is generally accepted that vitellogenin synthesis in trout (and other oviparous vertebrates) is primarily under the control of estrogens, and can be induced by exogenous estrogens even in male fish. Hence, the enhanced synthesis of vitellogenin in the trout maintained in sewage effluent must have been due to the presence of an estrogenic substance (or substances) present in the effluent.

The nature of the estrogenic compound(s) in effluent is not known. The *a priori* hypothesis was that ethynylestradiol, originating from use of the contraceptive pill, was implicated. Laboratory tests of the potency of this and other steroids were conducted. Attempts were also made to measure contaminant levels in sewage effluents by GC-MS and radioimmunoassay. Both of these attempts were unsuccessful and technical problems preclude unequivocal interpretation of the data (see also Aherne *et al.*, 1985). Notwithstanding this, the assessment of the efficacy of ethynylestradiol by injection or by immersion has shown it to be an extremely potent inducer of vitellogenesis, far exceeding the effect of estradiol-17β, the natural estrogen implicated in vitellogenesis. Concentrations of 10 ng l^{-1} in the water generated increases in plasma vitellogenin of a similar magnitude to those observed in the nationwide survey of STWs. Concentrations as low as 0.1 ng l^{-1} also caused a significant rise in plasma vitellogenin and this has been confirmed by more recent studies (Sheahan *et al.*, 1993). This places the artificial estrogen ethynylestradiol amongst the most potent of biologically active molecules.

Although various phytoestrogens and pesticides with low estrogenic potencies could contribute to an overall estrogenic effect, it has always seemed unlikely that they could be present in effluents at sufficiently high concentrations to be serious contributors to the phenomenon. Recently, however, a further possibility was suggested (A.J. Dobbs, personal communication, 1991), namely nonylphenols, which

can be present in sewage systems and which have been shown to be estrogenic in an estrogen-sensitive human cell assay (Soto *et al.*, 1991). Amongst the group of related alkylphenol-ethoxylates (APE), the nonylphenols are major degradation products of surfactants and detergents and can be present in large amounts in sewage. In a survey of STWs, Waldock and Thain (1986) concluded that concentrations of nonylphenols were generally low (<2 to 21 ng l^{-1}) in effluents but were present at higher levels in sludges (0.3 mg kg^{-1} dry weight). Levels (<25 to 314 ng l^{-1}) which were also higher than in STW effluent were observed in water samples taken from the mouth of the River Mersey (MAFF, 1991). Detailed assessment of the possible significance of APE is being conducted and will be reported elsewhere. The present position appears to be that the choice of hypothesis to account for the estrogenic activity in STW effluent lies between (a) ethynylestradiol, highly potent at nanogram per litre concentrations, but not yet detectable chemically, and (b) APE, which may be 4 or more orders of magnitude less potent than ethynylestradiol, but is more readily demonstrable in sewage systems.

The full implication of the discovery that effluents from STWs are estrogenic is not yet clear but studies on the reproductive impact for fish are underway. The demonstration that the estrogenic effect is dissipated downstream, and the largely negative results from abstraction points and potable water supplies do limit concern on public health grounds but wider study is desirable here too. Going back to the original observation of hermaphrodite fish, this is unlikely to be a consequence of the estrogenic effect of effluent. Changes to the primary sexual characters, the gonads, of fish occur only when estrogenic treatments are made at an early stage in the life of fish fry (Yamamoto, 1969). The fish in the STW lagoons were not hatched there, but introduced as mature fish.

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