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Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment*

Oestrogenic effects in fish in English rivers with evidence of their causation

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Abstract: In the mid-1980s, effluents from sewage-treatment works discharging into rivers in England and Wales were found to be oestrogenic, due to their ability to induce the production of a female specific egg-yolk protein precursor, vitellogenin (VTG), in male fish. Subsequent field studies, in which caged rainbow trout (*Oncorhynchus mykiss*) were placed at varying distances downstream of STW effluent discharges, demonstrated that the oestrogenic activity persisted in some receiving waters for considerable distances (up to 5 km) downstream from the point of sewage effluent input. More recently, populations of wild fish inhabiting English rivers have been shown to be intersex (a manifestation of endocrine disruption which can be induced by exposure to oestrogen). In these fish, a direct association between the incidence (and degree) of intersexuality, and the level of exposure to sewage-treatment effluent, has been demonstrated. Chemical analysis of STW effluents has identified natural steroidal oestrogens (namely, oestradiol-17β and oestrone), and in some cases the synthetic oestrogen, 17α-ethynyloestradiol, at concentrations sufficient to induce the oestrogenic responses observed in the caged trout studies. In STWs receiving influent from wool scouring mills, alkylphenolic chemicals (biodegradation products of alkylphenol polyethoxylates; APEOs) were found to be the major oestrogenic contaminants and they were present at concentrations high enough to induce feminising effects in fish (VTG induction and suppression of testis growth in males). This chapter documents the major findings on the research into the oestrogenic effects in fish in English rivers, and critically assesses the weight of evidence (from both field studies and *in vivo* laboratory studies) that support the contention that exposure to steroidal oestrogens and alkylphenolic chemicals may be responsible for the impairment of reproductive function in wild fish.

OESTROGENIC ACTIVITY IN STW EFFLUENTS AND RIVERS

It was more than twenty years ago that sexual disruption in fish was first reported**.** Thames Water Authority staff, acting on casual observations by anglers, found a low incidence (around 5%) of intersex, or hermaphroditism, in populations of wild roach (*Rutlilus rutilus*; a common lowland freshwater cyprinid fish) living in a sewage effluent settlement lagoon and just downstream of a STW effluent discharge in the River Lea catchment (South-East England) [1]. Roach, like most temperate freshwater fish, are gonochoristic (they have either a testis or an ovary), and hermaphrodites are believed to be very uncommon [2,3]. Despite this, their findings were filed as an internal report, and no further action was taken. Quite independently, in the mid 1980s, a joint study by Brunel University and the UK's Ministry of Agriculture, Fisheries and Food into the reproductive physiology of rainbow trout, found that the male fish held at a Ministry research station contained measurable amounts of the yolk protein precursor

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vitellogenin (VTG) in their plasma. The production of VTG is oestrogen-dependent and is normally restricted to females [reviewed in 4]; little, if any, VTG is usually present in plasma of male fish [5,6]. However, male fish also carry the VTG gene, and exposure to both natural and synthetic oestrogens can trigger its expression [7,8]. The finding of VTG in the plasma of male fish was, therefore, indicative of the presence of an 'oestrogen' in the water. The location of a STW works discharging into the river upstream from the research station raised the possibility that the effluent was the source of the oestrogenic contaminant.

In the late 1980s, an extensive study was undertaken to test the hypothesis that effluent from STWs was oestrogenic. In that study caged rainbow trout were placed in effluents from STWs (receiving domestic and/or industrial effluent) at 28 locations covering all 10 Water Authority Areas throughout England and Wales [9]. The fish were exposed to the effluent for 2 to 3 weeks and the induction of plasma VTG was determined by radioimmunoassay [10]. A further five sites, where the water supplies were not contaminated with STW effluent, were chosen as controls. At some test sites, the effluent was lethal to rainbow trout, but at the remaining sites where the fish survived, the effluents were all found to be strongly oestrogenic. The degree of the vitellogenic response in the exposed fish ranged from a 500-fold up to 50 000-fold increase in plasma levels of VTG at the different sites. In the most oestrogenic effluents, plasma VTG concentrations exceeded 100 mg/mL, which are higher than those normally found in fully mature females [4,11]. The variability in the magnitude of the response between the different sites was probably due to differences in the age and sex of the exposed fish, differences in the timing of fish deployment (effluent exposures were conducted at different times of the year) as well as the 'strength' of the effluent to which the fish were exposed. At some of these sites immature carp (*Cyprinus carpio*), a native cyprinid fish, were exposed to the effluent and they produced a similar, albeit less pronounced, vitellogenic response as the trout [9].

It is now known that the phenomenon of oestrogenic effluent is not confined to the United Kingdom. Similar studies, in which fish were exposed to effluent from STWs receiving primarily domestic inputs, have been conducted in Germany (using bream, *Abrama abramis*; pers. comm. S. Halm) and France (eels, *Anguilla anguilla*; pers. comm. R. Billard). In addition, exposure of fish to effluent from STWs receiving a mixture of both domestic and industrial influent have been conducted in the United States (carp [12]) and Norway (rainbow trout [13]). In all of these studies, VTG induction occurred in the exposed fish, albeit that the magnitude of response varied widely.

The widespread nature of oestrogenic effluent from STWs, prompted a series of investigations to determine whether the oestrogenic activity persisted in rivers downstream from the point source STW discharges. Sites on six English rivers, receiving input from STWs with primarily domestic influent (but with a small proportion of industrial influent) were chosen for study[14]. The rivers studied were the Lea, Arun and Kent Stour in southern England; the Chelmer and Essex Stour in eastern England; and the Aire in northern England. Adopting the same protocol as Purdom *et al*. (1994) for the effluent exposure studies, caged rainbow trout were placed at various distances downstream of STW discharges for a period of 3 weeks, and the plasma samples were subsequently assayed to determine VTG concentrations. No induction of VTG occurred in fish placed in the Essex Stour, Kent Stour or on the Chelmer. In contrast, a vitellogenic response was seen in fish on the Arun up to 1.5 km downstream, and on the Lea up to 4.5 km downstream of the discharges [14]. Simultaneous measurements of testicular weight (gonadosomatic index or GSI) showed that the rate of testis growth was reduced at some, but not all, of the sites on these rivers compared with the laboratory controls [14]. Of all the sites studied, the most marked effects occurred on the River Aire in Yorkshire, downstream of Marley STW. At all the sites studied along a 5 km stretch of river below Marley STW there were significant elevations in plasma VTG concentrations, which ranged from 25 mg/mL to 52 mg/mL [14]. Simultaneous measurements also showed that the hepatosomatic index (HSI; which may serve as an indicator of metabolic activity/load) was elevated, and testis growth was significantly depressed in the trout at all sites along the surveyed stretch of river. From the caged trout work, it was clear that in some English rivers the oestrogenic activity was not limited to areas immediately surrounding STWs outfalls, but rather persisted along significant stretches of the river.

WIDESPREAD SEXUAL DISRUPTION IN WILD FISH

Investigations followed the caged trout studies to determine whether the concentrations of oestrogenic chemicals present in Englands' rivers were sufficiently high enough to cause adverse reproductive effects in wild fish. In fish (as in all other oviparous vertebrates), oestrogens are not only responsible for induction of VTG, but they also play important roles in many other reproductive and developmental processes, including sexual maturation [15] and sexual differentiation [16]. Both the amount of endogenous natural oestrogen and the timing of its release into the blood are carefully controlled by the endocrine system, and inappropriate exposure to oestrogen [reviewed in 17], or oestrogen mimics [reviewed in 18] during sexual differentiation can induce sex reversal and/or intersexuality [19], whilst exposure during sexual maturation can inhibit gonadal growth and development [20].

An extensive field study was undertaken in which over 2000 roach of mixed age and sex from eight rivers, both upstream and downstream of STWs, and from 5 reference sites (mainly canals and lakes) throughout England and Ireland were sampled, and their reproductive status (through histology of the gonad and measurement of the concentrations of plasma VTG) were assessed [21]. The rivers selected varied with respect to the amount of sewage effluent they received. In addition, where ever possible, the sampling sites on rivers receiving STW effluent were selected where a physical barrier occurred (such as a weir) that separated the fish from the sites located upstream and downstream of the STWs (this would have limited the movement of fish between the two populations—fish downstream of the STWs could not migrate to the upstream population, although movement of fish from the upstream population to the one downatream could have occurred during periods of spate).

Histological examination of the gonads of the roach revealed that a large proportion of 'males' were in fact intersex (as defined by the simultaneous presence of both testicular and ovarian characteristics in the gonad). In populations of roach downstream of STW effluent discharges, between 16% (River Wreake/Eye, The Midlands) and 100% (both Rivers Aires, Yorkshire and Nene, Northamptonshire) of the 'males' were found to be intersex. In sites upstream of STW effluent discharges, the incidence of intersexuality was between 11.7% (River Lea) and 44.4% (River Nene). Intersex fish were also found at the control sites, however, the incidence was considerably lower (ranging from 4% in the laboratory fish up to 18% in one population of field control fish) compared with populations of roach in rivers receiving STW effluent. The low incidence of intersexuality seen in the control fish may be a natural phenomenon. However, we do not know whether the field control fish had been exposed to non-point sources of oestrogenic chemicals (for example, oestrogenic pesticides/herbicides in run-off from the surrounding farmlands). Notwithstanding this, in five of the eight rivers studied receiving STW effluents, the incidence of intersexuality was significantly higher compared with the control sites. Furthermore, the degree of intersexuality in the fish from the control sites was slight (often there were just a few primary oocytes in an otherwise normal testis), whilst in 'male' fish from rivers receiving STW effluent the proportion of gonadal tissue that was ovarian sometimes exceeded 50%. In these individuals the sperm duct was absent and was replaced by an ovarian cavity.

It was not possible to determine the genetic sex of the roach that were sampled, as no sex-specific probes are available for this, or any closely allied, species at this time. Evidence that the intersex fish were derived from genetic males was derived from the fact that the number of fish with normal testes in any population was inversely proportional to the number of intersex fish [21], and from the knowledge that sewage effluent discharges are predominantly oestrogenic [e.g. 9,14,15,22,23]. Moreover, the VTG concentrations in the intersex fish were generally intermediate between the concentrations measured in the males and females (and there was a positive correlation between the concentration of plasma VTG and the degree of intersexuality), supporting the contention of a feminisation of males [21].

In the roach field study, the proportion of intersex fish was found to be positively correlated with the concentration of the effluent at the different sampling sites. The concentration, or 'strength', of the effluent in the river at each sampling site was categorised according to both the population equivalent of the nearest upstream STW, and the average annual dilution factor of its effluent in the river [21]. Population equivalents are a measure of the strength of the influent entering a STW; where one population equivalent is the amount of biodegradable organic load which has a biochemical oxygen demand of 60g of oxygen per day [24]. The average dilution factor of the effluent in the river at the capture site was calculated using hydrometric data of monthly river flows and actual sewage flows, in order to provide an estimate of the dilution factor of effluent in the river over a period of several years (a time period encompassing the life spans of the fish captured where ever possible). In turn, the absolute concentration of effluent at each site was approximated by adjusting the population equivalent to allow for the degree of dilution of the effluent in the river. The results provided very strong evidence that it was the exposure of the roach to STW effluent (and the chemicals therein) that caused the disruption in reproductive development.

The effects reported in wild fish in English rivers are not limited to roach, as preliminary studies on a second species of fish, the gudgeon (*Gobio gobio*), collected from the same rivers as the roach, indicate that they display a similar pattern of intersexuality [our own, unpublished data]. The gudgeon is a cyprinid fish that has both a very different reproductive strategy and ecological niche compared with roach. To illustrate this, the gudgeon is an asynchronous spawner (producing gametes at several times during the reproductive season [25]), whereas roach spawn only once during the reproductive season, and the gudgeon lives on the bottom of the river, close to the river sediments, whereas roach live mid-water. Thus, reproductive disruption in fish in English rivers, resulting from exposure to STW effluent is not species specific. A number of *in vivo* and *in vitro* laboratory studies on oestrogens and their mimics in a variety of animals indicate that there is little, if any species differences in specificity [reviewed in 18], although species differences in sensitivity may occur [26].

In summary, the findings from populations of wild fish in English rivers strongly suggest that the concentration of sewage effluent in a river is a major causal factor in the evolution of intersexuality. Furthermore, as the majority of the sampling sites in the field study were several kilometres downstream from any point of sewage input, it is likely that the fish collected were truly representative of populations in typical English river ecosystems. The ecological implications of intersexuality on wild fisheries in the English rivers will depend on the physiological significance of intersexuality; that is, whether the reproductive competence of fish that are intersex is compromised. To address this question, the quality and quantity of gametes produced by these fish will need to be determined. Having said this, in severe cases of intersex, the sperm duct was found to be absent [21], and this would prevent the release of viable sperm, whilst the ability of these individuals to produce viable eggs is questionable. Nothing is yet known about the possible effects of exposure to STW effluents on reproductive development and/or their effects in female fish.

IDENTIFICATION OF THE OESTROGENIC SUBSTANCES IN EFFLUENT FROM STWS

The composition of an effluent varies according to the type of input (e.g. industrial and domestic) it receives. Over 60000 man-made chemicals are in routine use world-wide, which may be partially degraded during the sewage-treatment process to produce many more chemicals which then enter the aquatic environment. In addition, anywhere between 200 and 1000 new synthetic chemicals enter the market each year [27]. It is apparent, therefore, that effluents contain highly complex mixture of chemicals, and identifying the specific chemical(s) responsible for the adverse effects observed in the field is difficult. Testimony to this is the fact that that there are very few examples where an adverse affect in any aquatic wildlife species has been conclusively linked to a specific chemical. The search for the causative agents of the feminising effects in fish in effluents and rivers in England has focused on 'oestrogens', due to the nature of the 'oestrogen-dependent' effects observed. Effluents, however, may potentially contain a wide variety of oestrogens, including 'real' steroidal oestrogens, phyto-oestrogens, myco-oestrogens as well as synthetic oestrogen mimics. It has now been established that there are a wide variety of synthetic chemicals capable of mimicking oestrogens and these are structurally diverse [reviewed in 18, 28]. They include alkylphenolic chemicals, resulting from the breakdown of non-ionic surfactants [20,29,30], plasticisers such as Bisphenol-A and some phthalates [31,32], as well as certain pesticides and herbicides [33]. It has been argued that because many of the oestrogen mimics identified to date are weak (compared with steroid oestrogens), it is unlikely that they would cause biological effects. This is not necessarily true however, as certain xeno-oestrogens are in widespread regular use and are continuously entering the environment. It is therefore not surprising that many xeno-oestrogens have been reported to be present in the environment at high concentrations, where they may persist for long periods of time because their breakdown is slow. Moreover, many xeno-oestrogens are lipophilic and therefore bioaccumulate [reviewed in 18,28]. Most of these factors are true for the oestrogenic xenobiotic, nonylphenol, which has been identified in STW effluents entering English rivers (see below).

In a recent study a Toxicity Identification and Evaluation approach was successfully used to identify the agents responsible for the oestrogenic effects in fish exposed to STW effluents [23]. In this study, effluents from seven STWs in England were analysed that received primarily domestic input. The effluents analysed were Southend-on-Sea (southern England), where the effluent had only primary treatment before being discharged into the marine environment; Harpenden, Rye Meads, and Deephams STWs in the River Lea catchment, south-east England and Horsham STWs that discharges into the River Arun, Sussex (from caged trout studies, all of these effluents were known to be oestrogenic); Billing STWs that discharges into the River Nene, Northamptonshire and Naburn STWs that discharges into the River Ouse, Yorkshire. Effluent from Marley STW on the River Aire (near Leeds) was also analysed because this STW receives 7% of its influent from trade sources; a major contribution of which comes from the wool scouring industry [14,34]. All the effluents were fractionated into samples of decreasing complexity which were assessed for oestrogenic activity using a recombinant yeast screen containing the human oestrogen receptor. In the presence of oestrogenic chemicals, the yeast cell line produces an enzyme that mediates a change in the colour of a chromogenic substance in the medium [35]. The oestrogenic components (unknown at this time) were largely confined to the dissolved phase of the effluents, and these were successively sub fractionated, firstly using C18 columns, and secondly by HPLC, until the components in the oestrogenic fractions could be identified using GC-MS. Despite the complexity of the composition of domestic effluents, only a small number of oestrogenic compound were identified using this approach; namely the synthetic oestrogen 17α-ethynyloestradiol, the natural steroid oestrogens oestradiol-17β and oestrone, and alkylphenolic chemicals.

EVIDENCE THAT STEROIDAL OESTROGENS AND ALKYLPHENOLS IN STW EFFLUENT CAUSE ENDOCRINE DISRUPTION IN FISH

17α-Ethynyloestradiol

The concentration of 17α-ethynyloestradiol measured in the seven different STWs analysed ranged from non-detectable (in 4 of the STWs studied; namely Harpenden, Rye Meads, Deephams and Billing), to between 0.2 ng/L (Horsham STWs) and 7 ng/L (Southend-on-Sea STW [23]). The presence of 17α-ethynyloestradiol in STW effluent and their receiving waters was consistent with previous studies in England and other European countries, although the reported concentrations vary from 0.3 ng/L in a Dutch river [36], from less than 5ng/L up to 15 ng/L in English rivers [22] and up to 62 ng/L in German STW effluents [37]. As the effluents analysed in England (with the exception of Marley STWs) contained little, if any, agricultural input, it is likely that the 17α-ethynyl-oestradiol was anthropogenic in origin; derived mainly from the contraceptive pill. 17α-ethynyl-oestradiol is largely excreted in the urine as inactive conjugates (glucuronides and sulphates), although faecal elimination mainly occurs in uncongugated forms [38].

The amount of 'free' 17α-ethynyloestradiol measured in some sewage effluents and rivers, suggests that conjugated 17α-ethynyloestradiol (passing out of the body in the urine) is biotransformed back into the biologically active form during the STW process. This observation is supported by the fact that *Escherichia coli* (which is probably the most common bacteria found in sewage) synthesize large amounts of ß-glucuronidase [39,40], which is an enzyme capable of hydrolysing steroid glucuronides. Differences in the reported concentration of 17α-ethynyloestradiol measured in STWs and rivers in England and Europe may be, in part, a function of the amount of steroid decongugation that has taken place and/or differences in the steroidal extraction procedures employed in the various studies.

In fish, 17α-ethynyloestradiol is a very potent inducer of vitellogenesis, and exposure of male rainbow trout to concentrations of 0.1 ng/L and above in the water causes a rapid and pronounced synthesis of VTG [9]. Exposure of male trout to concentrations of 10 ng 17α-ethynyloestradiol/L, for a period of 3 weeks, resulted in maximal induction of plasma VTG [9]. Little is known about the reproductive consequences of exposure of oviparous animals to 17α-ethynyloestradiol. However, exposure to a single dose of 2 ng 17α-ethynyloestradiol/L in water was found to produce a 50% retardation of testicular growth and development in maturing male trout [31]. Furthermore, exposure of fathead minnows to 17α-ethynyloestradiol, at a concentration of 10 ng/L for a period of four weeks, causes a reduction in the number of eggs oviposited [41]. Thus, although the concentrations of 17α-ethynyloestradiol present in the riverine environment in England are likely to be very low (ng/L, or less), the extreme potency of this synthetic oestrogen means that even these concentrations can cause adverse biological effects.

Natural steroidal oestrogens

The natural steroidal oestrogens, oestradiol-17β and oestrone, were detected in effluents from STWs. Concentrations of oestradiol-17β ranged from 2.7 ng/L in Rye Meads STW effluent up to 48 ng/L in Southend STW effluent, and concentrations of oestrone ranged from 1.4 ng/L in Billing STW effluent up to 76 ng/L in Deephams STW effluent [23]. Similar concentrations have been reported in effluents from STWs in Germany where concentrations of up to 20 ng/L oestradiol-17β were measured [37]. Again the consistency of the findings in different STW effluents suggests that the common source of the oestrogenic compounds was domestic sewage. It is likely that the primary source of these oestrogens are women. Cycling women excrete between 10 μg and 100 μg of oestrogen daily, depending on the phase of their cycle. Pregnant women may secrete up to 30 mg of oestrogen a day. The metabolic processing of oestradiol-17β and oestrone in the body is similar to that of 17α-ethynyloestradiol (i.e. they are excreted primarily in conjugated forms), and the presence of 'free' oestrogen in the aquatic environment probably results from their deconjugation by bacteria in the environment (see above[18,38]). In Israel, natural steroidal oestrogens have been detected in raw and treated sewage at concentrations between 48 ng/L and 141 ng/L and between 39 ng/L and 153 ng/L, respectively. Moreover, appreciable concentrations of steroidal oestrogens have also reported to be present in river and lake water [42]. However, in this case, the oestrogens are believed to originate from intensive animal farming practices.

Recent laboratory studies using male trout, and roach (mixed sex) have shown that the concentration of steroidal oestrogens (oestradiol-17β and oestrone) present in STW effluents are sufficient to account for the observed induction of vitellogenin synthesis in the caged fish placed close to effluent discharges in those rivers. In rainbow trout, the threshold concentration for a response to a 3 week exposure to oestradiol-17β was between 1 and 10 ng/L [26]. Male roach appeared to be less sensitive to oestradiol-17β, as the threshold concentration of oestradiol-17β required to produce a vitellogenic response was between 50 ng/L and 100 ng/L [26]. In rainbow trout the threshold concentration for a response to oestrone was between 25 ng/L and 50 ng/L [26]. Recent studies on adult fathead minnow (*Pimephales promelas*) have demonstrated similar threshold concentrations for a response to oestradiol-17β and oestrone (100 ng/L and 31.8 ng/L, respectively [43]). Furthermore, in the male fathead minnow, exposures to higher doses of oestradiol-17β (100 ng/L) and oestrone (320 ng/L) resulted in a partial or total inhibition of testicular growth during a 21 day exposure [43]. Early life-stages of fathead minnows are probably more sensitive to oestradiol-17β compared to adult fish as a very recent study in which embryos, exposed from 24 h post fertilisation through to hatch and up to 30 days post hatch, demonstrated a threshold concentration for a vitellogenic response to oestradiol-17β of between 25 ng/L and 50 ng/L [44].

Studies in male salmonid fish have reported pronounced deleterious effects on reproductive development following exposure to high doses of steroidal oestrogens, ranging from inhibition of spermatogenesis to complete regression of the testes[31,45]. The responses of male fish to steroidal oestrogen vary according to the dose administered and the stage of testicular development at the time of exposure [31,45]. Care should be taken, therefore, when ascribing species specific sensitivities to oestrogen, as the stage of the reproductive cycle at the time of exposure may effect the magnitude of the response.

ALKYLPHENOLIC CHEMICALS

The major oestrogenic contaminants identified in the effluent from Marley STW (receiving an input from wool scouring plants) were APEOs [34]. Alkylphenols and their polyethoxylate derivatives have been used for over 40 years, in the manufacture of plastics, elastomers, agricultural chemicals, and pulping and industrial detergent formulations. Nonylphenol polyethoxylates (NPEOs) are the most common nonionic surfactants, (APEOs) constituting 82% of their production [46]. Alkylphenols, such as nonylphenol and related compounds, are by-products of the microbial breakdown of APEOs, such as occurs during sewage treatment [47]. Substantial amounts of alkylphenolic chemicals enter the aquatic environment, from waste water discharges and from sewage sludge [48]. Wool scouring plants, such as those located along the River Aire, use large amounts of APEOs to remove the grease from fleeces [49]. In 1994, the concentration of nonylphenol alone in the effluent of Marley STW was found to be in excess of 300 μg/L, resulting in a concentration of up to 180 μg/L in the river [34,50]. However, the concentration of nonylphenol in the sediment was 800–1000 times higher than in the water phase [34], due to its tendency to absorb to particulates. Between 24 μg/L and 53 μg/L of dissolved nonylphenol was measured in a stretch of River Aire where maximal vitellogenin induction and retardation of testicular growth occurred in the caged fish work [50]. Analysis of tissue samples from cyprinid fish living in the river downstream of Marley STW showed around a 50-fold and 500-fold bioconcentration of nonylphenol in muscle and liver tissues, respectively [34]. However, the levels of alkylphenolic chemicals found in the River Aire are not characteristic of other English rivers, where concentrations are typically less than 10 μg/L [48]. For example, in STW effluents discharging into the River Lea, the measured concentration of nonylphenol was between 0.2 and 9.0 μg/L, and octylphenol was present at around 0.4 μg/L [48]. The measured concentrations of nonylphenol in rivers in Europe are generally higher than those reported in the USA, where a survey of 30 rivers found that 70% of the sites had concentrations of 0.1 μg/L or less [51].

It should be emphasised that nonylphenol and octylphenol are only two of the possible degradation products of APEOs, and other alkylphenolic compounds may contribute to the overall effect seen in the River Aire and elsewhere. Indeed, short chain nonylphenol polyethoxylates (NPEOs) and nonylphenol carboxylates (NPECs) are also oestrogenic to fish (see below [31]). NPECs are much more water soluble than nonylphenol, and therefore, we would expect them to be present at much higher concentrations than nonylphenol in the water column. Despite this, very little information is available on the concentration of NPECs in the environment. Recently, however, NPECs were measured in paper mill effluents, municipal sewage effluents and river water in the USA. In this study the concentration of NP_1EC to NP_4EC ranged from below detection to 1300 μg/L in the effluents, and from below detection to 13.8 μg/L in the river water [52].

Only five alkylphenolic chemicals have been tested for oestrogenic activity in fish (or in any other animal) *in vivo*, namely octylphenol, nonylphenol, NP_2EO, NP_1EC and 4-*tert*-pentylphenol (TPP); and all were oestrogenic in fish [31,19]. Nonylphenol and octylphenol (in 3 week exposure trials) induced an elevation in plasma VTG at threshold concentrations of 10 μg/L and 3 μg/L, respectively [31]. The concentrations of nonylphenolic compounds measured in the River Aire therefore, were alone high enough to stimulate VTG synthesis in fish. In laboratory experiments, exposure of fish to concentrations of alkyphenols that induced VTG synthesis were also found to significantly inhibit the development of the testis. TPP has also been shown to cause feminisation of male carp exposed via the water over the period encompassing sexual differentiation [19]. In almost all these fish, exposure to TPP at concentrations between 0.32 mg/L and 1 mg/L resulted in the formation of an oviduct, and in the highest dose group some of the fish were identified as intersex (oocytes were present in the testis).

In summary, the information available on alkylphenolic chemicals suggests that their concentrations in the River Aire were/are high enough to cause reproductive effects in fish. Further evidence for their causation of reproductive effects was confirmed following a recent programme (enforced by the Environment Agency) to reduce discharges of oestrogenic alkylphenolic compounds into the River Aire. In 1995, nonylphenol concentrations in the effluent from Marley STW were reduced by approximately 80% compared with 1994, and by a further 15% by 1996 and these reductions resulted in a concomitant decrease in oestrogenic responses in caged fish [34].

CONCLUSIONS

In summary, the presence of natural and synthetic steroidal oestrogens, and in some instances alkylphenols, in STW effluents have been identified (and confirmed) as the key causative agents for the oestrogenic (vitellogenic) responses observed in caged (field study) and wild fish in English rivers. It is also likely that these agents are responsible for the evolution of intersexuality in wild fish, especially given the knowledge that effluent from STWs can make up a considerable proportion of the river flow. For example , effluent from STWs makes up between 50% and 80% of the annual flow in the River Lea [14]. In addition, fish living in many English rivers are likely to be exposed for considerable periods of time (and possibly all their lives) to a cocktail of oestradiol-17β, oestrone, 17α-ethynyloestradiol and alkyphenolic chemicals (and other oestrogenic chemicals) whose effects may be additive (or greater than additive) [26]**.**

River water quality in the United Kingdom is good compared with a number of other European countries, where sewage is often discharged into rivers and canals with little or even no treatment. Indeed in British STWs around 95% of the organic polluting load is generally removed by treatment prior to discharge. This level of treatment is amongst the highest level in OECD countries, and is exceeded in Europe only by Germany [53]. The biological problems associated with the presence of steroidal oestrogens in English Rivers could be alleviated if the STW effluent was diluted further prior to its discharge. However, in reality this problem will not be easily overcome, given the drought problems faced in recent years, and the ever increasing demand for clean water (approximately a 2% increase per year), which together have resulted in the low flows in many rivers in England. In comparison, problems associated with the discharge of oestrogenic alkylphenolic compounds are more easily addressed. Given the weight of evidence presented here, where high quantities of alkylphenolic compounds are in use (such as wool scouring industries), a programme of tighter regulation for their discharge and/or a switch to alternative (non-oestrogenic) surfactants should be introduced.

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APPENDIX: Abbreviations

VTG	vitellogenin
STW	sewage-treatment works
GSI	gonadosomatic index
HSI	hepatosomatic index
HPLC	high performance liquid chromatography
GC-MS	gas-chromatography mass-spectrometry
APEOs	alkylphenol polyethoxylates
NPEOs	nonylphenol polyethoxylates
NPECs	nonylphenol polycarboxylates
TPP	4-*tert*-pentylphenol