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The influence of a surfactant, linear alkylbenzene sulfonate, on the estrogenic response to a mixture of (xeno)estrogens *in vitro* and *in vivo*.

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Abstract

The effect of the presence of a surfactant on the activity of a mixture of environmental estrogens was assessed. In their natural habitat, fish are subject not only to exposure to mixtures of estrogenic compounds, as has been addressed in previous publications, but also to other confounding factors (chemical, physical and biological), which may, in theory, affect their responses to such compounds. To assess the potential for such interference, the commonly occurring surfactant, linear alkylbenzene sulfonate (LAS), was applied to the yeast estrogen screen at various concentrations, independently and together with a mixture of estrogens at constant concentrations. LAS enhanced the estrogenic activity of the mixture, an effect which became less pronounced over the course of time. This information was used to design an *in vivo* study to assess induction of vitellogenin in fathead minnows exposed to the same mixture of estrogens plus LAS. A similar trend was observed, that is, the response was enhanced, but the effect became less pronounced as the study progressed. However, the enhanced response *in vivo* only occurred at the highest concentration of LAS tested (362 µg/L), and was transient because it was no longer apparent by the end of the study. Although LAS is a significant contaminant in terms of both concentration and frequency of detection in the aquatic environment, these data do not suggest that it will have a significant impact on the response of fish to environmental estrogens.

The exposure of fish to single environmental estrogens has long been known to induce significant biological effects, such as increased vitellogenin concentrations and a reduction in reproductive potential (Sumpter, 2005). It is also acknowledged that such chemicals occur in combination in the environment, but only recently has data demonstrating the additive effects of complex mixtures of estrogenic chemicals *in vivo* been published (Brian et al, 2005). Simultaneously present in river waters are various confounding factors that may alter the response of fish to the mixture of estrogenic chemicals. One group of confounding factors includes chemicals which are not themselves estrogenic, but which may influence the effects of estrogenic agents by promoting or inhibiting uptake or metabolism. Information concerning the effects that such chemicals may have on the response of fish to environmental estrogens is scarce. It has been suggested that some environmental pollutants, especially surfactants and organic solvents, may facilitate the uptake of agents across cell membranes (Witte et al, 1995; Jacobi et al, 1996; Brown, 2007). It is important that we try to elucidate the risk posed by exposure to multiple chemicals which could act via different mechanisms, given the complex mixtures of substances present in sewage treatment works (STW) effluents and their receiving waters.

Linear alkylbenzenesulfonates (LAS) are the most commonly used synthetic anionic surfactants, with an estimated global consumption of 2.8 million tonnes in 1998 (Verge et al, 2000). As a result, they are one of the most frequently found xenobiotics in urban wastewaters; they occur in high concentrations in STW effluents, and consequently in their receiving waters. Predictions indicate that concentrations of LAS in STW influents may be as high as 16 mg/L (Holt et al, 2003). This means that, whilst they are highly degradable, with more than 95% removed during the treatment process, concentrations in effluents can still be in hundreds of µg/L (Holt et al, 2003). For example, concentrations of up to 444 µg/L have been detected in densely populated areas, although they are more commonly found in the range of tens of µg/L (Holt et al, 2003; Clara et al, 2007). Further accumulation of LAS in river waters is unlikely, since they are easily degraded in aerobic conditions, although they may potentially occur in higher concentrations in anaerobic sediments.

In the current study, initial experiments to assess the effect of LAS on the estrogenic response of a mixture of estrogens were conducted *in vitro* using the recombinant

yeast screen. The standard assay procedure, according to Routledge et al (1996), was followed. Three controls were employed: a positive control (estradiol), a negative (solvent only) control, and a mixture control (comprising the mixture of estrogens at a constant concentration). The mixture of estrogens used was the same as that used by Brian et al (2005), that is, estradiol, ethynylestradiol, nonylphenol, octylphenol and bisphenol-A. These were added to the medium such that the concentration of each chemical was equal to its EC20. These concentrations were chosen so that each chemical was present at an equal ratio, based on its individual potency. In addition, LAS was added at 12 different concentrations (serially diluted from 10 to 0.005 mg/L). The standard duration of the assay is 3 days, and the plates were read daily over this period.

The results of this yeast screen are shown in figure 1. After one day of incubation (Fig. 1a), the response of the mixture of estrogens plus LAS was higher (at 25% of the maximum response) than that of the positive control, despite the absorbance value of the mixture control being similar to that of the negative control. This was already an indication that LAS affects the speed of estrogenic response, at least in this assay. After two days incubation (Fig. 1b), the response of the mixture plus LAS had reached a maximal value in the assay, even though the absorbance value of the mixture alone was still only 35% of the maximum at this stage. By day three (Fig. 1c), the response to the mixture of estrogenic chemicals had also risen (to approximately 75% of the maximum), and the difference between the response to the mixture control and those of the mixture plus LAS was less striking, although still apparent.

There appear to be two possible explanations for this phenomenon; the first is that LAS enhances the rate of transfer of estrogenic compounds across the cell membrane, the second is that LAS enhances the apparent solubility of estrogenic compounds in the water, resulting in increased bioavailability of these substances. Many chemicals, including surfactants and solvents, have been shown to impact upon the permeability of cell membranes (Helenius and Simons, 1975; Jacobi et al, 1996; Cserhati et al, 2002). The binding of surfactants to proteins within the cell membrane can lead to structural modifications which may in turn disrupt the biological function of the membrane, generally by increasing its permeability (Helenius and Simons, 1975). Bioavailability of organic chemicals in the presence of

surfactants has been investigated by several authors. The increased apparent solubility of organic compounds has been described as being either a result of decreased interfacial tension or due to incorporation into surface micelles (Kile and Chiou, 1989). It is not thought that the latter theory is applicable to this experiment, however, as the concentrations of LAS used were below the critical micelle concentration (CMC).

This information, together with the results from the yeast screen, lead us to question whether such effects could occur *in vivo*, and consequently an experiment was designed in which the induction of vitellogenin in adult male fathead minnows exposed to a mixture of estrogens, as well as the same mixture of estrogens plus LAS, was assessed. The flow-through system used for this study was the same as that described in Brian et al (2005), and the dose response curves reported by Brian et al were used to calculate the mixture concentration applied in the current study. Fathead minnows were reared from stock maintained at Brunel University. The mixture of estrogens was prepared as a single stock in dimethylformamide (DMF); the DMF concentration in the fish tanks was 67 µL/L, which is below the concentration recommended by the OECD (100 µL/L) for solvent use in aquatic toxicity testing (OECD, 2000). LAS has a high water solubility and so LAS stock solutions were prepared in water, not solvent. This helped to limit the overall solvent concentration. The nominal concentrations of LAS in the tanks were 15, 30, 60, 125, 250, 500 ug/L. Tanks were equilibrated with the test chemicals for a period of 1 week prior to the introduction of the fish. A total of 16 fish were deployed to each treatment tank, and the exposure was maintained for a duration of 2 weeks. At the end of the first week, 8 fish were sampled, and the remaining 8 were maintained in experimental conditions until the end of the second week. Plasma VTG concentrations were determined using a homologous carp-VTG ELISA, which has been validated for measurement of fathead minnow VTG (Tyler et al, 1999).

Water samples were collected at weekly intervals for the determination of exposure concentrations. LAS was measured by LC-MS, which revealed that the concentrations were close to nominal in all tanks, except the highest concentration (nominal 500 µg/L), where the measured concentration was 362 µg/L. The concentrations of the estrogenic mixture components were also measured, as per

Brian et al (2005), which revealed that their levels were very consistent across the seven dosed tanks.

The *in vivo* data are presented in Fig. 2. The results show that there was an apparent increase in VTG in the fish exposed to 362 µg/L LAS after one week of exposure, although standard statistical methods (that is, ANOVA) did not detect these differences. The data were then analysed using the Jonckheere-Terpstra (JT) trend test, which is a nonparametric test for ordered differences among classes, and provides a more sensitive means of detecting subtle, dose-dependent effects (OECD, 2004). This revealed a significant trend in increasing concentrations of vitellogenin in the dosed tanks ($p<0.02$). In contrast, after two weeks of exposure this trend was no longer evident, due to the increased plasma vitellogenin concentrations in the fish exposed to the mixture of estrogens alone. Whilst the enhanced *in vivo* response when LAS was present was not as robust as that observed *in vitro*, largely due to the increased data variability associated with whole-organism studies, there are consistencies between the two data sets; both suggest that LAS enhances the speed at which the effects of environmental estrogens reach their plateaux in these estrogen-response assays. The nature of the parallel response (particularly the temporal aspects) observed *in vitro* and *in vivo* are striking, and suggests that, in future studies, emphasis should be placed on the characteristics of the response observed *in vitro*, such that *in vivo* studies can be designed accordingly.

There is evidence to suggest that surfactants may increase the rate of chemical uptake *in vivo*. For example, they have been shown to increase the flux of tritiated water across fish gills (Umezu, 1991). LAS was one of the surfactants tested in that study, which demonstrated a doubling of the influx of water in fish exposed to this chemical for just 15 minutes. Furthermore, cadmium transfer across the perfused gills of rainbow trout was found to be enhanced in the presence of LAS at environmentally-relevant concentrations (50 µg/L), although at high concentrations (34.8 mg/L) LAS had a negative impact on gill viability (Pärt et al, 1985). Similarly, it has been observed that anionic surfactants increase the rate of diffusion of urea across perfused rainbow trout gills (Partearroyo et al, 1992).

Alternatively, it is possible that the enhanced (albeit transient) estrogenic response of the fish occurred as a result of increased bioavailability. Recent data have shown

increases in DDE bioavailability of less than 10-fold by some surfactants, but by up to 74-fold by others (White et al, 2007). The variability experienced when assessing surfactant-mediated increases in contaminant bioavailability is apparently unpredictable and has worrying implications for the assessment of exposure of organisms to complex mixtures of environmental pollutants. Several studies have discussed the increased aquatic toxicity of hydrocarbons present in toxic oil spills following the addition of a dispersant (Singer et al, 1998; Ramachandran et al, 2004). The conclusion reached by the authors of both of these papers was that the increased toxicity of the crude oil was a result of the enhanced bioavailability of the toxic fraction, and not due to increased permeability of gill membranes.

Whilst it is not possible to ascertain the mechanism responsible for the effects of LAS *in vitro* or *in vivo* at this stage, further work may help to clarify this. For example, the use of a radiolabelled hormone in conjunction with LAS could enable tracing of the hormone in various fish tissues, and the influence of LAS on the distribution of the hormone within the fish. Such work may also help to elucidate why the effects we observed were more pronounced *in vitro* than *in vivo*.

Given all of the above evidence, we are confident that the results reported here do show a real phenomenon, that of increased estrogenic activity in the yeast screen and in fish in the presence of LAS. What is not so clear is the environmental significance of these data. Firstly, because increased vitellogenin induction *in vivo* was only observed in fish exposed to the highest concentration of LAS (362 µg/L). Although this is not an entirely unrealistic environmental concentration, levels this high are not frequently detected in the aquatic environment. Secondly, although LAS at the highest concentration used appeared to enhance the rate at which the estrogenic response plateaued in this assay, an enhanced response was no longer apparent after two weeks of exposure, suggesting that in this scenario, LAS is not a significant confounding factor. Finally, it is important to recognise that there may be other surfactants simultaneously present in the environment which may act additively with LAS, thereby leading to more pronounced effects on the responses of fish to estrogenic chemicals than observed with individual surfactants (as Brian et al (2005) found with estrogenic substances), and also that exposures may occur repeatedly and/or over extended periods. However, it was not possible to address these issues

(that is, the potential additive effects of different surfactants and long-term exposure scenarios) within the scope of the current study. Therefore, although the implications for risk assessment are difficult to anticipate given the unknown factors mentioned above, based on the available data our overall conclusion is that it seems likely that real-world concentrations of LAS will not have pronounced effects on the response of fish to estrogenic chemicals.

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Figure Legends

Figure 1

The effect of incubating recombinant yeast with a mixture of estrogens plus LAS for up to 3 days. In each figure, a positive (mixture of estrogens at a constant concentration) and a negative (solvent only) control are shown, as well as the positive control (estradiol). In addition, the effect of LAS plus the mixture and LAS with no other chemical added is depicted. Figures a, b and c show the results after 1, 2 and 3 days incubation, respectively.

Figure 2

Vitellogenin concentrations in plasma of exposed male fathead minnows. Fish were exposed to the mixture of estrogens alone (indicated by the hatched bar), or to the mixture of estrogens plus increasing concentrations of LAS (indicated by the solid bars). Vitellogenin concentrations in fish exposed to solvent only (the negative control) are depicted using hollow bars. (a) shows vitellogenin concentrations in fish exposed for one week and (b) shows results from fish sampled after two weeks of exposure. There were no significant differences between any of the treatments when ANOVA was applied, but the Jonckheere-Terpstra (JT) trend test revealed a significant trend of increasing concentrations of vitellogenin with increasing LAS concentration in the fish exposed for one week ($p<0.02$).