Pergamon

Marine Pollution Bulletin Vol. 38, No. 10, pp. 880–884, 1999

Printed in Great Britain

PII: S0025-326X(99)00036-3

0025-326X/99 $ - see front matter

Linear Alkylbenzene Sulfonates and Intermediate Products from their Degradation are not Estrogenic

JOSÉ MARÍA NAVAS†*, EDUARDO GONZÁLEZ-MAZO‡, ANDREA WENZEL§, ABELARDO GÓMEZ-PARRA‡ and HELMUT SEGNER†

†*Umweltforschungszentrum Leipzig-Halle GmbH, Chemische Ökotoxikologie, Permoserstr 15, D-04318 Leipzig, Germany*

‡*Departamento de Química Física, Facultad de Ciencias del Mar, Universidad de Cádiz, Campus Río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain*

§*Fraunhofer-Institut für Umweltchemie und Ökotoxikologie, Auf dem Aberg 1, D-57392 Schmallenberg, Germany*

A number of natural and man-made chemicals possess estrogenic activity, i.e., they induce receptor-mediated estrogen dependent responses in vertebrates. Recently, high concentrations of linear alkylbenzene sulfonates (LAS) and intermediate products of LAS biodegradation, sulfophenyl carboxylates (SPC), have been found in the Bay of Cádiz, Spain. Due to their similar chemical properties with the estrogenic alkylphenolpolyethoxylates (APnEO) it has been speculated that LAS and their metabolites have estrogenic effects. The present work addresses the question upon the possible estrogenicity of LAS and LAS metabolites by two *in vitro* assays: the yeast estrogen receptor assay and the vitellogenin assay with cultured trout hepatocytes. With both bioassays no estrogenic effects were observed for any of the compounds tested.

Keywords: Linear alkylbenzene sulfonates; Sulfophenyl carboxylates; Xenoestrogens; Rainbow trout hepatocytes; Estrogen receptor.

Introduction

An increasing number of man-made chemicals present in the environment are found to be capable of disturbing the endocrine system of animals, and man. In the last years, particular concern has been attracted by those chemicals able to act as estrogens. In vertebrates the primary natural estrogen is the 17 β-estradiol (E2). In oviparous vertebrates E2 stimulates the hepatic synthesis of vitellogenin (Vg). This glycolipophosphoprotein is transported from the liver to the oocytes where it is proteolytically processed to form the yolk proteins (Mommsen and Walsh, 1988; Wallace, 1985). The induction of Vg synthesis by primary cultured hepatocytes has been shown to be useful in the detection of estrogenic potential of xenobiotics (Pelissero *et al*., 1993; Sumpter and Jobling, 1995).

The estrogenic effects are mediated through the interaction of E2 with the estrogen receptor (ER). The E2–ER complex binds to DNA sequences that are responsive to estrogens, the so-called estrogen responsive elements (ERE), and thereby activates the transcription of a cis-linked target gene (Tsai and O'Malley, 1994).

A wide variety of substances have been shown to mimic the action of E2, including synthetic steroids such as those used in the contraceptive pill (Pelissero *et al*., 1993), many pesticides (Chambers, 1984; Eroschenko, 1981; Johnson *et al*., 1992), phytoestrogens (Pelissero *et al*., 1989, 1991) or phthalate plasticizers (Jobling *et al*., 1995). Soto *et al*. (1991) reported that the alkylphenols (AP) are weakly estrogenic in mammals. These compounds are the final products of the biodegradation of a family of non-ionic surfactants (alkylphenol-polyethoxylates, APnEO, $n = 9$–40) during sewage treatment (Giger *et al*., 1987) and they are persistent and present in substantial quantities in effluents and in river water. In subsequent studies it was observed that these chemicals are able to bind to the ER (Routledge and Sumpter, 1996; Petit *et al*., 1997) and to induce the synthesis of Vg in trout liver cells *in vitro* (Jobling and Sumpter, 1993) and *in vivo* (Jobling *et al*., 1996).

Another group of synthetic surfactants used mainly in the formulation of detergents and other cleaning products are the linear alkylbenzene sulfonates (LAS). Due to their widespread use there has been increasing concern about the environmental behaviour of these compounds. It has been determined that LAS chemicals are completely biodegradable products. During the

*Corresponding author. Tel.: +49-341-235-2272; fax: +49-341-235-2401; e-mail: jn@theo.uoe.ufz.de

biodegradation process of the LAS compounds, a methyl group at the end of the alkyl chain undergoes oxidation giving rise to a sulfophenylcarboxylic acid (SPC). Further oxidations of the alkyl chain generate new SPCs, with a shorter alkyl chain. In fresh water, these chemicals were reported to have a half-life of approximately 3 days (Larson and Payne, 1981) and thus, they are very quickly degraded in waste water treatment plants (Larson *et al*., 1993).

Until recently, the presence of LAS biodegradation products has not been reported in seawater. González-Mazo *et al*. (1997) described the presence of SPCs in water samples of a salt-marsh area to the south of the Bay of Cádiz (Spain, 36°30′N 6°15′W). Samples were taken from a channel of around 18 km long whose both mouths run into the Atlantic Ocean. The discharge outlet of the untreated urban effluents from San Fernando, a town of about 100 000 inhabitants, is located in this channel. A variety of SPCs were identified in this channel, most of them having an alkyl chain length of 7–11 carbon atoms (C7–C11). The concentration of these compounds (SPC, C7–C11) increased in proportion to the distance from the discharge point, attaining a maximal concentration of 120 μg/l at a distance of around 5 km of the discharge point, and then decreased progressively (González-Mazo *et al*., 1997).

Given the similarities in the chemical properties of LAS and SPC with APnEO, it was suggested that LAS and SPC may exert estrogenic effects on fish populations in the Bay of Cádiz, leading to alterations of the fisheries resources, traditionally very important in this region. This question was raised by the media and produced a passionate discussion (Comments published in ''El País'', 10 February 1998).

The objective of the present study was to evaluate the estrogenic potency of LAS and SPC. For this purpose, two *in vitro* screening assays for measurement of estrogenic activity were employed: the ER assay, with the recombinant yeast screen (Routledge and Sumpter, 1996; Petit *et al*., 1997) and the vitellogenin assay, with hepatocytes (Pelissero *et al*., 1993). Taking into account that the effects of LAS and SPC could be influenced by the length of the alkyl chain, as it was reported to occur with the epoxylate compounds (Jobling and Sumpter, 1993), we have assayed two homologues of the SPC, with a different length of the alkyl chain (C11 and C5).

Material and Methods

Xenobiotic treatments: LAS (LAS-C11, A.I. = 47%) was supplied by Petroquímica Española S.A. SPC5, and SPC11 were synthesised in Cádiz University.

Chemicals were dissolved in sterile distilled water and added to the culture media of yeast or hepatocytes. E2 (Serva, Germany) was used as positive control. E2 was diluted in ethanol and added to the media to achieve the final desired concentrations. Final concentration of the solvent was 0.1%. Controls received the solvent only. For the test compounds, serial dilutions were assayed. In the yeast assay the maximal concentration used for the three compounds was 600 μM. Maximal concentrations used in the hepatocytes assay were: 150 μM (50 mg/l) for LAS, 25 μM (7.4 mg/l) for SPC5, and 200 μM (72.8 mg/l) for SPC11.

Recombinant yeast estrogen screen

An estrogen-inducible screen developed in yeast (*Saccharomyces cerevisiae*) was used to observe receptor-binding activity of the test compounds. The yeast screen was gently provided by Glaxo (United Kingdom) and the assay was performed according to Routledge and Sumpter (1996). The yeast cells contain expression plasmids carrying the human ER sequences and the reporter gene (lac-Z, encoding the enzyme β-galactosidase). Upon binding an active ligand (E2 or an estrogenic xenobiotic) the activated ER causes the expression of the reporter gene.

Hepatocytes culture

Immature rainbow trout (*Oncorhynchus mykiss*) weighing around 300 g were supplied by a local trout farm and kept in recycled aerated water until use. Hepatocytes were isolated following a two-step perfusion technique as described by Scholz *et al*. (1997). As culture medium, modified M199 medium (Sigma, USA) was used. Cells were plated in 24-well culture plates (Falcon) precoated with Matrigel (0.1 mg protein ml^{-1}). Every well received 400 μl of the final suspension of cells (density: 2×10^5 cells cm^{-2}). During the first 24 h of treatment the medium was supplemented with 5% fetal bovine serum (Sigma, USA) to enhance the attachment of the cells. During exposure to E2 and the test compounds serum free medium was used. The plates were maintained at 15°C and 80% humidity. Twenty four hours after plating, half of the medium was substituted with fresh medium containing the desired concentrations of xenobiotics and E2. Control wells received medium alone. Treatments were applied by duplicate. The medium was renewed each 24 h. After plating for 96 h the medium was removed, aliquoted, and stored at −80°C until analysis of LDH and vitellogenin in medium. The cells were briefly washed with phosphate-buffered saline (PBS), pH 7.5, and the plates were frozen by using liquid nitrogen. They were maintained at −80°C until analysis of protein and LDH content of the cells.

Vitellogenin assay

Vitellogenin was analysed in medium using a non-competitive ELISA as previously described by Schrag *et al*. (1998).

LDH assay

Cytotoxicity was evaluated by measuring the release of lactate dehydrogenase (LDH) into the extracellular medium following the method described by Denizeau and Marion (1990).

Protein assay

The concentration of Vg detected in the medium was normalised to the quantity of cell protein present in the corresponding well. The protein concentrations were determined by means of the fluorescamine method (Kennedy *et al.*, 1995).

Results and Discussion

In the yeast screen, the different concentrations of the three test substances did not induce any difference in the absorbance with respect to the blanks (results not shown). This screen indicates that neither LAS nor SPCs interact with the human ER at the concentrations tested.

Figure 1 shows the Vg concentrations observed in media from primary hepatocytes after three days of treatment with E2 or with different concentrations of LAS, SPC5 and SPC11. The treatment with E2 induced strong production of Vg, indicating the functional responsiveness of the cells. In the medium of the control cells negligible Vg was detected. The three test substances, at all concentrations studied, did not induce an increase in the concentration of Vg over the levels detected in the controls.

The lack of Vg production in the xenobiotic treated cells could have been related with a cytotoxic effect of these substances. To exclude this possibility we have analysed the cytotoxicity of LAS and SPCs by measur-

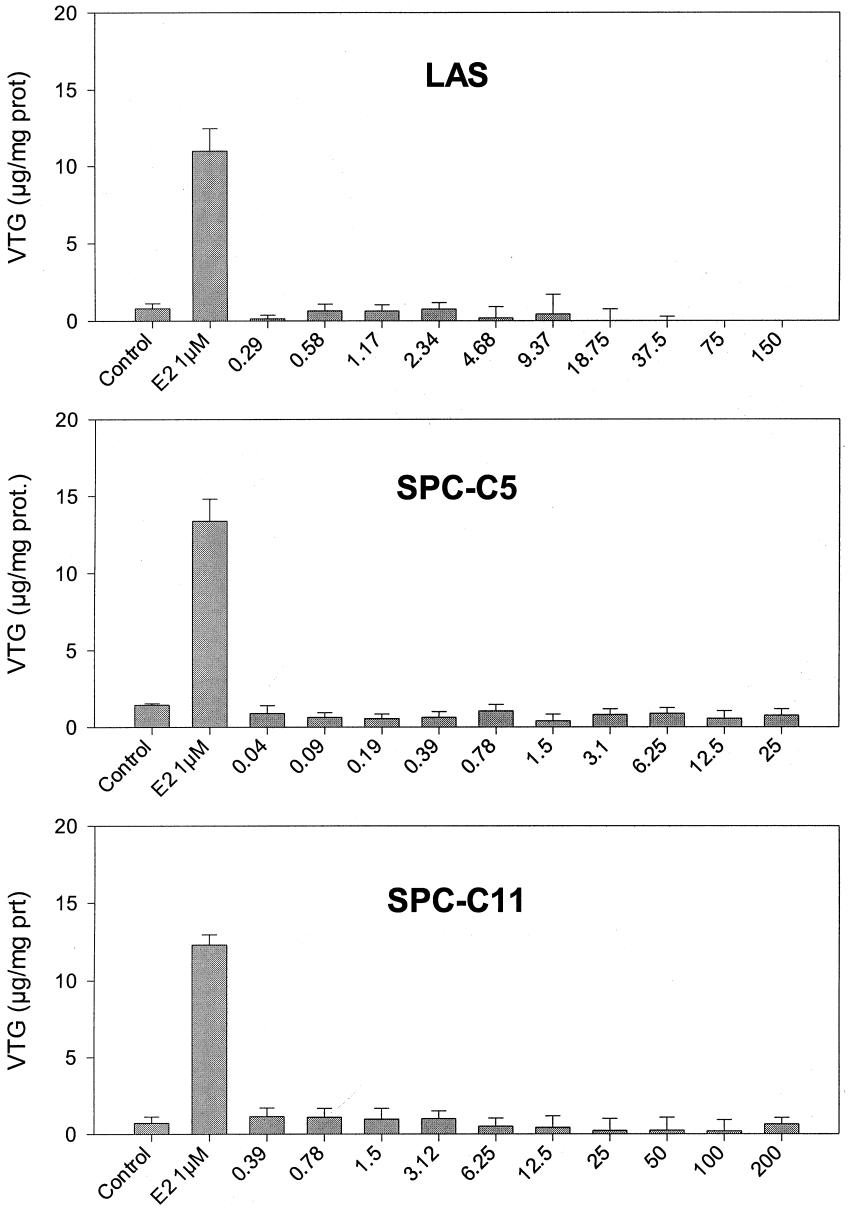

Fig. 1 Vitellogenin levels (μg^{-1}/g protein) observed in controls, and in the cells treated with 1 µM E2 and with the different concentrations (µM values indicated in the *x*-axis) of LAS (Linear Alkylbenzene Sulfonate), SPC5, and SPC11 (Sulfophenyl Carboxylate C5 and C11). Data are expressed as mean ± SEM ($n = 4$ fishes).

ing LDH leakage from the cells (Tyson and Green, 1987). As it can be observed in Fig. 2, only the three highest concentrations of LAS (37.5–150 μM) lead to an enhanced release of LDH from the cells into the medium indicating a loss of cell viability. The cytotoxic effect of LAS could be explained by the documented membrane alterations of permeability (Zaccone *et al.*, 1985). For the SPCs, however, none of the tested concentrations was associated with cytotoxic effects. Thus, the lacking response of Vg synthesis to LAS and SPC exposure obviously can not be explained by cytotoxicity of these compounds.

Recent studies have indicated the necessity to combine several *in vitro* test to asses the estrogenic potential of chemicals (Petit *et al.*, 1997; Zacharewski, 1997). In the present work, both *in vitro* assays have not been able to detect any estrogenic activity of the test compounds. The results of the yeast assay show that the test compounds obviously are not able to bind to the estrogen receptor what would be a prerequisite to induce receptor-mediated estrogenic responses. The hepatocytes assay, which has the fundamental advantage to exhibit the physiologic and metabolic functions of the intact cell (Segner, 1998), also failed to show an estrogenic activity of the test compounds. Thus, the lack of Vg production by the hepatocytes *in vitro* indicates that no cellular metabolites of the test substances were neither estrogenic.

In vitro systems often are less sensitive to estrogenic compounds than *in vivo* assays since they can not

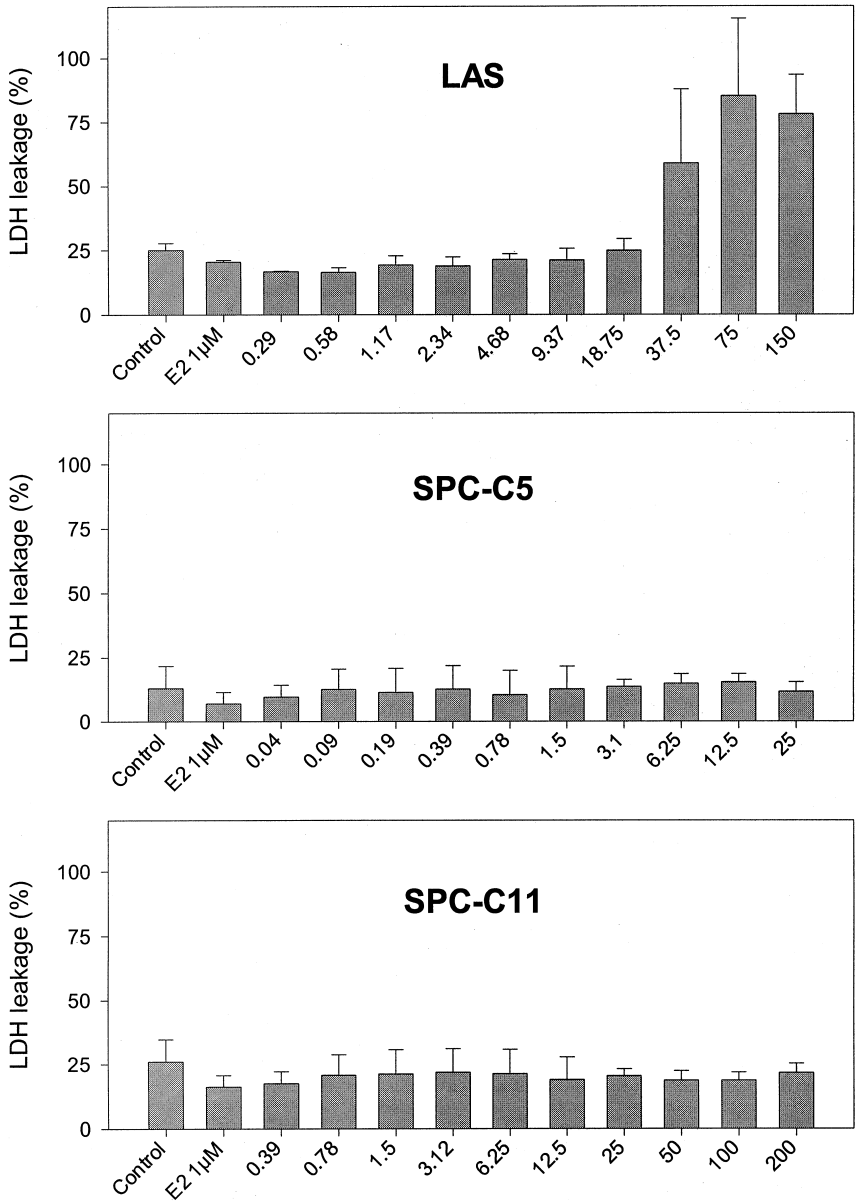

Fig. 2 Percent of LDH leakage from the control cells and from the cells treated with 1 μM E2 and with the different concentrations (μM values indicated in the *x*-axis) of LAS (Linear Alkylbenzene Sulfonate), SPC5, and SPC11 (Sulfophenyl Carboxylate C5 and C11). Data are expressed as mean ± SEM ($n = 3$ fishes).

account for the bioaccumulation of the chemicals. For example, the exposure of male rainbow trouts for three weeks to 30 μg/l of a variety of AP and APnEO induced a significant increase of plasma Vg levels with respect to the controls (Jobling *et al*., 1996). The same compounds needed concentrations of 100–1000 μg/l and three days of incubation (Routledge and Sumpter, 1996) in the yeast assay, and of 50 μM (around 10 mg/l) and two days of exposure (Jobling and Sumpter, 1993) in the hepatocytes assay to induce an estrogenic effect. However, the results from the two *in vitro* assays indicate that LAS and SPCs are mechanistically not able to interact with estrogen dependent pathways and, therefore, an estrogenic effect appears to be unviable even after chronic *in vivo* exposure and bioaccumulation.

In this work we evaluated possible estrogenic effects of LAS and of SPCs up to concentrations much higher than those observed by González-Mazo *et al*. (1997) in the Bay of Cádiz. With both *in vitro* test systems used, no estrogenic activity could be detected. Although there no exist previous studies about the bioaccumulation of these substances and their *in vivo* effects, the failure of LAS and SPCs to activate estrogen-dependent responses in both *in vitro* assays strongly suggest that these compounds are neither estrogenic *in vivo*.

The authors would like to thank Dr. C. Sarasquete and Dr. J. Blasco for their useful advice and discussion. Founded in part by the Environmental and Climate Program of the European Commission: Contract N° Env-4-CT965042 to José María Navas and the Contract ENV4-CT97-0494 from Waste Water Cluster.

Chambers, J. E. (1984) Oestrogenic effects of organochlorine insecticides in channel catfish. *Abstracts of papers of the American Chemical Society* **187**, 33.

Denizeau, F. and Marion, M. (1990) Toxicity of cadmium copper and mercury to isolated trout hepatocytes. *Canadian Journal of Fisheries and Aquatic Sciences* **47**, 1038–1042.

Eroschenko, V. P. (1981) Estrogenic activity of the insecticide Chlordecone in the reproductive tract of birds and mammals. *Journal of Toxicology and Environmental Health* **8**, 731–742.

Giger, W., Ahel, M., Koch, M., Laubscher, H. U., Schaffner, C. and Schneider, J. (1987) Behaviour of alkylphenol-polyethoxylate surfactants and of nitriloacetate in sewage treatment. *Water Science and Technology*, **19**, 449–460.

González-Mazo, E., Honing, M., Barceló, D. and Gómez-Parra, A. (1997) Monitoring long-chain intermediate products from the degradation of linear alkylbenzene sulfonates in the marine environment by solid-phase extraction followed by liquid chromatography/ionspray mass spectrometry. *Environmental Science and Technology*, **31,** 504–510.

Johnson, D. C., Sen, M. and Dey, S. K. (1992) Differential effects of DDT analogs, Chlordecone and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on establishment of pregnancy in the hypophysectomized rat. *Proceedings of the Society of Experimental Biology and Medicine* **199**, 42–48.

Jobling, S. and Sumpter, J. P. (1993) Detergent components in sewage effluent are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology* **27**, 361–372.

Jobling, S., Reynolds, T., White, R., Parker, M. G. and Sumpter, J. P. (1995) A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environmental Health Perspectives* **103**, 582–587.

Jobling, S., Sheahan, D., Osborne, J. A., Matthiessen, P. and Sumpter, J. P. (1996) Inhibition of testicular growth in rainbow trout. exposed to estrogenic alkylphenolic chemicals (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry* **15**, 194–202.

Kennedy, S. W., Lorenzen, A., James, C. A. and Collins, B. T. (1995) Efficient analysis of Cytochrome P4501A catalytic activity, porphyrins, and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. *Analytical Biochemistry* **226**, 362–370.

Larson, R. J. and Payne, A. G. (1981) Fate of the benzene ring of LAS in natural waters. *Applied and Environmental Microbiology* **41**, 621–627.

Larson, R. J., Rothgeb, T. M., Shimp, R. J., Ward, T. E. and Ventulio, R. M. (1993) Kinetics and practical significance of biodegradation of LAS in the environment. *Journal of the American Oil Chemistry Society* **70**, 645–657.

Mommsen, T. P. and Walsh, P. J. (1988) Vitellogenesis and oocyte assembly. In *Fish Physiology,* vol. XIA, eds W. S Hoar and D. J. Randall, Academic Press, New York, pp. 347–406.

Pelissero, C., Cuisset, B. and Le Menn, F. (1989) Influence of sex steroids in commercial fish meals and fish diets on plasma concentration of estrogens and vitellogenin in cultured Siberian sturgeon, *Acipenser baeri*. *Aquatic Living Resources* **2**, 161–168.

Pelissero, C., Bennetau, B., Babin, P., Le Menn, F. and Dunogues, J. (1991) The estrogenic activity of certain phytoestrogens in the Siberian sturgeon, *Acipenser baeri*. *Journal of Steroid Biochemistry and Molecular Biology* **38**, 293–299.

Pelissero, C., Flouriot, G., Foucher, J. L., Bennetau, B., Dunogues, J., Le Gac, F. and Sumpter, J. P. (1993) Vitellogenin synthesis in cultured hepatocytes; an *in vitro* test for the estrogenic potency of chemicals. *Journal of Steroid Biochemistry and Molecular Biology* **44**, 263–272.

Petit, F., Le Goff, P., Cravédi, J -P., Valotaire, Y. and Pakdel. F. (1997) Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. *Journal of Molecular Endocrinology* **19**, 321–335.

Routledge, E. and Sumpter, J. P. (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry* **15**, 241–248.

Scholz, S., Behn, I., Honeck, H., Hauck, C., Braunbeck, T. and Segner, H. (1997) Development of a monoclonal antibody for ELISA assay of CYP1A in primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Biomarkers* **2**, 287–294.

Schrag, B., Ensenbach, U., Navas, J. M. and Segner, H. (1998) Evaluation of xenoestrogenic effects in fish at different biological levels. In *Reproductive Toxicology*, ed J. del Mazo pp. 207–214 Plenum Press, New York.

Segner, H., (1998) Isolation and primary culture of teleost hepatocytes. *Comparative Biochemistry and Physiology* **120A**, 71–81.

Soto, A. M., Justicia, H., Wray, J. W. and Sonnenschein, C. (1991) P-nonylphenol: an estrogenic xenobiotic released from modified polystyrene. *Environmental Health Perspectives* **92**, 167–173.

Sumpter, J. P. and Jobling, S. (1995) Vitellogenesis as a biomarker for oestrogenic contamination of the aquatic environment. *Environmental Health Perspectives* **103**, 173–178.

Tsai, M. J. and O'Malley, B. W. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Reviews of Biochemistry* **63**, 451–486.

Tyson, C. A., Green, C. E. (1987) Cytotoxicity measures: choices and methods. In *The Isolated Hepatocyte: Use in Toxicology and Xenobiotic Transformations,* eds E. J. Rauckman and G. M. Padilla, pp. 119–158 Academic Press, Orlando.

Wallace, R. A. (1985) Oocyte growth in non-mammalian vertebrates. In *The Vertebrate Ovary*, ed R. E. Jones, pp. 469–502 Plenum Press, New York.

Zaccone, G., Cascio, P. L, Fasulo, S. and Licata, A. (1985) The effect of an anionic detergent on complex carbohydrate and enzyme activities in the epidermis of the catfish, *Heteropneustes fossilis*. *Histochemical Journal* **17**, 453–466.

Zacharewski, T. (1997) *In vitro* bioassays for assessing estrogenic substances. *Environmental Science and Technology* **31**, 613–623.