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In vitro acute toxicity of anionic surfactant linear alkylbenzene sulphonate (LAS) on the motility of gilthead (*Sparus aurata* L.) sperm

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Summary. This paper describes the acute toxicity of a known anionic surfactant, Linear Alkylbenzene Sulphonate (LAS), on the quality of gilthead *Sparus aurata* L. sperm. The parameter used to judge exposure effectiveness was sperm motility as well as its fertilizing ability after being combined with unexposed gilthead eggs.

Preincubation of sperm suspensions with concentrations of LAS of 0.1, 0.5, 1, 2 and 4 mg/L caused decrease in sperm motility and fertilizing ability. In this respect, percentages of motile sperm were respectively 89.8±9.8, 81.7±16.3, 69.5±21.3, 57.1±19.1 and 21.2±10.9%. With regard to the percentage of fertilization success, the results were 85.7±8.1, 75.1±20.2, 62.9±19.7, 52.7±19.2 and 14.2±7.9% respectively. At concentrations of LAS of 0.5 mg/L or higher, the differences in both percentage of motility and fertilizing ability with controls were significant ($p<5\%$).

Although extrapolation from the laboratory to the field requires caution, the results of this work demonstrated that low-level surfactant pollution may impact directly on reproduction of the free gametes (sperm) released into water. It may lead to a long-term decline and eventual extinction of gilthead populations in nature when they are located close to effluents that are either untreated or receive inadequate secondary treatment. It is also quite important because this species constitutes an important link in the food chain and its death via exposure to surfactants may imbalance the littoral ecosystem.

Key words: Pollution, Sufactant, *Sparus aurata,* Sperm, Motility, Fertilization

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Introduction

The worldwide uses of surfactants show that about 70% are anionic and among them, Linear Alkylbenzene Sulphonate (LAS) is the world's leading type in terms of volume consumption, with a total industrial production of 1.5 million tons (Perales et al., 1999). Its surface active properties make it important in hundreds of household and industrial cleaners, personal care products as well as a delivery aid in pharmaceuticals and in biochemical research involving electrophoresis (Singer and Tjeerdema, 1993). In addition, anionic surfactants such as LAS are important components of dispersants, whose use in oil-spill clean up programs has been contentious for many years (Hatcher and Larkum, 1982).

Due to their widespread use, surfactants are common constituents in municipal effluents and in the corresponding receiving fresh- and sea- water environments (Gonzalez-Mazo et al., 1999). In this respect, concentrations of LAS of 0.12 mg/L have been encountered in the Bay of Cadiz (Spain, 36º30' N, 6º15' W) at a distance of around 5 km from the discharge point of an untreated urban effluent (Gonzalez-Mazo et al., 1997).

Ecotoxicology is concerned with describing and predicting the behaviour of substances in the environment and the response of biological systems, and ultimately with assessing the risks associated with emissions (DelValls and Conradi, 1999). In this respect, faunal and chemical monitoring has frequently been used to assess environmental quality (Martin and Richardson, 1995). The use of biota as an indicator of pollution is advantageous over chemical analysis as they are ecologically realistic (Pocklington and Wells, 1992). Gilthead *Sparus aurata* L. was used as a bioassay test species because it is considered a widespread fish with a great economic-commercial interest in the fishing industry and in extensive aquaculture production of many southern European countries such as Spain (Arias et al., 1976). Furthermore, these days it is of great importance to evaluate the effects of pollution on fish

both for environmental protection and for socio-economic reasons (Lin and Hwang, 1998).

To date, ecotoxicological effects of anionic surfactants on aquatic species have been studied mainly in juvenile and adult life stages (Okuwosa and Omoregie, 1995; Ribelles et al., 1995; Rosety et al., 2000). The effects of this phenomenon on the early developmental stages and free gametes have received little attention (Ankley and Burkhard, 1991; Kusk and Petersen, 1997; Nipper et al., 1993; Rosety et al., 2001a,b). So, the need to implement toxicity bioassays to protect early life stages of aquatic organisms has encouraged the development of bioassays using gametes and larvae as test organisms (Larrain et al., 1999).

In fish, on the contrary to mammals, large volumes of sperm and thousands of oocytes can be easily obtained for in vitro fertilization studies. Further, the sperm cell toxicity test requires only 60 minutes of toxicant exposure whereas other marine embryo and larval development tests require 2 to 4 days of exposure, both having similar sensitivities (Dinnel, 1995).

For the reasons already mentioned, this experimental design was undertaken to evaluate the acute impact of several concentrations (0.1, 0.5, 1, 2 and 4 mg/L) of anionic surfactant LAS on the motility and fertilizing ability of gilthead sperm.

Materials and methods

LAS is sold in the form of a mixture of homologues in which the length of the alkyl chain varies between 10 and 14 carbon atoms (C10-LAS to C14-LAS). The proportions of these five homologues in the various commercial formulations depend on the specific application of the detergent product (Perales et al., 1999).

In our case, the anionic surfactant C10-13 linear alkylbenzene sulphonate, with a purity greater than 99%, was provided by Fluka. LAS was dissolved in deionized water to form a stock solution which was diluted with sperm-free seawater in order to obtain the desired concentrations. The seawater used for dilution and control tubes was filtered through a 0.5 μm cellulose filter prior to being used. Its physiological characteristics before adding LAS were as follows: salinity 33-35‰, pH 7.4, temperature 20±1 ºC, dissolved oxygen 8-8.6 mg/L, total hardness 100 mg CO_3Ca/L, surface tension 72.7 mN/m and absence of heavy metals.

Adult giltheads *Sparus aurata* L. were collected from a culture unit at San Fernando (Cadiz, Spain). Three males were chosen to collect the sperm stock for our bioassay. Milt was kept at 4 °C in a dry undiluted state until the beginning of both experiments. Samples contaminated with urine or blood were discarded. Sperm stock was diluted to the concentration of $7x10^7$ sperm/ml. Neubauer chamber was used for calculation of sperm concentration.

Billard and Cosson (1992) have described a two-step dilution process, whereby sperm is initially diluted in an extender, in which it remains inmotile, while the final dilution takes place on the microscope slide itself. Due to the very short duration of motility after dilution, the addition of LAS to the extender permits the study of pollutant effects over a longer period. Percentage of motile sperm was estimated subjectively under a microscope (x200 magnification) from the moment of the final dilution (Ciereszko and Dabrowski, 2000).

We also selected three reproductive females, varying in weight between 4 and 4.5 kg whose eggs had shown previously a 90% fertilization success or more, to contribute to the pool of eggs for each bioassay. Spawning was artificially induced by a photoperiod regime (Kadmon et al., 1985). Breeders were maintained at a constant temperature of 20±1 ºC and at 33-35‰ salinity.

Released eggs were filtered through a relatively coarse screen to remove debris and washed 3 times in 300 mL of seawater before being used in the bioassay. The egg stock was diluted to 2000 eggs/ml using a Sedgwick-Rafter chamber to count them. In the experiments to determine surfactant toxicity on sperm fertilizing ability, 0.1 mL of sperm suspensions were exposed for 1 hour in individual test tubes containing LAS in concentrations of 0 (controls), 0.1, 0.5, 1, 2 and 4 mg/L. To prevent the adhesion of sperms, the tubes were precoated by dipping in 1% polyvinyl alcohol solution (average molecular weight 30000-70000; Sigma) and dried at 60 °C (Perchec et al., 1995).

After this period, 1 ml of the unexposed egg suspension was added to each test tube. After 20 minutes exposure during which fertilization took place, the tests were stopped by adding 2 ml of a 10% solution of formaldehyde in seawater. Finally, fertilization rate in a 100-egg subsample per replicate was analyzed under a microscope.

Fertilization, defined as the presence of a fertilization envelope, was assessed by microscopic observation. While Dinnel et al. (1982) considered unfertilized eggs if they had partially formed membranes, we considered them fertilized since the test endpoint is the evaluation of the fertilizing capability of the sperm. In this respect, partial membranes indicate the existence of enough viable sperm to fertilize the eggs, in spite of the egg's inability to raise the whole fertilization membrane (Nipper et al., 1993).

It may be noted that the series of concentrations for both motility and fertilizing ability studies were tested in triplicate.

The test results were expressed as percentages of motility and fertilization (means ± SD). Point to point comparisons of control and experimental data were performed using Duncan's Multiple Range Test with a 95% degree of confidence. The "trimmed Spearman-Karber" method for estimating median lethal concentrations in toxicity tests (Hamilton et al., 1977, 1978) was performed in this study to determine the EC50 for the effects of LAS on the fertility success of gilthead sperm.

Results

Preincubation of sperm suspensions with different concentrations of LAS caused a reduction in the motility and a consequent lack of fertilization of gilthead sperm. The individual results of the several tests prepared are presented in Table 1.

In this respect, the percentage of motile sperm at 0.1, 0.5, 1, 2 and 4 mg/L of LAS were respectively 89.8±9.8, 81.7±16.3, 69.5±21.3, 57.1±19.1 and 21.2±10.9%. At concentrations of LAS of 0.5 mg/L or higher, percentages of motile sperm were significantly different from controls.

Sperm/egg ratios of 3500 were selected for subsequent experiments to evaluate the effects of LAS on the sperm fertilizing capability. Using this ratio, fertilization rate in controls was 92.5±4.7 %. In addition, the potential problem of high sperm concentrations masking the toxicity of LAS was avoided by mean of this ratio.

Percentage of fertilized eggs following 60 minutes exposure of *Sparus aurata* sperms to different concentrations (0.1, 0.5, 1, 2 and 4 mg/L) of LAS were 85.7±8.1, 75.1±20.2, 62.9±19.7, 52.7±19.2 and 14.2±7.9% respectively. At concentrations of LAS of 0.5 mg/L or higher, percentages of fertilization success were significantly different from controls. Besides, the EC50 value for fertilization of giltheads after sperm exposure to LAS was found to be 2.6 mg/L.

Discussion

Due to their widespread use of surfactants, it may be assumed that they are one of the major sources actant toxicity to the aquatic ecosystem in detail. To assess the potential hazards of these pollutants on fish reproduction we considered in agreement with (Kime et al., 1996), that fresh sperm might provide a useful tool as a convenient biomonitor of pollution.

Since it was also suggested that the motility of sperm might be used as a sensitive and readily available species-specific bioindicator of pollution (Kime, 1995), we have assessed the acute impact of LAS on the motility of gilthead sperm. Unlike mammals in which sperm is motile for some hours after ejaculation, fish sperm ceases all movement after 1-2 minutes (Billard and Cosson, 1992), which constitutes the main problem encountered in these studies. The results of our investigations showed that preincubation with concentrations of LAS of 0.5 mg/L or higher decreased sperm motility in a significant way ($p<5\%$). However further studies are required to determine how decreased sperm motility may relate to decreased fertility.

In agreement with Harrison and Wallace (1990), fertilization success was used as the criterion of this investigation because of its potential vulnerability to external influences and for the importance of repeated successful spawning years for the long-term maintenance of these populations in nature.

In the present study, an inhibitory effect of anionic surfactant LAS on fertilization success of gilthead sperm. was also found. In this respect, the exposure to LAS concentrations of 0.5 mg/L or higher for 60 minutes significantly reduced fertilizing capability of gilthead sperms.

Clear dose-response relationship for the reduction on motility and fertilizing ability of *Sparus aurata* sperm was observed for LAS. The first relationship was described previously by Kime et al. (1996) on catfish Clarias gariepinus sperm exposed to cadmium and zinc. The second one was reported by Vaschenko et al. (1999) on sperm of sea urchin *Anthocidaris crassispina* (Agassiz) treated with cadmium.

In a previous study, Nipper et al. (1993) reported the toxicity of an also known anionic surfactant, sodium dodecyl sulphate (SDS), on echinoderm *Lytechinus variegatus* Lmk. sperm. The EC50 in this experiment was found to be 2.9 mg/L of SDS. Although differences in experimental procedures and conditions between the studies urge to proceed with caution, these data suggest that the sensitivity of gilthead sperm to anionic surfactants was in a similar range to that of echinoderm *Lytechinus variegatus*.

While the mechanisms of surfactant toxicity on gilthead sperm remain to be ascertained, potential targets of its toxicity may very well include a decrease in surface tension (Prat and Giraud, 1964), destruction of biological membranes and subcellular organelles (Ribelles et al., 1995) as well as the alteration of the function of some enzymes (Thorhaug, 1992), etc.

Although extrapolation from the laboratory to the field requires caution, the results of this work concluded low-level surfactant pollution may impact directly on reproduction of the free gametes (sperm) released into water, causing sperm immobilization and a consequent lack of fertilization. It may lead to a long term decline and eventual extinction of gilthead populations in nature when they are located close to effluents that either are untreated or receive inadequate secondary treatment. It is also quite important because gilthead *Sparus aurata*, L constitutes an important link in the food chain and its death via exposure to surfactants may imbalance the littoral ecosystem.

Table 1. Motility and fertilizing ability of gilthead sperm preincubated for 60 minutes with different concentrations of LAS.

TREATMENT (LAS)	MOTILITY	FERTILIZING ABILITY
0 mg/L (control)	95.8±3.2	92.5±4.7
0.1 mg/L	89.8±9.8	85.7±8.1
0.5 mg/L	81.7±16.3 *	75.1±20.2 *
1 mg/L	69.5±21.3 *	62.9±19.7 *
2 mg/L	57.1±19.1 *	52.7±19.2 *
4 mg/L	21.2±10.9 *	14.2±7.9 *

*: significantly different from the control, $p<5\%$ (Duncan's Test).

References

Ankley G.T. and Burkhard L.P. (1991). Identification of surfactants as toxicants in a primary effluent. Environ. Toxicol. Chem. 11, 1235-1248.

Arias A.M., Drake P. and Rodriguez R.B. (1976). Los esteros de la salina de San Fernando (Cádiz, España) y el cultivo extensivo de peces marinos. In: L´ aquaculture du Bar et des Sparides. INRA. Paris.

Billard R. and Cosson M.P. (1992). Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool. 261, 122-131.

Ciereszko A. and Dabrowski K. (2000). In vitro effect of gossypol acetate on yellow perch (*Perca flavescens*) spermatozoa. Aquat. Toxicol. 49, 181-187.

DelValls T.A. and Conradi M. (1999). Advances in marine ecotoxicology: laboratory tests versus field assessment data on sediment quality studies. Cienc. Mar. 26, 39-64.

Dinnel P.A., Stober Q.J., Crumley S.C. and Nakatami R.E. (1982). Development of a sperm cell toxicity test for marine waters. In: Aquatic toxicology and Hazard assessment. Pearson J.G., Foster R.B. and Bishop W.E. (eds.). Fifth Conference, ASTM STP 766, American Society for Testing and Materials. Philadelphia. p 82.

Dinnel P.A. (1995). Evolution and present status of the sea urchin sperm test. Russ. J. Marine Biol. 21, 344-350.

Gonzalez-Mazo E., Honing M., Barcelo D. and Gomez-Parra A. (1997). Monitoring long chain intermediate products from the degradation of linear alkylbenzene sulfonates in the marine environmental by solid-phase extraction followed by liquid chromatography/ionspray mass spectrometry. Environ. Sci. Technol. 31, 504-510.

Gonzalez-Mazo E., Quiroga J.M. and Gomez-Parra A. (1999). Linear Alkylbenznes Sulphonates (LAS) as tracers of urban waste waters in shallow littoral ecosystems: a case study in the Bay of Cadiz. Cienc. Mar. 25, 367-379.

Hamilton M.A., Russo R.C. and Thurston R.V. (1977). Trimmed Spearman Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714-719. Correction (1978) 12, 417.

Harrison P.L. and Wallace C. C. (1990). Reproduction, dispersal and recruitment of scleractinian corals. In: Coral reefs. Ecosystems of the world. Dubinsky D. (ed). Elsevier. Amsterdam.

Hatcher A.I. and Larkum A.W.D. (1982). The effects of the short term exposure to Bass Strait crude oil and corexit 8667 on benthic community metabolism in Posidonia australis. Hook. F. dominated microcosms. Aquatic Botany 12, 219-227.

Kadmon G., Yaron Z. and Gordin H. (1985). Sequence of gonadal events and oestradiol levels in *Sparus aurata* L. under two photoperiod regimes. J. Fish. Biol. 26, 609-620.

Kime D.E. (1995). The effects of pollution on reproduction in fish. Rev. Fish. Biol. 5, 52-56.

Kime D.E., Ebrahimi M., Nysten K., Roelants I., Rurangwa E., Moore H.D.M. and Ollevier F. (1996). Use of computer assisted sperm analysis (CASA) for monitoring the effects of pollution on sperm quality of fish; application to the effects of heavy metals. Aquat. Toxicol. 36, 223-237.

Kusk K.O. and Petersen S. (1997). Acute and chronic toxicity of tributyltin and linear alkylbenzene sulfonate to the marine copepod *Acartia tonsa*. Environ. Toxicol. Chem. 16, 1629-1633.

Larrain A., Riveros A., Silva J. and Bay-Schmith E. (1999). Toxicity of metals and pesticides using the sperm cell bioassay with the sea urchin *Arbacia spatuligera*. Bull. Environ. Contam. Toxicol. 62, 749-757.

Lin H.C. and Hwang P.P. (1998). Acute and chronic effects of gallium chloride (Ga Cl3) on tilapia (*Orechromis mossambicus*) larvae. Bull. Environ. Contam. Toxicol. 60, 931-935.

Martin M. and Richardson B.J. (1995). A paradigm for integrated marine toxicity research?. Further views from the Pacific Rim. Mar. Poll. Bull. 30, 8-13.

Nipper M.G., Prosperi V.A. and Zamboni A. J. (1993). Toxicity testing with coastal species of the southeastern Brazil. Echinoderm sperm and embryos. Bull. Environ. Contam. Toxicol. 50, 646-652.

Okuwosa V.N. and Omoregie E. (1995). Acute toxicity of alkylbenzene sulphonate (ABS) detergent to the toothed carp, Aphyosemion gairdneri L. Aquacult. Res. 26, 755-758.

Perales J.A., Manzano M.A., Sales D. and Quiroga J.M. (1999). Linear Alkylbenzene Sulphonates: Biodegradability and Isomeric Composition. Bull. Environ. Contam. Toxicol. 63, 94-100.

Perchec G., Jeulin C., Cosson J., Andre F. and Billard R. (1995). Relationship between sperm ATP content and motility of sperm spermatozoa. J. Cell Sci. 108, 747-753.

Pocklington P. and Wells P.G. (1992). Polychaetes: Key taxa for marine environmental quality monitoring. Mar. Poll. Bull. 24, 593-598.

Prat R. and Giraud A. (1964). The pollution of water by detergents. O.E.C.D. Paris.

Ribelles A., Carrasco C., Rosety M. and Aldana M. (1995). Morphological and histochemical changes on the liver and pancreas of giltheads, *Sparus aurata* L induced by acute action of the anionic detergent sodium dodecyl sulphate. Histol. Histopathol. 10, 781-787.

Rosety M., Ribelles A., Rosety-Rodriguez M., Carrasco M., Ordoñez, F.J. and Rosety J.M. (2000). Morpho-histochemical study of the biological effects of sodium dodecyl sulphate on the digestive gland of the Portuguese oyster. Histol. Histopathol. 15, 1137-1143.

Rosety M., Ordoñez F.J., Rosety-Rodriguez M., Rosety J.M., Rosety I., Carrasco C. and Ribelles A. (2001a) Acute toxicity of anionic surfactants sodium dodecyl sulphate (SDS) and linear alkylbenzene sulphonate (LAS) on the fertilizing capability of gilthead (*Sparus aurata* L) sperm. Histol. Histopathol. 16, 839-843.

Rosety M., Ordoñez F.J., Rosety-Rodriguez M., Rosety J.M., Rosety I., Carrasco C. and Ribelles A. (2001b) Comparative study of the acute toxicity of the anionic surfactants alkyl benzene sulphonate (ABS) and sodium dodecyl sulphate (SDS) on gilthead, *Sparus aurata L*., eggs. Histol. Histopathol. 16, 1091-1095.

Singer M.M. and Tjeerdema R.S. (1993). Fate and effects of the surfactant sodium dodecyl sulfate. Rev. Environ. Contam. Toxicol. 133, 95-149.

Thoraugh A. (1992). Oil spills in the tropics and subtropics. In: Pollution in tropical aquatic systems. Connel D.W. and Hawker D. (eds). CRC Press. London.

Vaschenko M.A., Zhadan P.M., Malakhov V.V. and Medvedeva L.A. (1999). Toxic effect of mercury chloride on gametes and embryos in the sea urchin Strongylocentrotus intermedius. Russ. J. Marine Biol. 21, 300-307.

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