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Presence, biotransformation and effects of sulfophenylcarboxylic acids in the benthic fish *Solea senegalensis*

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Abstract

The presence of linear alkylbenzene sulfonates (LAS) and their degradation intermediates, sulfophenylcarboxylic acids (SPCs), with concentrations up to 100 ppb has been found in surface waters taken from the estuary of the river Guadalete (Cádiz, SW of Spain). Higher concentrations were found at the sampling site located adjacent to the discharge outlet of a wastewater treatment plant (WWTP). The concentrations decreased downstream to a few ppb as a result of dilution, sorption, and degradation processes, which were taking place along the estuary. Once the presence of both xenobiotics was confirmed in the environment, an *in vivo* assay was conducted to study their biotransformation and effects in the benthic fish *Solea senegalensis*. A flow-through system was employed, consisting of an exposure phase (120 h) with environmental levels of the surfactant (200, 500 and 800 μg/L of 2ØC$_{10}$LAS), followed by a depuration stage (72 h). The generation of SPCs has been quantified during these phases in both water and fish, with LAS biotransformation shown in all cases. The antioxidant enzymes catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), the phase II enzyme glutathione S-transferase (GST), and the phase III acid and alkaline phosphatases (AcP, ALP) were also estimated and utilized as biomarkers.

Keywords: Surfactants; Linear alkylbenzene sulfonate; Sulfophenylcarboxylic acids; Estuaries; Biotransformation; Biomarkers; Flow-through system; Fish

1. Introduction

Linear alkylbenzene sulfonates (LAS) are the most widely produced and used synthetic surfactants, mainly in the formulation of detergents and other cleaning products. About 98% of the total LAS used by the population is removed in sewage treatment plants (Feijtel et al., 2000) and the rest is discharged to aquatic ecosystems. A continuous input of these compounds is responsible for their presence in receiving waters and therefore their primary degradation products, sulfophenyl-carboxilyc acids (SPCs), also occur (González-Mazo et al., 1997; Marcomini et al., 2000; Yadav et al., 2001; León et al., 2002). Most studies for the determination of environmental levels of these metabolites have been carried out in fresh waters, and a wide range of concentrations has been reported: up to 53 μg/L in U.S. rivers (Trehy et al., 1996), from 0.5 to 3.1 μg/L in rivers in Taiwan (Ding et al., 1999) and 1.8 and 5.0 μg/L in the Rhine and Llobregat rivers respectively (Eichhorn et al., 2002). Coastal marine and estuarine waters subjected to wastewater discharges tend to show higher SPC concentrations, probably due to the slower biodegradability of LAS in these media (Vives-Rego et al., 1987): up to 120 μg/L in a tidal channel in Cadiz, Spain (González-Mazo et al., 1997), from 2.6 to 420 μg/L in a lagoon in Venice, Italy (Marcomini et al., 2000) and concentrations of several hundreds of ppb in different estuaries in the north of Spain (León et al., 2002). These papers also confirm that the highest SPCs values are often associated with treated or untreated wastewaters discharges into the environment; decreasing from the point source as a result of dilution, sorption, and degradation processes.

Organisms living in receiving waters are susceptible to LAS bioconcentration and biotransformation. In this context, LAS and SPC concentrations ranging from 0.35 to 8.76 μg/g, and from 0.02 to 0.63 μg/g, respectively, have been reported in several marine species (*Sparus aurata*, *Diplodus vulgaris*,

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Ruditapes semidecussatus, *Carcinus maenas*, *Liocarcinus vernalis* and *Cerastoderma edule*) collected in littoral ecosystems (Sáez, 2002). Furthermore, laboratory studies have detected the generation of polar LAS metabolites in the bile of fathead minnow and carp (Comotto et al., 1979; Newsome et al., 1995), in the urine of channel catfish (Schmidt and Kimerle, 1989), and have confirmed that the biotransformation of LAS in fishes is carried out by fish enzymes rather than by microorganisms in the gut. The kinetics of this biotransformation process have been also studied in *in vivo* assays in fathead minnow (Tolls et al., 2000), which indicate that biotransformation into SPCs is a process that significantly reduces the bioaccumulation potential of LAS.

The toxicity of SPCs is not known with any certainty; but it has been confirmed to be lower than that of LAS (LC 50 values for SPCs 120–240% higher than those for LAS were found by Kimerle and Swisher, 1977), and non-estrogenic effects were observed by Navas et al. (1999) for LAS and SPCs in *in vitro* assays.

Biomarkers based on specific enzyme activities are useful tools for evaluating these and others toxicological effects of xenobiotics, but there is a paucity of information related to surfactants. Studies available report the performance of *in vitro* assays for the non-ionic surfactants Triton X-100 and Tween 80 (Da Silva and Meirelles, 2004), *in vitro* and *in vivo* experiments for LAS with the clam *Ruditapes philippinarum* (Blasco et al., 1999) and mussels *Mytilus* sp. (Da Ros et al., 1995). However all these experiments were carried out employing static and semi-static assays, where there is no continuous water flow and xenobiotic inputs as in flow-through systems and in the real environment.

To our knowledge, there are no *in vivo* studies performed at environmental concentrations of LAS that relate the concentration of the surfactant in the organisms, the generation of the metabolite products (SPCs) and the toxic effects observed. For this reason, a survey was conducted in the estuary of the river Guadalete (SW of Spain) in order to determine the environmental LAS and SPCs concentrations in waters from this area, followed by the performance of an *in vivo* assay using a flow-through exposure system. LAS concentrations of the same order of magnitude as those detected in a previous sampling were used in the exposure water to simulate the subchronic effects of LAS and its transformation products in the benthic fish *Solea senegalensis*. Five different biomarkers were also selected to evaluate toxicological effects of LAS and SPCs. Catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR) are key components of the antioxidant defence system and useful tools for estimating oxyradical-mediated responses (Di Giulio et al., 1989). The phase II enzyme glutathione S-transferase (GST), which plays an important role in homeostasis as well as in detoxification and removal of many xenobiotic compounds, and the acid and alkaline phosphatases (AcP and ALP respectively), very important for regulation of various metabolic processes that occur by phosphorylation and dephosphorylation with kinases (Sparks and Brautigan, 1986), have also been monitored.

2. Materials and methods

2.1. Experimental

The survey was carried out along the estuary of the river Guadalete (SW of Spain) (Fig. 1). El Puerto de Santa María (100,000 inhabitants) and Jerez de la Frontera (200,000 inhabitants) are the two main towns situated on this estuary. There are wastewater treatment plants serving both towns, but while that of El

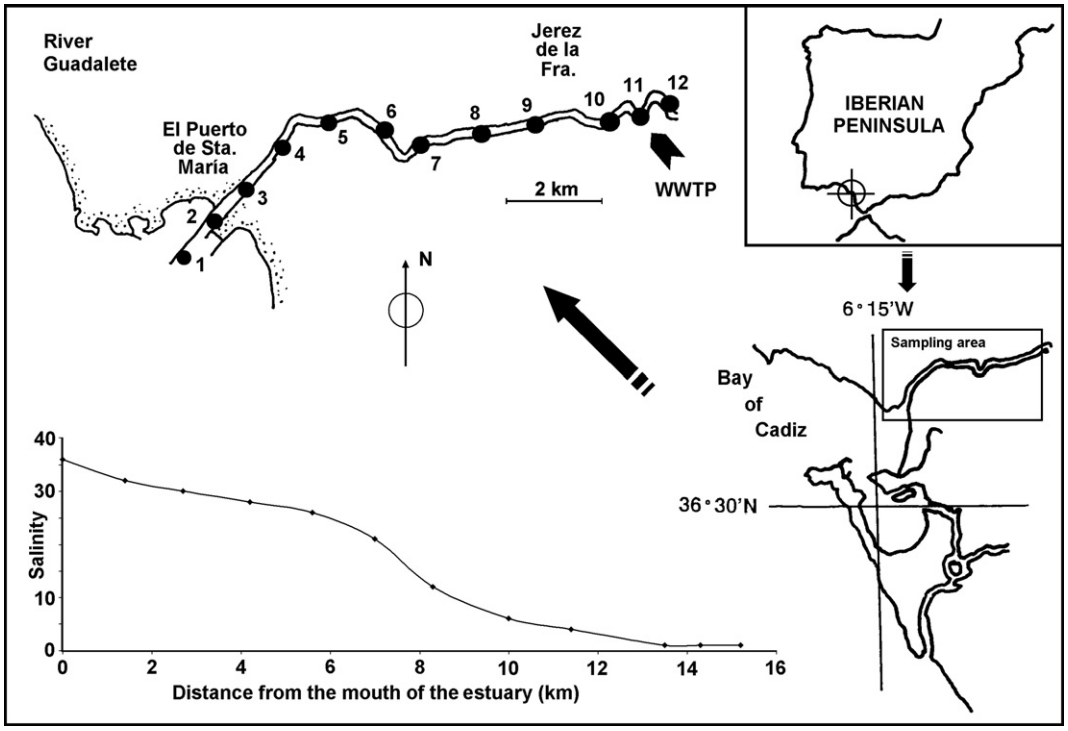

Fig. 1. Map of the sampling area showing the location of the sampling sites and the longitudinal salinity profile along the Guadalete estuary.

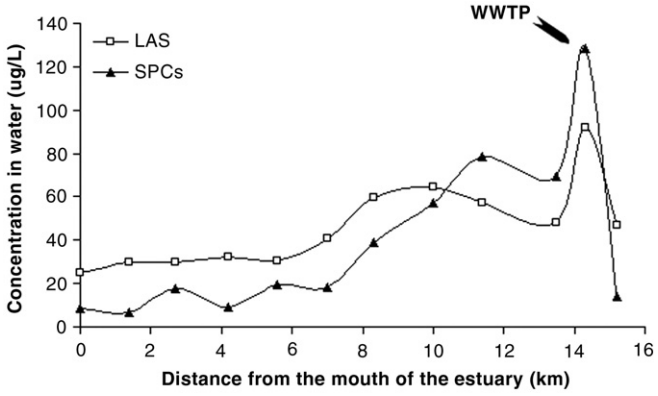

Fig. 2. Longitudinal variations of LAS and SPC concentrations (μg/L) along the Guadalete estuary.

Puerto de Santa María discharges into the ocean, that of Jerez de la Frontera has its discharge outlet located near site number 11. Water samples were collected from the surface on an ebbing tide using 2 L rinsed glass containers, at twelve sampling sites. Salinity was measured using an induction salinometer (Fig. 1) and 4% formaldehyde was added to the samples, which were stored in the dark at −20 °C until they were analyzed (in duplicate).

An *in vivo* assay has been carried out in a continuous flow-through system for a duration of 192 h (in accordance with OECD guideline 305) using seawater spiked with 2ØC$_{10}$LAS (100% purity and 99% active index), supplied by Petroquímica Española S.A. Different concentrations (200, 500 and 800 μg/L) were used during the exposure period (120 h), followed by a depuration period (72 h). The rate of water renewal during both phases was five times per day. A control tank was kept under the same conditions but without addition of surfactant. The organism chosen was the benthic fish *S. senegalensis* with a body weight between 3 and 6 g, specimens of which were purchased from the aquaculture facilities of our university.

2.2. Analytical methods

LAS and SPC concentrations in water were analysed using the procedure described by León et al. (2000). The same target compounds were determined in the whole organisms following the methodology developed by Álvarez-Muñoz et al. (2004) and Sáez et al. (2000) respectively.

2.3. Enzyme bioassays

The selected target tissue was the gill; dissection was performed at the end of the exposure and depuration phases, and the samples were stored until processing at −80 °C. About 0.5 g of gill tissue (pooled sets from three fishes) was homogenised after the addition of Henriksson buffer (50% glycerol, Tris–HCl 100 mM, 5 mM β-mercapto-ethanol, 0.5 EDTA, 0.02% BSA and Protease inhibitor) (1:4, w/v) and two centrifugations were performed. The first one, at 9000 ×*g* for 15 min at 4 °C, was used in order to obtain the post-mitochondrial fraction which is needed for the GPX assay, and the second at 50,000 g for 2 h at 4 °C, for the cytosolic fraction.

Reagents were purchased from Sigma-Aldrich (Steinheim, Germany) and the enzyme assays (CAT, GST, GR and GPX) were performed using the methodology followed by Mc Farland et al. (1999). The acid and alkaline phosphatases were determined according to Blasco et al. (1993) and the protein concentrations were estimated by the BCA method (Smith et al., 1985).

For the statistical analysis, the SPSS 11.5 for Win computer program was used. ANOVA and the *post hoc* Tukey test were used to test differences between groups. The differences were regarded as statistically significant when $p<0.05$.

3. Results and discussion

3.1. Environmental concentrations of LAS and SPCs

Fig. 2 shows LAS and SPC concentrations found in water along the estuary of the river Guadalete. The highest concentrations for both compounds (92 and 129 μg/L for LAS and SPCs, respectively) correspond to those measured at site number 11 (14th Km from the mouth). As shown in Fig. 1, the WWTP discharge outlet from the town of Jerez de la Frontera is situated adjacent to this station. This must represent the main input of LAS and SPCs into the estuary because concentrations for both compounds show a sharp decrease upstream (47 and 14 μg/L for LAS and SPCs respectively at the adjacent station number 12). Values for SPCs near the discharge outlet are higher than those for LAS as expected because LAS is degraded into SPCs inside the WWTP, and both sets of compounds are of the same order of magnitude as those found near the discharge points of other WWTPs (Trehy et al., 1996; Eichhorn et al., 2002).

Moving downstream, concentrations of both LAS and SPCs show a decrease due to dilution, adsorption–precipitation and degradation processes, as previously described by González-Mazo et al. (1998) and León et al. (2002) among other authors. At the mouth of the estuary only a few ppb of these compounds are detected in the water. A similar behaviour has been described previously in other estuaries along the Spanish coasts (León et al., 2002) where LAS and SPC concentrations decrease from values between 400 and 800 μg/L, near wastewater discharges, to undetectable at the mouth of the rivers and in the adjacent littoral zones.

3.2. Biotransformation of SPCs

Having confirmed the presence of LAS and SPCs in concentrations of several tens to hundreds of ppb in the estuarine waters, an *in vivo* assay

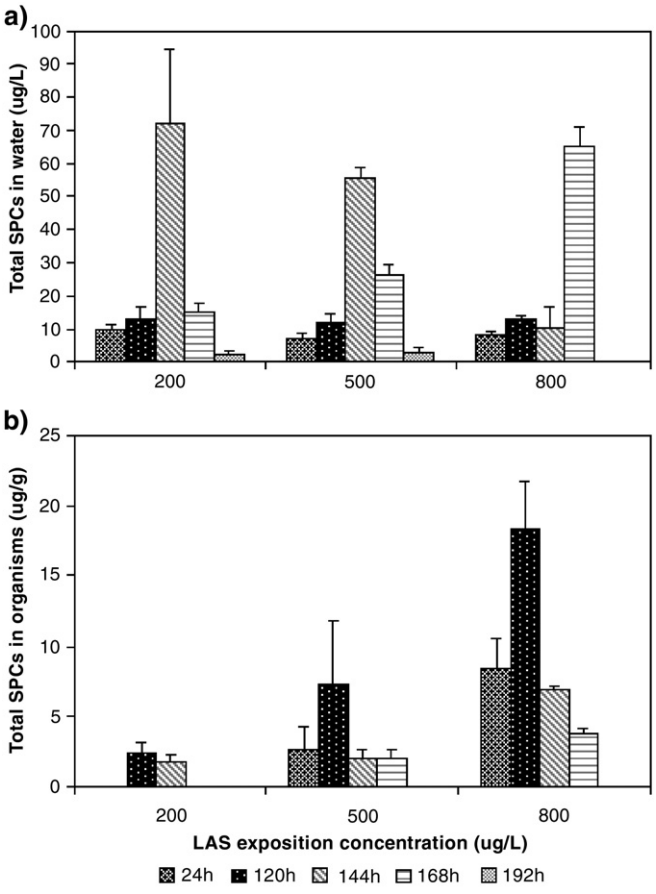

Fig. 3. Concentrations of total SPCs determined at three different LAS exposure concentrations (200, 500 and 800 μg/L) tested over time (24 and 120 h correspond to the exposure phase, and 144, 168 and 192 h correspond to the depuration phase) (a) in experimental water ($n=4$) and (b) in whole fish (*Solea senegalensis*) ($n=4$).

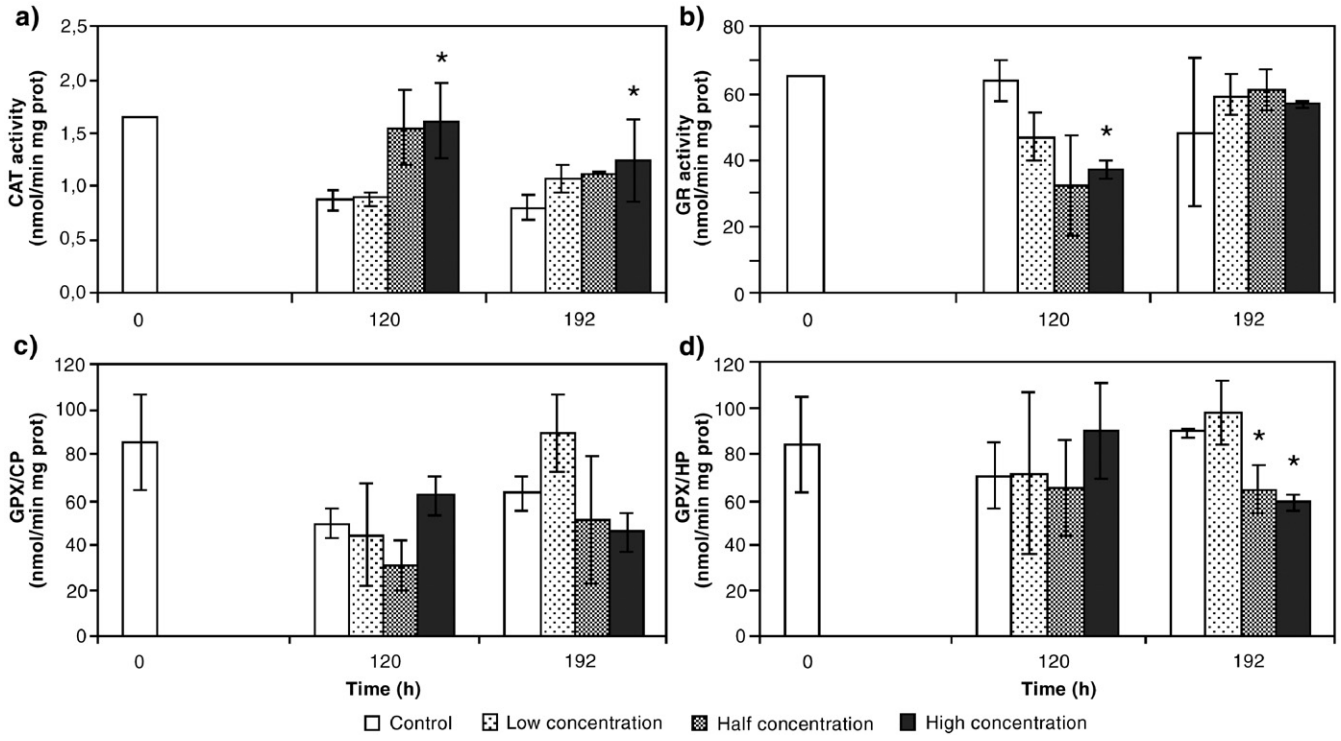

Fig. 4. Activities of antioxidant enzymes in gills of controls and fish exposed to three different LAS concentrations measured in the whole body of the organism, before the experiment (0 h), after the exposure phase (120 h) and at the end of the elimination phase (192 h). The activities are expressed as mean ± SD (n = 4). a) CAT, b) GR, c) GPX/CP, d) GPX/HP activity and an asterisk indicates a significant ($p < 0.05$) difference from the control.

was carried out in a continuous flow-through system in order to estimate biotransformation and toxicological effects of the two target compounds in *S. senegalensis*, a representative benthic fish from the area of study. The fish were exposed to a constant concentration of either 200, 500 or 800 μg/L of 2ØC$_{10}$LAS in a flow-through regime, and the concentrations of SPCs were measured at different times (24,120,144,168 and 192 h) during the experiment. The total concentration of SPCs determined in the experimental water (μg/L) and in whole body of *S. senegalensis* (μg/g) are shown in Fig. 3a and b respectively (as the sum of compounds C$_6$SPC to C$_{10}$SPC). During the exposure phase, the concentration of LAS degradation products in the experimental water is similar for the three exposure concentrations tested (Fig. 3a). A slight increase in the concentration of SPCs in water has been observed between the beginning of the assay (24 h) and the end of the exposure stage (120 h). This increase is due to the excretion of intermediate degradation products after the biotransformation of LAS by the fish and the microbial degradation of LAS in the water of the aquarium.

The elimination phase starts when the fishes are transferred to clean water. At 24 h after the start of this stage (144 h after the start of the experiment as a whole) the highest concentration of SPCs in water was detected in the tanks corresponding to 200 and 500 μg/L of LAS exposure (72 ± 22.4 and 55 ± 3.1 μg/L of SPCs respectively) (Fig. 3a). However, for the highest exposure concentration assayed (800 μg/L of LAS), the highest concentration of SPCs in water (65 ± 5.6 μg/L) was found 48 h after the start of this stage (after 168 h of the experiment as a whole). During the depuration phase the concentration of SPCs measured in the water must be due solely to the biotransformation processes in the fish because there was no inflow of LAS into the depuration tanks. At the end of the depuration stage (after 192 h), the concentration of SPCs in the water is lowest, near to zero, because the biotransformation of LAS by the organisms should be almost complete.

The highest concentration of SPCs in whole organisms increased with the increase in LAS exposure concentration and with the exposure period. Fig. 3b shows the concentrations of SPCs determined in the fish exposed to the highest concentration of LAS in water, 800 μg/L. As we can see in the same figure, for the three exposure concentrations tested, the highest concentrations of SPCs were observed at the end of the exposure stage (after 120 h) (2.34 ± 0.7; 7.31 ± 4.4 and 18.36 ± 3.4 μg/g, respectively). When the elimination period began, the concentration of SPCs in *S. senegalensis* decreased and this decrease was time- and concentration-dependent, the concentrations falling to undetected levels at the end of the experiment. This decrease is matched by the decreasing concentrations detected in the water. Therefore, these metabolites must originate from the biotransformation and subsequent excretion of SPC by the fish.

In Fig. 3b we can also see that for a LAS exposure level of 200 μg/L after 48 h of the elimination stage (168 h of the experiment), no SPCs were detected in the organisms. However, for 500 and 800 μg/L of 2ØC$_{10}$LAS exposition, no SPCs were detected after 72 h (192 h of the experiment).

3.3. Toxicological effects of LAS and SPCs

In order to relate the concentrations of LAS and SPCs measured in the organisms with their effects, the following biomarkers were studied: catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), and the acid and alkaline phosphatases (AcP and ALP respectively). The concentrations of SPCs have been reported above. LAS concentrations in the whole body of the fish were measured at the end of the exposure stage: 2.54 ± 0.7 μg/g, 7.18 ± 1.7 μg/g, 11.07 ± 2.4 μg/g, for exposure to 200 μg/L, 500 μg/l and 800 μg/L of 2ØC$_{10}$LAS respectively.

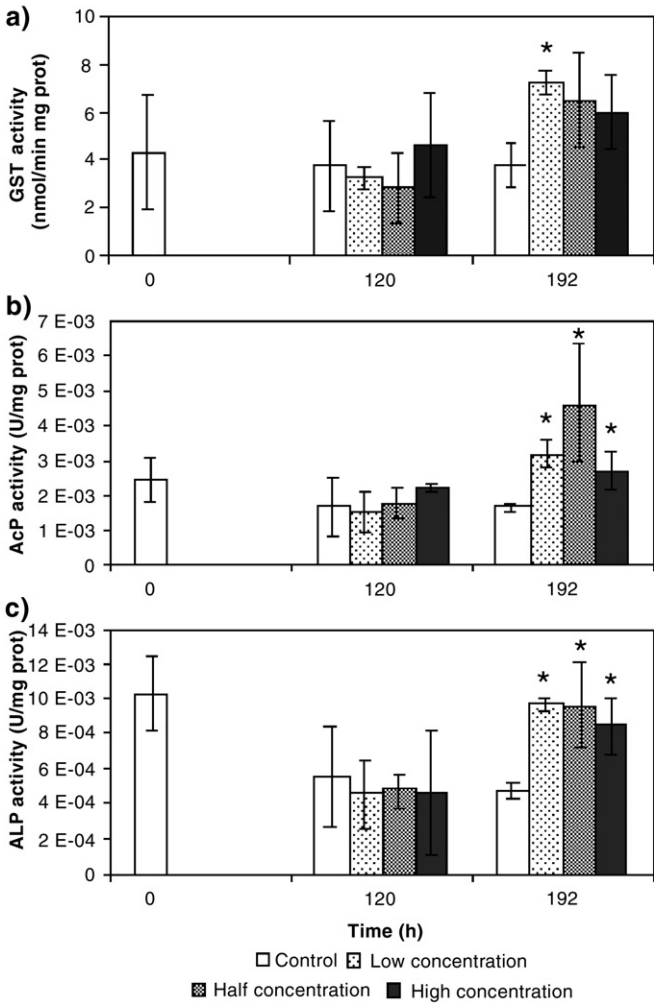

Fig. 5. Activities of (a) GST, (b) AcP, and (c) ALP in gills of *Solea senegalensis* exposed to LAS at three concentrations measured in the organism, before the experiment (0 h), after the exposure phase (120 h) and at the end of the elimination phase (192 h). The activities are expressed as mean±SD (n=4) and an asterisk indicates a significant (p<0.05) difference from the control.

The biomarkers were determined in the control group prior to the beginning of the experiment (0 h) to confirm the health of the organisms, and also in control and exposed fish at the end of the phases of exposure (120 h) and elimination (192 h). In Figs. 4 and 5 the activity of each biomarker studied is given at 0, 120 and 192 h at the three exposure concentrations tested during the first phase of the experiment, designated: low, half, and high concentration (200, 500 and 800 μg/L of 2ØC$_{10}$LAS).

The measured responses for oxidative stress are shown in Fig. 4. A significant increase (p<0.05) in CAT activity in the gills of *S. senegalensis* compared with the control group at each experimental stage (at 120 and 192 h) was found at the high exposure concentration (800 μg/L of 2ØC$_{10}$LAS exposure and 11.07±2.4 μg/g of LAS in the whole body measured at the end of the exposure phase). Similar results were also found in a study carried out with C$_{11}$-LAS on the clam *R. phillipinarum* where an increase of CAT activity was observed in the elimination stage (Álvarez-Muñoz et al., 2006). Da Ros et al. (1995) detected a slight increase of CAT activity in the digestive glands of mussels *Mytilus* sp. after 30 days exposure to LAS, and Da Silva and Meirelles (2004) reported an increase of CAT in the fish *Prochilodus scrofa* testing Triton X-100, a non-ionic surfactant.

A significant inhibition (p<0.05) of glutathione reductase GR was measured during exposure to the surfactant (Fig. 4b) comparing the high concentration group and the control. The same effect was observed by Rudneva-Titova and Zherko (1994) in red mullet exposed to PCBs. In the depuration phase no significant differences were found.

The results obtained with selenium-dependent (GPX/CP) and selenium-independent (GPX/HP) glutathione peroxidase are shown in Fig. 4c and d respectively. In the first case no significant differences were obtained whereas in the second there was a decrease (p<0.05) of the GPX/HP activity during the depuration phase. Da Ros et al. (1995) also found a similar effect for this enzyme in mussels exposed to LAS.

Fig. 5a shows the results obtained for GST activity. In the depuration stage there is an activation of this phase II enzyme which catalyzes the conjugation of electrophilic compounds with glutathione (GSH), resulting in the transformation of the compound. A significantly higher level of GST activity compared with the control group was observed in the low exposure concentration group (200 μg/L of 2ØC$_{10}$LAS exposure and 2.54±0.7 μg/g of LAS in the whole body measured at the end of the exposition phase). Increases in GST activity have been reported in several studies of different fishes exposed to PAHs, PCBs, OCPs and PCDDs (Van der Oost et al., 2003).

The AcP and ALP activities are shown in Fig. 5b and c respectively. In both cases a significantly higher activity (p<0.05) was found during the depuration period, comparing the control and the moderate concentration groups (500 μg/l of 2ØC$_{10}$LAS of exposure and 7.18±1.7 μg/g in the whole body measured at the end of the exposure phase).

Overall, significant inductions of CAT activity at 800 μg/L of LAS exposure, of AcP and ALP activity at 500 μg/L of LAS exposition and of GST activity at 200 μg/L were found during the elimination stage. The higher enzyme activities during this period are related to an increased SPC concentration in the water (Fig. 3a) and to a decreased concentration in the organisms in the elimination stage (Fig. 3b). GST was found to be the biomarker that is most sensitive to the lowest exposure concentration of LAS (200 μg/L 2ØC$_{10}$LAS), followed by AcP and ALP (sensitive to 500 μg/L 2ØC$_{10}$LAS) and lastly by CAT (sensitive to 800 μg/L 2ØC$_{10}$LAS). With respect to the inhibition of GR activity at 800 μg/L in the exposure phase, and of GPX/HP at the same LAS concentration exposure but in the depuration stage, this appears to be associated with the highest concentration of SPCs measured in the exposed organisms.

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