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Stress Biomarkers in Juvenile Senegal Sole, *Solea senegalensis*, Exposed to the Water-Accommodated Fraction of the "Prestige" Fuel Oil

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The "Prestige" oil spill occurred in November 2002 off the Galician Coast (NW Spain). Soon after the black tide episode, that caused an ecological disaster, several monitoring programs and projects evaluating the acute and long-term toxicity of the crude oil were initiated (see monograph Marine Pollution Bulletin 2006; vol. 53). Characteristics of the "Prestige" oil are described in Albaigés and Bayona (2003). Briefly, it consists of 50% polycyclic aromatic hydrocarbons (PAHs), mostly dominated by 2–3 ringed compounds which, contrarily in contrast to the higher molecular weight PAHs, are not reported as very toxic to fish (Seruto et al. 2005). Also, due to the heavy nature of this oil and its low solubility in water, the dispersion in sea water was very low and it mostly remained in situ forming oil patches adhered to rocks and sediment.

Fish, as with all aerobic aquatic organisms, face natural stress conditions when oxygen is not fully reduced, and intermediate reactive oxygen species (ROS) are formed. Enhanced ROS formation can also take place under exposure to some toxicants such as crude oil (Livingstone 2001). In particular, the water-accommodated fraction (WAF) of some crude oils has been responsible for oxidative stress situations in aquatic organisms (Cajaraville et al. 1992). Hence, in order to evaluate if exposure to the WAF of the "Prestige" crude would interfere with the antioxidant defense system of juvenile sole, *Solea senegalensis*, the activities of indicative enzymes were analyzed. These included: catalase (CAT), total glutathione peroxidase (t-GPX; sum of Selenium dependent and independent forms), glutathione reductase (GR) and DT-diaphorase (DTD), as part of the antioxidant defense system. However, when this natural protective system is saturated, damage to key molecules such as DNA, proteins or lipids can occur. The latter, termed lipid peroxidation (LP), was included in the present study as a biomarker of effect, indicating damage caused by ROS. The enzyme acetylcholinesterase (AChE) regulates nerve impulse transmissions and is used as a marker of exposure to neurotoxic substances (Galloway et al. 2006). Carboxylesterases (CbE) constitute an heterogeneous group of isozymes, dominant in the liver, that play a role in the metabolism and subsequent detoxification of many xenobiotics. In marine fish AChE and CbE are biomarkers used as indicators of neurotoxicity (Lionetto et al. 2004) and enhanced hepatic metabolism (Wheelock et al. 2005). The phase II conjugating enzyme glutathione *S*-transferase (GST) has also been included in the present work as a measure of the detoxification metabolism.

This study is part of a project aiming to evaluate the toxicity of the water-accommodated fraction (WAF) of the "Prestige" oil in juvenile *Solea senegalensis* after short-term (24, 48 and 72 h) exposure to environmentally realistic PAHs levels. The choice of this species was based on (1) its high economic interest in the affected region, (2) its benthic nature and, therefore, its adequacy for pollution monitoring programs and (3) its ease to rear under laboratory conditions.

Materials and Methods

Juvenile *Solea senegalensis* were provided by the Aquaculture facilities of the IRTA (Sant Carles de la Ràpita,

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Tarragona). Initial weight was 243 ± 61 mg, water conditions were *T*: 18.7°C, pO_2: 97% and salinity: 37.7 psu. Fish were transported to the ICM-CSIC facilities and left to acclimatize for at least 24 h before starting the exposure experiment. Exposure conditions in the lab facilities were: water *T*: 18.1°C ± 0.2, pO_2: 89.6% ± 1.0 and salinity: 37.5 psu ± 0.2. Fifteen individuals were placed in each of 4 L tanks: 1 control and three groups with, respectively, 1:5,000, 1:10,000 and 1:50,000 dilutions from the WAF (1:500) of the "Prestige" crude. This first 1:500 WAF was obtained following the protocol of Singer et al. (2000). For each tank, 50% of the water was renewed daily and, refilled with the adequate dilution of the 1:500 extract using sea water. A detailed description of the chemical analysis of the 1:500 fraction used for the respective dilutions can be found in Solé et al. (2007).

Juvenile Senegal sole were individually weighted and further homogenized using a Polytron® homogeniser in a 0.15 M KCl-KOH pH 7.5 buffer containing 1 mM EDTA in a 1:8 (sample weight: buffer volume) ratio. All analytical determinations were carried out on the supernatant resulting from a 13,500 g centrifugation (S13) during 5 min at 4°C. Assays were carried out in duplicate or triplicate at 25°C. *Acethylcholinesterase* (AChE) and *Carboxylesterase* (CbE) were measured using 25 µl of 1/10 diluted S13 fraction of the fish homogenate. Concentrations in the well were 1 mM ATC (acetylthiocholine) or 1 mM PTA (*S*-phenyl thioacetate) as respective substrates, and 180 µM DTNB. Reading was carried out on a $VERSA_{max}$ microplate reader for 5 min at 405 nm (ε = 13.6 $mM^{-1}cm^{-1}$). *Lipid peroxidation* (LP) was measured using 60 µl of the same S13 fraction and mixed with 650 µl of methanol/1-methyl-2-phenylindole in acetonitrile and 150 µl of HCl and this mixture incubated at 45°C for 40 min. Absorbance was read at 586 nm versus a standard solution of 1,1,3,3-tetramethoxypropane treated similarly. *Catalase* (CAT) activity was measured as the decrease in absorbance at 240 nm (ε = 40 $M^{-1}cm^{-1}$) during 1 min using 50 µl of sample (S13) and 50 mM H_2O_2 as substrate. *Total Glutathione peroxidase* (t-GPX) activity was measured by the NADPH consumption at 340 nm (ε = 6.2 $mM^{-1}cm^{-1}$) during the formation of reduced glutathione by commercial glutathione reductase (GR) using 2 mM cumene hydroperoxide (CHP) as substrate. Conditions in the well were 2.5 mM GSH, 0.29 mM NADPH, one unit of GR and 10 µl of sample. The reaction was started by adding 30 µl of CHP and lasted for 3 min. *Glutathione reductase* (GR) activity conditions for the assay were 1 mM GSSG and 0.1 mM NADPH in the well and 20 µl of sample. Reading was also performed on a microplate for 3 min at 340 nm. *DT-Diaphorase* (DTD) activity was measured as the dicoumarol inhibited part of NAD(P)H-DCPIP reductase activity using 20 µl of sample. Reading was carried out on a microplate for 3 min at 600 nm (ε = 21 $mM^{-1}cm^{-1}$). *Glutathione S-transferase* (GST) activity was measured using 25 µl the S13 fraction following a 1/10 dilution. 1-chloro-2,4-dinitrobenzene (CDNB) was used as substrate, the final reaction mixture containing 1 mM CDNB and 1 mM of GSH (reduced glutathione). Measurement was done in a microplate reader for 3 min at 340 nm following the methodologies described elsewhere (Solé et al. 2004, 2005; Fernández-Díaz et al. 2006). *Total protein content*. TPC, to which were related biochemical determinations, was determined by the method of Bradford (1976), using bovine serum albumin (0.05–1 mg/ml) as standard. Biochemical data are presented as mean ± SEM (n = 4 individual fish). A two way Analysis of Variance (ANOVA) was used to test differences between means, using time and dose as factor. Significance level was established at $p < 0.05$.

Results and Discussion

In Table 1, mean individual physical parameters such as total wet weight (TW), protein yield (PY), lipid peroxidation levels (LP) and enzymatic activities are presented. These parameters were similar for all exposure doses and times. However, PY in 70 dph fish was much lower than in younger specimens from the same species aged between 20 and 30 dph (Solé et al. 2004, 2005; Fernández-Díaz et al. 2006). All antioxidant enzyme activities including phase II GST, esterases and LP levels were unaffected by the WAF of the "Prestige" crude, regardless of dose or time exposure ($p > 0.05$). Catalase activity in 70 dph individuals was higher to those described for younger animals (Solé et al. 2004). However, t-GPX, DTD (NADH dependent form), GST and AChE activities were similar to those described in younger specimens aged 20–30 dph (Solé et al. 2004, 2005; Fernández-Díaz et al. 2006). We are not aware of any data available concerning GR or CbE activities in this species to enable comparisons, therefore, we have provided data for these activities in this species. LP levels attained in our specimens were similar to those described for younger individuals (up to 6 dph) and much lower than those reached during the metamorphosis period of up to 140 nmol MDA/g w.w. (Solé et al. 2004, 2005) which appears to be a more stressful event. Parameters of neurotoxicity such as AChE, and indicators of hepatic metabolism such as CbE, were also unaffected by the exposure and correlated well between themselves (r = 0.896; $p < 0.05$). The former decreasing in mussels collected in areas affected by another oil spill event as well as under lab exposure to crude oil (Moreira et al. 2004). We are not aware of any studies measuring CbE in relation to PAHs exposure either, but it has been pointed out as a

promising marker in relation to exposure to various organic pollutants in fish studies (Al-Ghais et al. 2000; Wheelock et al. 2005).

The lack of effect on stress markers in our short term study contrasts with observations in juvenile fish species of Atlantic cod (*Gadus morhua*) (Sturve et al. 2006) and, Atlantic cod and turbot (*Scophthalmus maximus*) exposed to waterborne North Sea oil (Martin-Skilton et al. 2006). Composition of both oils (North Sea oil-NSO- and Prestige crude) is rather similar as they are both rich in two ringed PAHs (89% and 91%, respectively) with 5–6 ringed PAHs being almost undetected in the former and representing only 1.5% of the total PAHs in the 1:500 dilution of the WAF used in our study. Several reasons may be put forward to explain these lack of responses. One is that levels of PAHs reached in our study were much lower than those attained in the former studies. That is, the maximum concentration of PAHs in our study, at the 1:5,000 dilution was of 19.3 μg/L (ppb) which contrasts with the 0.52 mg/L (ppm) of dissolved PAHs (representing the sum of the same 16 individual PAHs) in the water from the NSO study (Martin-Skilton et al. 2006). In fact, the same 0.52 mg/L of the NSO report were responsible for increased EROD activity, CYP1A levels and catalase, although no effect was seen in GR, GST or LP levels (Sturve et al. 2006). The PAH levels reached in our experiment were more in line with those experimentally obtained exposing Herring (*Clupea pallasi*) to the WAF of the *Exxon Valdez* oil. In that reported study, an hepatic mixed function oxygenase (MFO) induction was seen after 8 days exposure to 3.2 ppb of PAHs or after a higher dose of 58 ppb after only 48 h exposure (Thomas et al. 1997). Characteristics of both oils differ in the sense that the presence of higher molecular weight PAHs in the "Exxon Valdez" oil were better represented (Thomas et al. 1997).

From a realistic environmental point of view, the highest concentration of PAHs from the affected region was 0.46 μg/L (representing the sum of the same 16 PAHs and following an analysis similar protocol to ours) with the average being 0.15 μg/L (González et al. 2006). These levels are in line with the exposure doses from our study (19.3, 9.6 and 0.19 μg/L for the 1:5,000, 1:10,000 and 1:50,000, respectively). Similarly to our observations, PAH levels obtained from the water soluble fraction (WSF) of the same "Prestige" crude of about 70 μg/L (Navas et al. 2006) caused no toxicity associated to an RTG-2 cell line, the algae *Chlorela vulgaris* or the crustacean *Daphnia magna*. A 1:500 dilution of the WSF of the crude was the lowest dilution to produce a significant EROD induction in the RTG-2 cell line (Navas et al. 2006). No citotoxicity, using a bivalve embryogenesis bioassay, was associated to exposures to polluted "Prestige" sediment either (Franco

Table 1 Total wet weight (*TW*), protein yield (*PY*) and biomarkers of stress in *Solea senegalensis* 70 dph exposed to different dilutions of the Water Accommodated Fraction (*WAF*) of the *Prestige* crude oil

Dose & time	TW[a]	PY[b]	AChE[c]	CbE[c]	LP[d]	CAT[e]	t-GPX[c]	GR[c]	DTD[c]	GST[c]
24 h										
Control	268 ± 24.5	16.7 ± 0.9	73.8 ± 11.9	18.4 ± 4.1	20.5 ± 1.5	47.9 ± 2.6	52.1 ± 8.1	11.8 ± 1.7	1.6 ± 0.9	64.8 ± 3.1
1:5,000	255 ± 18.8	15.7 ± 2.2	71.5 ± 10.8	17.9 ± 3.6	17.3 ± 1.3	50.7 ± 4.9	59.8 ± 14.2	12.6 ± 4.1	2.0 ± 1.2	79.5 ± 17.9
1:10,000	268 ± 30.2	16.8 ± 1.0	86.7 ± 16.9	23.2 ± 4.5	21.2 ± 3.9	51.0 ± 3.6	51.8 ± 8.0	12.6 ± 2.0	1.8 ± 0.8	67.1 ± 1.8
1:50,000	231 ± 20.7	13.7 ± 0.3	98.0 ± 17.5	27.3 ± 4.6	18.6 ± 1.1	60.6 ± 10.7	61.9 ± 10.2	20.2 ± 3.2	2.0 ± 1.0	77.3 ± 5.4
48 h										
Control	252 ± 30.3	17.5 ± 1.7	99.9 ± 23.2	26.6 ± 5.6	20.7 ± 3.3	43.1 ± 11.0	48.9 ± 6.8	10.9 ± 1.2	1.7 ± 0.6	64.5 ± 5.2
1:5,000	274 ± 18.2	17.9 ± 1.2	100 ± 15.8	23.7 ± 4.1	18.7 ± 3.7	50.4 ± 10.6	49.2 ± 9.2	18.5 ± 3.1	1.6 ± 0.8	65.1 ± 4.7
1:10,000	321 ± 39.1	15.0 ± 1.7	105 ± 5.4	30.9 ± 3.0	22.3 ± 2.2	62.3 ± 10.8	60.8 ± 8.6	17.2 ± 1.7	2.0 ± 0.7	77.1 ± 7.3
1:50,000	257 ± 37.0	17.1 ± 0.8	110 ± 21.0	26.3 ± 2.9	19.9 ± 1.9	44.3 ± 6.7	49.9 ± 8.4	19.7 ± 4.3	2.4 ± 0.8	60.2 ± 4.0
72 h										
Control	330 ± 27.9	16.8 ± 1.6	110 ± 26.2	29.6 ± 10.7	22.9 ± 1.8	51.1 ± 5.6	57.7 ± 11.8	15.2 ± 1.5	2.1 ± 1.2	71.9 ± 8.8
1:5,000	271 ± 30.2	17.0 ± 2.4	111 ± 31.9	30.4 ± 8.7	21.1 ± 2.9	49.8 ± 6.4	51.8 ± 6.0	12.0 ± 0.9	1.6 ± 0.6	77.2 ± 12.3
1:10,000	255 ± 14.7	16.7 ± 1.1	117 ± 12.0	31.5 ± 4.9	20.7 ± 1.1	47.2 ± 3.9	53.9 ± 9.8	15.6 ± 2.2	2.1 ± 0.8	66.3 ± 4.9
1:50,000	271 ± 25.9	16.7 ± 1.2	124 ± 17.1	30.6 ± 6.7	20.1 ± 2.0	46.4 ± 6.4	52.4 ± 7.0	14.5 ± 1.1	1.4 ± 0.9	65.4 ± 4.8

Values are mean ± SEM (n = 4); ANOVA test; $p > 0.05$ in all parameters and conditions

[a] mg wet weight

[b] μg prot/mg w.w

[c] nmol/min per milligram prot

[d] nmol MDA/g.w.w

[e] μmol/min per milligram prot

et al. 2006). Additionally, natural fito- and zooplankton communities did not seem affected by the spill (Varela et al. 2006), nor were the feeding patterns of four key species in the affected area modified as a consequence of the accident (Sánchez et al. 2006). Nevertheless, in a field study with two species of flatfish, taken from the polluted site, effects on antioxidant defences and xenobiotic metabolising enzymes were observed, partially related to tar aggregate concentrations. However, a cause-effect relationship due solely to the "Prestige" oil spill could not be established because of the existence of chronic pollutants from local industries already present in some of the sampled areas (Martínez-Gómez et al. 2006).

Other factors to consider are the route and time of exposure. In our study it was solely through water as the fish were not fed during the short 3-day exposure. However, in the field, in addition to water exposure, animals also accumulate PAHs through their diet. Results similar to ours were observed in a related species, the sole *Solea solea*, which was exposed to waterborne pyrene and unfed for a period of 7 days (Budzinski et al. 2004). In this reported experiment, pyrene levels in water rapidly decreased from 1 ppm to 367 ppb in 2 h and it was quickly metabolised by the fish causing no apparent toxicity. Pyrene levels at the highest concentration (1:5,000) in our experiment were of 28 ppb and were maintained by daily replacing 50% of the tank water with a fresh WAF solution.

In our study, the acute toxicity attributed to short term exposure of environmental realistic doses of the WAF from the crude, did not cause oxidative stress or neurotoxicity in juveniles of the commercial species *S. senegalensis*. Nevertheless, we can not exclude the possibility that long-term chronic exposures to low levels of the crude oil can induce toxicity and compromise sole reproductive status as has been pointed out in other benthic fish species (Monteiro et al. 2000; Martin-Skilton et al. 2006; Sturve et al. 2006; Martínez-Gómez et al. 2006).

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