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Genotoxic and biochemical responses in caged eel (*Anguilla anguilla* L.) after short-term exposure to harbour waters

V.L. Maria, A.C. Correia, M.A. Santos*

Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

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Abstract

European eel (*Anguilla anguilla* L.) were caged and exposed in situ for 8 and 48 h to the Aveiro offward fishing harbour water (HW) and to clean seawater under laboratory conditions (Control). Eel liver biotransformation (Phase I) was measured as ethoxyresorufin-*O*-deethylase (EROD) activity, cytochrome *P*450 (*P*450) and glutathione *S*-transferase (GST) activity (Phase II). Genotoxic responses were determined as blood, liver and kidney DNA strand breaks as well as erythrocytic nuclear abnormalities (ENAs).

HW failed to significant increase liver EROD, GST activities and ENA frequency. Nevertheless, *P*450 content was significantly increased after 8 and 48 h exposure. Genotoxicity measured as DNA integrity decrease was found in blood after 8 and 48 h exposure to HW, whereas in liver and kidney, it was observed after 48 h exposure to HW.

Blood, kidney and liver genotoxicity may be due to the presence of polycyclic aromatic hydrocarbons (PAHs) which are genotoxic compounds and the main HW organic contaminants.

Keywords: Genotoxicity; DNA strand breaks; ENAs; EROD; *P*450; *Anguilla anguilla* L.

1. Introduction

The impact of industrial effluents on marine ecosystems is complex, because exposure to its contaminants, directly or indirectly, impairs the organisms via induced altered metabolism and leading eventually to a decreased viability (Everaarts et al., 1998). Polycyclic aromatic hydrocarbon (PAH) contamination, resulting from oil tanker accidents, major spillage, industry and municipal waste discharges (Kennish, 1992) are among the different types of pollutants normally attributable to anthropogenic activities. Among mixed-function oxidases (MFOs), liver ethoxyresorufin-*O*-deethylase (EROD) activity, a Phase I biotransformation enzyme, has been extensively studied and adopted as a valuable indicator of water PAH and other organic xenobiotic contamination (Stegeman and Lech, 1991; Addison et al., 1994; Pacheco and Santos, 1998, 2001). According to Tuvikene et al. (1999), responses such as catalytic activities associated to cytochrome *P*450 (*P*450) seem to be inhibited, whereas glutathione *S*-transferase (GST) induction is the only measurable response. Fish *P*4501A induction associated with DNA damage studies in fish exposed to PAH xenobiotics, proved to be also a powerful way of monitoring aquatic pollution (Goksøyr and Förlin, 1992; De Flora et al., 1993).

Several environmental interactions can alter the genotoxic potential of complex industrial effluents (Claxton et al., 1998; Maria et al., 2002c). The genotoxicity of industrial pollutants related to effluent complex mixtures, such as the harbour waters (HW), needs to be monitored (Zoll-Moreaux and Ferrier, 1999). Therefore, DNA damage measured as adduct formation (Stein et al., 1990; El Adlouni et al., 1995) and strand breaks (Everaarts and Sarkar, 1996; Everaarts et al., 1998) were previously presented as useful for the monitoring genotoxicity in the environment (Shugart et al., 1992). The assessment of environmental contamination has been carried out using caged fish exposed in situ to polluted waters (Rice et al., 1994; Pacheco and Santos, 2001).

Anguilla anguilla L. is a representative species of the Aveiro Lagoon that despite its description as a bottom dwelling fish, can move along the entire water column thus being a versatile indicator species with a high economic value, easy to capture and to maintain in laboratory (Costa et al., 1992). Previous research concerning this species physiological and genotoxic responses to the Aveiro

* Corresponding author.
E-mail addresses: vmaria@bio.ua.pt (V.L. Maria), acorreia@bio.ua.pt (A.C. Correia), monteiro@bio.ua.pt (M.A. Santos).

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Lagoon industrial effluents and their isolated contaminants, measured as blood plasma cortisol, glucose and lactate, liver alanine amino transferase (ALT) and EROD activity and erythrocytic nuclear abnormalities (ENAs) (Santos et al., 1990; Pacheco and Santos, 1998, 1999, 2001), proved its sensitivity as well as its resistance to those adverse conditions.

In Portugal, Aveiro Lagoon, the long-distance harbour presents a PAH contamination ranging between 0.0075 and 0.0086 ng ml^{-1} (Pacheco and Santos, 2001) due to intense industrial sewage harbour activity. In this perspective, the present research work concerns a field study with caged eels exposed in situ to harbour waters, during 8 and 48 h, to relate genotoxic responses such as blood, liver and kidney DNA strand breaks as well as ENAs with biotransformation measured as liver EROD, GST activity and *P*450 content. ALT activity was also determined for the indication of liver hepatocyte plasma membrane disruption (Santos et al., 1990; Pacheco and Santos, 1999).

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade, obtained from Sigma (USA), Roche (Germany) and E. Merck-Darmstadt (Germany).

2.2. Fish

European eel (*A. anguilla* L.), with an average weight of 50 g (silver eel), were collected from the Aveiro Lagoon—Murtosa (Portugal). The eels were transported in anoxia, acclimatized to laboratory conditions in aerated, recirculated, filtered and artificial seawater in 80-l aquaria for 1 week, at 20 °C under a natural photoperiod [light/dark (hours) = 12]. Fish were not fed during recovery. The 1-week recovery period after capture revealed that all biomarkers are according to control levels found in reference sites (Santos and Pacheco, 1996).

2.3. Biochemical analyses

2.3.1. Liver EROD assay

Microsomes were obtained according to the methods of Lange et al. (1992) and Monod and Vindimian (1991) as adapted by Pacheco and Santos (1998). Liver EROD activity was measured as described by Burke and Mayer (1974). Results were expressed as pmol/min/mg of microsomal protein.

2.3.2. P450 content

*P*450 content was quantified by measuring the 490- to 450-nm absorbance spectrum as described by Hermens et al. (1990).

2.3.3. GST assay

Liver GST activity was determined as described in Habig et al. (1974) and Lemaire et al. (1996), with 1-chloro-2,4-dinitro-benzene (CDNB) as substrate. The assay prepared in the cuvette was carried out in a 2-ml mixture of 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB and 0.2 mM GSH. The reaction was initiated by sample addition. The increase in absorbance at 340 nm was recorded at 25 °C for 3 min.

2.3.4. Liver ALT assay

ALT activity was measured according to Reitman and Frankel (1957) in the supernatant resulting from liver microsomal isolation (Santos et al., 1990; Pacheco and Santos, 1999).

2.3.5. Protein measurement

Microsomal protein content and supernatant protein concentration were determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt) as a standard.

2.4. Genotoxicity responses

Genotoxicity was tested using the ENAs and the DNA alkaline unwinding assay. ENA test was carried out in eel's mature erythrocytes, according to the procedures of Schmid (1976), Carrasco et al. (1990) and Smith (1990) as adapted by Pacheco and Santos (1996). Each group final result was presented as the mean value (‰) of the sums for all the individual lesions observed and scored in 1000 cells per fish blood smear. Blood and liver DNA integrity measurements (%) were performed according to Rao et al. (1996), with minor modifications, where liver and blood DNA was extracted from liver and blood tissues by Genomic DNA Purification Kit, Fermentas, USA. Data from blood and liver DNA alkaline unwinding technique were expressed as *F* value (%) double-stranded DNA over double stranded DNA plus single-stranded DNA.

2.4.1. Statistical analysis

The results are expressed as mean ± standard error (S.E.) and statistical analysis was performed using a two-tailed Student's *t* test (Bailey, 1959).

2.5. Experimental protocols

Eels were not fed during the exposure period to harbour water and to clean water (control group). Adult eels were exposed during 8 and 48 h, in cages, to the following conditions:

1– The eels were exposed during high tide to the Aveiro harbour waters (HW)—offward fishing Port (Aveiro estuary). Fish were transported in cages from laboratory to the harbour location in anoxia (30 min), and plunged

near the sediment. The total volume of each net cage was 80 dm^3. The experiment was carried out in October, at approximately 21 °C water temperature.

2– Similarly to the harbour condition, control eels (C) were kept in net cages (volume = 80 dm^3), into aerated, filtered and artificial seawater at 20 °C under laboratory conditions (natural photoperiod), and submitted to the equivalent previous anoxia period (30 min).

Each exposure condition was carried out using test groups of five eels each ($n = 5$). Fish were killed by decapitation; their blood, kidney and liver were sampled. Blood smears were prepared for ENA assay. Each liver was divided into two halves where one was immediately frozen in liquid nitrogen and stored at −20 °C for later liver EROD, *P*450, GST and ALT determination, while the other half was placed in TNES-urea (8 M) buffer with proteinase K solution (final concentration, 0.8 mg/ml) for DNA isolation procedure (Genomic DNA Purification Kit). Fresh blood samples, after centrifugation and plasma separation, were stored overnight at 4 °C with TNES-urea (8 M) buffer for DNA isolation. Blood, liver and kidney DNA extraction was carried out for the DNA strand breaks assay.

3. Results and discussion

3.1. Genotoxicity responses

A significant decrease in *A. anguilla* L. blood DNA integrity was found after 8 ($p < .02$) and 48 ($p < .05$) h exposure to HW (Fig. 1a). However, a significant decrease in liver ($p < .05$) and kidney ($p < .01$) DNA integrity was only observed at 48 h exposure to HW, compared to their controls (Fig. 1b,c). A significant decrease in the eel's liver DNA integrity after 48 h ($p < .01$) compared to 8 h exposure to HW was also observed (Fig. 1b). The highest DNA integrity decrease was found in blood (16.5%), followed by kidney (16.3%) and liver (15%), after 48 h exposure to HW (Fig. 1a–c).

Despite the absence of significant ENAs frequency differences, between control and exposed eels, the results exhibit an increased ENA tendency after 48 h exposure to HW (Fig. 2).

Harbour waters contain (Pacheco and Santos, 2001) various progenotoxic PAHs that may be environmentally biotransformed into reactive genotoxic compounds leading to genotoxic effects in aquatic species (James and Kleinow, 1994; Maria et al., 2002a).

The eels' liver S9 capacity of converting a progenotoxic substance, such as BaP, into a genotoxic compound with mutagenic effects, was previously demonstrated by the Ames test (Maria et al., 2002b).

An early blood DNA strand breaks increase after 8 h compared to a delayed liver and kidney increase after 48 h exposure to HW in caged *A. anguilla* L., is probably due to the direct action of genotoxic and progenotoxic compounds such as PAHs/PAH metabolites present in the HW, and consequent blood DNA damage. Maria et al. (2002a) previously found that the eels' blood DNA integrity decreased after 8 h exposure to 0.9 and 2.7 μM BaP, suggesting the presence of PAH progenotoxic equivalent concentrations in the HW, where our experimental animals were exposed.

According to Maria et al. (2002c), the eel's DNA blood damage measured as strand breaks was also observed after 8, 16 and 24 h exposure to 50% diluted pulp mill effluent as well as at 16 and 24 h exposure to 25% diluted pulp mill

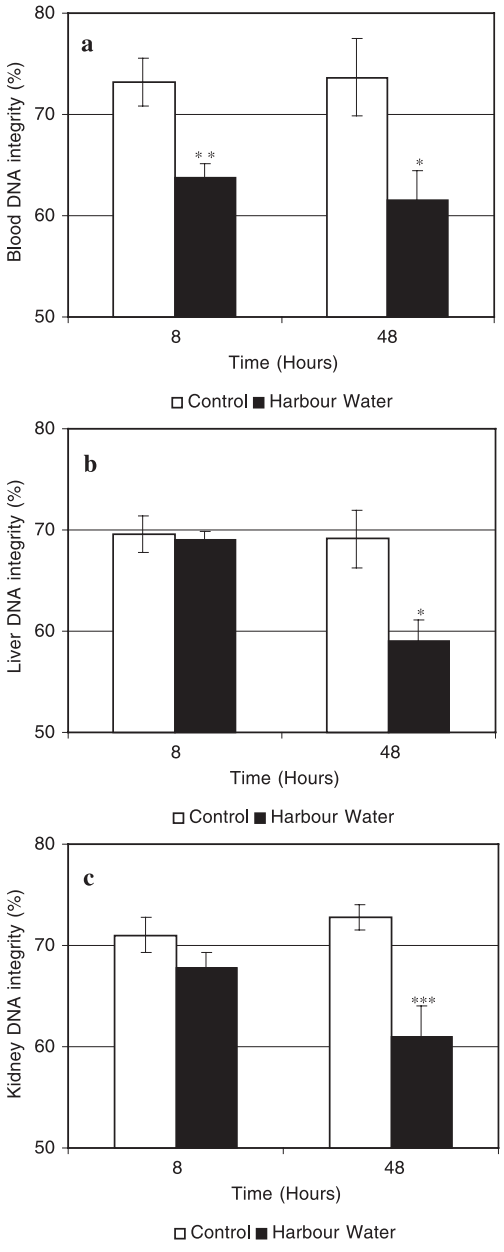

Fig. 1. Blood (a), liver (b) and kidney (c) DNA integrity (%) in adult eel (*A. anguilla* L.) exposed to harbour waters in field at 8 and 48 h. Values represent mean ± S.E. Differences from control: *$p < .05$; **$p < .02$; ***$p < .01$.

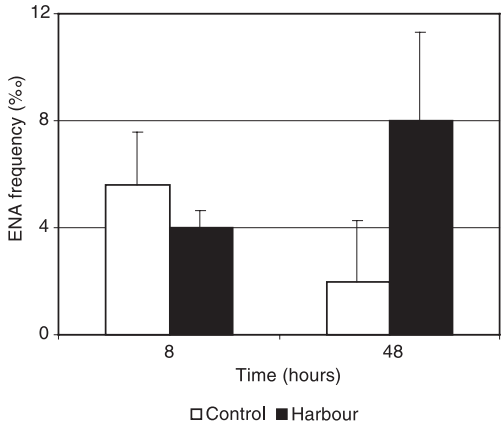

Fig. 2. ENA frequency (‰) in adult eel (*A. anguilla* L.) exposed to harbour waters in field at 8 and 48 h. Values represent mean ± S.E. No statistical differences from control.

effluent, demonstrating once again that the dilution of a complex effluent results in a delayed DNA-damaging effect. Other authors such as Devaux et al. (1998) also observed that chub (*Leuciscus cephalus*) caught in Rhône River close to an industrial area presented an increased DNA damage in their erythrocytes.

PAH reactive intermediates have the potential to interact with any nucleophilic sites within the cell, including those on proteins, DNA and induce a genotoxic response. It should be noted that the formation of reactive intermediates might not lead to cellular toxicity if there is a balance between the rates of their formation and detoxification (Shugart, 1996). A most efficient phase II detoxification process may be responsible for a delayed liver and kidney DNA integrity decrease at 48 h because the experimental results of Ahmad et al. (2002) demonstrated an increased liver GSH content after 8 h exposure and further liver lipid peroxidation (LPO) after 48 h exposure to HW concomitantly with a GSH liver content decrease. Despite blood DNA strand breaks increase after 8 and 48 h exposure to HW, an ENA frequency increase was not found. However, Maria et al. (2002c) demonstrated *A. anguilla* L. ENA increase after 8, 16 and 24 h exposure to pulp mill effluent in laboratory conditions. ENA frequency absence in HW-exposed eels may be due either an efficient removal by the spleen and/or an inhibition in erythrocytic production during this exposure period. A slight non-significant elevation of ENA frequency noticed after 48 h exposure suggests either the necessity of an extended exposure length or/and the use of different HW dilutions.

According to White (2002), the absence of information about the behaviour of toxic substances in complex mixtures is often avoided by assuming that the toxicity of a mixture is simply the sum of the expected effects from each mixture component, that is, no synergistic or antagonistic interactions.

In previous field studies with caged *A. anguilla* L., neither significant liver EROD activity nor ENA increase was observed after 1 and 3 days of environmental exposure to HW (Pacheco and Santos, 2001). However, laboratory studies with the same species exposed for 1 day to low tide harbour water (LTHW) demonstrated the presence of liver EROD inducers, whereas after 3 days of exposure to high tide harbour water (HTHW), the presence of ENA inducers was revealed (Pacheco and Santos, 2001). According to Shugart (1996) animal aquatic species exposed either to natural field or laboratory conditions seem to respond differently. Therefore, some difficulties on the field data interpretation may arise, as fish may be affected by the influence of environmental factors such as air and water temperature, water flow and currents on the relative amounts of hydrocarbons in the water (Neff, 1990; Pacheco and Santos, 2001). However, the ENA significant increase found by Gravato et al. (2000) in the juvenile sea bass, after 6 h exposure to HT and LT HW under laboratory conditions, also confirmed the presence of genotoxic compounds in the HW and seems to be related with species differences.

3.2. Biotransformation responses

Though not statistically different from control groups, liver EROD activity slightly increased in eels after 8 h exposure to HW (Table 1). However, liver *P*450 content significantly increased at 8 ($p < .01$) and 48 ($p < .01$) h exposure, compared to control levels (Table 1).

Previous results, concerning liver *P*450 content increase after 8 and 48 h exposure to HW despite no EROD activity increase may be due to their high PAH concentration. According to Gooch et al. (1989) and Haasch et al. (1993), high PAH concentrations (BaP) and PAH-like compounds (BNF) can also inhibit liver EROD catalytic activity. This was also supported by Gravato et al. (2000) who demonstrated an increased liver *P*450 content in sea bass (*Dicentrarchus labrax*) after 6 h to HTHW, whereas no significant liver EROD activity increase was found.

Table 1
Liver EROD activity (pmol/min/mg protein), *P*450 content (nmol/mg/protein), GST activity (nmol/min/mg protein) and ALT activity (U/g) in adult eel (*A. anguilla* L.) exposed to harbour waters in field at 8 and 48 h

Parameters	Time (h)	Control	Harbour waters
Liver EROD activity	8	1.076 ± 0.102	1.850 ± 0.485
(pmol/min/mg protein)	48	0.750 ± 0.187	0.996 ± 0.344
*P*450 content	8	0.154 ± 0.0211	0.384[a] ± 0.0623
(nmol/mg protein)	48	0.197 ± 0.00252	0.261[a] ± 0.0157
Liver GST activity	8	71.791 ± 3.836	100.951 ± 12.305
(nmol/min/mg protein)	48	71.803 ± 3.886	83.202 ± 3.248
Liver ALT activity (U/g)	8	1.678 ± 0.0605	1.669 ± 0.130
	48	1.413 ± 0.1115	1.443 ± 0.0655

Values represent mean ± S.E.[b]

[a] Differences from control: ***$p < .01$.

[b] Each exposure condition was carried out using test groups of five eels each ($n = 5$).

Several inducers of CYP1A synthesis can also inhibit MFO catalytic activity [EROD or aryl hydrocarbon hydroxylase (AHH)] (Stegeman and Hahn, 1994). According to Goksøyr et al. (1991), elevated *P*450 1A protein was found in fish from one site containing elevated contaminant despite no EROD activity increase. Sea bass liver microsomal EROD activity was also inhibited in vitro by low BNF (0.001 μM) and BaP (0.01 μM) concentrations. Moreover, BNF and BaP doses ranging from 0.1 to 2.7 μM inhibited at least 85% of liver EROD activity (Gravato and Santos, 2002). However, Rice et al. (1994) found a significant relationship between caged speckled sanddabs MFO responses and sediment hydrocarbon concentrations in a field study to monitor the potential hydrocarbon impact at Moss Landing. In the present field studies with caged *A. anguilla* L., HW exposure and liver EROD activity increased failure, suggesting either inhibition or inactivation mechanisms by the harbour water complex mixture which include elevated PAH concentrations (Pacheco and Santos, 2001; Gravato et al., 2000) and significant levels of heavy metals (Hall et al., 1988).

In vitro studies concerning previously exposed *D. labrax* to organic xenobiotics such as BNF (Viarengo et al., 1997; Oliveira et al., 2002) have demonstrated liver microsomal EROD activity inhibition by heavy metals. Other compounds, such as tributyltin (TBT) has been recognised as potent in vitro liver microsomal EROD activity inhibitor in fish (Fent and Stegeman, 1993) having been found in the Aveiro Lagoon water and sediment (Barroso et al., 1998). Liver GST activity was not altered for any exposure condition (Table 1), which suggests a failure on Phase II conjugation process.

3.3. Liver damage

Liver ALT activity results were not altered after any HW exposure condition (Table 1) demonstrating that there was no liver hepatocyte plasma membrane disruption after 48 h exposure despite liver LPO increase (Ahmad et al., 2002, personal communication). The equivalent result was also observed by Pacheco and Santos (2001) in eels after 1 and 3 days of exposure to high tide HW and low tide HW. Gravato et al. (2000) also demonstrated no significant liver ALT activity alteration in sea bass (*D. labrax*) after 6 h exposure to HTHW and LTHW.

4. Conclusion

This study reveals that there are genotoxic and/or progenotoxic compounds among harbour water contaminants, which are able to induce early genotoxicity as DNA strand breaks in eel's blood cells and a delayed genotoxicity in liver and kidney. In this field short-term exposure to HW, a significant ENA response was not observed. The genotoxicity increase in liver and kidney at 48 h exposure to HW is probably due to a failure in the detoxification process where some of the unconjugated-reactive HW metabolites may be responsible for DNA damage. Liver *P*450 content increase in eels despite the failure in liver EROD activity induction, is probably due to a complex mixture of high PAHs concentrations in harbour water contamination, which is able to induce the cytochrome synthesis, preventing, however, the liver EROD activity increase.

To avoid misleading experimental results concerning the failure in liver EROD and ENA induction in monitoring field studies by effluent complex mixture, further studies should be complemented by shortening the interval between 8 and 48 h as well as prolonging it over 48 h exposure.

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