Anguilla anguilla L. liver ethoxyresorufin O-deethylation, glutathione S-tranferase, erythrocytic nuclear abnormalities, and endocrine responses to naphthalene and b-naphthoflavone

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Abstract

The effects of naphthalene (NAP) and b-naphthoflavone (BNF) on phase I biotransformation and genotoxicity in Anguilla anguilla L. were evaluated. Phase II biotransformation and cortisol levels were also assessed in NAP-treated fish. Two groups of eels were exposed to either a NAP or a BNF concentration range (0.1–2.7 mM) for different exposure periods (2–72 h). An early significant ethoxyresorufin O-deethylation (EROD) activity inhibition was observed, especially for the highest NAP concentrations at 2–6 hexposure and for BNF at 2 hexposure. However, a significant EROD activity increase was detected from 16 to 72 h exposure for NAP and from 4 to 72 h exposure for BNF. The cytochrome P450 (P450) content was not dose related. However, with regard to BNF exposure, P450 was the first biomarker to respond. Liver alanine transaminase (ALT) activity was measured as an indicator of hepatic health condition. ALT results demonstrated that the EROD activity decrease, previously described for NAP, was not related to tissue damage. Nevertheless, the highest BNF concentrations were demonstrated to induce liver damage and to impair the EROD activity response. An increased genotoxic response, measured as erythrocytic nuclear abnormalities (ENA), was observed during the first 8 h NAP exposure. However, for exposures longer than 8 h, ENA frequency returned to the control levels. This response profile may reflect a considerable DNA repair capacity and/or a metabolic adaptation providing an efficient NAP biotransformation and consequent detoxification. BNF revealed no ENA alterations for all concentrations and exposure lengths. In the NAP experiment a causal relationship between immature erythrocytes (IE) and ENA frequency disappearance was not found. BNF results with regard to IE frequency revealed an ability to alter the balance between erythropoiesis and removal of erythrocytes. Liver glutathione S-transferase activity was significantly induced after 2 and 48 hNAP exposure. A cortisol-impaired response seems to occur from 4 to 24 hNAP exposure, demonstrating an endocrine disruption. However, an adaptation process seems to occur after 48 h, since the plasma cortisol had a tendency to increase. The present findings confirm the usefulness of the adopted biomarkers. The ecological risk associated with aquatic contamination by NAP was also confirmed by the present data.

1. Introduction

A large number of xenobiotic compounds have been produced in the 20th century as a result of petroleum exploitation, with the aquatic environment being the ultimate sink for most of them. Therefore, the vast majority of aquatic animals have become increasingly exposed to industrially derived xenobiotic pollutants, suchas polycyclic aromatic hydrocarbons (PAHs) (George, 1994). These widespread environmental contaminants, originating mainly from combustion processes, oil spills, land runoff, and domestic and industrial wastes, are of international concern due to their persistence in the environment and mutagenic/carcinogenic potential. Naphthalene (NAP), the simplest PAH, is frequently encountered in soil and in aquatic environments (Irwin et al., 1997).

Fishreadily take up lipophilic organic contaminants suchas NAP from the environment and possess a variety of cellular mechanisms for protection against the deleterious effects of suchchemicals (Peters et al., 1997). These mechanisms include biotransformation, which converts organic contaminants to water-soluble and excretable metabolites. However, biotransformation enzymes may also convert certain xenobiotics to intermediates, which are more toxic than the parent compounds. Biotransformation can generally be simply divided into phases I and II: phase I is the alteration of the original foreign molecule so as to add a functional

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group, which can be conjugated in phase II. Biotransformation studies in fishrevealed that PAHs exposure induces cytochrome P450 (P450)-dependent enzymatic activities such as ethoxyresorufin O-deethylation (EROD) (phase I). Therefore, the EROD activity determination has been adopted as a valuable indicator allowing the detection of PAHs at presumably toxic levels.

A common mechanism for protection against biotransformed electrophilic xenobiotics is conjugation with glutathione (GSH) in phase II detoxification. This reaction is catalyzed by glutathione S-transferase (GST) and accounts for one of the primary routes of NAP detoxification (Mitchell et al., 2000). Since phase II detoxification enzymes are often inducible in aquatic animals, it has been suggested that its activity might also be a useful index of organic compounds exposure (Gallagher et al., 2001).

Some authors have successfully employed aquatic organisms in cytogenetic studies, for chromosome aberrations, sister chromatid exchange, micronuclei (Peschet al., 1981; Kligerman, 1982), and erythrocytic nuclear abnormalities (ENA), which include micronuclei (supernumerary nucleus) and other nuclear abnormalities quantification (Pacheco and Santos, 1997, 2002; Aylló n and Garcia-Vasquez, 2001).

Fishendocrine system can be adversely affected by toxic chemicals. Therefore, biochemical alterations and consequent effects on specific hormonal functions may constitute important stress biomarkers (Hontela, 1997). In this context, changes in interrenal function, measured as plasma cortisol, are recognized as important fish stress biomarkers (Santos and Pacheco, 1996).

The toxic effects of NAP in fish have been poorly studied. The first reported studies concern lethality experiments (Boylan and Tripp, 1971; Anderson et al., 1974). Levitan and Taylor (1979) found a general plasma cortisol increase in Fundulus heteroclitus exposed to NAP under different salinities. BothNAP genotoxic potential measured as ENA increase in Dicentrarchus labrax (Gravato and Santos, 2002) and an oxidative stress induction in Anguilla anguilla L. (Ahmad et al., 2001) have been observed.

b-Naphthoflavone (BNF) has been considered the most potent P450 mixed-function oxydase (MFO) inducer among a number of synthetic flavonoid compounds (McKillop and Case, 1991). This compound is commonly used to evaluate fishbiotransformation responses due to its MFOs responses, considered similar to those displayed by PAHs inducers, such as benzo(a)-pyrene (BaP) (Okey, 1990; Novi et al., 1998). Thus, in a biotransformation context, BNF is regarded as a PAH-type inducer. Previous investigations with A. anguilla revealed that BNF is a strong EROD inducer both in adult eels intraperitoneally injected (Pacheco and Santos, 1998) and in glass eels (leptocephalic stage) exposed to BNF-contaminated water (Pacheco and Santos, 1997). McKillop and Case (1991) reviewed the information available relating to BNF mutagenicity and carcinogenicity. Their literature search produced no primary reports on the mutagenicity/carcinogenicity of BNF, suggesting that no definitive study had been carried out specifically to assess these BNF effects. Furthermore, the majority of the studies were carried out in mammals; thus, doubt with regard to its potential in other animal groups remains. Recently, the BNF mutagenicity potential was demonstrated in fishby Pacheco and Santos (1997) who observed a significant ENA increase in glass eels. In this perspective, further fishstudies are needed to clarify the BNF genotoxic potential in fish.

This research concerns the study of the effects of NAP on liver biotransformation, measured as P450 content, EROD (phase I), and GST activity (phase II), and on genotoxicity, measured as ENA. The immature erythrocytes (IE) frequency was determined to assess changes in the erythropoiesis/erythrocytic catabolism balance and its influence on ENA expression. Liver alanine transaminase (ALT) activity was measured as an indicator of hepatic health condition. The endocrine disruption was evaluated as plasma cortisol concentration.

Additionally, BNF was tested as a reference compound for biotransformation parameters (phase I) and to assess its genotoxic potential in fish, as ENA.

2. Material and methods

2.1. Test animals

A. anguilla L. (yellow eel), withan average weight of 30 g, were collected from the Aveiro lagoon, Murtosa. The eels were transported in anoxia and acclimated to laboratory conditions for 1 week prior to experimentation. During recovery and experimental periods, eels were kept in 80-L aquaria at 201C under a natural photoperiod, in recirculating, aerated, filtered, and dechlorinated tap water. Fish were fed neither under laboratory adaptation nor during the experimental procedure.

2.2. Chemicals

NAP, 7-ethoxyresorufin, dithionite, 1-chloro-2,4-dinitro-benzene (CDNB) and BNF were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). NADPH was purchased from Roche (Germany).

2.3. Experimental design

Two different lots of eels were exposed either to a NAP or a BNF concentration range—0 (control), 0.1,

0.3, 0.9, and 2.7 mM—for 2, 4, 6, 8, 16, 24, 48, and 72 h. The appropriate amount of NAP or BNF was previously dissolved in 1 mL of dimethyl sulfoxide (DMSO) and added to the experimental aquaria. The same volume of DMSO was added to the control aquaria. During the exposure time the water was not replaced.

Fishwere killed at eachsampling point and their blood and liver were collected. Liver was immediately frozen in liquid nitrogen and stored at −201C until homogenization. Blood was used for smear preparation and for plasma isolation using an Eppendorf centrifuge (14,000 rpm). The following parameters were determined for NAP and BNF experiments withthe exceptions of plasma cortisol and liver GST activity which were measured only for the NAP experiment.

2.4. Biochemical analyses

2.4.1. Liver EROD activity

Liver microsomal fraction was 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 in microsome suspension as described by Burke and Mayer (1974).

2.4.2. Liver cytochrome P450 content

Cytochrome P450 content was determined using the dithionite-reduced carbon monoxide difference spectrum between 450 and 490 nm, as previously described by Hermens et al. (1990).

2.4.3. Liver GST activity

GST activity was determined in the cytosolic fraction as described by Lemaire et al. (1996), using CDNB as substrate (Habig et al., 1974). 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 addition of 10 mL sample. The increase in absorbance at 340 nm was recorded at 251C for 3 min.

2.4.4. Liver ALT activity

The liver ALT activity was measured in the cytosolic fraction according to the method of Reitman and Frankel (1957).

2.4.5. Protein measurement

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

2.4.6. Plasma cortisol

Plasma cortisol quantification was performed using a diagnostic enzyme-immunological kit (Boehringer Mannheim GmbH No. 1288946).

2.5. ENA frequency

Genotoxicity was tested using the ENA assay. The nuclear abnormalities were scored in 1000 mature erythrocytes (ME) per fish, according to the criteria of Schmid (1976), Carrasco et al. (1990), and Smith(1990), adapted by Pacheco and Santos (1996). According to these authors, nuclear lesions were scored into one of the following categories: micronuclei, lobed nuclei, dumb-bell-shaped or segmented nuclei, and kidney-shaped nuclei. The final result was expressed as the mean value (%) of the sum for all the individual lesions observed.

2.6. IE frequency

IE were scored for each of the 1000 erythrocytes (immature+mature) per fish. Results were presented as a frequency (%) resulting from the expression

$$\text{IE frequency } \delta[\text{No: IE}/(\text{No: IE} + \text{No: ME})] \times 1000:$$

The distinction between ME and IE was based on the criteria established by Hibiya (1982) and Smith(1990).

2.7. Statistical analysis

Mean ± standard error (SE) was calculated for each experimental group, and data were analyzed for significance of differences between control and exposed groups according to the two-tailed Student t test (Bailey, 1959). Experiments were carried out using test groups of five eels (n = 5).

3. Results

3.1. NAP experiment

3.1.1. Liver EROD activity

A significant inhibition in liver EROD activity was observed during the first 6 h exposure (Fig. 1), especially for the highest NAP concentrations. However, a general significant liver EROD increase was detected from 16 to 72 hexposure. The maximum liver EROD activity level (7.06 pmol/min/mg protein) was reached at 48 h exposure to 2.7 mM.

3.1.2. Liver P450 content

A general decreasing tendency for liver cytochrome P450 content was observed for the first 6 h exposure to all NAP concentrations (Fig. 2), despite the absence of

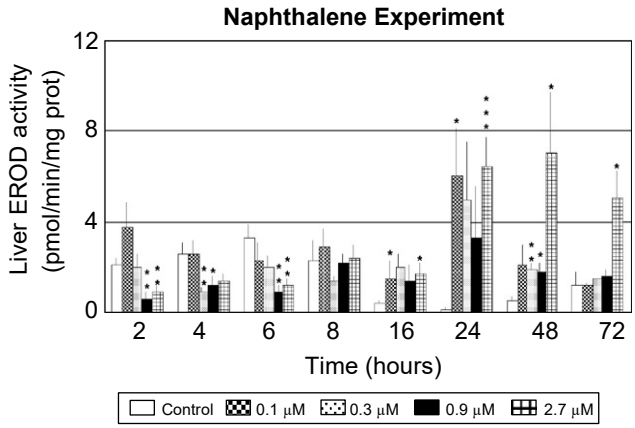

Fig. 1. A. anguilla liver EROD activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: $^{*}P_{O}0{:}05$; $^{**}P_{O}0{:}01$; $^{***}P_{O}0{:}001$:

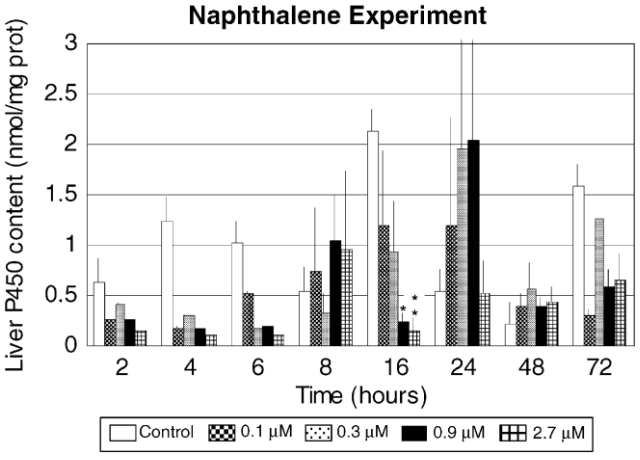

Fig. 2. A. anguilla liver P450 content after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: $^{*}P_{O}0{:}05$; $^{**}P_{O}0{:}01$:

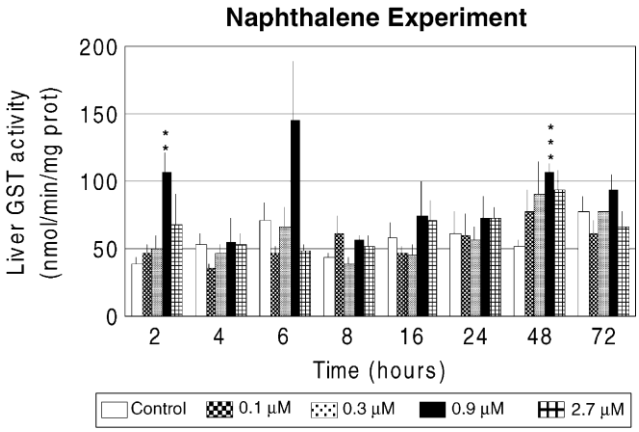

Fig. 3. A. anguilla liver GST activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: $^{**}P_{O}0{:}01$; $^{***}P_{O}0{:}001$:

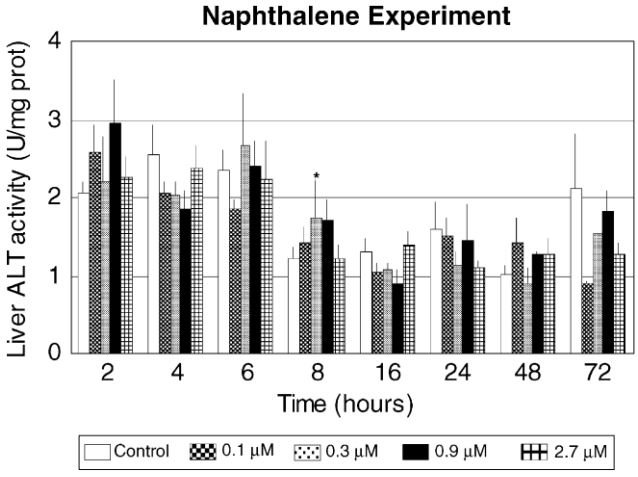

Fig. 4. A. anguilla liver ALT activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: $^{*}P_{O}0{:}05$:

any statistical significance. A significant decrease was observed at 16 hexposure to 0.9 and 2.7 mM ($P_{O}0{:}05$ and $P_{O}0{:}01$; respectively).

3.1.3. Liver GST activity

The GST activity demonstrated a significant induction only at 2 ($P_{O}0{:}01$) and 48 h($P_{O}0{:}001$) exposure to 0.9 mM NAP (Fig. 3). However, this concentration induced a general GST increase during the whole experiment.

3.1.4. Liver ALT activity

In general, no significant alterations were observed in liver ALT activity during the whole NAP exposure period (Fig. 4), except for 0.3 mM at 8 hexposure where a significant increase was detected ($P_{O}0{:}05$).

3.1.5. ENA frequency

At 2 hexposure to NAP, significant ENA increases were observed for 0.3 and 2.7 mM ($P_{O}0{:}01$); an increased ENA frequency was observed at 4 hexposure for 0.9 and 2.7 mM ($P_{O}0{:}05$) and at 6 hfor 0.3 mM ($P_{O}0{:}05$), whereas at 8 h all the tested concentrations significantly increased ENA (0.1 mM, $P_{O}0{:}01$; 0:3 mM, $P_{O}0{:}01$; 0.9 mM, $P_{O}0{:}01$; 2.7 mM, $P_{O}0{:}05$) (Fig. 5). ENA frequency returned to control levels after exposures longer than 8 h.

3.1.6. IE frequency

At 4 ha significant increase was observed for 0.1 ($P_{O}0{:}05$) and 0.3 mM ($P_{O}0{:}001$), whereas at 24 h a significant decrease ($P_{O}0{:}05$) was detected for 0.1 mM (Fig. 6).

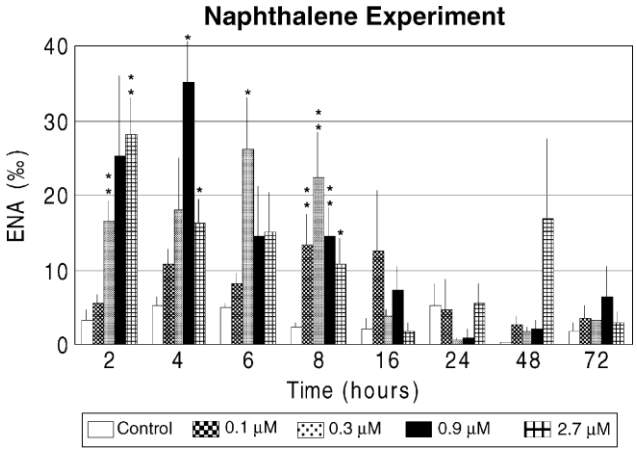

Fig. 5. A. anguilla ENA frequency after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: *P<0:05; **P<0:01:

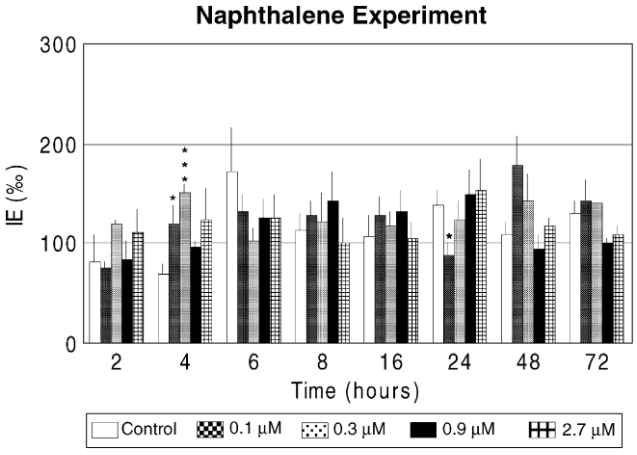

Fig. 6. A. anguilla IE frequency after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: *P<0:05; ***P<0:001:

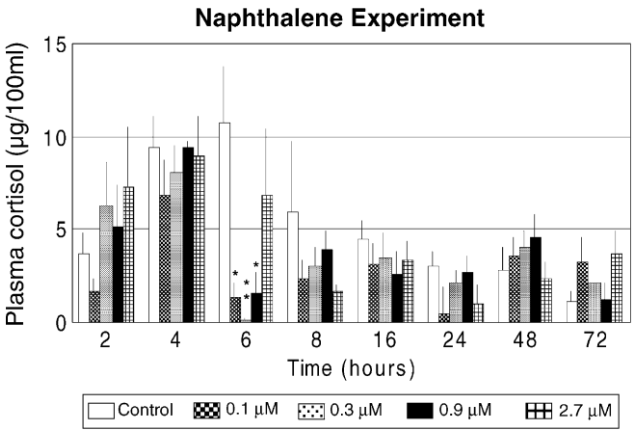

Fig. 7. A. anguilla plasma cortisol levels after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: *P<0:05; **P<0:01:

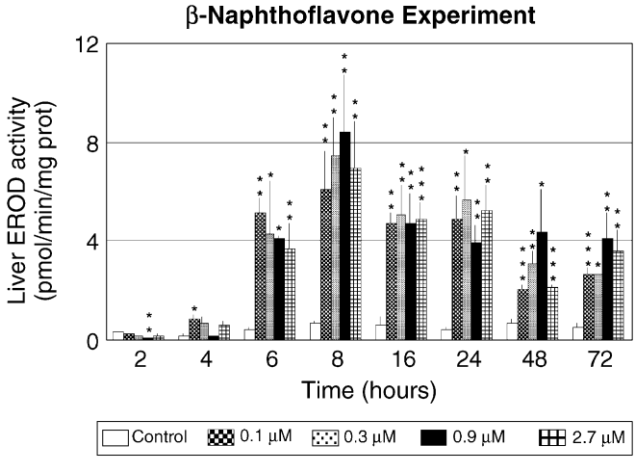

Fig. 8. A. anguilla EROD activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to b-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: **P<0:01; ***P<0:001:

3.1.7. Plasma cortisol

A plasma cortisol level decreasing trend was observed from 4 to 24 h(Fig. 7). This tendency was confirmed at 6 hwhere a significant decrease was observed for 0.1 mM (P<0:05), 0.3 mM (P<0:01), and 0.9 mM (P<0:05). After 48 hthe previous tendency disappeared and an opposite trend was observed.

3.2. BNF experiment

3.2.1. Liver EROD activity

The A. anguilla response after 2 hexposure suggests an initial inhibition effect which became evident by the complete absence of EROD activity increase and mainly by the significant activity decrease observed for 0.9 mM BNF (P<0:001) (Fig. 8). The first significant EROD increase was observed after 4 hexposure (P<0:05) but just for the lowest BNF concentration (0.1 mM). During the exposure period between 6 and 72 h, all the concentrations were able to significantly increase EROD activity. For eachexposure concentration, a global analysis of EROD results reveals a time-related tendency to increase from 2 to 8 hexposure, where it reaches the maximum values.

3.2.2. Liver P450 content

This biomarker exhibited a fast significant increase at 2 h0.1 mM BNF exposure (P<0:01) (Fig. 9). This parameter demonstrated a general tendency to increase

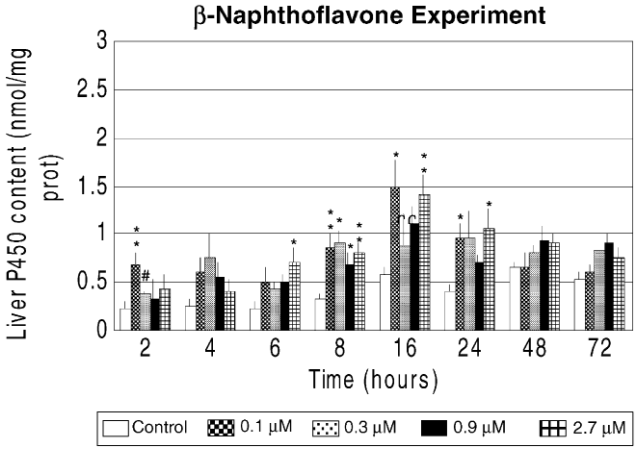

Fig. 9. A. anguilla liver P450 content after 2, 4, 6, 8, 16, 24, 48, and 72 hexposure to b-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: *PO0:05; **PO0:001; #PO0:1 mm vs 0.3 mm.

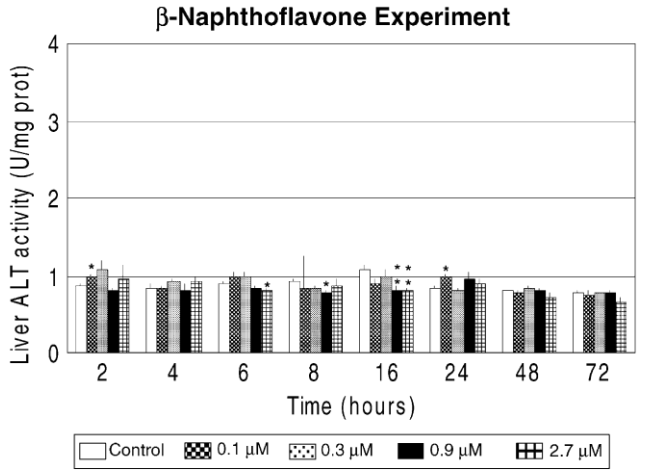

Fig. 10. A. anguilla liver ALT activity after 2, 4, 6, 8, 16, 24, 48, and 72 hexposure to b-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: *PO0:05; **PO0:01:

at all BNF exposure lengths. However, significant responses were observed only at dispersed points and there was an unclear dose–response relationship. A response profile similar to that described for EROD activity was observed, i.e., the maximum values were obtained after 8 and 16 hexposure, followed by a tendency to decline. Similar to the EROD response, an inhibition effect seems to occur in P450 content, particularly for short exposures, as confirmed by a significant decrease observed at 2 hfor 0.3 mM, compared to 0.1 mM (PO0:05). Nevertheless, a decline of this inhibitory action was observed as result of an increased exposure length.

3.2.3. Liver ALT activity

The lowest BNF concentration (0.1 mM) was able to significantly increase ALT activity, as demonstrated at 2 and 24 hexposure (PO0:05) (Fig. 10). However, the highest BNF concentrations were able to induce liver damage sporadically, as it occurred for 0.9 mM at 8 (PO0:05) and 16 h(PO0:01) exposure, and for 2.7 mM at 6 (PO0:05) and 16 h(PO0:01) exposure.

3.2.4. ENA frequency

The genotoxic response revealed no alterations for all tested concentrations and exposure times (Fig. 11).

3.2.5. IE frequency

The IE frequency was markedly affected by BNF exposure (Fig. 12). The results revealed significant decreases for 24 hand longer exposures. At some points in time, such as 24 h, a dose–response relationship seems to be evident; i.e., the IE frequency decreases as the BNF dose increases.

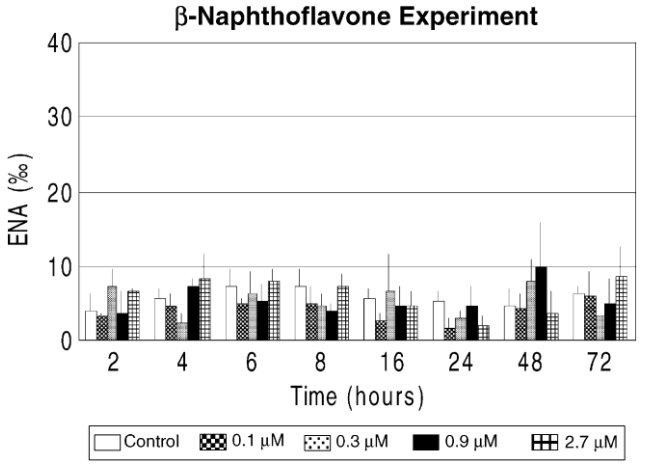

Fig. 11. A. anguilla ENA frequency after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to b-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment).

4. Discussion

To our knowledge there are very few studies on the effects of NAP on biotransformation, genotoxicity, and endocrine parameters in fish.

It is well known that xenobiotics classified as PAH-type compounds induce hepatic microsomal cytochrome P450-dependent reactions in fish(Fouché court et al., 1999). Measurements of aryl hydrocarbon hydroxylase (AHH) and EROD (phase I) activities in fish appear to be the most sensitive means for determining water contamination by PAHs. These phase I enzyme activities occur at low and sometimes undetectable levels in control or untreated fish(Fö rlin et al., 1994). Several studies have demonstrated liver EROD induction in fish

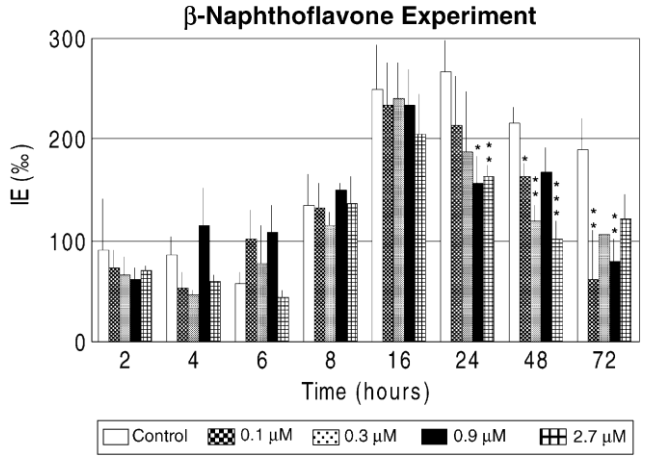

Fig. 12. A. anguilla IE frequency after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to b-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 mM). Values represent the means and SE (n ¼ 5/treatment). Differences from control: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

exposed to PAHs suchas dibenzo[a]pyrene (Vetter et al., 1985) and BaP (Wolkers et al., 1996). Nevertheless, Bols and coworkers (1999) failed to obtain EROD activity induction by NAP in a trout liver cell line.

The results of the present study indicated that NAP could modulate the biotransformation metabolism, expressed as liver EROD activity increase. However, compared to the positive control (BNF), NAP induced a later EROD rise (16–72 h).

Despite the EROD induction potential, the current NAP and BNF results also revealed an inhibitory capacity detected especially for the highest concentrations (0.9 and 2.7 mM) during the first 6 h NAP exposure and at 2 hBNF exposure (0.9 mM). This inhibitory effect may be explained by the high xenobiotic exposure concentrations. Previous studies demonstrated that several CYP1A1 inducers could also inhibit the MFO catalytic activity (EROD or AHH) at high concentrations (Stegeman and Hahn, 1994). Suchdouble performance was found in fishexposed, either in vivo or in vitro, to high doses of polychlorinated biphenyl (PCBs) congeners (Boon et al., 1992; Hahn et al., 1993), BaP (Goddard et al., 1987), and BNF (Haaschet al., 1993). The majority of cytocrome P450 enzyme inhibitors act either by competitive inhibition or by mechanism-based inactivation in which the inhibitor is metabolized by the cytocrome P450 into a product that covalently modifies the active site and thereby inactivates the enzyme (Stegeman and Hahn, 1994). In the present study, EROD activity and P450 content presented a concomitant inhibitory tendency during the first hours of exposure, especially in the NAP experiment. However, in this experiment a significant EROD activity increase corresponds to a significant decrease in cytochrome P450 content (16 h).

The liver EROD decrease observed during the first 6 h NAP exposure was not followed by a liver ALT decrease. Moreover, liver ALT activity at 8 hexposure to 0.3 mM NAP revealed a significant increase. Thus, the observed EROD activity decrease is not explained by liver lesions.

Liver EROD induction observed after 16-hNAP and 4-h BNF exposures might be explained by either a progressive fishacclimation and physiological adjustment, reducing the inhibition efficiency, or by a decline of the xenobiotic levels in the aquaria.

Globally, the current phase I results proved that liver EROD activity rather than total cytochrome P450 is a good biomarker for PAH and PAH-like compounds exposure, providing an early biological warning signal.

Increases in GSH, glutathione reductase, and GST levels have been linked to organism resistance and adaptation to a variety of physical and chemical stresses found in the environment (Gallagher et al., 2001). Considering the current NAP results, a liver GST activity tendency to increase was observed from 2 to 72 h, being significantly expressed at 2 and 48 h. GST induction could reflect a stress response to the chemical, resulting in a phase II conjugation process, where the metabolites formed in phase I are combined with endogenous molecules to form conjugates. Nevertheless, it is difficult to establisha direct correspondence between GST, EROD, and P450 content responses.

In agreement withour results, a hepatic GST activity increase was previously observed in NAP-treated mice (Mitchell et al., 2000). Additionally, the same response was observed in BaP-treated fish(Lemaire et al., 1992; George, 1994). Some authors suggested that the absence of GST activity variation could be followed by induction of other conjugases, such as UDP–glucoronyl transferase (Novi et al., 1998).

The A. anguilla ENA response to bothcompounds was clearly different. A significant ENA frequency increase from 2 to 8 hexposure to NAP was observed, demonstrating an early genotoxic effect. However, BNF did not reveal any ENA increase for all the exposure durations.

PAHs mutagenic characteristics are strongly related to their biotransformation, especially the initial oxidative metabolism carried out by cytochrome P450 monooxygenases. However, considering the early ENA induction observed in the NAP experiment concomitant to an EROD decrease, it might be suggested that NAP is genotoxic itself, without the need of bioactivation. Since NAP is the simplest PAH, having only two fused aromatic rings, its entrance into the cell is facilitated and a large number of these molecules might rapidly injure the DNA. The increased EROD activity observed from 16 to 72 hNAP exposure coincides withan ENA decline. Furthermore, a significant GST increase occurred at 48 h exposure. These facts indicate that the

phase I activation did not carry negative consequences for erythrocytic DNA and the whole detoxification process (phases I and II) seems to be effective, preventing ENA appearance. This explanation is supported by a previous human study (Tingle et al., 1993) in which liver cytochrome P450 enzymes metabolized NAP to a cytotoxic and protein-reactive, but not genotoxic, metabolite (probably an epoxide). This is rapidly and efficiently detoxified by microsomal epoxide hydrolase forming stable metabolites.

The observed NAP genotoxic potential is in agreement withprevious results of Gravato and Santos (2002) in Dicentrarchus labrax and Delgado-Rodriguez et al. (1995) in Drosophila melanogaster.

The hypothesis presented above for the NAP genotoxic mechanism may also be applicable to the current ENA/EROD results in the BNF experiment, since an EROD induction was observed from 4 to 72 hexposure without a simultaneous ENA response. This is in agreement withthe results of Pacheco and Santos (1997) who observed in glass eels an ENA appearance at 6 and 9 days BNF exposure following a time-related tendency to decrease in EROD activity. Additionally, withrespect to the previous findings, it must be suggested that longer BNF exposures should be used to assess its genotoxic potential.

Previous investigations suggested that the expression of genotoxic effects, suchas micronuclei or other ENA, could be masked by a direct erythropoiesis inhibition (Das and Nanda, 1986; Dinnen et al., 1988) and/or by an increased erythrocytic catabolism (Pacheco and Santos, 1997). Alterations in the balance of erythropoiesis/erythrocytic catabolism could be assessed by IE frequency changes. In the NAP experiment a causal relationship between IE frequency and ENA disappearance was not found. However, erythrocytic catabolism needs to be studied. In the BNF experiment IE frequency data indicated an ability to alter the balance between erythropoiesis and erythrocytes removal. Therefore, it was evident that ENA expression from 24 to 72 hwas affected by an IE frequency decrease, becoming more obvious at longer exposures to high BNF doses. Consequently, a causal relationship between the previous disruption observed at 24, 48 and 72 h and the absence of ENA increase may be suggested. If the erythrocytes are not produced or are catabolized more intensely, the ENA appearance can be masked despite the presence of genotoxic metabolites.

The endocrine system plays a central role in fish stress mechanisms. Cortisol, in particular, is a crucial hormone in the physiological response to stressors. Previous studies demonstrated that acute exposures to xenobiotics, namely PAHs (Thomas and Rice, 1987), in addition to capture and handling (Vijayan et al., 1997), elevate plasma cortisol. Nevertheless, effects of chronic exposures to pollutants are less well understood (Hontela, 1997). Fish exposed to heavy metals exhibited an initial plasma cortisol rise followed by a decline to control levels (Pratap and Wenderlaar, 1990). According to Hontela (1997), fishchronically exposed to PAHs, PCBs, and heavy metals were unable to increase cortisol in response to a capture stress. The authors explained this fact by an exhaustion of the cortisol-producing endocrine system, possibly as a result of its prolonged hyperactivity. Other authors demonstrated that the interrenal function might also be affected by short-term exposures. Eels exposed either to bleached kraft pulp mill effluent for 4 h(Santos and Pacheco, 1996) or to diesel water-soluble fraction for 3 hto 3 days (Pacheco and Santos, 2002) exhibited an impairment in the expected plasma cortisol increase, as a response to the stress of capture and handling.

In the current study, a cortisol-impaired response seems to occur from 4 to 24 hNAP exposure, demonstrating an interrenal disruption after short-term exposures. However, an adaptation process seems to occur after 48 hNAP exposure, since the plasma cortisol levels revealed a tendency to increase in treated fish. These results are in agreement with the findings of Pacheco and Santos (2001) who detected a similar response profile. In opposition, Levitan and Taylor (1979) found a general plasma cortisol increase in F. heteroclitus exposed to 4 mg/L NAP for 2–12 h. This disagreement may be explained by the use of different fishspecies and/or by the concentration levels adopted (about 10-fold lower in the current work).

There is some evidence that acute exposures could exert adrenal cytotoxicity, impairing the adrenal function and the ability to secrete cortisol (Jö nsson, 1994). Nevertheless, if this cytotoxic effect is related to the observed interrenal impairment in the present study, a recovery occurs after 24 hNAP exposure. Further investigations are needed to clarify the mechanisms involved in the cortisol responses, focusing mainly on the inhibitory processes in short-term exposures.

5. Conclusions

The current results led to the following conclusions: (1) Naphthalene (NAP) is an aquatic contaminant, which induces early genotoxic damage in erythrocytes and a later liver ethoxyresorufin O-deethylation (EROD) induction. (2) NAP is a less potent EROD inducer than the positive control (BNF). (3) NAP is more genotoxic than BNF. (4) The increased ENA frequency induced by NAP is not directly related to high EROD activity. Furthermore, ENA seems to be expressed when the detoxification mechanisms are depressed. (5) The adoption of a multibiomarker battery focused on biotranformation, genotoxicity and endocrine levels should be a good biomonitoring strategy,

facilitating the understanding of the mechanisms involved in fishtoxic responses.

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