

Increased formation of carcinogenic PAH metabolites in fish promoted by nitrite

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Abstract

Nitrite (NO_2^-), a highly reactive chemical species, accumulates in coastal waters as a result of pollution with nitrogenous waste and/or an imbalance in the bacterial processes of nitrification and denitrification. The present study probed the impact of nitrite (NO_2^-) on the metabolism of polycyclic aromatic hydrocarbons (PAHs) in fish. In a laboratory experiment, exposure of the euryhaline fish, *Oreochromis mossambicus* to industrial effluents containing PAHs in the presence of NO_2^- enhanced the activity of the biotransformation enzyme, cytochrome P4501A1 determined as 7-ethoxyresorufin O-deethylase (EROD), by nearly 36% compared to the value observed in the absence of NO_2^- (50.2 ± 6.74 pmol resorufin $\text{min}^{-1} \text{g}^{-1}$ liver). Fixed wavelength fluorescence measurements in bile revealed maximum enhancement to have occurred in the metabolites of benzo(a)pyrene, a carcinogenic PAH. Lasting, sublethal physiological deterioration was apparent in fish exposed simultaneously to an oil refinery effluent and NO_2^- , from the unremittingly decreasing liver somatic index, even after the withdrawal of the contaminants.

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1. Introduction

The coastal waters are the ultimate depository of most anthropogenic pollutants. Accordingly, two major environmental problems are encountered here viz., accumulation of pollutants and eutrophication. Prominent among the ubiquitous organic contaminants occurring in the marine environment are the polycyclic aromatic hydrocarbons (PAHs), which arise mainly from combustion- and oil-related anthropogenic activities (Neff, 1985). Exemplified by the recalcitrant benzo[a]pyrene, PAHs are of extreme ecotoxicological importance being responsible for such toxic effects as DNA adduct formation and carcinogenesis (Stegeman and Lech, 1991). By and large, pollution research is centred on the effects of discrete contaminants on organisms. A major issue, however, is that organisms do not encounter a single factor at a time but a suite of stresses, including environmental factors, acting together. Under field conditions the metabolism and toxicity of contaminants are liable to be altered by the presence of multiple contaminants / stresses. Reports indicate that PAHs could have more than additive (synergistic) effects with other pollutants (Chaloupka et al., 1993).

One major natural stress in the marine environment is eutrophication-related hypoxia. Several estuarine and coastal areas worldwide are increasingly experiencing eutrophication and subsequent oxygen depletion from an over-supply of nutrients (Nixon, 1990). Hypoxia alters the biogeochemical cycling of nutrients in the affected waters through denitrification, leading to short-term accumulation of nitrite (NO_2^-). The concentration of NO_2^- in aquatic ecosystems can, thus, be elevated much above the normal as a result of pollution with nitrogenous waste and/or an imbalance in the

bacterial nitrification and denitrification processes. NO_2^- is a highly reactive chemical which is more toxic to freshwater organisms than to marine animals (Eddy et al., 1983). Brackish and marine fish are not particularly susceptible to NO_2^- toxicity since chloride salts compete with NO_2^- for uptake at the gill lamellar surface. The interactive effects of NO_2^- and xenobiotics such as PAHs are poorly described; yet, NO_2^- has been found to significantly increase the toxicity of PAHs such as phenanthrene leading to severe hepatic damage in estuarine fish (Shailaja and Rodrigues, 2003a) and DNA damage in hepatic cells (Shailaja et al., unpublished).

The objective of the present study was to investigate how NO_2^- affects the biological impact of PAH - containing industrial effluents in fish. The euryhaline fish, *Oreochromis mossambicus* were exposed, in separate experiments, to two industrial effluents viz., refinery- and distillery effluent in the presence of NO_2^- . The concentrations of the two test toxicants (PAH and nitrite) used were well within the range of field-measured levels (COMAPS, 2002). Biotransformation of PAH in the exposed fish was followed with the help of biomarkers namely, 7-ethoxyresorufin O-deethylase (EROD) activity and biliary PAH metabolites. The liver somatic index (LSI) was used to determine the physiological status of the fish.

2. Materials and methods

2.1. Exposure protocol

Juvenile *Oreochromis mossambicus* (8.6 to 11g) obtained from a local estuarine fish farm were acclimatized in the laboratory for 7d in tap water that had been stored for at least 2 weeks to remove all traces of residual chlorine and amended with reagent grade

NaCl (final salt concentration 15g L^{-1}) so as to nullify the effect of nitrite in the experimental tanks. The exposure experiments (6-8 fish per tank) were conducted for 14 d in 40-L glass aquaria with continuous aeration using (a) a refinery effluent treated up to the secondary level and (b) a distillery effluent, diluted respectively, to 1% and 0.1% in water described as above, to yield a total PAH concentration of $\sim 4\text{ppb}$ [as determined in hexane extracts by spectrofluorimetry using pure chrysene (Sigma, USA) as the standard and excitation/emission wavelengths 310/360nm]. An aqueous solution of sodium nitrite (1ml) was added to two tanks containing refinery- and distillery effluents, respectively, to obtain a final NO_2^- concentration of $1\mu\text{M}$. In the experiment involving the refinery effluent only, fish (n=6) after 14 days of contaminant exposure were transferred to clean water for a further period of one week.

Control experiments were conducted with fish (n=12) in water with/without added NO_2^- . The test medium was renewed every alternate day. Commercial feed was provided *ad libitum* during the period of the experiment.

2.2. Hepatic biomarkers

At the conclusion of each experiment, livers were excised, weighed and the liver somatic index (LSI) determined as percentage ratio of liver weight to body weight. As the liver samples were too small to be processed individually for enzyme activity, they were pooled (3-4 fish livers each) before homogenization. The post-mitochondrial supernatant (PMS) was prepared according to Gunther et al. (1997). EROD activity was determined in PMS by the spectrophotometric method described by Klotz et al. (1984) using

NADPH as the electron donor. The enzyme activity denoted by an increase in absorbance at 572 nm was measured in a Jasco spectrophotometer (Model V-530) and reported in terms of pmol resorufin $\text{min}^{-1} \text{g}^{-1}$ liver. Limit of detection of the photometric assay was $9.18 \text{pmol ml}^{-1} \text{min}^{-1}$.

2.3. Biliary PAH metabolites

Bile samples were collected with a hypodermic syringe from the gall bladder and stored frozen at -20°C until analysis. Fluorescent aromatic compounds (FAC) were determined in the bile, diluted 1:3000 with 48% ethyl alcohol (AR grade), by fixed wavelength fluorescence (FF) measurements. Naphthalene-, pyrene-, phenanthrene- and B(a)P-type metabolites were analysed at the specified excitation / emission wavelength pairs of 290/335nm, 341/383nm, 260/380nm and 380/430nm, respectively (Krahn et al.,1987,1993). Bile absorption at 380nm was used to determine significant changes in biliverdin concentrations.

2.4. Statistical analysis

Statistical evaluations involving the Student's *t*-test were performed using Excel 2000 spreadsheet software (Microsoft). $P < 0.05$ was considered as statistically significant.

3. Results

*3.1. Exposure of *O. mossambicus* to distillery effluent and NO_2^-*

Analysis of the biliary FACs by fixed wavelength fluorescence in the distillery effluent-treated fish showed that the most responsive wavelength pair was $\text{FF}_{380/430}$ (Fig.

1) which corresponds to conjugated B(a)P- like metabolites. Hepatic EROD activity, biliary B(a)P metabolites and LSI data of *O. mossambicus* exposed to 0.1% distillery effluent and effluent plus NO_2^- (1 μM) are shown in Fig.2. CYP1A – mediated EROD activity in the fish treated with the effluent (PAH content ~ 4 ppb) displayed an enhancement of $\sim 233\%$ over the control value of 16.3 ± 3.4 pmol resorufin $\text{min}^{-1} \text{g}^{-1}$ liver. Co-treatment with NO_2^- led to a further 36% increase in the EROD activity and 12.5% in $\text{FF}_{380/430}$ compared to the effluent-treated fish, the increase being statistically significant ($P < 0.05$).

Liver somatic index of the fish remained unaffected upon exposure to distillery effluent. However, simultaneous treatment with NO_2^- reduced LSI by about 23% compared to the control (Fig.2).

3.2. Exposure to refinery effluent and NO_2^-

The major PAH metabolites in the bile in *O. mossambicus* exposed to secondary-treated refinery effluent were B(a)P-related (Fig. 1). $\text{FF}_{380/430}$ of bile corresponding to B(a)P-like metabolites was nearly 35% higher than in the unexposed fish and rose further by 22% when the fish were exposed to PAH along with NO_2^- (Table 1). However, in both the treatment groups the post-exposure values of the B(a)P metabolites determined after a 7-day depuration period in clean water were close to normal (Table 1).

Mean LSI values of *O. mossambicus* exposed to the effluent were similar to the control fish value (4.35 ± 0.64) both during and post-exposure (4.69 ± 0.11 and 4.47 ± 0.47 , respectively). But in fish exposed concurrently to effluent and NO_2^- , LSI value

during exposure (3.44 ± 0.99) was ~20% lower than the control fish value and continued to drop even after the pollutants had been withdrawn. The 7th day post-exposure LSI value was 2.67 ± 0.54 i.e., ~63% less than the control fish value.

4. Discussion

4.1. Effect of NO_2^- on the biotransformation of PAH in effluents

The process of biotransformation of PAHs to polar metabolites is catalysed initially by the cytochrome P450-dependant MFO enzymes (CYP1A1 in fish). A clear relationship has been observed between EROD activity (a surrogate for CYP1A1) and exposure to PAH in *O. mossambicus* (Shailaja and Rodrigues, 2003; Ueng et al., 1992; Shailaja and D'Silva, 2003; Chen et al., 2001). In the present study, EROD induction in *O. mossambicus* was evident on exposure to very low concentrations of a PAH-containing distillery effluent. Fish exposed to 1:1000 diluted distillery effluent showed a 3-fold higher EROD activity (significance >95%) compared to the clean-water control group (Fig. 2) whereas in fish exposed to dilute (1:100) secondary-treated refinery effluent the EROD activity was 1.3 times that of control fish (Shailaja and Rodrigues, 2001).

The ability of *O. mossambicus* to metabolise PAH present in both distillery and refinery effluents was evident also from the marked increases in bile FACs. Biliary FF values obtained in fish exposed to the distillery effluent (Fig. 1) corresponded predominantly to B(a)P-like metabolites which have maximum fluorescence at the excitation/emission wavelengths of 380/430 nm (Krahn et al., 1987) and compared to the control, they were 14.6% higher in the treated fish. Both MFO induction and biliary

B(a)P-like metabolites went up further when the fish were exposed simultaneously to NO_2^- , denoting an increase in PAH metabolism (Fig. 2). Similarly, in fish exposed to refinery effluent alone, significantly higher B(a)P-related metabolites were noted (Fig. 1), the $\text{FF}_{380/430}$ values being ~35% higher than in the unexposed fish and rising by another 22% in the presence of NO_2^- (Table 1). The observed increases in the latter case were, however, nullified by 1 week of depuration. The level of PAH metabolites in the bile is influenced by exposure to PAH as well as the flow of bile through the gall bladder; the latter being mainly dependent on the feeding status of the organism. In the present study, the animals were fed throughout to better mimic natural conditions. No significant changes in biliverdin concentrations were observed during the exposure period in any group although fish exposed to effluent plus NO_2^- displayed poorer appetite.

4.2. Biological effects of PAH plus NO_2^-

4.2.1. Physiological effect

Liver somatic index (LSI) has frequently been used as a biomarker for examining fish exposed to environmental contaminants. LSI values are generally elevated in vertebrates experiencing induction of hepatic microsomal P-450 for detoxification of organic compounds (Huuskonen and LindströmSeppä, 1995). In accordance with this, mean LSI values of *O. mossambicus* exposed to distillery effluent showed 15.5% enhancement over the control value. But significant ($P < 0.05$) decrease in the LSI values that is, 23% and 20% occurred when the fish were exposed simultaneously to NO_2^- plus distillery / refinery effluent, respectively. These observations agree with the results of our previous study in which a pure PAH compound namely, phenanthrene was used in the

presence of NO_2^- (Shailaja and Rodrigues, 2003). Additionally, the earlier study had demonstrated the occurrence of sublethal hepatocellular injury associated with cell death in the exposed fish. The present investigation has shown that exposure to PAH in the presence of NO_2^- could cause lasting, sublethal physiological deterioration of the fish since, after 7 days of recovery in clean water, the decreasing trend in the LSI continued even as the bile PAH metabolites returned to background levels (Table 1).

4.2.2. Potential genotoxic effect

PAHs need metabolic activation to become genotoxic. Based on our observation that NO_2^- upregulates induction of cytochrome P450 activity by PAH and leads to enhancement of metabolism of benzo(a)pyrene, a carcinogenic PAH, we can logically conclude that the presence of NO_2^- in the environment will influence the overall carcinogenicity of PAH-containing effluents.

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Table 1. Results of benzo(a)pyrene-related fixed fluorescence (B[a]P-FF) and liver somatic index (LSI) in *Oreochromis mossambicus* exposed to refinery effluent in the presence / absence of nitrite. Values are mean \pm SD and marked with an asterisk when significantly different ($P < 0.05$; *t*-test) from the control value

Treatment	Assay	
	B(a)P-FF ^a	LSI(%)
Control (n=6)	97.81 \pm 11.29	4.35 \pm 0.64
Exposure		
(n=7) effluent	293.73 \pm 31.24 ^b	4.69 \pm 0.11
(n=6) effluent+NO ₂ ⁻	359.64 \pm 5.21 ^b	3.44 \pm 0.99 ^b
Post-exposure		
(n=5) effluent	92.57 \pm 2.93	4.47 \pm 0.47
(n=5) effluent+NO ₂ ⁻	107.74 \pm 1.61	2.67 \pm 0.54 ^b

^a in arbitrary units

Fig.1. Fluorescence intensity (in arbitrary units) of biliary PAH metabolites of *Oreochromis mossambicus* exposed to refinery effluent (1%) and distillery effluent (0.1%). (Naph – naphthalene; B(a)P – benzo(a)pyrene; Pyr – pyrene and Phe – phenanthrene). Values are mean \pm SD (n = 6 to 8).

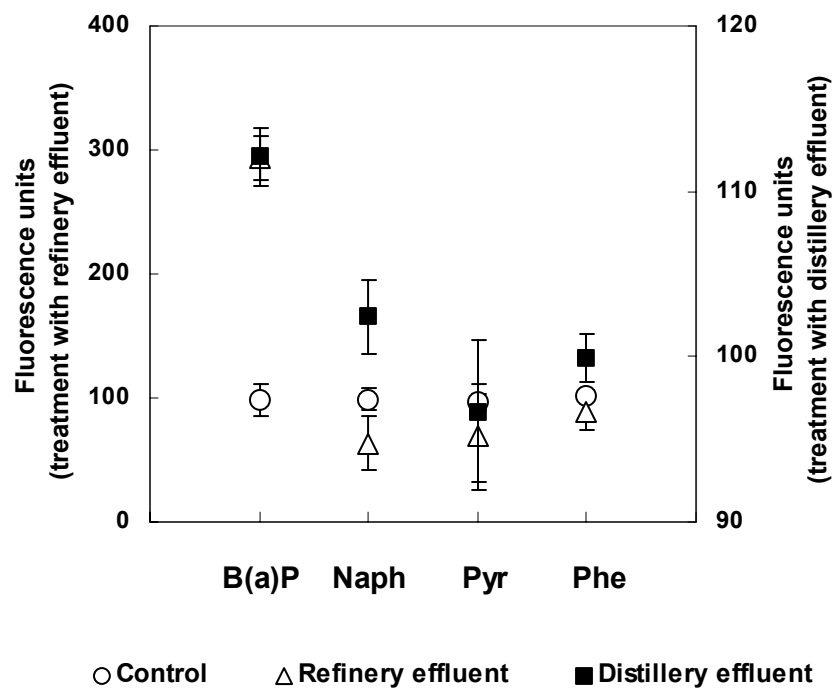
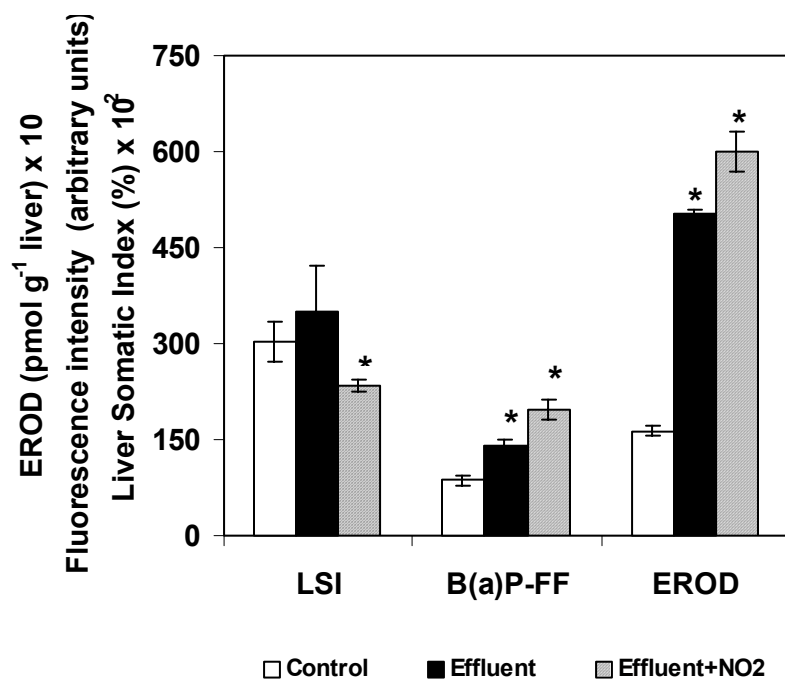


Fig. 2. Mean (\pm SD) values of 7-ethoxyresorufin O-deethylase (EROD) activity, liver somatic index (LSI) and biliary benzo(a)pyrene-related metabolites in *Oreochromis mossambicus* exposed to 0.1% distillery effluent in the presence / absence of nitrite. Values marked with an asterisk were significantly different ($P < 0.05$, t-test) from the control values. EROD values were measured in composite liver samples ($n = 3$ or 4).



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