

Formation of genotoxic nitroPAH compounds in fish exposed to ambient nitrite and PAH

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Abstract

Mutagenic nitrated polycyclic aromatic hydrocarbons (nitroPAHs) have been known to arise in the environment through direct emissions from combustion sources and nitration of PAHs, primarily in the atmosphere. Here we report the formation of nitroPAH compounds in fish contaminated with PAH, and exposed to nitrite (NO_2^-) in the ambient water. Electrospray ionization mass spectrometric analysis of the bile of the euryhaline fish, *Oreochromis mossambicus* exposed simultaneously to field-relevant, sub-lethal concentrations of phenanthrene ($1 \mu\text{g g}^{-1}$) and NO_2^- ($1 \mu\text{M}$) and collision-induced dissociation of selected ions revealed the presence of two strongly genotoxic nitroPAH metabolites namely, phenanthrene-6-nitro-1, 2-dihydrodiol-3, 4-epoxide (m/z 273) and dihydrodihydroxy acetylamino nitrophenanthrene (m/z 359). These two metabolite peaks present only in the bile of fish exposed simultaneously to phenanthrene and NO_2^- constituted, respectively, about 3.1 and 2.7% of the highest peak among the putative unconjugated phenanthrene metabolites in the mass spectrum. The presence of the oxidized phenanthrene metabolite, dihydroxyphenanthrene (m/z 233) in fish exposed to phenanthrene alone as well as phenanthrene plus NO_2^- suggested that oxidation of phenanthrene precedes nitration in the sequence of reactions leading to the formation of the observed nitrophenanthrene metabolites. However, the route of PAH administration seems to determine the nature of metabolites formed. Nearly 92% of the hepatic cells of the fish exposed to phenanthrene in the presence of NO_2^- were found to have suffered extensive DNA fragmentation on comet assay.

Key words: nitrite, coastal waters, nitroPAH, fish, genotoxicity

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Introduction

Potentially harmful chemical pollution and excess nutrient runoff remain top concerns in coastal areas. Coastal organisms are exposed to an assortment of environmental chemicals, natural as well as anthropogenic. PAHs are one of the classic anthropogenic organic pollutants encountered in the marine environment, while the highly reactive nitrite (NO_2^-) is produced naturally, mainly through biological processes such as denitrification (including assimilatory and dissimilatory nitrate reduction) and nitrification dominated by ammonia oxidation. NO_2^- accumulates in coastal waters under conditions that promote an imbalance in the above processes such as oxygen (O_2) deficiency or pollution with nitrogenous waste. In recent years, pelagic O_2 deficiency has intensified, essentially due to eutrophication, in many estuaries and coastal seas around the world (Nixon, 1995). An important biogeochemical consequence of this pelagic O_2 deficiency is the activation of alternate (anaerobic) pathways of respiration, mainly denitrification. The western continental shelf of India experiences a suboxic zone formation due to upwelling during SW monsoon (June-September) (Naqvi *et al.*, 2000), which is presumably exacerbated by the burgeoning fertilizer use (consumption of fertilizers in the region has increased by a factor of 7 since the early 1970s), contributing additional nitrogen loading from land. As a result, large accumulations of the denitrification intermediates notably, NO_2^- ($\sim 1 \mu\text{M}$ near surface and $> 6 \mu\text{M}$ near bottom) and nitrous oxide occur in the water column (Naqvi *et al.*, 2000; 2005).

Although more toxic to freshwater fish than to their marine counterpart (Eddy *et al.*, 1983), NO_2^- can increase the toxicity of PAHs leading to severe hepatic damage in estuarine fish (Shailaja and Rodrigues, 2001), impact the oxidative (Phase I) metabolism of PAHs and enhance the formation of carcinogenic metabolites in fish exposed to refinery effluent (Shailaja *et al.*, 2005). Mice injected with pyrene and subjected to

inhalation exposure to nitrogen dioxide (NO₂) form highly mutagenic nitrogenated metabolites of pyrene (Kano *et al.*, 1990). But whether marine organisms (e.g., fish) similarly produce endogenous nitrated PAHs (nitroPAHs) when co-exposed to PAHs and reactive NO₂⁻ in ambient water is not known. The main objective of the present study was to determine the manner in which NO₂⁻ present in seawater affects the toxicokinetics of PAH metabolism in fish by exposing the euryhaline fish, *Oreochromis mossambicus* (tilapia) to a combination of phenanthrene, a 3-ring PAH and NO₂⁻ dissolved in water to yield field-relevant, sub-lethal concentrations of the two stressors, and characterising the biliary metabolites.

Materials and Methods

a) Exposure protocol: Juvenile *O. mossambicus* (9.3 to 11 g, n=24) obtained from a local estuarine fish farm were acclimatized in the laboratory for 7d in stored tap water at room temperature (~25° C) amended with reagent grade NaCl (final salt concentration 15 g l⁻¹) to nullify the effect of nitrite in the experimental tanks. In one set of experiments, *O. mossambicus* (n=6 per tank) were treated with phenanthrene (purity >96%; Sigma, USA) dissolved in dimethylformamide and added to the tank with and without nitrite for 6 days. The final concentrations of phenanthrene and nitrite (added as analytical grade sodium nitrite) in the exposure medium were 1 mg l⁻¹ and 1 μM, respectively. Control fish (n=6) were exposed to equivalent concentrations of nitrite or phenanthrene only.

In another set of experiments, *O. mossambicus* (n=6) were administered a single intra-peritoneal injection (1 μg g⁻¹) of phenanthrene dissolved in sunflower oil (100 μl) and exposed to dissolved nitrite (1 μM) for 6 days as before. An equal number of control fish received equivalent amounts of phenanthrene without being exposed to nitrite.

The above experiments were conducted in several 30 L glass aquaria with continuous aeration and the water being changed every 24 h. Commercial feed was provided ad libitum during the period of experiment.

b) Comet Assay for DNA strand-break determination: Isolated hepatic cells of *O. mossambicus* (control and exposed) were subjected to modified single cell gel electrophoresis or comet assay (Singh *et al.*, 1988; Fairbairn *et al.*, 1995). To obtain the cells, a small piece of the liver was washed with an excess of ice-cold Hank's Balanced Salt Solution (HBSS) and minced quickly into approximately 1-mm³ pieces while immersed in HBSS, with a pair of stainless steel scissors. After several washings with cold phosphate-buffered saline to remove red blood cells, the minced liver was dispersed into single cells using a pipette (Lal and Singh, 1995). In brief, the protocol for electrophoresis involved embedding the isolated cells in agarose gel on microscopic slides, lysing them with detergent at high salt concentrations (overnight in the cold), treatment with alkali to denature the DNA (20 min) and electrophoresis under alkaline conditions (30 min) at 300mA, 25V. The slides after electrophoresis were stained with ethidium bromide and examined using a fluorescence microscope (Olympus BX60 F-3) with a green filter at 40x magnification. For each experimental condition, ~100 cells (about 25 cells per fish) were examined to determine the percentage of cells with DNA damage that appear like comets. Randomly selected, non-overlapping cells were visually assigned a score on an arbitrary scale of 0 - 3 (i.e., class 0 = no detectable DNA damage and no tail, class 1 = tail with a length less than the diameter of the nucleus, class 2 = tail of length between 1x and 2x the nuclear diameter and class 3 = tail longer than 2x the diameter of the nucleus), based on perceived comet tail length migration and relative proportion of DNA in the nucleus (Kobayashi *et al.*, 1995; Collins *et al.*, 1997). A total damage score for each slide was derived by multiplying the number of cells assigned to

each class of damage by the numeric value of the class and summing up the values.

Slides were analysed by one observer to minimise the scoring variability.

c) Electrospray ionization mass spectrometry (ESI-MS): ESI-MS analyses were performed on a QTOF XL mass spectrometer (Applied Biosystems Instrument, Canada) using an ion spray source with the following settings: ion spray voltage 5500 V, nebuliser gas (N₂) 20 (arbitrary units) and curtain gas (N₂) 20 (arbitrary units). The declustering potential (DP) and the collision energy were optimized around 15V and 3V, respectively during infusion experiment and 15 and 40 V respectively for MS/MS experiments. Composite untreated bile samples, after dilution with methanol : water (1:1), were analysed by direct ESI-MS. The sample was introduced at a constant flow rate of 10 ml min⁻¹ into the electrospray source using an integrated syringe pump. Data acquisition and processing were carried out using mass Lynx NT version 3.5. The MS survey range was m/z 0 - 950 (+ve mode) [or m/z 220-680 (-ve mode) when used] with scan duration of 1 s. Each mass spectrum was recorded over a period of 1 s and accumulated for 5 minutes for both single analyzer profiles and collision-induced dissociation (CID) experiments. For each of the ion species examined, the lock mass in each product ion mass spectrum was the observed monoisotopic mass/charge (m/z) ratio of the precursor ion. The samples were analysed mainly in the positive ion mode. The discrepancies occasionally observed in the values for protonated molecular ions to the extent of 1 or 2 amu were due to the instrument not being calibrated each time.

Selected ions with m/z values corresponding to putative metabolites were further subjected to MS/MS or collision-induced dissociation (CID) with N₂ gas and the subsequent product ion signal masses analyzed to produce a product ion mass spectrum.

Results and Discussion

Genotoxic effects of co-exposure to phenanthrene and NO₂⁻ in O. mossambicus.

Phenanthrene is found in the highest concentrations in aquatic samples but, unlike other PAHs such as benzo(a)pyrene and chrysene, it is not a pro-carcinogen (IPCS, 1998). Yet, the hepatic cells of *O. mossambicus* exposed simultaneously to phenanthrene and NO₂⁻ in 3 different experiments showed considerable DNA damage when evaluated by comet assay for DNA strand break in nuclei from individual cells. In a representative experiment (Table 1), DNA fragmentation was observed in nearly 92% of the hepatic cells as compared to 9% in the control fish exposed to NO₂⁻ alone and 23% in fish exposed to only phenanthrene. The liver was chosen for study because it is the major organ for organic xenobiotic metabolism and biotransformation enzymes.

While genomic injury is the initial step in the triggering of mutagenesis and carcinogenesis, the carcinogenic potential of PAHs is linked to the formation of ultimate carcinogens generated by metabolic biotransformations. In order to characterize the compounds formed by the interaction between NO₂⁻ and PAH in *O. mossambicus*, the bile of the exposed fish was subjected to ESI-MS and some selected ions therein were further analysed by CID. Untreated bile was analysed for the presence of free metabolites as they are potentially genotoxic. The positive ion mass spectra of the bile from fish exposed to water-borne phenanthrene alone and phenanthrene (via i.p. injection/water) plus NO₂⁻ are shown in Fig. 1a, b and c, respectively.

Identification of biliary nitrophenanthrene metabolites in O. mossambicus.

Diols are one of the major metabolites of phenanthrene formed in fish (Varanasi *et al.*, 1986; Livingstone, 1998; Pangrekar *et al.*, 1995, 2003), about 20% of them being present as free metabolites in the bile. Putative unconjugated metabolites of

phenanthrene could, therefore, be expected to have m/z values in the range of 200 to 400. We identified two biliary metabolites namely, phenanthrene-6-nitro-1, 2-dihydrodiol-3, 4-epoxide and a dihydrodihydroxy acetylamino nitro-phenanthrene, based on the mass of the MH^+ ions and the product ion spectrum generated by CID. These metabolite peaks (m/z 273 and m/z 359) present in the bile of fish exposed to both phenanthrene and NO_2^- (Fig. 1b), constituted, respectively, about 3.1 and 2.7% of the highest peak in the considered m/z range. With a collision energy of 45eV, the CID spectrum of dihydrodihydroxy acetylamino nitrophenanthrene (disodium) ion of m/z 359 yielded product ion signals of significant intensity at m/z 313, 255, 176, 59, 46, 38.9 and 22.9 (Fig. 2a). Of these, the last three fragments could be attributed to NO_2 , potassium and sodium ions, respectively, and the signal at m/z 313 resulted from the elimination of the NO_2 radical from the molecular ion (i.e., $M^+ - 46$). Removal of the acetylamino ($-NHCOCH_3$) group from the fragment of m/z 313 produced the peak corresponding to m/z 255, while the signal noted at m/z 59 is characteristic of a protonated acetylamino group. The product ion signal observed at m/z 176 represented the structure resulting from the loss of two $-ONa$ moieties from the fragment corresponding to m/z 255. Based on the logical fragmentation pattern, the metabolite was identified as the disodium salt of dihydrodihydroxy acetylamino nitrophenanthrene. However, the regiodistribution of the $-NO_2$, $-NHCOCH_3$ and $-OH$ groups on the molecule is unclear.

We determined a second biliary metabolite peak (m/z 273, Fig. 1b and c) as corresponding to phenanthrene-6-nitro-1, 2-dihydrodiol-3, 4-epoxide from its CID-MS/MS product ion spectrum with signals at m/z 215, 129, 114, 99 and 46 (Fig. 2b). The fragment at m/z 215 resulted from the combined loss of ($NO_2 + 2H_2O + Na$) from the molecule while the peak at m/z 129 comprised a portion of the phenanthrene ring system ($C_6O_3H_6$) carrying the hydroxy and epoxy groups. Loss of a molecule of water from this ion resulted further in the signal at m/z 114. Another portion of the

phenanthrene ring system containing the $-\text{NO}_2$ group ($\text{C}_4\text{H}_3\text{NO}_2+2\text{H}^+$) yielded the signal at m/z 99 even as the signal observed at m/z 46 represented the NO_2 fragment.

NitroPAHs are mostly produced either during incomplete combustion processes (pyrosynthesis) or as a result of reactions taking place in the atmosphere involving PAHs adsorbed on air particulates and gaseous nitrogenous species (Atkinson and Arey, 1994; Sasaki *et al.*, 1995; Ciccioli *et al.*, 1996). First detected in atmospheric particulate matter in the 1970s (Pitts *et al.*, 1978), they are studied mainly as potential genotoxic risk factors present in the atmosphere, based on their strongly mutagenic and carcinogenic properties (Möller *et al.*, 1993). In the water phase, nitroPAHs can result from a photochemical reaction of PAH with nitrite (NO_2^-) donating the nitro group (Ohe, 1984; Suzuki *et al.*, 1987). Endogenous formation of nitroPAH compounds, however, has not been reported so far in fish although the ability of 1-nitropyrene to form DNA adducts in fish has been demonstrated both *in vitro* and *in vivo* in brown trout (*Salmo trutta*) and turbot (*Scophthalmus maximus*) (Mitchelmore *et al.*, 1998). From the mass spectra in the present study it appears that a number of phenanthrene metabolites (not fully identified) are present in the bile, many of them as conjugates (m/z value >400). The formation of the various nitro- metabolites, very likely depends on the route of PAH administration as suggested by the fact that dihydrodihydroxy acetylamino nitrophenanthrene (m/z 359) was not observed in the mass spectrum of the bile of fish co-administered phenanthrene and NO_2^- through water (Fig. 1c).

The basis for the observed genotoxic effects in the hepatic cells of *O. mossambicus* exposed to phenanthrene in presence of NO_2^- is not certain but, presumably, the nitrophenanthrene metabolites were responsible given that very little damage occurred in the case of control fish exposed to either phenanthrene or NO_2^- alone. Mitchelmore and Chipman (1998a) have reported concentration-dependent increases in DNA strand breaks (using the "comet" assay) in isolated brown trout

(*Salmo trutta*) hepatocytes incubated *in vitro* with 1-nitropyrene. Both the nitrophenanthrene metabolites identified by us are capable of inducing DNA strand breaks in *O. mossambicus* since hydroxylated and diol epoxide metabolites of nitroPAHs are potent genotoxic compounds (Fu *et al.*, 1982; Möller, 1994; Li *et al.*, 1994; Chae *et al.*, 1993). Conjugation reactions involving acetylation also activate chemical carcinogens (Grant *et al.*, 1992), and, according to McCann *et al.* (1975), hydroxylated acetylaminoPAHs (e.g., OH-acetylamino fluorene) do exhibit a low mutagenic potency. Also, interestingly, the major part of the urinary mutagenicity in the case of 2-nitrofluorene-treated rats has been found associated with the unconjugated fraction of metabolites consisting of a range of hydroxylated acetylamino fluorenes and hydroxyfluorenes (Möller *et al.*, 1987; Kielhorn *et al.*, 1993).

Possible mode of formation of nitrophenanthrene metabolites in O. mossambicus.

A tentative metabolic pathway for the formation of nitrophenanthrene and its metabolites identified by ESI-MS/MS is presented in Fig. 3. We speculate that, in the sequence of reactions leading to the formation of the nitrophenanthrene metabolites, ring oxidation of phenanthrene precedes nitration since the presence of the oxidized metabolite, dihydroxyphenanthrene (m/z 233) was noted in fish exposed to phenanthrene alone as well as phenanthrene plus NO_2^- (Figs. 1a-c) and confirmed by CID-generated product ion spectrum (Fig. 2c). Results of studies conducted in several animals (hamsters, mice etc.) to elucidate the mechanism of *in vivo* nitration of pyrene have also suggested that PAH undergoes hydroxylation in the liver before being nitrated (Miyanishi *et al.*, 1996). Nitration of the hydroxyl groups by NO_2^- possibly involves the action of a peroxidase (Budde *et al.*, 2001). The further sequence of reactions likely includes ring oxidation of the nitrated PAH (resulting in the formation of phenanthrene-6-nitro-1, 2-dihydrodiol-3, 4-epoxide; m/z 273) and nitroreduction and acetylation (leading to the formation of

dihydrodihydroxy acetylamino nitrophenanthrene; m/z 359). The ring oxidation of the nitrated PAH involves the biotransformation enzyme CYP1A whose induction by a mixture of PAH and nitroPAH is additive (Fent, 2001). This is also supported by our previous observations of nearly 100% and ~270% increase in EROD activity compared to normal in *O. mossambicus* co-exposed to phenanthrene and NO_2^- and a PAH-containing effluent and NO_2^- , respectively (Shailaja and Rodrigues, 2001; Shailaja *et al.*, 2005).

It is known that interactions with other pollutants can play important modulatory roles in the expression of PAH toxicity (Chaloupka *et al.*, 1993). But this study presents the only observation so far of potentiation of mutagenicity of a non-carcinogenic PAH (e.g., phenanthrene) in fish by ambient NO_2^- . Elevated NO_2^- levels are generally limited to certain hypoxic habitats and habitats receiving nitrogenous pollutants; however, the ubiquitous nature and strong bio-concentration capacities of PAHs in the marine environment increase the likelihood that the type of interactions discussed will occur. Also, given the importance of coastal systems and the increasing pressure on them, it would be important to investigate the immediate as well as long-term effects of such interactions on ecosystem health for, in the final reckoning, human health depends on thriving ocean ecosystems.

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References:

Atkinson, R., and Arey, J. (1994). Atmospheric chemistry of gas-phase polycyclic

- aromatic hydrocarbons: formation of atmospheric mutagens. *Environ. Health Perspect.* **102**, 117-26.
- Budde, C.L., Beyer, A., Munir, I.Z., Dordick, J.S., and Khmel'nitsky, Y.L. (2001). Enzymatic nitration of phenols. *J. Mol. Catalysis B: Enzymatic.* **15**, 55-64.
- Buhler, D.R. and Williams, D.E. (1989). Enzymes involved in metabolism of PAH by fishes and other aquatic animals: oxidative enzymes (or Phase I enzymes). *In Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment.* (Ed. Varanasi, U.), CRC Press, Boca Raton, pp. 151-184.
- Chae, Y. H., Yun, C. H., Guengerich, F. P., Kadlubar, F. F., and El-Bayoumy, K. (1993). Roles of human hepatic and pulmonary cytochrome P450 enzymes in the metabolism of the environmental carcinogen 6-nitrochrysene. *Cancer Res.* **53**, 2028-2034.
- Chaloupka, K., Harper, N., Krishnan, V., Santostefano, M., Rodriguez, L. V., and Safe, S. (1993). Synergistic activity of polynuclear aromatic hydrocarbon mixtures as aryl hydrocarbon (Ah) receptor agonists. *Chem.Biol.Interact.* **89**, 141-158.
- Ciccioli, P., Cecinato, A., Brancaleoni, E., Frattoni, M., Zacchei, P., Miguel, A.H., and de Castro Vasconcellos, P. (1996). Formation and transport of 2-nitrofluoranthene and 2-nitropyrene of photochemical origin in the troposphere. *J. Geophys. Res.* **101**, 19567- 19582.
- Collins, A., Dusinska, M., Franklin, M., Somorovska, M., Petrovska, H., Duthie, S., Fillion, L., Panayiotidis, M., Raslova, K., and Vaughan, N. (1997). Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environ.Mol. Mutagen.* **30**, 139-146.
- Eddy, F. B., Kunzlik, P. A., Bath, R. N. (1983). Uptake and loss of nitrite from the blood of rainbow trout, *Salmo gairdneri* Richardson and Atlantic salmon, *Salmo salar* L. in fresh water and in dilute seawater. *J. Fish. Biol.* **23**, 105-116.
- Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995) Comet assay: a comprehensive review. *Mut. Res.* **339**, 37-59.
- Fent, K. (2001). Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P450 1A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicol. In Vitro* **15**, 477-488.
- Fu, P.P., Chou M. W., Yang S. K., Beland, F. A., Kadlubar, F. F., Casciano, D. A., Heflich, R. H., and Evans, F. E. (1982). Metabolism of the mutagenic

- environmental pollutant, 6-nitrobenzo(a)pyrene: metabolic activation via ring oxidation. *Biochem. Biophys. Res. Commun.* **105**, 1037-1043.
- Grant, D. M., Josephy, P. D., Lord, H. L. and Morrison, L. D. (1992) Salmonella typhimurium strains expressing human arylamine N-acetyltransferases: metabolism and mutagenic activation of aromatic amines. *Cancer Res.*, **52**, 3961–3964.
- IPCS. (1998). Selected non-heterocyclic polycyclic aromatic hydrocarbons. In *Environmental Health Criteria 202*. International Programme on Chemical Safety, WHO Monograph.
- Kanoh, T., Fukuda, M., Hayami, E., Kinouchi, T., Nishifuji, K., and Ohnishi, Y. (1990). Nitro reaction in mice injected with pyrene during exposure to nitrogen dioxide. *Mutat. Res.* **245**, 1-4.
- Kielhorn, J., Wahnschaffe, U., and Mangelsdorf, I. (2003). Selected nitro- and nitroxy-polycyclic aromatic hydrocarbon. In *Environmental Health Criteria UNEP/ILO/WHO Monograph 229*
- Kobayashi, H., Sugiyama, C., Morikawa, Y., Hayashi, M., and Sofuni, T. (1995). A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis assay. *MMS Commun.* **3**, 103-115.
- Lal, H., and Singh, N.P. (1995). Acute low intensity microwave exposure increases DNA single-strand breaks in rat brain cells. *Bioelectromagnetics*, **16**, 207-210.
- Li, E. E., Heflich, R.H., Bucci, T.J., Manjanatha, M.G., Blaydes, B. S., and Delclos, K. B. (1994). Relationships of DNA adduct formation, K-ras activating mutations and tumorigenic activities of 6-nitrochrysene and its metabolites in the lungs of CD-1 mice. *Carcinogenesis* **15**, 1377-1385.
- Livingstone, D. R. (1998). The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. *Comp. Biochem. Physiol.* **120**, 43-49.
- McCann, J., Choi, E., Yamasaki, E., and Ames, B. (1975). Detection of carcinogens as mutagens in the salmonells/microsome test: assay of 300 chemicals. *Proc. Natl. Acad. Sci. USA.* **72**, 5135-5139.
- Mitchelmore, C. L., Livingstone, D. R. and Chipman, J. K. (1998). Conversion of 1- nitropyrene by brown trout (*Salmo trutta*) and turbot (*Scophthalmus*

- maximus*) to DNA adducts detected by ^{32}P - postlabelling. *Biomarkers* **3**, 21–33.
- Mitchelmore, C. L., and Chipman, J. K. (1998a). Detection of DNA strand breaks in brown trout (*Salmo trutta*) hepatocytes and blood cells using the single cell gel electrophoresis (comet) assay. *Aquat. Toxicol.* **41**, 161–182.
- Miyanishi, K., Kinouchi, T., Kataoka, K., Kanoh, T., and Ohnishi, Y. (1996). In vivo formation of mutagens by intraperitoneal administration of polycyclic aromatic hydrocarbons in animals during exposure to nitrogen dioxide. *Carcinogenesis* **17**, 1483-1490.
- Möller, L., Rafter, J., and Gustafsson, J. A. (1987). Metabolism of the carcinogenic air pollutant 2-nitrofluorene in the rat. *Carcinogenesis* **8**, 637-645.
- Möller, L., Lax, I., and Eriksson, L.C. (1993). Nitrated polycyclic aromatic hydrocarbons: a risk assessment for the urban citizen. *Environ. Health Perspect.*, **101**, 309-15.
- Möller, I., (1994). In vivo metabolism and genotoxic effects of nitrated polycyclic aromatic hydrocarbons. *Environ. Health Persp.* **102**, 139 –146.
- Naqvi, S. W. A., Jayakumar, D.A., Narvekar, P.V., Naik, H., Sarma, V.V.S.S., DeSouza, W., Joseph, S., and George, M. D. (2000). Increased marine production of N_2O due to intensifying anoxia on the Indian continental shelf. *Nature*, **408**, 346 – 349.
- Naqvi, S. W. A., Naik, H., Jayakumar, D. A., Shailaja, M. S., and Narvekar, P. V. (2005). Seasonal oxygen deficiency over the western continental shelf of India. In NATO ASI Series , (L. Neretin, B. B. Jorgensen and J. W. Murray, Eds.), Kluwer – Springer (in press).
- Nixon, S. W. (1995). Coastal marine eutrophication: A definition, social causes, and future concerns. *Ophelia* **41**, 199-219.
- Ohe, T. (1984). Mutagenicity of photochemical reaction products of polycyclic aromatic hydrocarbons with nitrite. *Sci. Total Environ.* **39**, 161–175.
- Pangrekar, J., Kandaswami, C., Kole, P., Kumar, S., and Sikka, H.C. (1995). Comparative metabolism of benzo(a)pyrene, chrysene and phenanthrene by brown bullhead liver microsomes. *Mar. Environ.Res.* **39**, 51-55.
- Pangrekar, J., Kole, P. L., Honey, S. A., Kumar, S., and Sikka, H. C. (2003). Metabolism of phenanthrene by brown bullhead liver microsomes. *Aquat.*

- Toxicol.*, **64**, 407-418.
- Pitts Jr., J. N., van Cauwenberghe, K.A., Grosjean, D., Schmid, J.P., Fitz, D.R., Belser Jr., W.L., Knudson, G.B., and Hynds, P.M. (1978). Atmospheric reactions of polycyclic aromatic hydrocarbons: facile formation of mutagenic nitro derivatives. *Science* **202**, 515-519.
- Sasaki, J., Arey, J. and Harger, W. (1995). Formation of mutagens from the photooxidations of 2-4-ring PAH. *Environ. Sci. Technol.* **29**, 1324-1335.
- Shailaja, M. S., and Rodrigues, A. (2003). Nitrite-induced enhancement of toxicity of phenanthrene in fish and its implications for coastal waters. *Estuar. Coast. Shelf Sci.* **56**, 1107- 1110.
- Shailaja, M. S., Rajamanickam, R., and Wahidulla, S. (2006). Increased formation of carcinogenic PAH metabolites in fish promoted by nitrite. *Environ. Pollut.* **143**, 174-177.
- Singh, N.P., McCoy, M.T., Tice, R.R., and Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **175**, 184-191.
- Suzuki, J., Hagino, T., and Suzuki, S. (1987) Formation of 1-nitropyrene by photolysis of pyrene in water containing nitrite ion. *Chemosphere*, **16**: 859-867.
- Varanasi, U., Nishimoto, M., Reichart, W. L., and Eberhart, B.-T. L. (1986). Comparative metabolism of benzo(a)pyrene and covalent binding to hepatic DNA in English sole, starry flounder, and rat. *Cancer Res.* **46**, 3817-3824.

Table 1. Visual Scores of DNA damage. The total score was obtained by multiplying the number of cells in each class by the numeric value assigned to the class, and ranged from 0 (all undamaged) to 300 (all maximally damaged).

Treatment	No. of cells		Class ^b				Total Score	DNA Damaged Cells (% Mean)
	Analysed ^a	Comet	0	1	2	3		
Nitrite (Control N)	100	9	91	2	0	7	23	9
Phenanthrene (Control P)	100	23	77	16	2	5	35	23
Phenanthrene + nitrite (Test Group)	104	96	8	0	0	96	277	92.3

^a From 4 slides; 1 slide/fish scored

^b Class 0 = no tail, 1 = tail length < diameter of nucleus, 2 = tail length between 1x and 2x the diameter of nucleus, 3 = tail length > 2x the diameter of nucleus

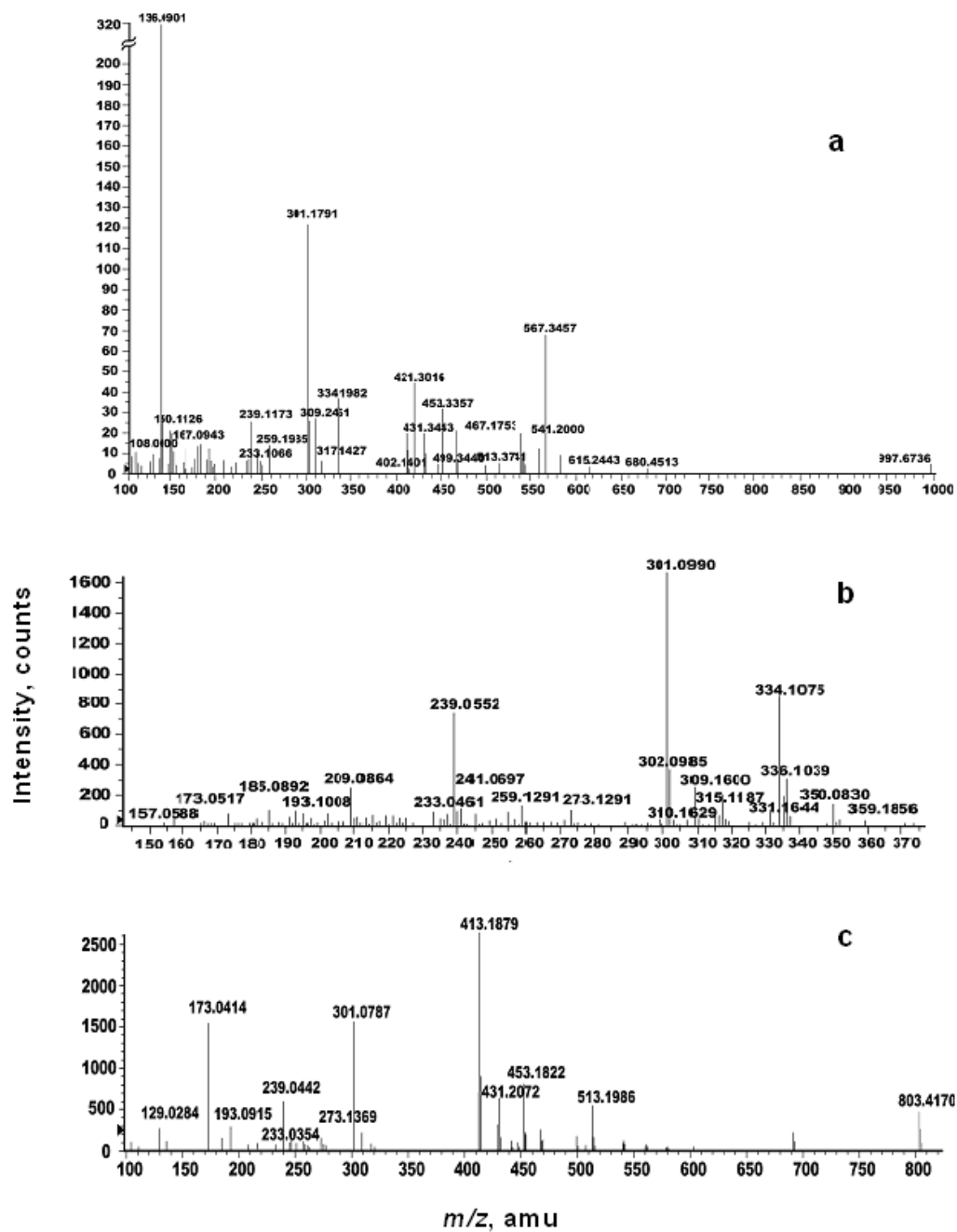


Fig. 1. Positive ESI-mass spectra showing biliary metabolites of *O. mossambicus* (a) administered phenanthrene through intra-peritoneal injection, (b) injected with phenanthrene and exposed to nitrite in water, and (c) co-exposed to phenanthrene and nitrite through water.

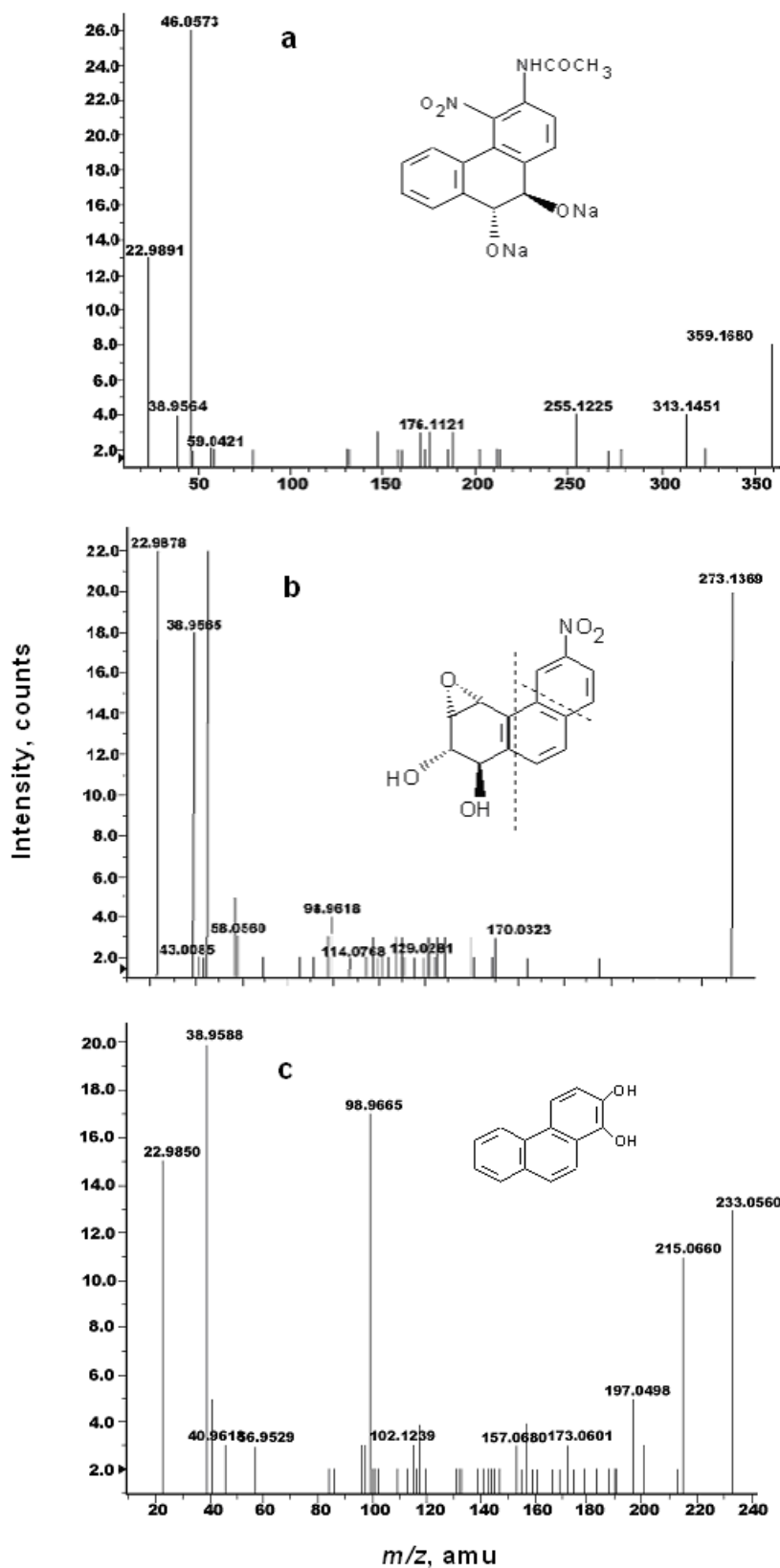


Fig. 2. Collision-induced dissociation spectrum of (a) dihydrodihydroxy acetylamino nitrophenanthrene, disodium salt (m/z 359, Fig. 1b), (b) phenanthrene-6-nitro-1,2-dihydrodiol-3,4-epoxide (m/z 273, Fig. 1b and c), and (c) (1,2/3,4/9,10)-dihydroxyphenanthrene (m/z 233, Fig. 1a-c).

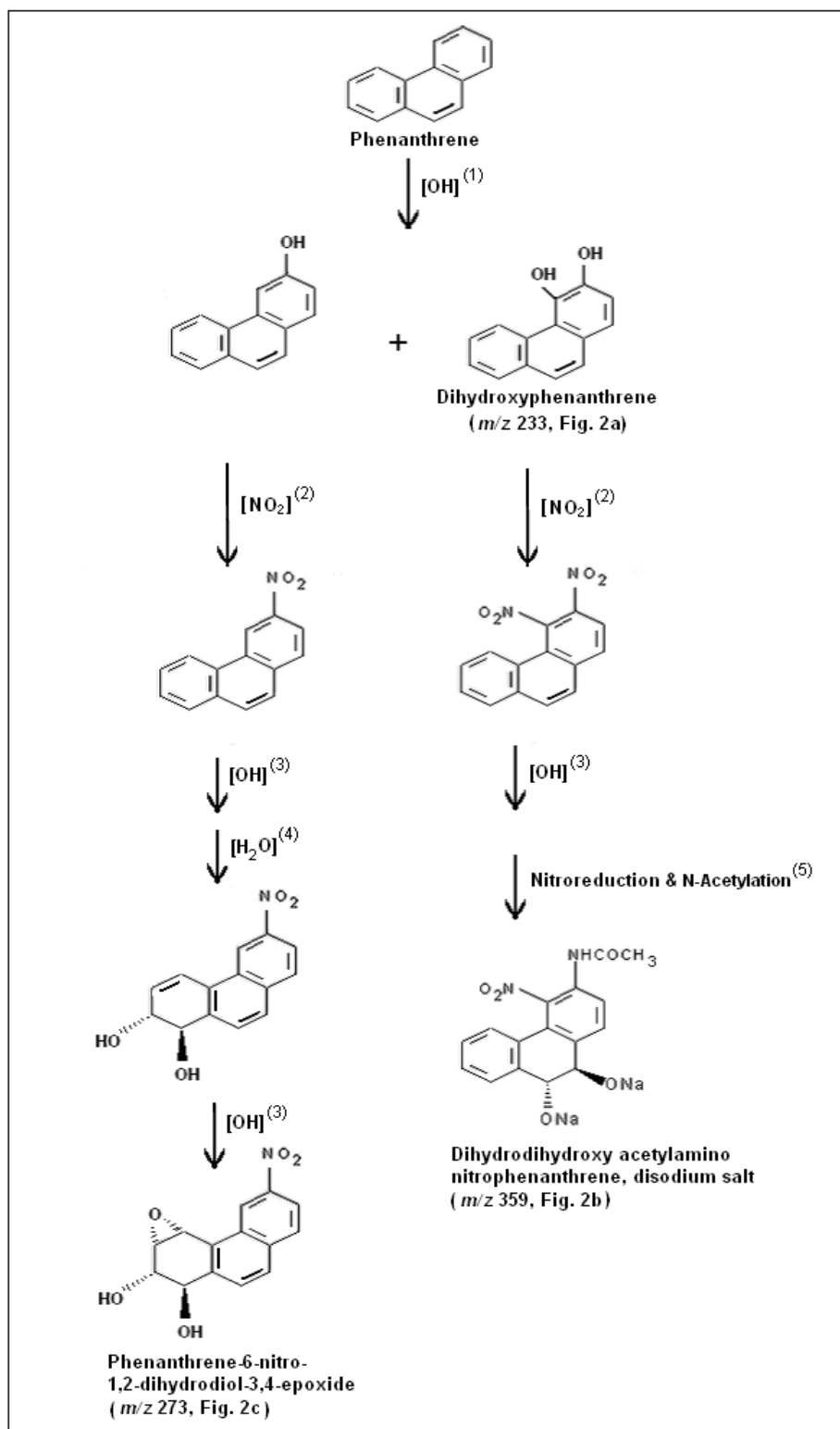


Fig. 3. Suggested metabolic pathway of formation of nitrophenanthrenes and some identified metabolites (1,2/3,4/9,10) - dihydroxyphenanthrene (*m/z* 233), phenanthrene -6-nitro-1, 2-dihydrodiol-3, 4-epoxide (*m/z* 273), and dihydrodihydroxy acetylamino nitrophenanthrene, disodium salt (*m/z* 359) in *O. mossambicus* exposed to phenanthrene in presence of nitrite. ⁽¹⁾ oxygenation reaction catalysed by CYP1A, ⁽²⁾ nitration, possibly catalysed by a peroxidase (Budde *et al.*, 2001); ⁽³⁾ ring oxidation of nitrophenanthrene(s) by CYP1A (Fent, 2001); ⁽⁴⁾ hydrolysis by epoxide hydrolase (Kielhorn *et al.*, 2003) ⁽⁵⁾ catalysed consecutively by a nitroreductase and an N-acetyltransferase (Kitamura and Tatsumi, 1996)

