NITROARENE POLLUTION IN ESTUARINE ENVIRONMENT AND THEIR RISKS ON AQUATIC ORGANISMS

(ニトロアレーンによる沿岸環境汚染とそのリスク評価に関する研究)

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ABSTRACT

Nitroarenes (NPAHs) are persistent in the environment and are reported to be carcinogenic and mutagenic. In order to investigate NPAHs pollution in waters and their biological effects, laboratory and field experiments were done.

This study consisted of four main parts with six different sub-studies to answer these objectives. Part 1 involved the investigation of NPAHs level. Study 1 was conducted to determine the level of NPAHs contamination in the surface road soil from 13 different regions. Among the regions, Thailand had the highest total NPAHs while India and Cebu (Philippines) had the highest carcinogenic 1-nitropyrene, other carcinogenic NPAHs were also detected such as 2-nitroflourene, 4-nitropyrene and 6-nitrochrysene. The main factors that affected varying NPAHs concentrations could be the type of automobile fuel used, traffic situation, urbanization and industrialization. Study 2 was conducted to investigate NPAHs contamination in road soils near the river, river sediments and water. Results showed that NPAHs concentration was higher in road soils. A positive correlation was obtained between NPAHs concentration in road and river sediments.

Part 2 involved the investigation of bioaccumulation and genotoxicity of NPAHs. Studies 3 and 4 were conducted to investigate if NPAHs were bioaccumulated through dietary or waterborne sources by marbled flounder *Pleuronectes yokohamae*. In Study 3, bioconcentration of NPAHs by *P. yokohamae* was investigated. Results showed that NPAHs are bioconcentrated with BCF between 4 to 422 and half-lives less than 6 days. In Study 4, *P. yokohamae* were exposed to dietary NPAHs. Results showed that NPAHs were accumulated but not biomagnified. In addition, micronuclei (MN) and other nuclear abnormalities (NA) were induced in exposed groups as compared to control. These studies suggest that NPAHs were accumulated from water borne sources and dietary NPAHs induced genotoxicity in *P. yokohamae*.

Part 3 involved the field experiments. Study 5 was conducted to investigate bioaccumulation of NPAHs and genotoxicity in tilapia *Oreochrombis niloticus* exposed to waters from Butuanon River upstream and downstream, Cebu, Philippines. NPAHs were accumulated and MN and NA were induced in *O. niloticus* exposed to the water from downstream. Other than NPAHs, PAHs and heavy metals were also investigated but were not possible to cause genotoxicity in *O. niloticus.* This study suggests that NPAHs can be accumulated and possibly one of the agents that can cause genotoxic effects in *O. niloticus*.

Part 4 involved the evaluation of hazards of environmental relevant concentration. Study 6 aimed to assess the overall effects of relevant NPAHs concentration in terms of oxidative stress and genotoxicity in tilapia *O. niloticus* exposed to waterborne 1-nitropyrene. Results showed that glutathione peroxidase, oxidative damage, and induction of MN and NA were significantly increased in exposed groups. These results suggested that *O. niloticus* was under oxidative stress and then induced MN and NA due to NPAHs genotoxicity. The biomarkers used in this study seem to be useful for the assessment of nitroarenes exposure or contamination in aquatic organisms.

Based on these results, it seems NPAHs pose a risk to aquatic organisms. However, monitoring of NPAHs in aquatic environment should be continued to determine further risks. In addition, further investigations should be conducted on the biological effects of NPAHs to completely understand their fate in aquatic organisms.

要　旨

ニトロアレーン（以下 NPAHs）は環境中に残留する物質のグループであり、そのいくつかに発ガン性などのあることが報告されている。これらは粒子状物質に吸着し、水環境を含めて環境の至る所で検出されている。本研究では、水環境中 NPAHs 汚染の状況とそれらの水生生物に対する影響を明らかにするため、室内およびフィールドでの実験を実施した。

パート 1 では、東南アジアなどを含む 13 地域の道路堆積物中 NPAHs（16 種）の分析を行った。タイで最も高い総 NPAHs 濃度が検出されたが、発ガン性が最も高いとされる 1-nitropyrene の濃度はインドおよびフィリピンのセブで高かった。発ガン性が疑われるその他の NPAHs として、2-nitroflourene、4-nitropyrene および 6-nitrochrysene も検出された。NPAHs の種類と濃度に影響を及ぼすものは、自動車燃料の種類、交通事情、都市化および産業化ではないかと考えられる。道路堆積物とその近くの河川底質および水中 NPAHs 汚染について調査した結果、道路堆積物と河川底質の間に正の相関が認められ、道路堆積物で最も高い濃度が検出された。

パート 2 では NPAHs の生物濃縮とその遺伝子毒性について調べた。NPAHs を添加した海水にマコガレイを暴露した結果、いずれの NPAHs とも生物濃縮され、それらの半減期は 6 日以下であった。一方、NPAHs を添加した餌を給餌したマコガレイでは NPAHs の生物濃縮は認められなかったが、暴露区で赤血球の小核（MN）および核形態異常（NA）の出現率上昇が観察された。水中から魚類に濃縮される NPAHs によって MN あるいは NA などの遺伝子毒性が引き起こされるものと考えられる。

パート 3 では、フィリピンセブ市内のボツワヌ川河川水でテラピアを飼育し、フィールドでの NPAHs 生物濃縮と遺伝子毒性の観察を行った。NPAHs はテラピアに生物濃縮され、下流の河川水で飼育した試験魚赤血球に MN および NA の有意な出現率上昇が観察された。河川水中の多環芳香族炭化水素化合物（PAHs）あるいは重金属類の濃度から考えて、これらの物質が MN および NA の出現率上昇をもたらしたとは考えにくく、上記の遺伝子毒性は魚類に生物濃縮された NPAHs が原因の一つと考えられる。

パート 4 では、環境中から検出される濃度の NPAHs の毒性評価を実験的に行った。環境中から検出されるレベルの 1-nitropyrene を添加した淡水でテラピアを飼育した結果、魚体中に生物濃縮されたことが確認され、MN および NA の出現率上昇、酸化ストレスに関連するバイオマーカー（glutathione peroxidase、過酸化脂質など）値の上昇が観察された。これらの結果から、1-nitropyrene に暴露したテラピアは酸化ストレス下にあったため、MN および NA の出現率上昇などの遺伝子毒性を発症したものと考えられる。これらの酸化ストレスバイオマーカーは、NPAHs に暴露された水生生物に対する有用なバイオマーカーであることが示唆された。

以上の結果から、NPAHs は水生生物に対してリスクを有していることが考えられる。したがって、環境中 NPAHs のモニタリングあるいはその生物影響についてさらに研究する必要がある。

PART 1

GENERAL INTRODUCTION

Chapter 1

General Introduction

Nitrated polycyclic aromatic hydrocarbons, nitroarenes or NPAHs are derivatives of polycyclic aromatic hydrocarbons (PAHs), which contain two or more fused aromatic rings made of carbon and hydrogen atoms as shown in Figure 1. The figure shows the structural formulas of some of the parent PAHs. Table 1 also shows the nomenclature, molecular formulas, relative molecular mass and CAS numbers of some NPAHs. NPAHs occur in the environment as a mixture together with parent PAHs and other organic compounds and are usually present in smaller quantities than the parent PAHs.

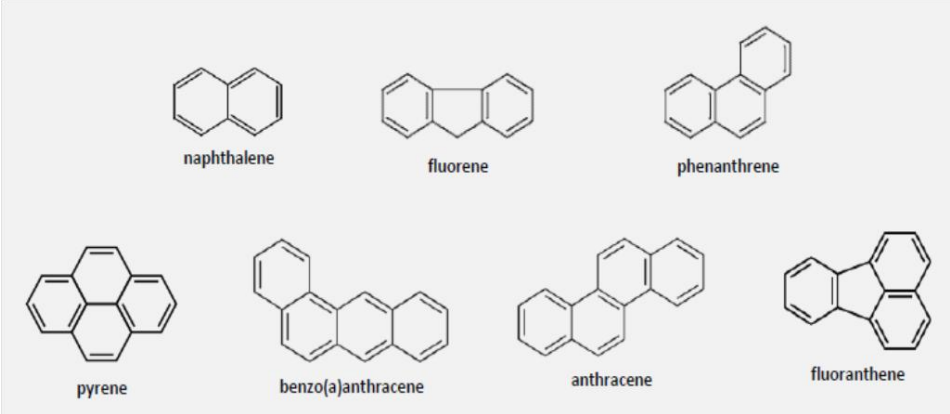

Figure 1.1 General structure of parent PAHs

NPAHs are insoluble or slightly soluble in water but are mostly soluble in organic solvents such as acetone, or benzene. Table 1.2 lists some of the physical and chemical properties of NPAHs used in this study. At ambient temperatures, NPAHs are yellowish to orange solids that tend to sublime (White, 1985). In the environment, NPAHs occur in the vapor phase or absorbed and/or adsorbed to particulate matter, depending upon their vapor pressure and the ambient conditions.

NPAHs are formed as a result of incomplete combustion of organic material and as a result of atmospheric reactions. Sources of NPAHs include diesel and gasoline exhaust, airplane emissions, combustion of heating oil like kerosene heaters, fuel gas and LPG

burners (Tokiwa et al., 1985; Kinouchi et al., 1988), and fumes from cooking oils (Wu et al., 1998).

Table 1.1 Nomenclature, molecular formulas, molecular mass and CAS numbers of some NPAHs (modified from EHC 229)

Parent PAHs	Nitro derivative	Molecular formula	Relative molecular mass	CAS number
Two-ring PAHs				
Naphthalene	1-nitronaphthalene	$C_{10}H_7NO_2$	173	86-57-7
	2-nitronaphthalene	$C_{10}H_7NO_2$	173	581-89-5
	1,5-dinitronaphthalene	$C_{10}H_6N_2O_4$	218	606-37-1
Three-ring PAHs				
Fluorene	2-nitrofluorene	$C_{13}H_9NO_2$	211	22250-99-3
	2,7-dinitrofluorene	$C_{13}H_8N_2O_4$	256	5405-53-8
Phenanthrene	2-nitrophenanthrene	$C_{14}H_9NO_2$	223	17024-18-9
	9-nitrophenanthrene	$C_{14}H_9NO_2$	223	954-46-1
Four-ring PAHs				
Fluoranthene	3-nitrofluoranthene	$C_{16}H_9NO_2$	247	13177-28-1
Pyrene	1-nitropyrene	$C_{16}H_9NO_2$	247	5522-43-0
	4-nitropyrene	$C_{16}H_9NO_2$	247	57835-92-4
	1,3-dinitropyrene	$C_{16}H_8N_2O_4$	292	75321-20-9
	1,6-dinitropyrene	$C_{16}H_8N_2O_4$	292	42397-64-8
	1,8-dinitropyrene	$C_{16}H_8NO_4$	292	42397-64-9
Benzo[a]anthracene	7-nitrobenz[a]anthracene	$C_{18}H_{11}NO_2$	273	20268-51-3
Chrysene	6-nitrochrysene	$C_{18}H_{11}NO_2$	273	7496-02-8

Table 1.2 Physical and chemical properties of some NPAHs (EHC 229, 2003)

Parent PAHs: nitro derivative	Melting point (^{o}C)	Solubility in water, mg/L (25^{o}C),	Log K_{ow}	Log K_{oc}
Two-ring PAHs				
Naphthalene	**81**	**31.7**	**3.4**	
1-nitronaphthalene	56.5	34	2.5	3.02
2-nitronaphthalene	76	26	2.78	3.09
1,8-dinitronaphthalene	171-172		2.52	
Three-ring PAHs				
Fluorene	**115**	**1.98**	**4.18**	
2-nitrofluorene	158	0.216	4.08	3.16
2,7-dinitrofluorene	334	0.28	3.35	
Phenanthrene	**100.5**	**1.29**		
2-nitrophenanthrene	119-120		4.23	
9-nitrophenanthrene	116-117			
Four-ring PAHs				
Fluoranthene	**108.8**	**0.26**	**5.22**	
3-nitrofluoranthene	166		5.15	
Pyrene	**150.4**	**0.135**	**5.18**	
1-nitropyrene	153	0.017	5.29	4.48
4-nitropyrene	190-192	0.017		4.48
1,3-dinitropyrene	295-297		4.44	
1,6-dinitropyrene	309-310		4.44	
1,8-dinitropyrene	299-300		4.44	
Benz[a]anthracene	**160.7**	**0.014**	**5.61**	
7-nitrobenz[a]anthracene	161-162		5.34	
Chrysene	**253.8**	**0.002**	**5.91**	
6-nitrochrysene	208		5.41	

Major sources of atmospheric NPAHs are of two groups. The first group comes from the diesel engine exhaust such as 1,3-, 1,6-, and 1,8-dinitropyrenes, 1-nitropyrene,

and 6-nitrochrysene. The second group is those formed in the atmosphere such as 2-fluoranthene and 2-nitropyrene (Perrini, et al. 2005).

NPAHs are formed in the atmosphere from PAHs and involving two processes: 1) through nitration during combustion process and 2) through atmospheric formation from PAHs either gas-phase reactions or heterogeneous gas-particle interaction of parent PAHs adsorbed into particles with nitrating agents (EHC 229; Perrini et al., 2005). Gas phase reactions in NPAHs occur during daytime and nighttime involving different reaction mechanisms as shown in Figures 1.2 and 1.3.

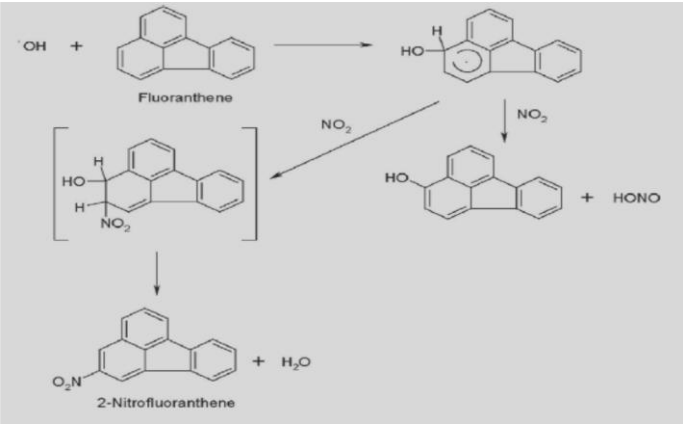

Figure 1.2 Daytime formations of NPAHs

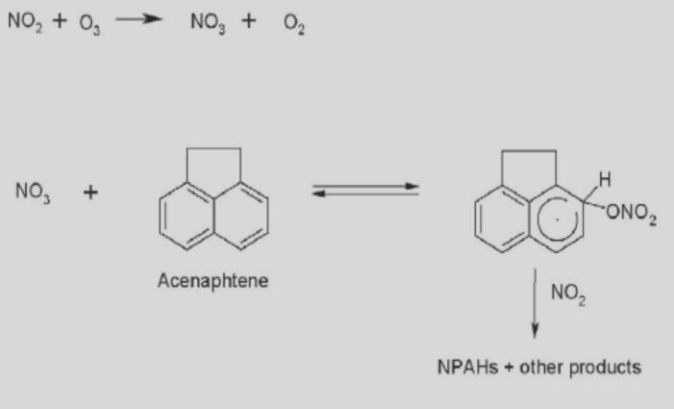

Figure 1.3 Nighttime formations of NPAHs

Generally, daytime gas-phase reaction involves reaction of a hydroxyl radical with PAHs, followed by reaction with nitrogen dioxide and the loss of water molecule, while in nighttime gas-phase reaction involves a nitrate radical addition to PAHs followed by

reaction with nitrogen dioxide and loss of nitric acid. Table 1.3 shows NPAHs that are formed during daytime and nighttime from their parent PAHs. That is, low molecular weights NPAHs like 1- and 2- nitronaphthalenes are directly formed in the atmosphere during daytime and nighttime.

Table 1.3 NPAHs formed from the gas phase reactions of PAHs known to be present in ambient air with hydroxyl radicals and nitrate radicals and their yields (EHC 229, 2003)

PAH	Daytime reactions	Nighttime reactions
Naphthalene	1-nitronaphthalene (0.3%) 2-nitronaphthalene (0.3%)	1-nitronaphthalene (17%) 2-nitronaphthalene (7%)
Fluorene	3-nitrofluorene (~1.4%) 1-nitrofluorene (~0.6%) 4-nitrofluorene (~0.3%) 2-nitrofluorene (~0.1%)	
Phenanthrene	Two nitro isomers (Trace amount)	Four nitro isomers (Trace amount)
Pyrene	2-nitropyrene (~0.5%) 4-nitropyrene (~0.06%)	4-nitropyrene (~0.06%)
Fluoranthene	2-nitrofluoranthene (~3%) 7-nitrofluoranthen (~1%) 8-nitrofluoranthene (~0.3%)	2-nitrofluoranthene (~24%)

*values in parenthesis are amount of NPAHs produced in percent

The transport and distribution of NPAHs depend on their physicochemical properties, but data for NPAHs are limited. However, their behavior is expected to be similar to that of the parent PAHs (IPCS, 1998). The main environmental transport of NPAHs is the atmosphere. NPAHs are either formed in the atmosphere from PAHs or emitted directly into the atmosphere during combustion processes. They can be transported in the vapor phase or adsorbed into particulate matter. NPAHs with liquid-phase vapor pressures that are greater than 10^{-4} Pa at ambient air temperature, such as two-ring NPAHs will exist partially in the gas phase (Atkinson & Arey, 1994). However, NPAHs with low vapor pressure will condense out on the surface of ambient particles. For example, 1- and 2-nitronaphthalene are expected to be found predominantly in the gas phase (Arey et al., 1987).

PAHs are ubiquitous in the environment, so NPAHs are also expected to behave the same. For example, 2-nitrofluoranthene and 2-nitropyrene were found out to be formed by atmospheric transformation (Ciccioli et al., 1995, 1996).

In the atmosphere, NPAHs may be distributed into fine and coarse fractions of atmospheric particulates. For example, 1-nitropyrene was detected in coarse fraction (2.5-10μm) and fine fraction (0.01-2.5μm [PM2.5]), while 2-nitrofluoranthene was mostly found in the fine particulate (Cecinato et al., 1999). Generally, most NPAHs are found in particulate fraction (Hayakawa, et al., 1999b).

NPAHs are not expected to be accumulated in the hydrosphere because of their low aqueous solubility. Furthermore, NPAHs sorption coefficients are high, thus they are adsorb strongly to the organic fraction of soils and sediments. Therefore, leaching through underground water is not a major route of transport for NPAHs.

NPAHs in the environment were identified and detected in different environmental matrices in air as vapor phase or air particulates, land such as road soils in urban and rural areas and water environment such as river water and sediments or sea water and sediments.

Most of the studies reported the presence of NPAHs in air environment. Murahashi and others reported concentration of several NPAHs such as 1-nitropyrene, 2-nitrofluorene, 6-nitrochrysene, 2-nitropyrene, 4-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene in both particulate matter and in rain. The concentrations in precipitations were in the range of 4.7-3,700 pg/L. The results obtained suggested that the NPAHs in the precipitations comes from the airborne particulate and are then dissolved in the aqueous phase (Murahashi et al., 1995; 1997).

Liu and others conducted a study about the distribution of PAHs and their derivatives in airborne particulates of East Asia, particularly in China, Japan, Korea and Russia. Results showed that Japan is second to China in terms of nitro-PAHs that are present in airborne particulates (Liu et al., 2006). A similar study conducted by Tang and others determined the PAHs and NPAHs in urban air particulates and their relationship to emission sources in the Pan-Japan sea countries, such as Shenyang (China); Vladivostok (Russia); Seoul (South Korea); and Kitakyushu, Kanazawa, Tokyo and Sapporo (Japan).

Four NPAHs, such as 1,3-, 1,6-, & 1,8-dinitropyrene and 1-nitropyrene were identified. For dinitropyrenes, 1,3- & 1,5-dinitropyrene concentrations were highest in Shenyang, while 1,8-dinitropyrene was highest in Sapporo. Furthermore, 1-nitropyrene concentration was also highest in Sapporo (Tang, et al., 2003; 2005).

Another study conducted by Hayakawa and others, compared PAHs and NPAHs in airborne particulates collected in downtown and suburban in Kanazawa, Japan. Two PAHs (pyrene and benzo[a]pyrene) and four NPAHs (1-nitropyrene, 1,3-, 1,6-, and 1,8-dinitropyrene) were determined. Results have shown that NPAHs and PAHs in suburban area have lower concentration when compared to downtown area, this may be due to the faster photodegradation of NPAHs during the atmospheric transportation from the downtown area to the suburban area (Hayakawa et al., 2002).

NPAHs were also detected in several food samples, such as: grilled and smoked meats (Joe et al., 1986), grilled corn, pork and chicken (Hayashi et al., 1986), vegetables, fruits and spices (Schlemitz et al., 1996). Results have shown that NPAHs concentrations were higher in smoked food and spices but lower in other food samples. These concentrations were 0.1-10.2 μg/kg for 2-nitronaphthalene, 0.8-350.7μg/kg for 2-nitrofluorene and 0.2-14.1 μg/kg for 2-nitropyrene (Schlemitz et al., 1996)

In aquatic environment, 1-nitropyrene concentration of 1 ng/L was reported from river water in Yodo, Japan. Samples were collected both at downstream and upstream location from a wastewater treatment plant, it was suggested that the latter was the source of 1-nitropyrene in the river (Ohe et al., 1996). Other NPAHs such as 1- & 2-nitronaphthalene, and 1,3- & 1,5-dinitronaphthalene were also detected in river water in Japan at concentrations of 1.3, 11.7, 1.7 and 3.2 ng/L, respectively (Takahashi et al., 1995). Vincenti et al., 2001, also reported NPAHs in Antarctic water and snow. Furthermore, a recent study conducted by Ozaki and others, reported eight NPAHs (9-nitroanthracene, 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene, 1,8-dinitropyrene, 2-nitrofluoranthene,

3-nitrofluoranthene and 6-nitrochrysene) in sea sediments and atmospheric particulate in Hiroshima Bay Area, Japan. Results showed that 2-nitrofluoranthene has the highest concentration between 18-39 ng/g sample in sea sediments. Generally, NPAHs concentration in sea sediments were lower compared to NPAHs in atmospheric particulate (Ozaki et al., 2009).

Data on the acute toxicity of NPAHs are only limited. A study on toxicity was reported for 1-nitronaphthalene with an LC_{50} (96h) of 9.0 mg/L for fathead minnow (*Pimephales promelas*). The test was conducted in a static renewal system, and was carried out using water with a hardness of 30-35 mg calcium carbonate/liter, pH 7.2-7.9 and a temperature of 22±1^{o}C (Curtis et al., 1981). Furthermore, it was found out that this NPAHs inhibited the growth of the ciliate *Tetrahymena pyriformis*, with an EC_{50} (60h) of 17.3 mg/L, after exposure in a static system at 28^{o}C for 60 h. The 95% confidence interval was 14.72-21.26 mg/L (Schultz et al., 1985). Lysak and others reported a 24-h LC_{100} value of 25 mg/L for rainbow trout (*Oncorhynchus mykiss*) exposed to 1-nitronaphthalene in a static renewal test system at a temperature ranging from 16 to 21.5^{o}C. The corresponding 48-h LC_0 concentration was 5 mg/L. Mortality was reported in fish to be 7.5-15 mg/L for 48 h (Lysak et al., 1972).

Since NPAHs has high affinity for organic phase than water, they are expected to be accumulated in aquatic organism. A study conducted for bioaccumulation of 2-nitrofluorene by *Daphnia magna* follows a first order kinetics. Results showed a bioconcentration factor of 170 when exposed to a 2-nitrofluorene concentration of 0.124 mg/L for up to 8 h (Gang & Xiaobai, 1994).

Biotransformation studies on aquatic species were also conducted. (Marsh and others 1992) reported that post-mitochondrial supernatants (S9) of marine invertebrates from three phyla: mussel (*Mytilus edulis*), crab (*Carcinus maenas*), and starfish (*Asteria*

rubens) activated 1-nitropyrene to products that were mutagenic to *S. typhimurium* strain TA98NR (Marsh et al., 1992).

Some studies have been concerned with the effect of NPAHs on the metabolism of some aquatic species, for example, the subcellular and tissue distribution of two- and one electron NADPH-dependent nitroreductase activity in marine invertebrates from three phyla: mussel (*Mytilus edulis*), crab (*Carcinus maenas*), and starfish (*Asteria rubens*) (Hetherington et al., 1996). Another study conducted by (Kitamura and Tatsumi, 1997) reported that 1-nitropyrene (8.3 mg/L) was metabolized to 1-aminopyrene, N-acetyl-1-aminopyrene and N-formyl-1-aminopyrene after 48 hours by goldfish (*Carassius auratus*) via nitro reduction pathway. Ueda and others also reported that 2-nitrofluorene is metabolized and excreted as 2-aminofluorene and its acetylated metabolites (Ueda et al., 2001).

A study on the effect of 1-nitropyrene to DNA was investigated by Mitchelmore and others. A 100μmol/L 1-nitropyrene produced a concentration-dependent increase in DNA strand breaks using the comet assay test in isolated brown trout (*Salmo trutta*) hepatocytes incubated in vitro, but no significant effects were found in blood cells (Mitchelmore & Chipman, 1998). The same was also reported for benzo[a]pyrene (24.7 at 100 μmol/L) and1-nitropyrene (54.7 at 200 μmol/L) using isolated mussel (*Mytilus edulis L.*) digestive gland cells (Mitchelmore et al., 1998a).

The ability of 1-nitropyrene to form DNA adducts in fish was investigated in vitro and in vivo using brown trout (*Salmo trutta*) and turbot (*Scophtalmus maximus*) and compared with that in Wistar rat. Hepatic S9 fractions from brown trout, uninduced and induced with β-naphthoflavone-induced rat were incubated with calf thymus DNA and 1-nitropyrene. With all S9 fractions, the presence of three distinct 1-nitropyrene-related DNA adducts was detected using ^{32}P post-labeling (Mitchelmore et al., 1998b).

Shailaja and others reported the formation of genotoxic NPAHs compounds in *Oreochromis mossambicus* after exposure to ambient nitrite and PAH (phenanthrene). Two strongly genotoxic NPAHs metabolites, namely phenanthrene-6-nitro-1,2-dihydrodiol-3,4-epoxide and dihydrodihydroxy acetylamino nitrophenanthrene were identified (Shailaja et al., 2006).

NPAHs have been reported to show genotoxic activity and mutagenicity. In addition, it has been found out that PAH`s are indirect mutagens while nitro-PAHs are direct-acting mutagens (Perrini et al., 2005). A study conducted by Ohe and others, identified 1-nitropyrene as a direct-acting mutagen in Yodo River, Japan using the Umu Test (Ohe et al., 1996). Hayakawa and others determined the distribution and mutagenicity of 1,3-, 1,6-, 1,8-dinitropyrene and 1-nitropyrene in airborne particulates collected in Kanazawa, Japan. Results showed that these NPAHs contribute to mutagenicity using *S. typhimurium* by the Ames Test (Hayakawa, et al., 1995a).

Mutagenic activities of NPAHs were also studied in surface soil samples in several areas of Japan. Watanabe and others, isolated and identified five mutagenic components (1,6-, 1,8-dinitropyrene, 1,3,6-trinitropyrene, 3,9-dinitrofluoranthene and 3,6-dinitrobenzo[e]pyrene using the Ames/Salmonella assay from surface soil in residential areas in Kyoto City (Watanabe et al., 2007). Other NPAHs isolated and identified that showed mutagenicity were 3-nitrobenzanthrone (Watanabe et al., 2003) and 1,3-dinitropyrene (Watanabe et al., 2000) in Kinki region.

NPAHs metabolites exhibited considerable mutagenicity for Salmonella tester strains (TA102 and TA96) which have adenine-thymine base pairs at the mutational target (Massaro et al., 1983). Furthermore, Murahashi and others identified a mutagen 3-nitrobenzanthrone from rain water collected in Kyoto, Japan (Murahashi et al., 2003a).

Several analytical methods have been used to detect NPAHs in different environmental matrices, employing different extraction and clean up procedure until analysis with simple to advance instruments.

The sampling of NPAHs is similar to that of PAHs. Ambient air is sampled by collecting particulate matter on special filters by means of high-volume samplers. Vapor-phase NPAHs are commonly collected on solid sorbents such as polyurethane foam.

Solvent extraction is followed by clean-up using liquid chromatography with silica gel or alumina, high-performance liquid chromatography or solid-phase extraction. The NPAHs fraction must be separated from the PAH fraction and oxygenated PAH fraction by HPLC on silica. Methods used for separation and detection of NPAHs include gas chromatography with a variety of detectors, HPLC with fluorescence, chemiluminescence or electrochemical detector, and mass spectrometric techniques.

Another approach to analysis of complex mixtures is bioassay-directed chemical analysis, where mutagenically active fractions are bioassayed and characterized until the major class or specific compounds potentially responsible for the mutagenicity are identified. The use of bacterial tester strains selectively sensitive to NPAHs has led to the identification of NPAHs as potent mutagens in complex mixtures from diverse sources.

Most extraction of NPAHs in particulate matter is generally carried out by soxhlet or sonication in organic solvents such as dichloromethane, benzene or toluene, but recently supercritical fluid extraction (SFE) has been reported. An SFE extraction method for NPAHs based on a two step extraction has been developed by Castells and others using CO_2 and toluene-CO_2 as extraction fluids (Castells et al., 2003). SFE extraction has some advantages over soxhlet and ultrasonic extraction, since both the extraction time and the solvent consumption are very low, on the other hand the recoveries for NPAHs are also low.

Jinhui and others developed a simple method to analyse NPAHs in particulate matter suitable for routine analysis since it needs a simple GC equipped with an electron capture detector (ECD). The method employs derivatization of NPAHs to fluorinated derivatives (Jinhui et al., 2001).

Since the nitro group is electron withdrawing, the NPAHs form more easily negative ions rather than positive ions when analyzed by a chemical ionization such as mass spectrophotometer: Bezabeth and others compared the electron impact (EI) and negative ion chemical ionization (NICI) peak areas for NPAHs extracted from a diesel particulate-related standard reference (SRMs) and found out that the NICI mode yielded two orders of magnitude greater sensitivity than the EI mode (Bezabeth et al., 2003).

Gas chromatography with flame ionization (GC-FID) or nitrogen and phosphorous selective detection (GC-NPD) has also been used (Librando, et al., 1993).

NPAHs give a very low fluorescence when irradiated with UV light, so in order to analyse them by HPLC/FLD (fluorescence detector) it is necessary to reduce them into aminoPAHs in the presence of sodium hydrogen sulfide. Hayakawa and others proposed a system equipped with a reducer column packed with a Pt/Rd-coated alumina for an on-line use in HPLC/chemiluminescence detector and detected trace levels of 1,3-, 1,6-, 1,8-DNP and 1-NP in airborne particulate (Hayakawa et al., 2001). NPAHs can also be reduced electrochemically using HPLC/EC/FLD as reported by Kuo et al., 2003.

Research Objectives

The main goals of the research were to investigate risks of NPAH contamination to aquatic organisms and determine possible biomarker for NPAHs pollution in aquatic environment.

Overview of the Study

NPAHs are reported to be carcinogenic and mutagenic; and some are classified and evaluated by IARC based on their carcinogenicity. Several studies has been conducted regarding their environmental concentration in atmosphere and aquatic organisms but few studies has been reported regarding their concentration in water environment.

The study consisted of four main parts with six separate studies were conducted, either in the field or laboratory. At first, since there is limited or no prior knowledge regarding concentrations of NPAHs in land environment, Study 1 was conducted to identify and determine NPAH concentrations in surface road soils. In this study, surface road soils were collected in different regions including Japan and Philippines. NPAH concentrations were also compared between regions according to kind and concentration of NPAHs present. Study 2 was conducted to investigate relationship between road soils near river banks, river sediments and river water in terms of NPAHs concentration. After knowing that NPAHs are persistent in the environment, Study 3 and 4 was conducted to investigate if NPAHs are bioaccumulated through dietary or waterborne sources by marbled flounder *Pleuronectes yokohamae* in laboratory conditions. A mixture of NPAHs was exposed to *P. yokohamae* and their genotoxicity was also investigated. Study 5 was conducted to investigate bioaccumulation of NPAHs and genotoxicity of tilapia *Oreochrombis niloticus* exposed to river water from Butuanon River, Cebu, Philippines. Knowing that NPAHs are bioaccumulated by *P. yokohamae* and *O. niloticus*, it is important to investigate the effect of NPAHs in fish. One of the immediate effects of pollution is oxidative stress. Study 6 was conducted to clarify the cause of genotoxicity in *O. niloticus* exposed to river water and investigate oxidative stress and genotoxicity once NPAHs are accumulated. Oxidative stress biomarker such lipid peroxidation, oxidation of protein, oxidation of DNA and glutathione peroxidase activity were used to investigate stress.

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PART II

ENVIRONMENTAL CONCENTRATION

Chapter 2

A survey of nitroarene level in road surface soil collected from Asian regions

Abstract

Present study was undertaken to determine the level of NPAHs contamination in the surface soil from 13 different regions. Sixteen priority nitroarenes were analyzed in <2mm surface soil fraction. Identification and quantification of NPAHs was done by high performance liquid chromatography coupled with mass spectrophotometer/mass spectrophotometer (LC-MS/MS). Total NPAHs concentration ranged from 111 to 16700 pg/g dry weight, with mean concentration of 2030 pg/g. In addition, lower total NPAHs concentration were found in Iloilo, Malaysia, Singapore and Vietnam, while higher total NPAHs concentration were found in France, Germany, India, Cebu in Philippines and Thailand. Among the sampling regions selected, Thailand has the highest total nitroarenes while India and Philippines, has the highest carcinogenic 1-nitropyrene in pg/g concentration level. Other carcinogenic NPAHs were also detected such as 2-nitroflourene, 4-nitropyrene and 6-nitrochrysene. NPAHs concentration levels observed in the present study were found to vary from region to region. One of the main factors that resulted to varying NPAHs concentrations could be type of fuel used, traffic situation, urbanization and industrialization.

Introduction

PAH derivatives, namely oxygenated and nitrated PAHs (OPAHs and NPAHs) had significantly been less addressed (Albinet et al., 2007; Albinet et al., 2008; Feilberg and Nielsen, 2001; Schnelle-Kreis et al., 2001) while they seem more toxic than their parent compounds (Durant et al., 1996). In the atmosphere, PAHs are mostly emitted by incomplete combustions of organic matter (Finlayson-Pitts and Pitts, 2000) whereas OPAHs and NPAHs are both, emitted primarily by combustion sources and are formed in the atmosphere by photo-oxidation processes of parent PAHs with atmospheric oxidants such as O_3, NOx, and OH radicals (Albinet et al., 2007; Vione et al., 2004; Zhang et al., 2011). Gasoline and diesel engine exhaust has been classified as group 2A and 2B, respectively (IPCS, 2003).

NPAHs were found in atmospheric particles and are considered to be carcinogenic or human carcinogens (IPCS, 2003). Several nitroarenes are believed to be far more mutagenic or carcinogenic than parent PAHs. NPAHs can be a significant fraction of direct-acting mutagenic compounds of atmosphere and can be a result of human exposure to respiration. NPAHs were found in various environmental compartments with PAHs. While their concentration is far lower than parent PAHs, they possibly have significant toxicity. NPAHs are thought to be emitted through atmospheric combustion processes such as diesel, gasoline engines, or industrial combustion processes (Paputa-Peck et al. 1983; IPCS, 2003), or composed from parent PAHs by hydroxyl radical addition followed by the reaction with nitrogen dioxide and by nitrate radical addition to the PAHs. After dispersion into the atmospheric environments, they may also deposit on ground surfaces, and discharge into and pollute water environments. In Chapter 3, the relationship of NPAHs concentration between road soil and river sediment were investigated, thus NPAHs concentration in road soil were analyzed.

Materials and Methods

Chemicals

The following NPAHs standards: 1] 1- and 2-nitronaphthalenes (1- and 2-NN); 1,5-dinitronaphthalene (1,5-DNN) and 2-nitroflourene (2-NF) were from Tokyo Kasei Kogyo Co. (Japan); 2] 2,7-Dinitrofluorene (2,7-DNF), 1-nitropyrene (1-NP), 3-nitroflouranthen (3-NFr), and 6-nitrochrysene (6-NC) were from Sigma-Aldrich (USA); 3] 3- and 9-Nitrophenanthrenes (3-NPh and 9-NPh); 1,3-, 1,6-, and 1,8-dinitropyrenes (1,3-DNP, 1,6-DNP, and 1,8-DNP) and 7-nitrobenzo(a)anthracene (7-NBaA) were from AccuStandard Inc. (USA); 4] 4-nitropyrene (4-NP) were from Dr. Ehrenstorfer GmbH (Germany) and 5] 1-nitropyrene-d9)1-NP-d9) from Central Chemicals Inc. (Canada).

Other chemical such as acetone, dichloromethane, diethyl ether and hexane were all pesticide grade reagents and purchased from Wako Pure Chemical Ind. (Japan). Acetonitrile and methanol were HPLC grade from Wako. Other chemicals, such as acetic acid anhydride, diethyl ether, formic acid, sodium hydroxide (NaOH), sodium hydrogen sulfide (NaHS), sodium chloride (NaCl) and silica gel (Wako gel) were analytical grade and also purchased from Wako. Silica gel containing 3% water was prepared by heating wako gel at 160^{o}C for 16 h and adding 3% (w/w) distilled water (Milli-Q) to dried silica gel with vigorous shaking for 1 h.

Sampling site

A total of 91 road surface soils were sampled in different regions, that is 14 in America (New York, Washington DC, Los Angeles, Florida), 2 in France (Paris), 2 in Germany (Berlin), 6 in Hongkong (Hongkong), 4 in India (Kolkotta), Japan (6 in Kagoshima and 2 in Hiroshima), 5 in Malaysia (Kuala Lumpur), Philippines (12 in Cebu and 5 in Iloilo), 13 in Singapore (Singapore), 11 in Thailand (Bangkok) and 9 in Vietnam (Ho Chi Minh).

Surface road soils were collected using a hand shovel. Collected samples were placed in clear glass bottles provided with cover and brought to laboratory. Samples were sieved using 2mm siever. Soils with diameter less than 2mm were stored below -70^{o}C until analysis.

Extraction and analysis of NPAHs in sediments

NPAHs were analyzed according to Uno et al. (2011). Briefly, about 5 g dry samples, was subjected twice to ultrasonic extraction with 20 mL dichloromethane-methanol mixture (3:1,v/v) for 15 min. The combined extract was further concentrated to 5 mL under a stream of N_2 gas and added with 15 mL Milli-Q water, 10 mL hexane-dichloromethane mixture (3:1) and finally saturated with NaCl. The resulting solution was then shaken for 15 min, and centrifuged for 10 min at 2300 rpm (1010 x g) at 4^{o}C. The organic layer was collected, dehydrated with anhydrous sodium sulfate, and completely dried using N_2 gas. The residue was then dissolved in 1 mL hexane for clean-up procedure. Clean-up was performed on extracts using a silica gel column (10cm length, 0.5cm i.d.; 3% moisture) and eluted with 60% acetone-hexane mixture followed with 5% acetone-hexane mixture. The eluates were combined and spiked with 1-nitropyrene-d_9 as an internal standard. Derivatization was performed by reduction with 3 mL 20% NaHS at 95^{o}C for 60 min and acetylation with acetic acid anhydride at 65^{o}C for 45 min. The acetylated NPAHs were extracted with 10 mL dichloromethane and the extracts were evaporated to dryness under a stream of N_2 gas. After dryness, 0.1 mL of 80% acetonitrile in water was added. NPAHs in this solution were measured employing a HPLC (Agilent Technologies, 1200 Series, USA) with mass spectrometry/mass spectrometry detector (Applied Biosystems, API-2000, USA) in positive electron scanning ionization mode with Inertsil C8-3 (4.6mm i.d. x 150mm, 5µm) as column (GL Sciences Inc., Japan).

Recovery Experiment

Recovery experiment was done using a mixture of standard NPAHs with individual concentration of 100 ng/mL. About 1g of washed and dried sediment was spiked with 100µL of 100 ng/mL of each target NPAHs. The procedure for extraction and analysis were discussed above.

Data Analyses

Most of the data processing was done using Microsoft Excel 2007 edition and results are presented as mean of two experiments per sampling site.

Results and Discussion

Recovery of NPAHs

Recovery rates (%) of 15 target NPAHs were investigated and the results are shown in Table 2.1. Recovery rates (%) are within 75 to 110% for target NPAHs with having the lowest and highest recovery, respectively; however, 1,3-DNP has a lower recovery rate with 23.5%. The results showed that the method can be applied to real environmental samples.

Table 2.1 Recovery rates and detection limit of target NPAHs in sediments (n=3)

Compounds, abbreviation	Molecular Mass	% Recovery rate ±SD	Detection Limit (pg/g)
1-nitronaphthalene, 1-NN	173	97.6±0.14	2.5
2-nitronaphtthalene, 2-NN	173	97.5±0.26	2.5
1,5-dinitronaphthalene, 1,5-DNN	218	74.7±0.42	7.5
2-nitroflourene, 2-NF	211	108±0.85	1.25
3-nitrophenanthrene, 3-NPh	223	97.4±0.49	2.5
1-nitropyrene, 1-NP	247	99.4±0.59	1.25
4-nitropyrene, 4-NP	247	101±0.59	1.5
3-nitroflouranthene, 3-NFr	247	96.1±0.26	17.5
1,6-dinitropyrene, 1,6-DNP	292	99.3±0.99	12.5
1,8-dinitropyrene, 1,8-DNP	292	110±0.99	0.75
7-nitrobenzo(a)anthracene, 7-NBaA	273	96.1±0.26	1.5
6-nitrochrysene, 6-NC	273	98.7±1.45	1

Concentration of NPAHs

A total of 10 NPAHs were detected in road soils collected from different regions. The distribution of individual NPAHs in each region with their concentration are shown in Figures 2.1 to 2.11. Table 2 shows the minimum and maximum concentrations of individual NPAHs detected in each sampling regions. Low molecular weight NPAHs such as 1-NN, 2-NN and 1,5-DNN were highest in Thailand with maximum concentration of 5260, 6100 and 2970 pg/g dry weight, respectively, while lowest maximum concentration of less than 1000 pg/g dry weight were detected in Hiroshima (Japan), Iloilo (Philippines), India, Kagoshima (Japan), Malaysia and Vietnam.

In addition, 2-NF and 9-NPh were highest in France, Germany and USA samples with maximum concentration of 172, 131 and 151 pg/g dry weight; and 548, 413 and 281 pg/g dry weight in 2-NF and 9-NPh, respectively. Furthermore, high molecular weights NPAHs such as 1-NP, 4-NP, 3-NFr, 7-NBaA and 6-NC were highest in India, Cebu (Philippines) and USA. Specifically, 1-NP was highest in Cebu (Philippines) and India with concentrations of 3670 and 2230 pg/g dry weight, respectively. Cebu (Philippines) has highest 7-NBaA and 6-nC level, with concentrations of 756 and 49 pg/g dry weight, respectively. Moreover, Table 2 also shows the total maximum NPAHs concentration was highest in Thailand (16,700 pg/g dry weight), followed by Cebu, Philippines (9010 pg/g dry weight), USA (5100 pg/g dry weight), India (4690 pg/ g dry weight), France (4670 pg/g dry weight), Germany (4230 pg/g dry weight), Hongkong (2500pg/g dry weight), Singapore (2290 pg/g dry weight), Kagoshima, Japan (1600pg/g dry weight), Hiroshima, Japan(1120pg/g dry weight), Vietnam (723 pg/g dry weight), Iloilo, Philippines (637pg/g dry weight) and Malaysia (574pg/g dry weight). 3-Nfr and 1-NP concentration in this study are within the reported concentration of 30-800 pg/g found in surface soil from the city of Basel, Switzerland (Niederer, 1998). 2-NF and 1-NP in this study were lower than reported concentration in river sediments detected in Suimon river (Japan) (Sato et al.,

1985). Furthermore, other reported concentrations of NPAHs were reported, such as 1-NP in sewage sludge at 680pg/g (Fernandez et al., 1992); 6-NC in sediments from urban littoral stations at 520pg/g (Fernandez et al., 1992); and 1-NN and 2-NN in incinerator ash at 2,860 and 3460 pg/g dry (Librando et al., 1993).

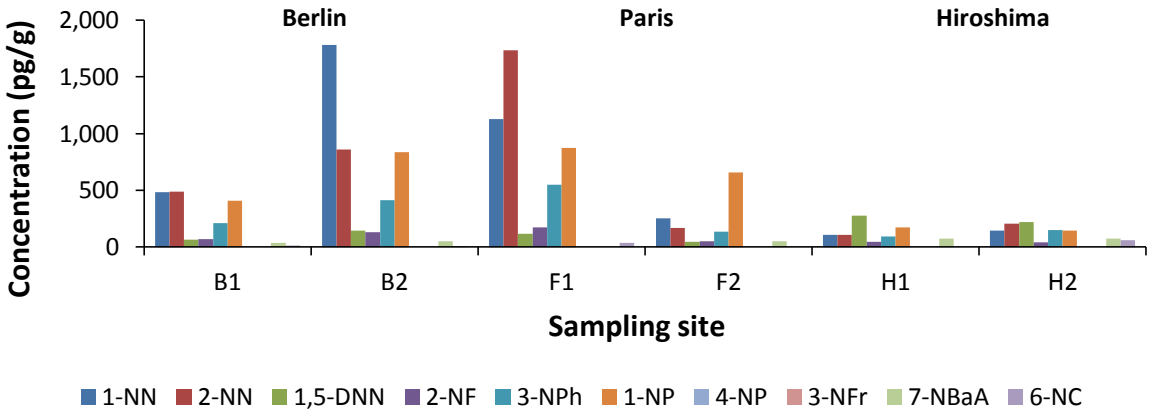

Figure 2.1 Distribution of individual NPAHs in Berlin, Paris and Hiroshima

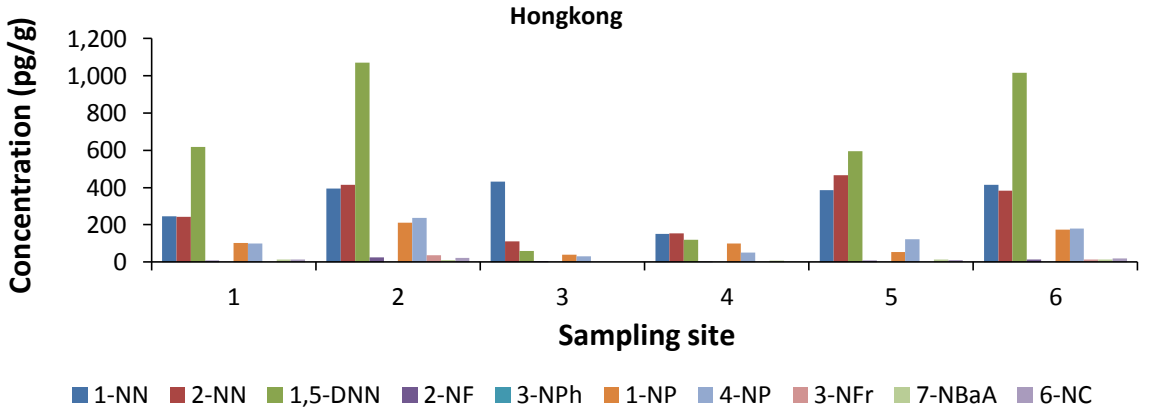

Figure 2.2 Distribution of individual NPAHs in Hongkong

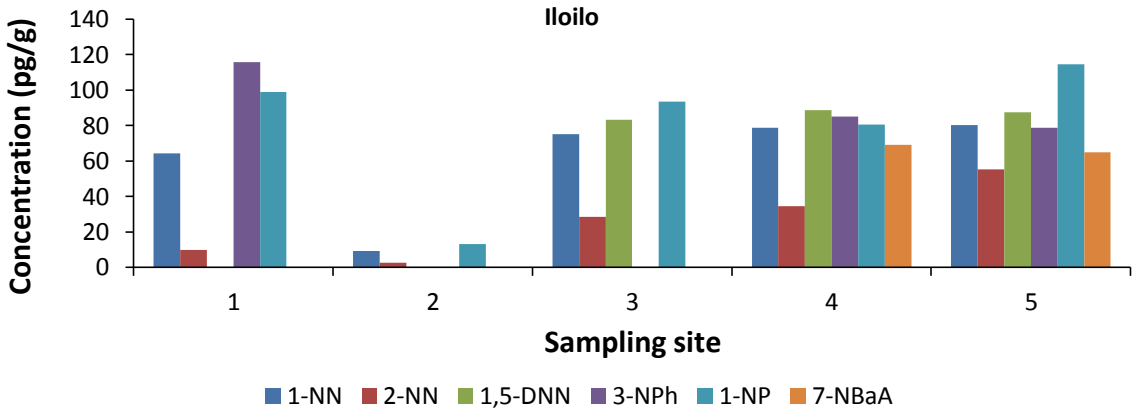

Figure 2.3 Distribution of individual NPAHs in Iloilo, Philippines

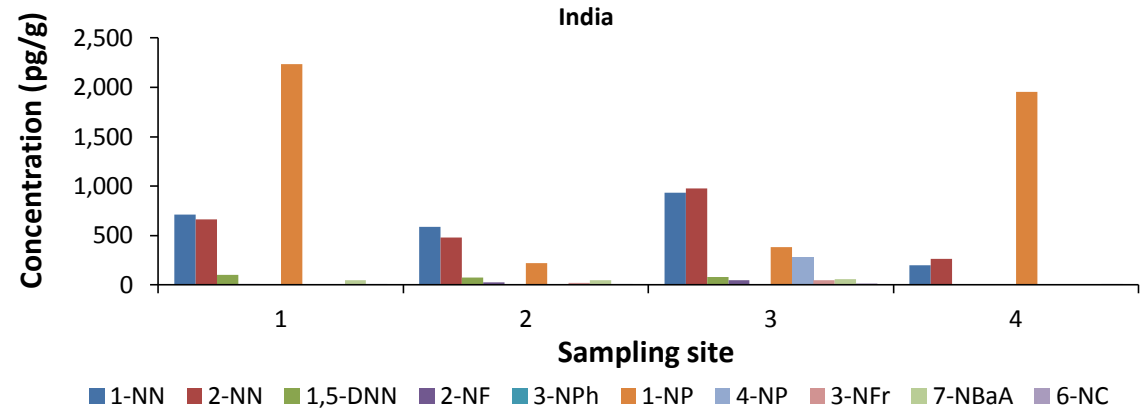

Figure 2.4 Distribution of individual NPAHs concentration in India

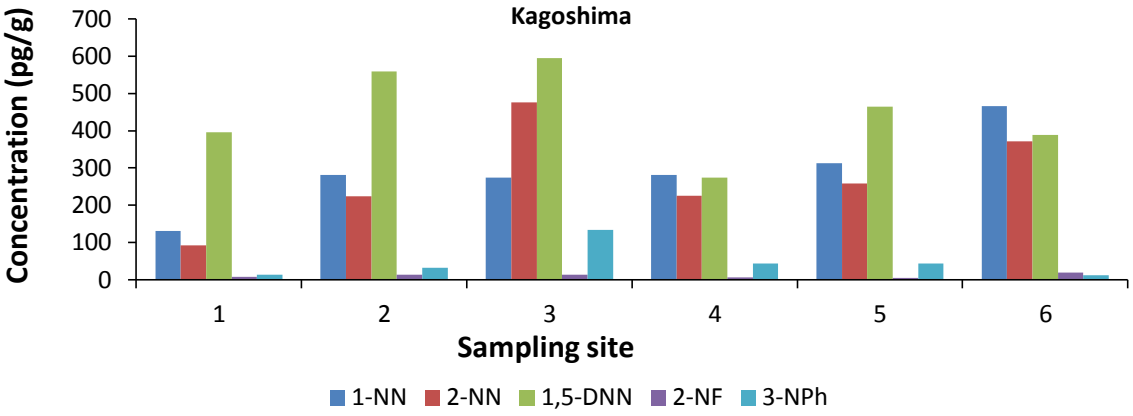

Figure 2.5 Distribution of individual NPAHs concentration in Kagoshima, Japan

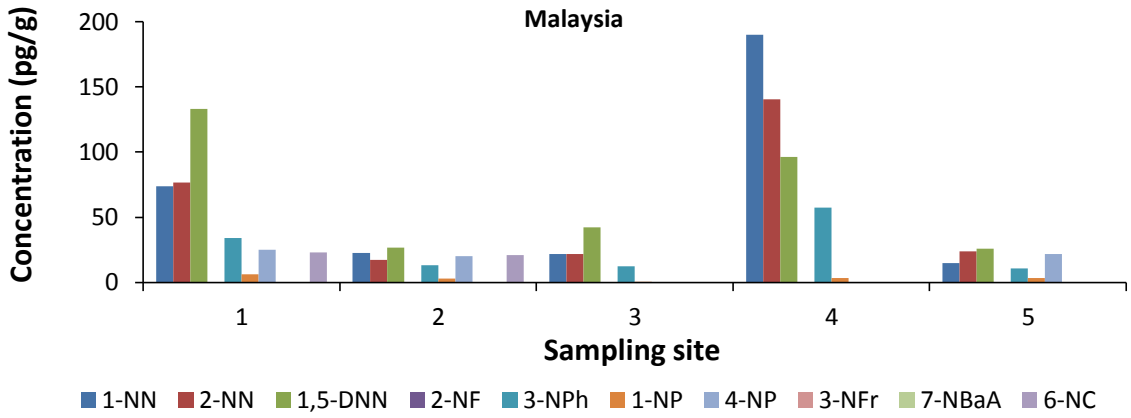

Figure 2.6 Distribution of individual NPAHs concentration in Malaysia

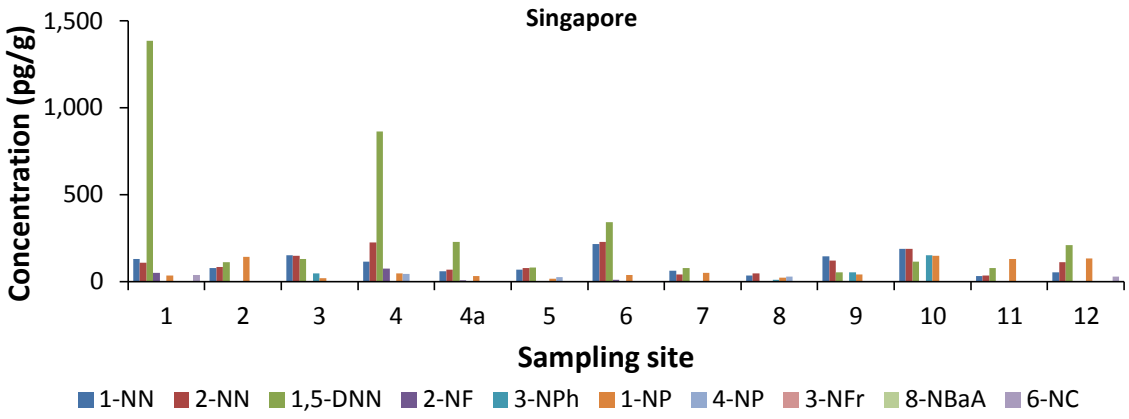

Figure 2.7 Distribution of individual NPAHs in Singapore

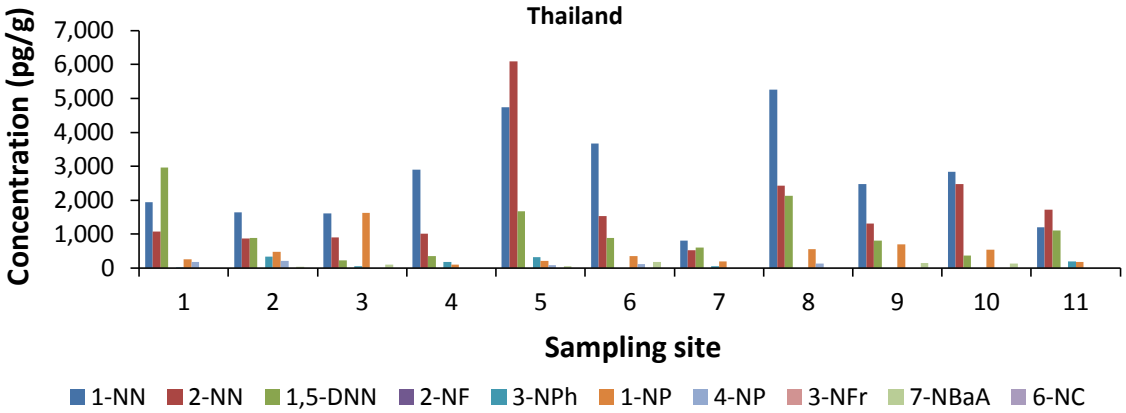

Figure 2.8 Distribution of individual NPAHs in Bangkok, Thailand

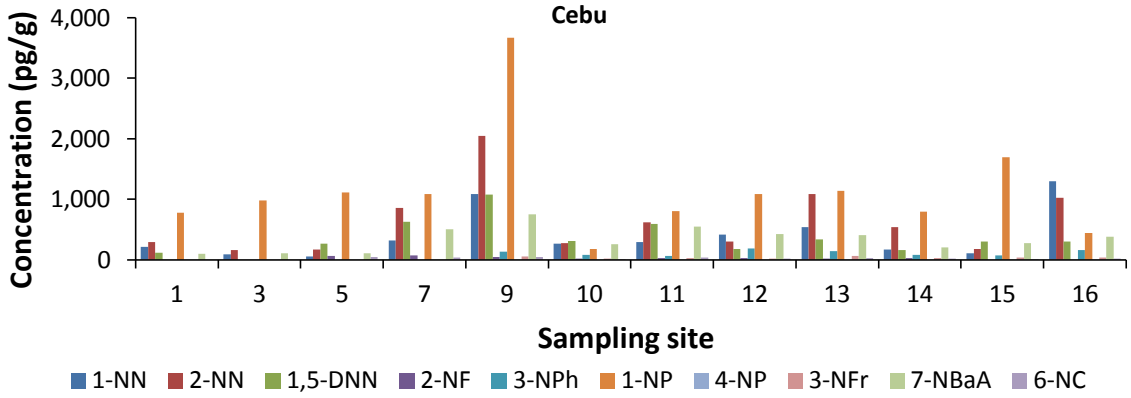

Figure 2.9 Distribution of individual NPAHs in Cebu, Philippines

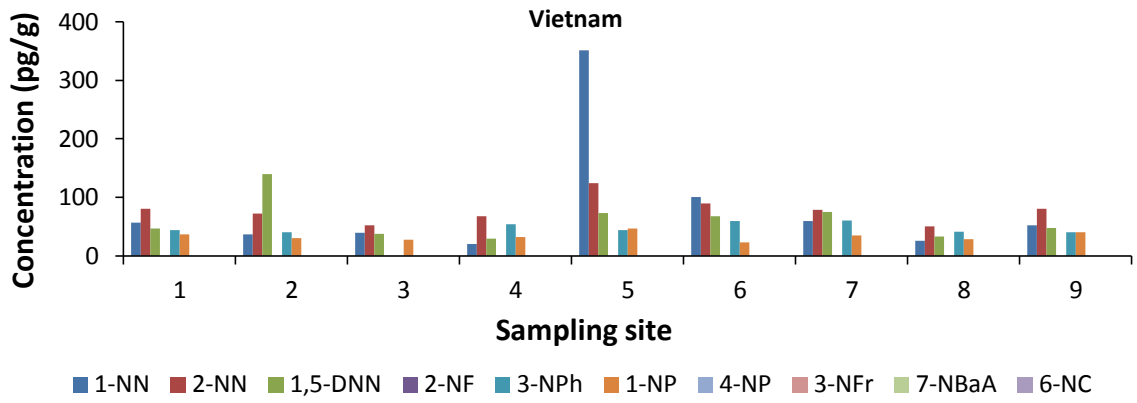

Figure 2.10 Distribution of individual NPAHs in Ho Chi Minh, Vietnam

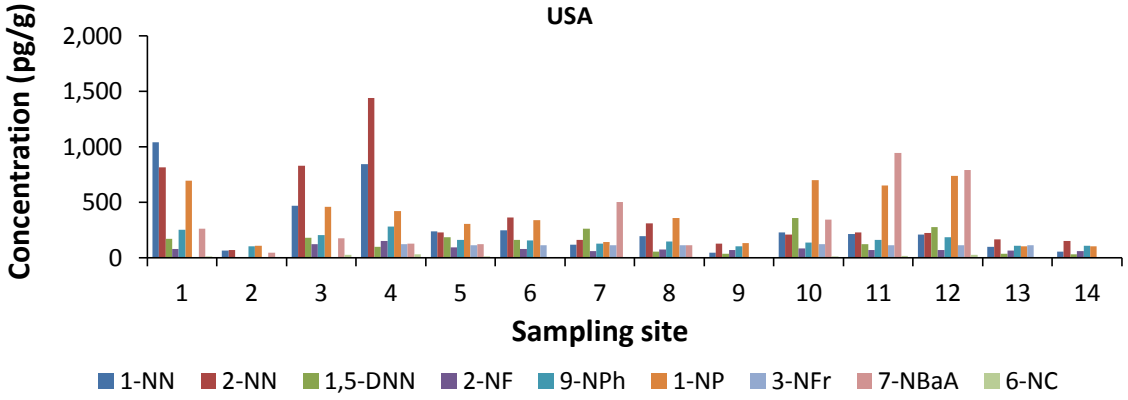

Figure 2.11 Distribution of individual NPAHs in USA

Table 2.2 Minimum (min) and maximum (max) concentration of NPAHs (pg/g dry) in each sampling region

NPAHs		France	Germany	Hiroshima	Hongkong	Iloilo	India	Kagoshima	Malaysia	Cebu	Singapore	Thailand	Vietnam	USA
	min	251	485	108	152	9	198	130	15	52	32	809	26	42
1-NN	max	1130	1780	143	414	80	932	465	190	1090	215	5260	351	1040
	min	170	489	106	111	3	263	92	17	161	36	521	51	66
2-NN	max	1740	861	206	467	55	976	476	141	2050	228	6100	124	1440
	min	45	63	218	59	83	77	274	26	114	54	218	29	31
1,5-DNN	max	114	145	277	1070	89	103	594	133	1080	1390	2970	140	355
	min	48	69	42	3	<1.25	7	6	<1.25	24	6	<1.25	<1.25	60
2-NF	max	172	131	43	24	116	45	18	<1.25	72	76	<1.25	<1.25	151
	min	133	209	94	<2.5	79	<2.5	11	11	69	11	53	40	101
3-NPh	max	548	413	147	<2.5	114	<2.5	42	56	185	150	331	61	281
	min	658	409	144	40	13	221	<1.25	1	182	20	91	23	100
1-NP	max	875	838	17	210	<1.25	2230	<1.25	6	3670	149	1630	47	734
	min	<1.50	<1.50	72	31	<1.50	281	<1.50	20	<1.50	24	88	ND	110
4-NP	max	<1.50	<1.50	73	237	<1.50	281	<1.50	25	<1.50	43	210	ND	123
	min	<0.75	<0.75	60	14	<0.75	18	<0.75	<0.75	21	<0.75	<0.75	<0.75	46
3-NFr	max	<0.75	<0.75	60	36	<0.75	47	<0.75	<0.75	58	<0.75	<0.75	<0.75	944
	min	51	36	<1.50	8	65	7	<1.50	<1.50	99	<1.50	33	<1.50	5
7-NBaA	max	51	48	<1.50	15	69	56	<1.50	<1.50	756	<1.50	177	<1.50	30
	min	37	7	<1	10	<1	9	<1	21	11	28	<1	<1	<1
6-NC	max	37	10	<1	22	<1	16	<1	23	49	37	<1	<1	<1

Correlation coefficient for individual NPAHs with total NPAHs

Table 3 shows correlation values (r) among 10 individual NPAHs and total NPAHs. The compounds 1-NN, 2-NN, 1,5-DNN and 9-NPh gave strong correlation (r>0.5). These compounds are normal combustion products of gasoline and diesel fuels, especially with nitronaphthalenes. Napthalenes, a parent PAHs of nitronapthalenes was reported as the major PAHs found in coke oven, highway tunnel, and gasoline engine samples (Khalili et al., 1995). In addition, 2-NF, 1-NP, 4-NP, 3-NFr, 7-NBaA and 6-NC gave lower correlation (r<0.5). The lower correlation suggests that some of the NPAHs may come from different sources. Niederer, 1998 explained that the degree of correlation may also depend on the variable length of stay of pollutants in the matrix; that is, contaminants in soil maybe the result of emissions of past decades, whereas those found in atmosphere reflect immediate emissions. Thus, lower correlation of NPAHs in soil samples maybe the results of a longer degradation period and differences in decomposition rates of NPAHs

Table 2.3 Correlation between NPAHs detected in surface road soils (n=91)

	1-NN	**2-NN**	**1,5-DNN**	**2-NF**	**3-NPh**	**1-NP**	**4-NP**	**3-NFr**	**7-NBaA**	**6-NC**
1-NN										
2-NN	**0.834**									
1,5-DNN	**0.601**	**0.546**								
2-NF	-0.079	0.051	-0.129							
3-NPh	**0.237**	**0.388**	-0.020	**0.611**						
1-NP	**0.172**	**0.276**	0.096	**0.169**	0.166					
4-NP	**0.245**	**0.191**	**0.401**	0.150	0.077	-0.064				
3-NFr	-0.072	-0.039	-0.062	**0.317**	**0.242**	0.124	**0.291**			
7-NBaA	0.123	**0.259**	0.144	0.028	0.059	**0.662**	-0.142	-0.020		
6-NC	-0.058	0.110	0.160	**0.180**	0.041	**0.491**	-0.021	-0.088	**0.615**	
ΣNPAHs	**0.891**	**0.910**	**0.685**	0.070	**0.344**	**0.493**	**0.277**	0.059	**0.394**	**0.209**

** numbers in bold letters are significant p<0.05*

Sources of NPAHs

Some source specific ratios of NPAHs were investigated in order to assess the relative importance of primary sources versus secondary sources arising from PAH

atmospheric degradation for the three sampling sites. The ratio of 2-NFr or 3-NFr or both on 1-NP concentrations is generally used to evaluate the relative contribution of primary or secondary sources (Bamford and Baker, 2003, Ciccioli et al., 1989, Ciccioli et al., 1996, Feilberg and Nielsen, 2001, Marino et al., 2000 and Zielinska et al., 1989a). 2-NFr or 3-NFr is only produced from the gas phase reaction between fluoranthene and NO_2 initiated by OH^- during the day and initiated by NO_3 during the night (Arey et al., 1986 and Atkinson et al., 1987). 1-NP is strictly arising from direct emissions (it has never been observed to result from any known gas phase reaction) (Arey, 1998, Nielson, 1984 and Paputa-Peck et al., 1983). Assuming reasonably for 2-NF and 1-NP the same removal rates and the same photolysis rates (Fan et al., 1996b, Feilberg and Nielsen, 2000 and Kamens et al., 1994), a value for the 2NF/1NP ratio less than five shows the relative extent of primary emission sources whereas a ratio value larger than five will highlight the importance of the secondary formation of NPAHs (Ciccioli et al., 1996). Since, 2- or 3-NFr is not produced from direct emissions, it can be concluded that their non detection in soil samples indicate that most NPAHs found in soil samples comes from direct emission and this can be confirmed by the detection of high level of 1-NP. In this study, the ratio of 3-NFr/1-NP is lower than 5 in all sites suggesting that NPAHs from all the sampling regions came from primary emission sources.

Conclusion

NPAHs were detected in surface road soils in all sampling regions with varying concentrations. Carcinogenic NPAHs were also detected signifying high risk for genotoxicity to aquatic organisms and human beings. NPAHs concentrations are possibly influenced by vehicular emissions, the type of fuel used and urbanization and industrialization. In addition, NPAHs in road soil collected (India and Cebu) might be transported into aquatic environment and affect aquatic life.

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Chapter 3

Nitroarenes concentration in road surface soil, river sediment and river water

Abstract

This study investigates the level of NPAHs in road soil near river bank, river sediment and river water and to draw a conclusion on the relationship between NPAHs concentration and sample location. NPAHs were analysed using LC-MS/MS with derivatization. Results showed that NPAHs concentration were higher in road as compared to river sediments. Butuanon River is more polluted with NPAHs as compared to Guadalupe, Kotsuki and Mahiga rivers. The presence of residential, commercial and industrial establishments, other than vehicular emissions could attribute to differences in concentration and composition of NPAHs. In addition, there is a direct relationship between NPAHs concentration in road, river sediment and river water.

Introduction

PAH derivatives, namely oxygenated and nitrated PAHs (OPAHs and NPAHs) had significantly been less addressed (Albinet et al., 2007; Albinet et al., 2008; Feilberg and Nielsen, 2001; Schnelle-Kreis et al., 2001) while they seem more toxic than their parent compounds (Durant et al., 1996). NPAHs were found in atmospheric particles and are considered to be mutagens or human carcinogens (IPCS, 2003). Several nitroarenes are believed to be far more mutagenic or carcinogenic than parent PAHs. NPAHs can be a significant fraction of direct-acting mutagenic compounds of atmosphere and can be a result of human exposure to respiration. NPAHs were found in various environmental compartments with PAHs. While their concentration is far lower than parent PAHs, they possibly have significant toxicity. NPAHs are formed through atmospheric processes such as diesel, gasoline engines, or industrial combustion processes (Paputa-Peck et al. 1983; IPCS, 2003), or composed from parent PAHs by hydroxyl radical addition followed by the reaction with nitrogen dioxide and by nitrate radical addition to the PAHs. After dispersion into the atmospheric environments, they may also deposit on ground surfaces, and discharge into and pollute water environments. Though many researchers have investigated the behaviors of nitroarenes in atmospheric environments (Arey et al. 1987; Ciccioli et al. 1996; Marino et al. 2000; Bamford and Baker 2003; Vasconcellos et al. 2008), there has been less investigation of nitroarenes in land and water environments.-In addition, 1-NP was also detected in river water of Yodo River, Japan with concentration of 1ng/L (Ohe et al., 1996) and in Kanazawa River, Japan with concentration between 0.004-0.110 pg/mL (Murahashi et al., 2001).

This study intended to investigate the relationship between NPAHs concentration in road soil and river sediment.

Materials and Methods

Chemicals

The following NPAHs standards: 1] 1- and 2-nitronaphthalenes (1- and 2-NN); 1,5-dinitronaphthalene (1,5-DNN) and 2-nitroflourene (2-NF) were from Tokyo Kasei Kogyo Co. (Japan); 2] 2,7-Dinitrofluorene (2,7-DNF), 1-nitropyrene (1-NP), 3-nitroflouranthen (3-NFr), and 6-nitrochrysene (6-NC) were from Sigma-Aldrich (USA); 3] 3- and 9-Nitrophenanthrenes (3-NPh and 9-NPh); 1,3-, 1,6-, and 1,8-dinitropyrenes (1,3-DNP, 1,6-DNP, and 1,8-DNP) and 7-nitrobenzo(a)anthracene (7-NBaA) were from AccuStandard Inc. (USA); 4] 4-nitropyrene (4-NP) were from Dr. Ehrenstorfer GmbH (Germany) and 5] 1-nitropyrene-d9)1-NP-d9) from Central Chemicals Inc. (Canada).

Other chemical such as acetone, dichloromethane, diethyl ether and hexane were all pesticide grade reagents and purchased from Wako Pure Chemical Ind. (Japan). Acetonitrile and methanol were HPLC grade from Wako. Other chemicals, such as acetic acid anhydride, diethyl ether, formic acid, sodium hydroxide (NaOH), sodium hydrogen sulfide (NaHS), sodium chloride (NaCl) and silica gel (Wako gel) were analytical grade and also purchased from Wako. Silica gel containing 3% water was prepared by heating wako gel at 160^{o}C for 16 h and adding 3% (w/w) distilled water (Milli-Q) to dried silica gel with vigorous shaking for 1 h.

Sampling site

River water, river sediments and road soils near river bank were collected from the lower stream of Butuanon River, Guadalupe River, Kotsuki River and Mahiga River. Butuanon River is located in Mandaue City, Philippines and is influenced by residential, commercial and industrial establishments where they discharge the waste water. In addition, the river is also affected by moderate to heavy traffic. Guadalupe River is located near the coastal side of Cebu City, Philippines and is mainly influenced by residential and less traffic conditions. Kotsuki River is located in Kagoshima City, Japan and is

influenced by residential and commercial establishments. Mahiga River is also located in Cebu City, Philippines and river is influenced by residential, commercial and moderate traffic situation.

Road soils near river banks and river sediments were collected as in Chapter 2. About 20L of river water were collected from Butuanon and Kotsuki river. The collected river water was placed in glass bottles and brought to laboratory for extraction.

Extraction of NPAHs in river water

Collected river water was filtered using vacuum filtration. River water collected from Butuanon River was extracted using blue rayon method. About 0.5g blue rayon per liter of water was suspended for 24 hrs in tank containing river water. Blue rayon was then removed, washed, dried and stored for further analysis. Furthermore, NPAHs in blue rayon was extracted using 80mL methanol:ammonia (50:1) per 0.5 g blue rayon. The extract was dried completely using N_2 gas. The residue was then dissolved in 1mL hexane for clean-up, derivatized and quantification procedure. In addition, river water collected from Kotsuki River was extracted using solvent extraction. About 200mL hexane was added to 1L of river water. The mixture was stirred for 30min using a magnetic stirrer. After 30min, the hexane layer was removed and dehydrated with anhydrous sodium sulfate. The extraction was performed twice. The extract was dried completely using N_2 gas. The residue was then dissolved in 1mL hexane for clean-up, derivatized and quantification procedure.

Extraction and analysis of NPAHs in sediments

NPAHs were analyzed according to Uno et al. (2011). Briefly, about 5 g dry samples, was subjected twice to ultrasonic extraction with 20 mL dichloromethane-methanol mixture (3:1,v/v) for 15 min. The combined extract was further concentrated to 5

mL using N_2 gas and added with 15 mL Milli-Q water, 10 mL hexane-dichloromethane mixture (3:1) and finally saturated with NaCl. The resulting solution was then shaken for 15 min, and centrifuged for 10 min at 2300 rpm (1010 x g) at 4^{o}C. The organic layer was collected, dehydrated with anhydrous sodium sulfate, and completely dried using N_2 gas. The residue was then dissolved in 1 mL hexane for clean-up, derivatization and quantification procedure.

Clean-up was performed on extracts using a silica gel column (10cm length, 0.5cm i.d.; 3% moisture) and eluted with 60% acetone-hexane mixture followed with 5% acetone-hexane mixture. The eluates were combined and spiked with 1-nitropyrene-d_9 as an internal standard.

Derivatization and quantification

Derivatization was performed by reduction with 3 mL 20% NaHS at 95^{o}C for 60 min and acetylation with acetic acid anhydride at 65^{o}C for 45 min. The acetylated NPAHs were extracted with 10 mL dichloromethane and the extracts were evaporated to dryness under a stream of N_2 gas. After dryness, 0.1 mL of 80% acetonitrile in water was added. NPAHs in this solution were measured employing a HPLC (Agilent Technologies, 1200 Series, USA) with mass spectrometry/mass spectrometry detector (Applied Biosystems, API-2000, USA) in positive electron scanning ionization mode with Inertsil C8-3 (4.6mm i.d. x 150mm, 5µm) as column (GL Sciences Inc., Japan).

Recovery experiment

Recovery experiment was done using a mixture containing standard NPAHs. Recovery in sediment samples was done using 1g of washed and dried sediment. The prepared sediment was spiked with 100 ng/mL of each target NPAHs using acetone as solvent. The mixture was then allowed to dry to evaporate the solvent. The procedure for

extraction and analysis were previously. In addition, recovery in water samples was done using 1L of Milli-Q pure water spiked with 100 ng/mL of each target NPAHs using acetone as solvent. Extraction of NPAHs from water was performed using solvent extraction or blue rayon method. Solvent extraction was done by adding 200mL hexane per liter of water and stirring for 30 minutes. The hexane layer was removed and added with anhydrous sodium sulfate. The extraction procedure was done twice. The extract was then evaporated to dryness using nitrogen gas and dissolved in 1ml hexane for clean, derivatization and quantification as discussed above. Blue rayon method was done by suspending 0.5g blue rayon per liter of water for 24 hrs with mild aeration. After 24 hrs, blue rayon was removed, washed and dried. About 0.5g dried blue rayon was added with 80mL methanol:ammonia (50:1) and shaken for 30 min. The solvent was removed after shaking and transferred to a clean flask with anhydrous sodium sulfate. The extraction procedure was done twice. The extract was then evaporated to dryness using nitrogen gas and dissolved in 1ml hexane for clean, derivatization and quantification as discussed above.

Data Analyses

Most of the data processing was done using Microsoft Excel 2007 edition and results are presented as mean of two experiments per sampling site.

Results and Discussion

Recovery of NPAHs

Recovery rates and detection limit of target NPAHs were investigated and the results are shown in Table 3.1. Recovery rates (%) are within 75 to 110% for target NPAHs with having the lowest and highest recovery, respectively; however, 1,3-DNP has

a lower recovery rate with 23.5%. The results showed that the method can be applied to real environmental samples.

Table 3.1 Recovery rates and detection limit of target NPAHs in sediment and water sample

	Recovery (%)			Detection Limit	
NPAHs	Sediment	Water (solvent)	Water (blue rayon)	Sediment (pg/g)	Water (ng/L)
1-NN	98	98	6	2.5	0.002
2-NN	98	95	5	2.5	0.002
1,5-DNN	75	103	7	7.5	0.007
2-NF	108	93	53	1.25	0.002
3-NPh	103	99	65	2.5	0.002
1-NP	99	98	79	1.25	0.001
4-NP	101	98	85	1.5	0.002
3-NFr	96	100	112	0.75	0.001
1,6-DNP	99	84	52	17.5	0.017
1,8-DNP	110	98	59	12.5	0.012
6-NC	99	76	100	1	0.001
7-NBaA	96	94	74	1.5	0.001

Concentration of NPAHs

A total of 10 NPAHs were detected in river water, river sediments and road soils. Table 3.2 shows the NPAHs detected and their concentration in river water collected from Butuanon and Kotsuki rivers. Total NPAHs concentration is higher in lower stream of Butuanon river than Kotsuki river with concentrations of 1.05 and 0.779 pg/mL respectively.

Table 3.2 Average concentration of NPAHs (pg/L) in river water

NPAHs	Butuanon	Kotsuki
1-NN	218	379
2-NN	220	277
1,5-DNN	<7.5	106
2-NF	100	<1.25
3-NPh	134	<2.5
1-NP	86	17
4-NP	<1.25	<1.25
3-NFr	40	<0.75
7-NBaA	67	<1.5
6-NC	189	<1
ΣNPAHs	1000	779

Carcinogenic NPAHs such as 1-NP was detected in both rivers, while 2-NF, and 6-NC were detected in Butuanon only. In addition, 1-NP was also detected in river water of Yodo River, Japan with concentration of 1ng/L (Ohe et al., 1996) and in Kanazawa River, Japan with concentration between 0.004-0.110 ng/L (Murahashi et al., 2001). These results showed that Butuanon is more polluted with NPAHs as compared with Kotsuki River and Kanazawa River. This is probably due to the presence of commercial, industrial and heavy traffic situation in Butuanon River. In addition, high molecular weights NPAHs were also detected in Butuanon River.

Table 3.3 shows the detected NPAHs and their concentration from river sediment collected from Butuanon, Guadalupe, Kotsuki and Mahiga rivers. Total NPAHs concentration is higher in Butuanon 741pg/g, while Kotsuki has the lowest concentration 120 pg/g. In terms of NPAHs composition, low and high molecular weight NPAHs were detected in Butuanon and Mahiga river, while low molecular weight NPAHs were only detected in Guadalupe and Kotsuki river. In addition, carcinogenic NPAHs such as 1-NP was detected in sediments collected from all rivers. 1-NP and 2-NF were also detected in sediment of Suimon River with concentrations of 1,500 and 25,200 pg/g, respectively (Sato et al., 1985). These concentrations were higher than our measured concentration in all river sediments.

Table 3.3 Average concentration of NPAHs (pg/g) in river sediment

NPAHs	Butuanon	Guadalupe	Kotsuki	Mahiga
1-NN	45	72	49	44
2-NN	150	98	64	27
1,5-DNN	330	45	<7.5	94
2-NF	101	<1.25	<1.25	88
3-NPh	<2.5	<2.5	<2.5	<2.5
1-NP	14	27	7.1	13
4-NP	<1,5	<1.5	<1.5	<1.5
3-NFr	<0.75	<0.75	<0.75	<0.75
7-NBaA	74	<1.5	<1.5	<1.5
6-NC	<1	<1	<1	24
ΣNPAHs	714	242	120	290

Table 3.4 shows the NPAHs detected and their concentration in road soils collected from Butuanon, Guadalupe, Kotsuki and Mahiga rivers. Total NPAH concentrations were 3500, 1350, 1400 and 1400 for Butuanon, Guadalupe, Kotsuki and Mahiga rivers, respectively. NPAHs concentrations in road sediments were higher than that of river sediment and river water. This may be due to direct emission of NPAHs from vehicles. Especially, 1-NP is directly emitted from diesel powered vehicles.

1-NP concentration in road soil in this study are within the reported concentration of 30-800 pg/g found in surface soil from the city of Basel, Switzerland (Niederer, 1998). Furthermore, other reported concentrations of NPAHs were reported, such as 1-NP in sewage sludge at 680pg/g (Fernandez et al., 1992); 6-NC in sediments from urban shore at 520pg/g (Fernandez et al., 1992); and 1-NN and 2-NN in incinerator ash at 2,860 and 3460 pg/g dry (Librando et al., 1993).

Table3.4 Average concentration of NPAHs (pg/g) in road soil

NPAHs	Butuanon	Guadalupe	Kotsuki	Mahiga
1-NN	316	89	290	213
2-NN	854	161	275	293
1,5-DNN	632	<7.5	779	114
2-NF	72	<1.25	10.6	<1.25
3-NPh	<2.5	<2.5	46.3	<2.5
1-NP	1090	984	<1.25	778
4-NP	<1.5	<1.5	<1.5	<1.5
3-NFr	<0.75	<0.75	<0.75	<0.75
7-NBaA	506	112	<1.5	99
6-NC	35	<1	<1	<1
ΣNPAHs	3,500	1,350	1,400	1,400

In general, individual and total NPAH concentration was higher in road sediments, followed by river sediment and water samples. Our result is in agreement with Krein et al., 2000 that PAHs concentrations in river sediments are lower than road soils. Relative low level of NPAHs in sediments maybe caused by resuspension of sediments and water current erosion to sediment during rainy or flood season. Differences in contaminant

composition by individual NPAHs are expected to be due different NPAHs input and characteristics of NPAHs. First, river water receives direct NPAH inputs from various sources, including waste water discharge, runoff and atmospheric fallout. Secondly, low molecular weight NPAHs gradually decreased by degradation and adsorption. Thirdly, complex water current condition during rainy season changed the state of water column process including dissolution, adsorption, desorption, degradation and deposition. As a result, the water, and sediment samples had different composition of NPAHs. In addition a strong positive correlation was obtained between concentration of low molecular weight ($r=0.597$), high molecular weight ($r=0.866$) and total NPAHs concentration ($r=0.959$) between road soils and river sediments. This finding showed that main sources of NPAHs in Butuanon, Guadalupe, Kotsuki and Mahiga Rivers come from road soils other than fallout. In addition, according to Chen et al., 2004, the difference pattern of contaminant abundance in surface waters, sediments and soils were contributed to two factors. First, water column and soil receives direct inputs from various sources. Second, high molecular mass more easily undergo sorption in sediment and are resistant to degradation.

Conclusion

The study showed that road soil is one of the indicator for NPAHs pollution, and possibly one of the sources of NPAHs detected in river water and sediments originating from vehicle exhaust.

The input into the aquatic system is possibly controlled by the location of sampling site (presence of automobiles, and others) and physical and chemical nature of NPAHs.

The presence of NPAHs in aquatic environment (river sediment and water) can possibly affect aquatic organisms.

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PART III

BIOACCUMULATION AND GENOTOXICITY

Chapter 4

Bioconcentration of waterborne NPAHs in marbled flounder *Pleuronectes yokohamae*

Abstract

Studies on the accumulation of waterborne NPAHs by fish are not well known. In this study, bioconcentration of NPAHs by marbled flounder exposed to a mixture of six NPAHs was investigated in a continuous flow-through system for a period of seven days exposure followed immediately with seven days elimination. Results show that NPAHs steady-state conditions were established from day 1 to 4 of exposure period. Estimated bioconcentration factors (BCFs) ranged from 4 to 422 with 6-nitrochrysene and 2-nitrofluorene having the lowest and highest BCFs, respectively. Half-lives were from 0.96 to 5.59 days with 2-nitroflourene and 6-nitrochrysene being the shortest and the longest, respectively. The present study showed that waterborne NPAHs were accumulated by marbled flounder but the BCFs were lower than reported values of their parent PAHs. Furthermore, the difference in BCFs between NPAHs and PAHs could be attributed to the biotransformation processes and steric hindrance of these compounds.

Introduction

NPAHs are polycyclic aromatic hydrocarbons containing nitro group, formed directly or indirectly from their parent polycyclic aromatic hydrocarbons (PAHs) during combustion of petroleum products or from vehicular and/or industrial emissions (Nielsen 1984) or during interaction of PAHs with nitrogen oxide in the atmosphere (Atkinson and Arey 1994). Several studies indicated that NPAHs are mainly adsorbed on small particles in ambient air (PM2.5) and are widely distributed in all environments including water systems (Bamford et al. 2003). Most studies regarding environmental monitoring of NPAHs were mainly focused in ambient air (Bamford et al. 2003; Hasei, et al. 2006; Hayakawa et al. 1995; Kuo et al. 2003; Vasconcellos et al. 2008). NPAHs, together with their parent PAHs are persistent in the atmosphere and can be transported into the aquatic environment as fallouts, mainly during rainfall. Hence, their presence in aquatic environments has also been reported, though limited, such as in marine sediments (Ozaki et al. 2010), river water (Ohe et al. 1996; Murahashi et al. 2001), and seawater (Murahashi et al. 2001). A recent study also reported residues of NPAH in mussels and oysters collected from Osaka Bay, Japan with total concentrations ranging from 2380 to 24688 and from 2672 to 25961 pg/g dry weight, respectively (Uno et al. 2011). The presence of NPAHs in aquatic organisms implies that these compounds can be accumulated and can cause genotoxic damage as shown below (Mitchelmore et al. 1998a; Mitchelmore et al. 1998b). However, there is no report regarding the degree of their accumulation by aquatic organisms, thus, the present study was conducted.

Some researchers reported lower toxicity for NPAH, such as 1-nitronaphthalene with LC_{50} (96h) of 9.0 mg/L to fathead minnow (Curtis et al. 1981). However, NPAHs have strong genotoxic potencies on mammals which are similar or higher with benzo(a)pyrene (Wislocki et al. 1986; Busby et al. 1988). In addition, DNA damage was detected in brown trout (Mitchelmore et al. 1998b) and mussels (Mitchelmore et al. 1998a)

exposed to 1-nitropyrene. Therefore, some NPAHs can be genotoxic on aquatic organisms as well as mammals. NPAHs are even reported to be probable genotoxic substances to humans (Landvik et al. 2007).

NPAHs, like any other organic chemicals have affinity for lipid phase, thus, they are expected to be accumulated by aquatic organisms. Bioaccumulation parameters, such as bioconcentration factors (BCFs), are important for the prediction of bioavailability of chemicals by aquatic organisms. However, BCFs of NPAHs in fish, some of which are an important human food source, are not well known compared to their parent PAHs. Several studies regarding accumulation and bioconcentration of parent PAHs have been reported as compared to NPAHs. For example, (Jonsson et al. 2004) reported bioconcentration for naphthalene, phenanthrene and pyrene in *Cyprinodon variegates*, while, (Cheikyula et al. 2008) reported accumulation of phenanthrene and pyrene in *Paralichthys olivaceus* and *Pagrus major*, but PAHs accumulation was higher in *P. major*. As mentioned earlier, there is no information regarding bioaccumulation of NPAHs by aquatic organisms, thus, it is important to investigate their bioaccumulation because of their strong genotoxic properties. On the other hand, there are no data on accumulation of NPAHs in fish, and it is therefore important to investigate their bioconcentration and their related factor because of their strong genotoxic characters. In general, one of the main absorption routes for chemicals is respiration for fish and likely NPAHs could also be absorbed through the same route.

Marbled flounder, *Pleuronectes yokohamae* is one of commercially important fish in Japan and is distributed in Japan from southern Hokkaido to Kyushu, in the Gulf of Pohai and Yellow Sea, and the northern part of the East China Sea (Sakamoto 1984), inhabiting sandy-mud bottoms less than 100 m deep and preying mainly on benthic animals. Studies reported the transfer of PCBs (Kobayashi et al. 2010) and o,p'-DDE and tri- to penta-CBs (Sakurai et al. 2009) from marine sediment to marbled flounder. Thus, the flounder might be susceptible for accumulation of NPAH.

In the present study, marbled flounder were exposed to a mixture of waterborne NPAHs and their bioaccumulation, uptake, elimination, and half-life were investigated. The examination of accumulation potencies of NPAH with strong toxicities in fish is important, because NPAH could affect not only fish through accumulation of NPAH, but also human beings which consume fish that could possess greater risk to us. But there are very few studies about their distribution and behavior in water systems, and accumulation and effects in aquatic organisms. This is the first study to report on accumulation of waterborne NPAHs by fish.

Materials and Methods

Chemicals

Chemicals used such as standard NPAHs and others were discussed in Chapter 2 and 3 of previous study.

Exposure of NPAHs in marbled flounder

Marbled flounder were purchased from a regional government aquaculture facility in Kudamatsu City Yamaguchi, Japan and reared in a 500-L sand-filtered seawater flow through system with aeration for 5 months in the laboratory. The fish were regularly fed with a commercial fish diet, C-1000 formula (Kyowa Hakko, Tokyo, Japan) during the rearing period.

A mixture of NPAHs containing 1,8-DNP, 1-NP, 3-NFr, 6-NC, 2-NF and 3-NPh were used for exposure tests in low and high concentrations. For the low concentration exposure, nominal concentrations of 1,8-DNP, 1-NP, 3-NFr, 6-NC, 2-NF, and 3-NPh were 15, 50, 50, 50, 50, and 500 ng/L, respectively. For the high concentration exposure, nominal concentrations of 1,8-DNP, 1-NP, 3-NFr, 6-NC, 2-NF, and 3-NPh were 150, 500, 500, 500, 500, and 5000 ng/L, respectively. The concentration of each NPAH was based

on water solubility (Yaffe et al. 2001). Control fish were reared in NPAH-free water for the entire exposure period.

The exposure tanks for low and high exposure consist of a glass aquarium measuring 35 x 44 x 43 cm in a flow-through (20-24L/h sand-filtered water) system with mild aeration. NPAHs stock solution dissolved in methanol were continuously pumped into mixing container at a rate of 0.1 mL/h using an automatic pump (KD Scientific, USA) and mixed with flowing sea water in a mixing container before reaching the water medium. The concentration of 1,8-DNP, 1-NP, 2-NFr, 6-NC, 2-NF, 1-NN and 3-NPh in stock solution was 3.5, 12, 12, 12, 12, , 12 and 120 mg/L for low exposure group and 35, 120, 120, 120, 120, 120 and 1200 mg/L for high exposure group, respectively. The stock concentrations were calculated to obtain the desired nominal concentration after mixing with sea water and into the water medium. After the fish had been exposed to waterborne NPAHs for 7 days, they were subsequently reared in NPAH-free seawater for 7 more days in the elimination period. Exposure period was chosen based on reports showing that concentrations of 2-, 3-, and 4-ring parent PAHs in fish tissue peak at approximately after 3 d of exposure for sheepshead minnows, *Cyprinodon variegates* (Jonsson et al. 2004) and for juvenile turbot, *Scophthalmus maximus* (Baussant et al. 2001).

A total of 57 fish were used for the bioconcentration experiment with 9, 24, and 24 individuals each for the control, low-concentration exposure, and high-concentration exposure, respectively. Mean body weights and mean total lengths of test fish were 112.6±19.1 g and 17.38±0.7 cm, respectively. During the exposure and elimination period, diet was not provided to prevent uptake of NPAHs from food and adsorption of NPAHs to food. No mortality was observed during the duration of the experiment. Dissolved oxygen (DO), pH, salinity and temperature of test waters were monitored daily.

Three fish were sampled in each tank after 1, 2, 4 and 7 d of the exposure period and after 1, 2, 4 and 7 d of the elimination period, while 3 fish from control tanks were collected on

0, 7 and 14 d. Fish were washed with distilled water and their body weights and lengths were measured before frozen at -20^{o}C until NPAH analysis. Samples of test waters were collected for measurement of NPAH concentrations after 1, 2 and 6 d of the exposure period and after 1, 2 and 6 d of the elimination period. In addition, samples of seawater in the control tank were collected at 0, 6, and 13 d of the experiment period.

Extraction of NPAHs in fish

NPAHs were analyzed following Uno et al. (2011). Fish samples were freeze dried and homogenized by grinding. About 1-2 g dry weight samples (approximately 5 g in wet weight), was subjected twice to ultrasonic extraction with 20 mL dichloromethane-methanol mixture (3:1,v/v) for 15 min. The combined extract was further concentrated to 5 mL using N_2 gas and added with 15 mL Milli-Q water, 10 mL hexane-dichloromethane mixture (3:1) and finally saturated with NaCl. The resulting solution was then shaken for 15 min, and centrifuged for 10 min at 2300 rpm (1010 x g) at 4^{o}C. The organic layer was collected, dehydrated with anhydrous sodium sulfate, and completely dried using N_2 gas. The residue was then dissolved in 1 mL hexane. Clean up, derivatization, and LC-MS/MS measurement were performed on the extract as described below.

Extraction of NPAHs in seawater

Seawater was filtered using vacuum filtration with 47mm i.d. Whatman glass microfilter (Whatman International Ltd., England). One liter of filtered water was subjected twice to extraction with 200 mL hexane using a mechanical shaker. The hexane extract was dehydrated with anhydrous sodium sulfate and then concentrated to 5 mL under a stream of N_2 gas. Clean up, derivatization, and measurement were performed on the concentrated extract as described below.

Derivatization and LC-MS/MS measurement

The details of derivatization and LC-MS/MS measurement were based on (Uno et al. 2011). Briefly, clean-up was performed on extracts using a silica gel column (10cm length, 0.5cm i.d.; 3% moisture) and eluted with 60% acetone-hexane mixture followed with 5% acetone-hexane mixture. The eluates were combined and spiked with 1-nitropyrene-d_9 as an internal standard. Derivatization was performed by reduction with 3 mL 20% NaHS at 95^{o}C for 60 min and acetylation with acetic acid anhydride at 65^{o}C for 45 min. The acetylated NPAHs were extracted with 10 mL dichloromethane and the extracts were evaporated to dryness under a stream of N_2 gas. After dryness, 0.1 mL of 80% acetonitrile in water was added. NPAHs in this solution were measured employing a HPLC (Agilent Technologies, 1200 Series, USA) with mass spectrometry/mass spectrometry detector (Applied Biosystems, API-2000, USA) in positive electron scanning ionization mode with Inertsil C8-3 (4.6mm i.d. x 150mm, 5µm) as column (GL Sciences Inc., Japan). The detection limits (LODs) of the NPAHs used in solvents (10 µL) were as follows: 0.25, 0.50, 0.25, 0.15, 0.20 and 2.5 pg/µL for 2-NF, 3-NPh, 1-NP, 3-NFr, 6-NC and 1,8-DNP, respectively.

Data Analyses

An OECD guideline was used to describe BCF, uptake (k_1), elimination (k_2), and half-life ($t_{1/2}$) of NPAHs (Bioconcentration: Flow-through Fish Test, OECD 305E 1996; Uno et al. 1997; Jonsson et al. 2004). In this study, BCF of NPAHs were calculated and reported from the ratio of k_1 and k_2, except for nitroarenes that did not achieved steady state, in this case BCF was calculated from the ratio of its peak concentration (dry weight) in fish to water concentration. This model is described by

$$\frac{dC_f}{dt} = k_1 C_w - k_2 C_f \quad (1)$$

which gives, after integration when C_w is constant,

$$C_f = k_1/k_2\, C_w \left(1 - e^{-k_u t}\right) \text{ (2)}$$

where, C_f, concentration of NPAHs in fish (pg/g wet); C_w, concentration of NPAHs in water (ng/L); k_1, uptake rate ($nLg^{-1}day^{-1}$); t, time (d) and k_2, elimination rate (d^{-1}) of chemicals in fish.

When the steady-state is approached in fish, Equation 2 may be reduced to

$$C_f = k_1/k_2\, C_w \text{ (3)}$$

Under steady-state conditions, the bioconcentration factors (BCFs) can be calculated from the ratio of the chemical concentration in the fish to that in water (C_f/C_w), or from the ratio of the uptake rate constant to depuration rate constant (k_1/k_2) as

$$\frac{C_f}{C_w} = \frac{k_1}{k_2} = \text{BCF (4)}$$

Elimination rates (k_2) for each NPAHs were determined by the slope of the linear regression line for plots of ln C_f versus time to a first order decay as

$$\ln C_f = \ln C_f^0 - k_2 t \text{ (5)}$$

where C_f^0 is the concentration of the compound in the fish at the beginning of the elimination period. These values were then used to determine the uptake rate constant (k_1) in terms of dry weight by fitting the data to the model (Eqn 2) by means of non linear regression analysis. In addition, half-life ($t_{1/2}$) was evaluated using

$$t_{\frac{1}{2}} = \frac{\ln 2}{k_2} \text{ (6)}$$

Results

Parameters and NPAHs concentration in test waters

During the exposure and elimination periods, the mean and standard deviation of the water temperature, dissolved oxygen, pH and salinity in all tanks were 17.3±0.23^{o}C, 6.6±0.11 mg/L, 7.50±0.05 and 35±0.1ppt, respectively. The measured NPAHs concentrations in test waters were lower than their nominal concentrations (Table 4.1).

This could be possibly due to its evaporation in seawater. However, their day to day variations in concentration were considerably insignificant during the duration of the exposure period. In addition, all NPAHs were belowe detection limit in test waters after exposure period and in seawater of the control tank during the experiment period.

Table 4.1 Mean concentrations of NPAHs in seawater during the exposure period

	Nominal concentration (ng/L)		Exposure concentration (ng/L)	
Target NPAHs	Low	High	Low (n=3)	High (n=3)
2-NF	50	500	36.8 (6.33) [a]	210 (53.1)
3-NPh	500	5000	233 (65.2)	1410 (295)
1-NP	50	500	14.3 (3.04)	99.2 (13.7)
3-NFr	50	500	14.7 (3.58)	118 (12.5)
6-NC	50	500	7.34 (2.81)	61.8 (7.22)
1,8-DNP	15	150	1.45 (0.137)	5.65 (1.76)

[a] Values in parentheses are the standard deviation.

Bioncentration parameters of NPAHs in marbled flounder

Figures 4.1-1 and 4.1-2 indicate the accumulation and elimination of waterborne NPAHs by marbled flounder. Most NPAHs were detected in exposed groups until 11 d of the experiment period.

In low-concentration exposure group, 1,8-DNP and 6-NC were not detected after 11 d, while in high-concentration exposure group, all NPAHs were detected up to 14 d of the experiment period. In addition peak level concentrations of NPAHs in whole fish were 13800, 52200, 2150, 641, 271 and 546 ng/kg dry weight in low and 37500, 45800, 7270, 1910, 288 and 1510 ng/kg dry weight in high exposures for 2-NF, 3-NPh, 1-NP, 3-NFr, 6-NC and 1,8-DNP, respectively. These peak concentrations of NPAHs in whole fish were higher than the water column concentration suggesting bioconcentration.

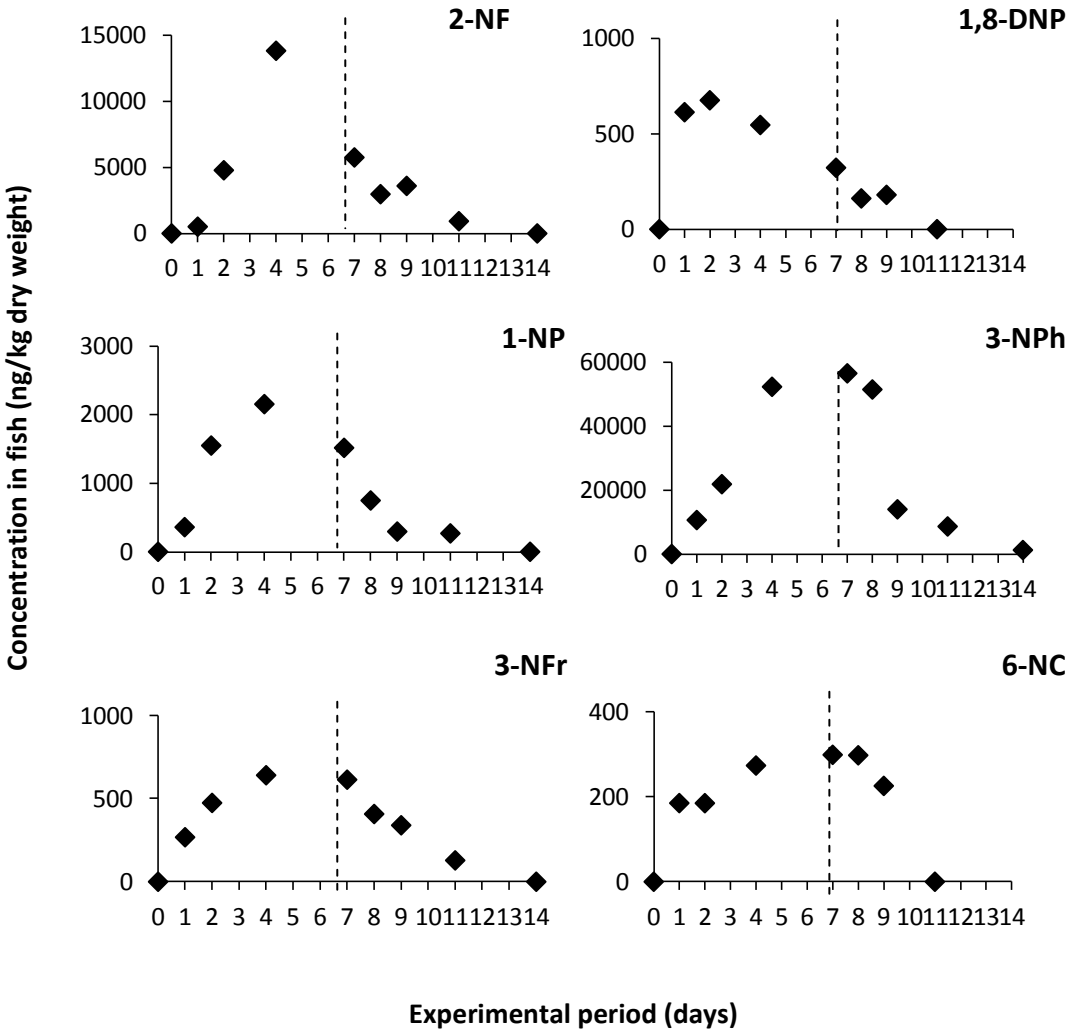

Fig 4.1-1 Mean NPAH concentrations in whole fish (ng/kg dry weight) throughout the experiment period for water-borne low NPAHs exposure. (Vertical dashed line indicates where the uptake phase ends and the depuration phase begins.)

NPAHs in low- and high-concentration exposure achieved steady-state in fish body from 1 to 4 d of the experiment period except for 1,8-DNP with steady-state after 1 d for both exposures. 2-NF did not show a clear steady-state in low exposure, thus, the peak concentration of 2-NF after 4 d was considered as a steady state peak.

NPAH concentrations in fish were gradually decreased during elimination period. In addition, there is no difference in BCFs of most NPAHs calculated from the ratio of k_1 and k_2 when compared with BCFs calculated from the ratio of peak body residue concentration and water concentration, except for 2-NF.

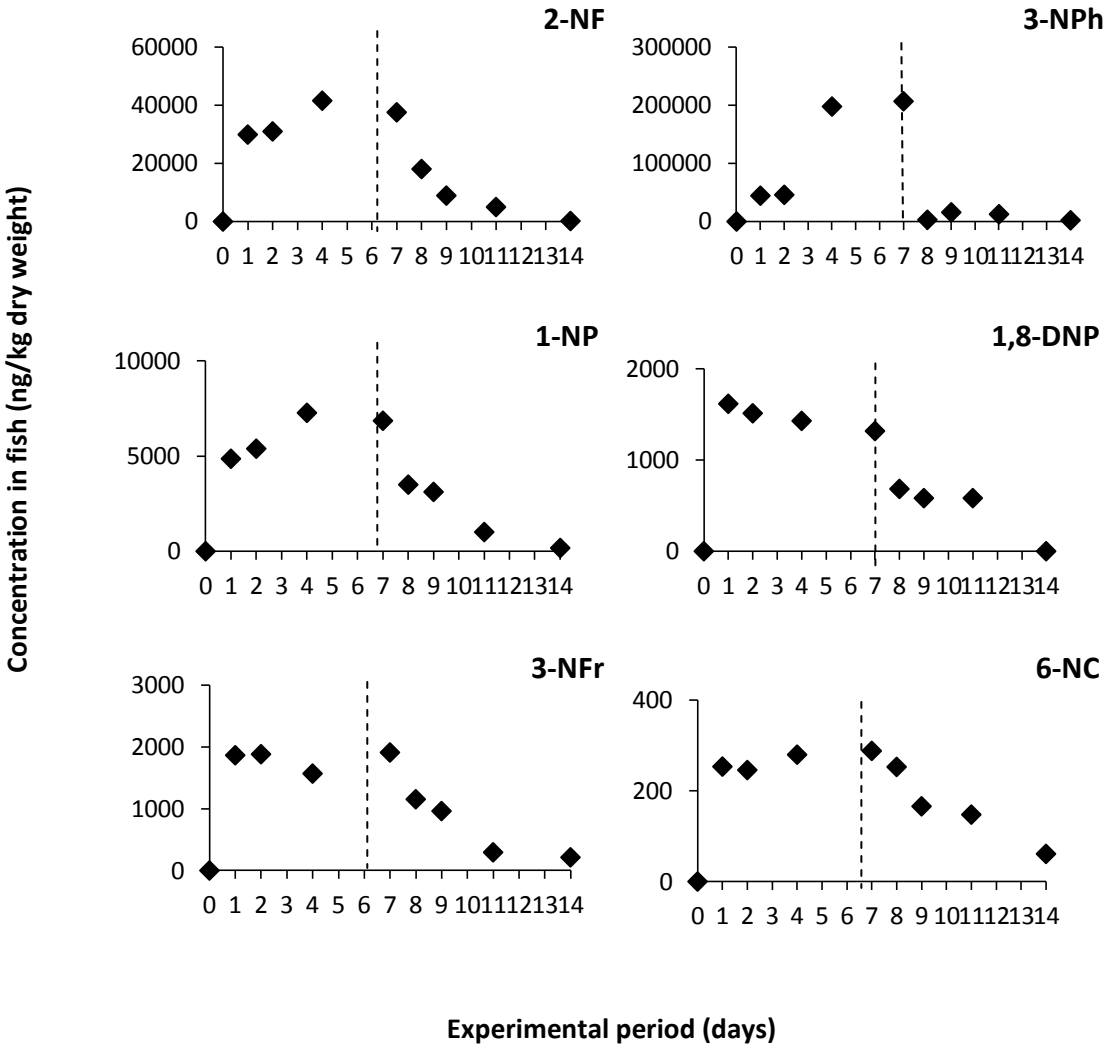

Fig 4.1-2 Mean NPAH concentrations in whole fish (ng/kg dry weight) throughout the experiment period for water-borne high NPAHs exposure. (Vertical dashed line indicates where the uptake phase ends and the depuration phase begins.)

From their concentration variations, bioconcentration factors, uptake and elimination rates, and half-life periods in marbled flounder were estimated and summarized in Table 4.2 for both exposure groups. The highest uptake rate (k_1) was for 2-NF and the lowest was for 6-NC in both exposures. Furthermore, elimination rates (k_2) of 2-NF, 3-NPh, and 1-NP tended to be higher, and 6-NC and 1,8-DNP was lower. Elimination rate (k_2) in both exposures was generally highest in 2-NF, 3-NPh and 1-NP but lower in 3-NFr, 6-NC and 1,8-DNP, while half-life ($t_{1/2}$) periods were shortest in 2-NF, 3-NPh and 1-NP but longest in 3-NFr, 6-NC and 1,8-DNP.

Table 4.2. Bioconcentration factors, uptake and elimination rates, and half life periods of NPAHs nitrated polycyclic aromatic hydrocarbons in marbled flounder

NPAHs	Molar Mass	Log K_{ow}[a]	Low NPAHs exposure				High NPAHs exposure				BCF of parent PAHs
			k_1 (nL/g/day)	k_2 (day^{-1})	BCF	$t_{1/2}$ (day)	k_1 (nL/g/day)	k_2 (day^{-1})	BCF	$t_{1/2}$ (day)	
2-NF	211	4.08	160	0.426	375	1.63	136	0.721	189	0.961	2230[b]
3-NPh	223	4.23	128	0.552	232	1.26	75.5	0.528	143	1.31	8351[c]
1-NP	247	5.29	65.2	0.432	151	1.6	37.3	0.526	71	1.32	1495[c]
3-NFr	247	5.15	16.5	0.384	43	1.81	4.77	0.318	15	2.18	379[d]
6-NC	273	5.41	4.84	0.124	39	5.59	0.864	0.216	4	3.21	83 to 104[e]
1,8-DNP	292	4.44	116	0.275	422	2.52	46	0.177	260	3.92	1495[c]

[a] From IPCS 2003.
[b] de Voogt et al., 1991
[c] Jonsson et al., 2004
[d] in liver Gerhart and Carlson 1978
[e] in liver Miller et al., 1982

Estimated BCFs ranged from 4 to 39 for 6-NC and 260 to 422 for 1,8-DNP being the lowest and highest BCF respectively in both exposure groups. However, BCFs were higher by 50% in low exposure group compared to high exposure group. In comparison, BCFs were in this order: 6-NC<3-NFr<1-NP<3-NPh<2-NF<1,8-DNP.

Discussion

In the present study, waterborne NPAHs were rapidly absorbed and accumulated by marbled flounder, with steady-state of individual NPAH in whole fish observed after 1 and 4 d in low and high exposure groups. This tendency was similar to reported accumulations of parent PAHs in fish. For example, peak level concentrations of two-, three-, and four-ring parent PAHs in fish tissue were obtained after 3 d of the exposure period to dispersed crude oil in sheepshead minnows, *Cyprinodon variegates* (Jonsson et al. 2004) and of juvenile turbot, *Scophthalmus maximus* (Baussant et al. 2001). As a result, uptake and elimination of NPAHs in fish could depend on their parent PAH structures. However, half-lives of parent PAHs were higher as compared to our reported half-lives for NPAHs, for example, flourene, phenanthrene and flouranthene were 7, 9 and 6 d, respectively in rainbow trout (Niimi et al., 1986). Even though toxicokinetic patterns of NPAHs are similar with PAHs, there is still a need to investigate for NPAHs, because these compounds are formed indirectly from PAHs and they are persistent in the

environment. However, their environmental concentration levels are lower, but they have strong genotoxic properties as compared to PAHs, thus NPAHs can be a potential risk to aquatic organisms and even to humans as uptake them from seafood.

The BCF indicates the potential accumulation of chemicals in aquatic organisms. In this study, large differences in calculated BCF between low and high exposure group were observed. The observed plot between log BCF and log K_{ow} almost reached a steady level or no change from log K_{ow} of 4.04 to 4.44 and decreases linearly from log K_{ow} 4.44 to 5.41 in both low ($r=0.8128$, $p<0.10$) and high ($r=0.8055$, $p<0.10$) exposure groups (Fig. 4.2). These p- values were not significant even though high correlation was obtained, this may be due to the limited number of points in the plot. In addition, the observed deviation in the plot can also be due to ionization, degradation or metabolism of many chemicals as discussed by (Nendza and Müller 2010).

The log K_{ow} of target NPAHs falls in the boundary between low and high log K_{ow}, thus a plateau and decreasing linearity maybe observed. Similar trends were also obtained by (Mizukawa et al. 2009), when they investigated bioconcentration of polybrominated diphenyl ethers (PBDEs) through lower-trophic-level coastal marine food web. They observed that for PBDEs with log $K_{ow} < 7$ were positively correlated and with log $K_{ow} >7$ were negatively correlated. The decreasing BCF with increasing log K_{ow} is also described in several laboratory studies with sediment and benthic invertebrates (Oliver 1987; Landrum 1989; Di Toro et al. 1991). The negative correlation can be due to the restriction of permeation to the cell membrane due to larger molecular size.

Furthermore, as shown in Table 4.2, the BCFs of NPAHs were considerably lower compared to their parent PAHs, for example phenanthrene, a parent PAHs of 3-NPh has a BCF range from 8351 to 146,600 in *C. variegates*; *S. maximus*; *M. edilus* (Baussant et al. 2001; Jonsson et al. 2004) and pyrene, a parent PAH of 1-NP and 1,8-DNP has a BCF range from 1,495 to 334, 300 in sheepshead minnow, *C. variegates* (Jonsson et al. 2004)

and *M. edilus* (Baussant et al. 2001). One major reason for their differences in BCFs between NPAHs and PAHs can be due to their differences in biotransformation processes. NPAHs like 2-NF and 1-NP are metabolized via reduction reaction, i.e. acetylation and amination as compared to PAHs which metabolized via oxidation (Kitamura and Tatsumi 1996; Ueda et al., 2001). This metabolic route may be the same for all NPAHs, because they have a nitro group, which is common structure for all NPAHs, is one of the major targets for metabolism. Furthermore, steric interferences due to the nitro group on the benzene rings of NPAHs, are higher than their parent PAHs, thus, their uptake could be difficult in fish body. In addition, studies have shown that compounds containing oxy- and nitro- groups can degrade more quickly and are the most unstable compounds (Manzetti, 2012). The nitro- groups in NPAHs are probably responsible for lower fish body residue as they are easily transformed to other possible metabolites, which could result to lower BCFs in NPAHs as compared to PAHs.

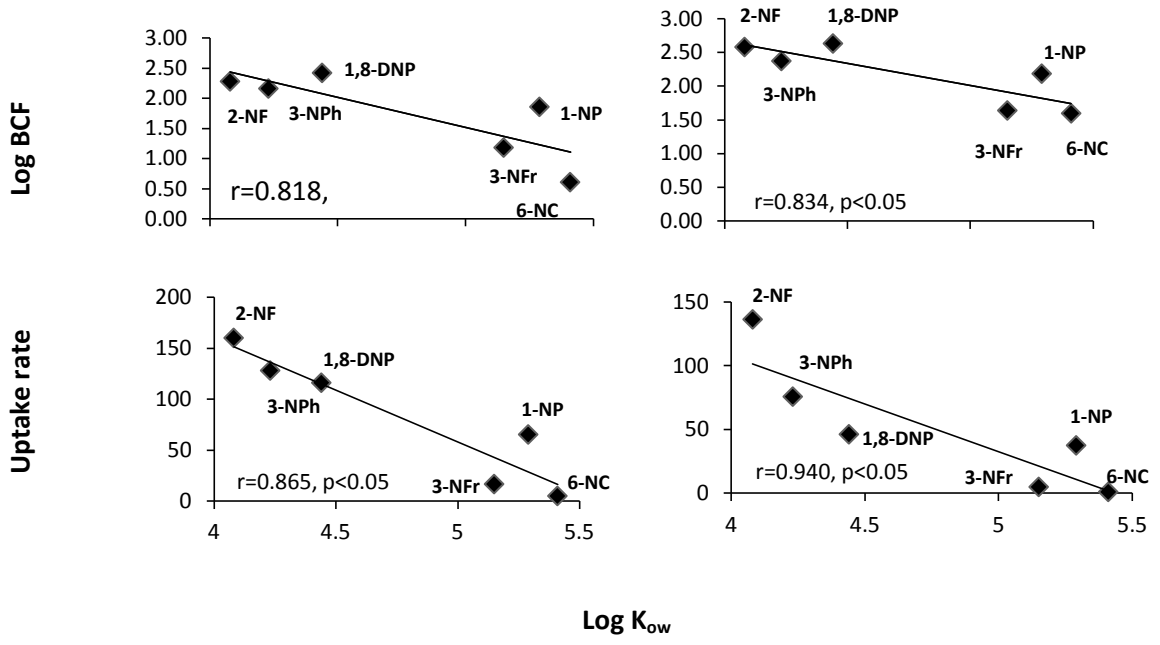

Fig 4.2 Plot of log K_{ow} versus log BCF and uptake rate of NPAHs in low and high exposure

Generally, it is well known, that the uptake rate of a chemical increases as their lipophilic character increases (Kong et al. 2007). However, the present study showed that

the rate of uptake (k_1) of waterborne NPAHs decreases as their lipohilic character increases and was comparable for the two exposure concentrations (Table 4.2).

(Gobas et al. 1988), mentioned that the decrease in uptake rate (k1) with increasing lipohilic character can be due to the limited uptake of very hydrophobic chemicals in fish. This relationship was also observed by (Landrum 1989) where PAHs, such as phenanthrene, anthracene and pyrene were exposed an amphipod, *P. hoyi*. (Bremle et al. 1995) also mentioned that, the uptake rates increases with log K_{ow}, but above a certain value the uptake rates decreases. Furthermore, the uptake rates for NPAHs were significantly related to their log K_{ow} at low ($r=0.865$, $p<0.05$) and high ($r=0.940$, $p<0.05$) exposures (Fig. 4.2).

This is the case with 6-NC having a log K_{ow} of 5.41 resulting to a lower uptake among the target NPAHs. This maybe because 6-NC has a higher molecular weight that tends to be difficult to absorbed by fish because of steric hindrance (Barron 1990). As reflected on 6-NC faster elimination rate in the present study, faster biotransformation of NPAHs also can result to lower BCF, but there is a need to further investigate the negative correlation between BCF and K_{ow} for NPAHs. Furthermore, the large difference between maximum and minimum for uptake rate, k_1, suggest that BCF may be influenced by the uptake rate comparing with smaller difference of k_2 in low and high exposures.

NPAHs such as 1-NP have been reported to be present in the water of Yodo River, Japan with concentration of 1ng/L (Ohe et al. 1996) and sea water collected at Sea of Japan (Murahashi et al. 2001) with concentration of 0.2 to 0.5 pg/L. DNA damage was detected in digestive gland cells of mussels (Mitchelmore et al. 1998a) and in hepatocytes of brown trout (Mitchelmore et al. 1998b) treated with 1-nitropyrene at short-term exposure concentrations of 1~100μM. These reported effective concentrations were conducted *in vitro* and short-term exposures, however, we do not know the genotoxicity of

1-NP and other NPAHs in aquatic organisms using a long-term exposure experiment. Thus, there is a need to further investigate their genotoxicity.

Furthermore, these compounds are known to be carcinogenic compounds especially 6-nitrochrysene, which is a strong carcinogenic chemical that has 10 times potency equivalency factor (PEF) for cancer induction relative to that of benzo(a)pyrene (Busby et al. 1988; Wislocki et al. 1986). In addition, NPAHs can form additional effects when combined with PAHs or other known carcinogenic substances (Moller et al. 1993). Hence, we are suspecting that this strong carcinogenic potency could still affect aquatic organisms.

Conclusion

The present study showed that waterborne nitroarenes can be accumulated by marbled flounder even at lower concentration. Toxicokinetic parameters of nitroarenes seemed to have similar trends with their parent PAHs, but can be influenced by the following factors, log K_{ow}, molecular structure and size, and biotransformation processes. Further investigation is recommended on the mechanisms of uptake and elimination, effects to other type of aquatic organisms and other possible accumulation sources, such as dietary borne. Further investigation is important, because the main sources of nitroarene exposure are the automobile exhaust gases and they are unintentionally generated, they widely spread with the ambient flow after generation, and they have genotoxic potencies similar or higher with benzo(a)pyrene even though they have lower concentration in the environment as compared to their parents PAHs.

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Chapter 5

Micronuclei and other nuclear abnormalities induction in erythrocytes of marbled flounder, *Pleuronectes yokohamae*, exposed to dietary NPAHs

Abstract

Marbled flounder *Pleuronectes yokohamae* were fed with a formulated diet spiked with 7 NPAHs during a 7-day intake period after which they were fed with control diet during the next 7 days as elimination period. Results showed that NPAHs concentrations in fish body were increased but levels were lower than those in the dietary and did not show signs of accumulation. Genotoxicity was manifested in the significantly higher frequencies of micronuclei (MN) and other nuclear abnormalities (NA) formation in erythrocytes with higher frequencies of Σ(MN+NA) at day 7 in lower and higher dietary exposure groups at 42.33 and 45.66 per 1000 erythrocytes, respectively and 5.66 per 1000 erythrocytes in the control.

Introduction

NPAHs are emitted directly from combustion of petroleum products including vehicle exhaust and industrial emissions (Nielsen, 1984). Originating from the reaction of parent PAHs and nitrogen oxide in the atmosphere (Atkinson and Arey, 1994), NPAHs together with their parent PAHs are persistent in the air and are transported into the aquatic environment as fallouts chiefly during rain and which eventually reach the coastal environment. In the water system, these NPAHs could be absorbed and accumulated in aquatic organisms through the water and the food web. Previous study showed that NPAHs are accumulated and bioconcentrated from waterborne NPAHs (Bacolod et al. 2013 in print). However, there is no information regarding accumulation of NPAHs from dietary sources. In addition, studies have shown that NPAHs are probable carcinogenic substances (Landvik et al. 2007) with similar or higher genotoxic potencies than benzo(a)pyrene (Busby et al. 1988; Wislocki et al. 1986). Genotoxicity of NPAHs was shown when 1- nitropyrene induced DNA damage in brown trout (Mitchelmore et al. 1998b) and in mussels (Mitchelmore et al. 1998a). These were results of exposure via gill only and not through dietary exposure which are mostly unknown.

MN (micronuclei) and NA (nuclear abnormalities) in fish erythrocyte are used as genotoxicology biomarkers for detecting chromosomal damage. MNs are ovoid or circular bodies that separate from the nuclei but both stain alike. In addition, these are small fragments of chromosomes or whole chromosomes that separate from the main nuclei during mitosis in erythrocytes (Cavas and Ergene-Gozukara, 2005). Their presence reflects the structural and/or numerical chromosomal aberrations arising during mitosis (Fenech et al. 1999). Other than MN, the presence of NA has been reported in several studies in cells of fish exposed to genotoxic substances (Ayllon and Garcia Vazquez, 2000; Cavas and Ergene-Gozukara, 2003b). In general, these abnormalities are considered to be indicators of genotoxic damage and, therefore, they may complement MN scoring in

routine genotoxicity surveys (Cavas and Ergene-Gazukara, 2005). NA formations include the following: presence of two nuclei (binuclei); lobes in nuclear membrane (lobed nuclei); nuclear membrane pressing into the nucleus (notched nuclei); or presence of extensions (blebbed nuclei). Other studies have described the presence of NA aside from MN, in cells of fish exposed to genotoxic substances (Ayllon and Garcia Vazquez, 2000; Cavas and Ergene-Gozukara, 2003b).

The marbled flounder *Pleuronectes yokohamae* occurs in Japan from southern Hokkaido to Kyushu; in the Yellow Sea; the Gulf of Pohai; and the northern part of the East China Sea (Sakamoto 1984) inhabiting sandy-muddy bottoms less than 100 m deep. Previously we reported bioconcentrations of NPAHs by *P. yokohamae (*Bacolod et al. 2013 in print) but genotoxicity from exposure to dietary NPAHs which is a major uptake route for fish is not well known. This study investigated potential genotoxicity of NPAHs manifested in MN and NA formation in marbled flounder through dietary exposure.

Materials and Methods

Reagents

Standard NPAHs were purchased from various companies as follows: 1] 1-nitropyrene (1-NP), 3-nitrofluoranthene (3-NFr), and 6-nitrochrysene (6-NC) (Sigma-Aldrich, USA); 2] 2-nitrofluorene (2-NF) and 1-nitronaphthalene (1-NN) (Tokyo Kasei Kogyo Co., Japan); 3] 3-nitrophenanthrene (3-NPh) and 1, 8-dinitropyrene (1,8-DNP) (AccuStandard Inc., USA); and 4] 1-nitropyrene-d_9 (as an internal standard) (Central Chemicals Inc., Canada). Acetone, dichloromethane, diethyl ether and hexane were all pesticide-grade reagents (Wako Pure Chemical Ind., Japan). Acetonitrile and methanol were HPLC-grade (Wako). Analytical-grade chemicals such as acetic acid anhydride, diethyl ether, formic acid, sodium hydroxide (NaOH), sodium hydrogen sulfide (NaHS), sodium chloride (NaCl) and silica gel (Wako gel) were also purchased from Wako. Silica

gel containing 3% water was prepared by heating Wako gel at 160^{o}C for 16 h and adding 3% (w/w) Milli-Q water to dried silica gel with vigorous shaking for 1 h. May-Grunwald and Giemsa stain were from Merck (Germany).

Dietary Exposure

Marbled flounder fingerlings were purchased from a regional government aquaculture facility in Kudamatsu City Yamaguchi, in Japan and reared in a 500L flow-through tank with aeration for 5 months in the laboratory with sand-filtered seawater until individuals grew to a weight of about 10 g. The fish were regularly fed with a commercial fish diet, C-1000 formula (Kyowa Hakko, Tokyo) before exposure to the treatment media. The feeding rate was at 2% of the average fish body weight per day.

The stock solution containing a mixture of 7 NPAHs (1-NN, 2-NF, 3-NPh, 1-NP, 3-NFr, 1,8-DNP and 6-NC) was prepared using 3:1 mixture of diethyl ether and acetone as solvent. The solution was then spiked into the fish food (C-1000 formula) to obtain nominal concentrations of 10 (low) and 100 (high) ng/g of the 7 NPAHs. These concentrations were based on reported concentration between 2.38 to 24.7 ng/g NPAHs in bivalves at Osaka bay, Japan (Uno, et al., 2011). The spiked diets were then completely dried in a draught chamber for 24 hours to remove all diethyl ether and acetone.

A total of 57 marbled flounder having body weights of 8.27 ± 0.30 g and total body lengths of 80.7 ± 1.41mm were used in the experiment, with 9 individuals for control, 24 for low concentrations and 24 for high concentrations. These were maintained in a 40 L (90 x 45 x 25 cm) glass aquarium with a 20-24L/h flow-through sand-filtered seawater system with mild aeration and water temperature at 20.3±0.2^{o}C.

Each exposure group was fed the NPAHs spiked diet for the first 7 days (intake period) and the control diet for the next 7 days (elimination period). The feeding rate was at 2% of the average fish body weight per day. In the control group, fish was fed with

control diet for 14 days of the experiment. The diet weights introduced into the aquaria were adjusted daily depending on the number of fish remaining. Dissolved oxygen (DO), pH, salinity and temperature were likewise monitored daily.

Three sample fish from each exposure group were collected for MN/NA examination and NPAHs analysis on days 1, 2, 4, 7 and on days 8, 9, 11, 14 during the intake and elimination periods respectively; and on days 0, 7, 14 from the control group. Due to limited number of test organisms, sampling days for control was lessen, thus, statistical test between control and exposed group was determined at days 0, 7 and 14. The sample fish were anaesthetized by immersion in a 0.5 ml/L solution of 2-phenoxyethanol.

Water samples for the measurement of NPAHs concentrations were taken on days 1, 2, 4 and on days 8, 9, 11 during the intake and elimination period in each exposure group, and on days 0, 7, 14 in the control group. During the experimental period, the mean and standard deviation of the water temperature, dissolved oxygen, pH and salinity in all test groups were 20.3±0.2^{o}C, 6.8±0.1 mg/L, 7.60±0.1 and 30.6±0.4 respectively.

Blood collection & MN determination

For MN and NA determination blood was collected from the caudal blood vessel using a heparinised syringe with 25-26 gauge needle (Terumo, Japan). Sampling days for MN and NA investigation were the same with fish sampling. Blood smears were prepared based on the process of Cheikyula *et al*. (2009) with slight modifications. A blood smear was air-dried for 24 hours, fixed in absolute methanol for 30 seconds, washed with distilled water, and air-dried for another 24 hours before staining. The dried smear was added with May-Grunwald solution and stained with 10% Giemsa stain in phosphate buffer. Duplicate smears were done for each fish.

MN and NA were identified based on Al-Sabti and Metcalfe (1995) and Fenech *et al.* (2003) using a Nikon Eclipse E600 microscope at 1000 magnification. A total of 1000

erythrocytes were observed per slide. The final frequency was obtained after getting the average MN & NA of two slides from one fish, and then finally getting the average MN and NA from three fish, n=3.

Analysis of NPAHs

Analysis of NPAHs in fish and water samples including extraction, clean-up and quantification were discussed in Chapter 3 (Bioconcentration of NPAHs).

Data analyses

MN and NA frequencies were reported as number of MN and/or NA observed per 1000 cells. Analysis of variance（ANOVA）with post hoc Dunnett`s test at $p<0.05$ or the non-parametric Games-Howell test were carried out using the SPSS 11 statistical package (SPSS Inc, USA) to determine differences in control and exposure MN & NA.

Accumulation parameters such as biomagnification factor, elimination rate, assimilation efficiency and half-life were determined following Fisk et al. (1998). The biomagnifications factors (BMF) were estimated from the ratio of concentration of individual NPAHs in fish to the concentration of individual NPAHs in food determined at highest accumulated concentration. From BMF and elimination rate constant (k_2), dietary assimilation was determined using

$$BMF = \alpha F/k_2 \qquad (1)$$

where F, is the feeding rate; α, is the dietary assimilation; and k_2, depuration rate constant.

Elimination rates (k_2) for each NPAHs were determined by the slope of the linear regression line for plots of ln C_f versus time to a first order decay as

$$\ln C_f = \ln C_f^0 - k_2 t \qquad (2)$$

where C_f^0 , is the concentration of the compound in the fish at the beginning of the elimination period. In addition, half-life ($t_{1/2}$) was evaluated using

$$t_{\frac{1}{2}} = \frac{\ln 2}{k_2} \qquad (3)$$

Results and Discussion

NPAHs accumulation

The measured NPAHs concentrations in the test diets are presented in Table 5.1. Target concentrations achieved were 95% and 71% in low and high exposures, respectively.

Table 5.1 Mean measured NPAHs concentration in the test diets

	NPAHs concentration (ng/g)		
Chemicals	Control	Low Exposure	High Exposure
1-NN	<0.005	9.85	89.5
2-NF	<0.002	9.50	81.3
3-NPh	<0.005	10.9	106
1-NP	<0.002	10.7	91.1
3-NFr	<0.001	10.0	82.2
1,8-DNP	<0.025	10.1	91.1
6-NC	<0.002	9.61	70.9

In addition, target NPAHs were not detected in control fish diet. In low exposure groups, most NPAHs were below detection limits in fish even during the exposure period thus; accumulation parameters such as assimilation, elimination rate, half-lives and BMF could not be estimated. These results may be attributed to biotransformation which had a faster rate than the uptake of NPAHs, or due to the lower concentration as observed by Cheikyula *et al*. (2008) where *P. major* did not accumulate parent PAHs from dietary exposure at lower concentrations.

Fig. 5.1A, 1B and 1C shows a typical accumulation for 2-nitroflourene, 2-NF, 3-nitrophenanthrene, 3-NPh and 6-nitrochrysene, 6-NC, respectively, in marbled flounder at

high exposure group. For target NPAHs, the concentrations 1-nitropyrene, 1-NP, 2-nitroflourene, 2-NF and 1-nitronaphthalene, 1-NN peaked at day 2 during exposure period and were detected until day 14 during the elimination period, while 3-NPh peaked at day 8 during elimination period but detected until day 14. In addition, the concentrations of 1, 8-DNP, 6-NC and 3-NFr peaked at day 2, but were detected until days 3, 4 and 8, for 1,8-DNP, 6-NC and 3-NFr, respectively, and were not detected during the elimination period.

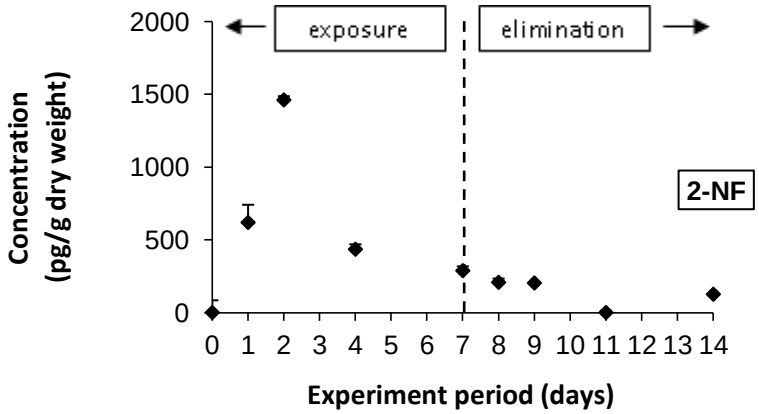

Fig. 5.1 A Whole fish 2-NF concentrations in marbled flounder accumulating and eliminating high dietary-borne NPAHs. (Vertical dashed line indicates where the uptake phase ends and the depuration phase begins.)

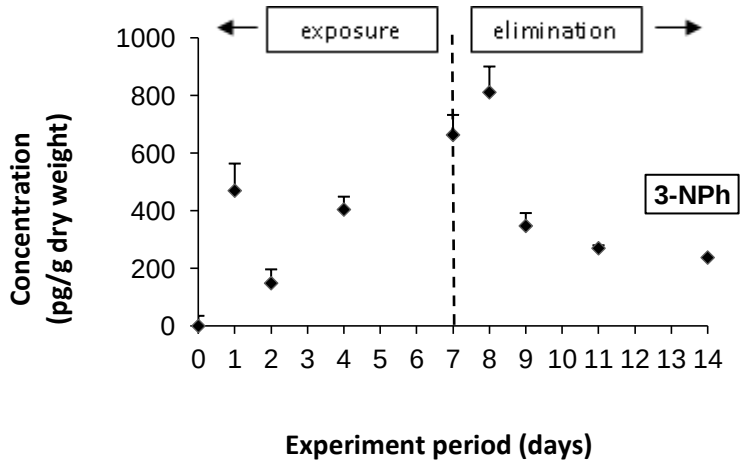

Fig. 5.1B Whole fish 3-NPh concentrations in marbled flounder accumulating and eliminating high dietary-borne NPAHs (Vertical dashed line indicates where the uptake phase ends and the depuration phase begins.)

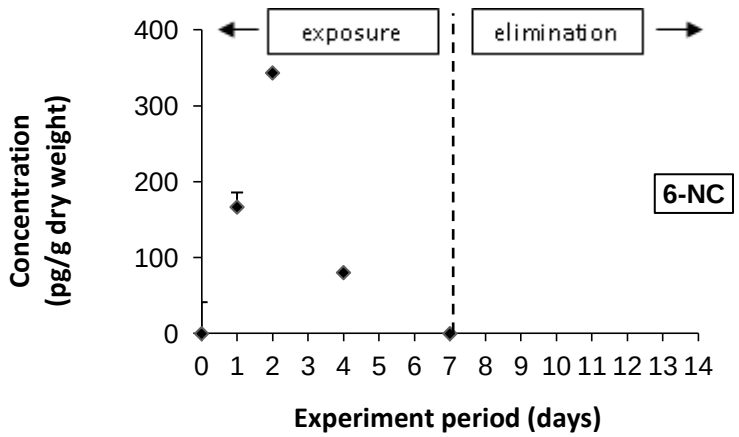

Fig. 5.1C Whole fish 6-NC concentrations in marbled flounder accumulating and eliminating high dietary-borne NPAHs. (Vertical dashed line indicates where the uptake phase ends and the depuration phase begins.)

As shown in the figures, almost all target NPAHs did not clearly show steady state in fish throughout the experiment, thus, we use the maximum concentration observed during the uptake phase to estimate biomagnifications. In our previous study (Bacolod et al. 2013 in press), we also observed maximum concentration for NPAHs between days 1 to 4 in marbled flounder exposed to waterborne NPAHs.

BMFs were estimated from the ratio between peak NPAHs concentration in fish and concentrations in diet. The calculated BMFs were less than 1.0, suggesting that NPAHs were not accumulated in fish from dietary exposure (Table 5.2). The non-accumulation of dietary NPAHs can be due to the type of exposure. That is, NPAHs from dietary sources are bioavailable only when ingested and digested by fish for assimilation, absorption and elimination as metabolites.

In addition, the non-accumulation of NPAHs may be due to the possible induction of reduction and faster elimination of NPAHs as can be seen in Fig 4.1A and 1C. Furthermore, NO_2-group in NPAHs which can easily metabolize parent NPAHs to corresponding reduction products such as amino NPAHs, as reported by Kitamura and Tatsumi (1996) and Ueda *et al*., (2001) may affect non-accumulation of chemicals.

Other accumulation factors, such as assimilation efficiency, elimination and half-life were estimated only for 1-NN, 2-NF, 3-NPh and 1-NP because they were detected only until day 14 of the experiment period (Table 5.2).

Table 5.2 Assimilation efficiency (α), elimination rate (k_d), half-life ($t_{1/2}$) and biomagnification factors (BMF) of nitrated polycyclic aromatic hydrocarbons (NPAHs) in marbled flounder

NPAHs	Molar Mass	Log K_{ow}[a]	α	k_d(d^{-1})	$t_{1/2}$ (d)	BMF
1-NN	173	2.50	0.33	0.34	2.07	0.020
2-NF	211	4.08	0.17	0.17	3.99	0.020
3-NPh	223	4.23	0.05	0.17	4.08	0.006
1,8-DNP	292	4.44	ND	ND	ND	0.016
3-NFr	247	5.15	ND	ND	ND	0.006
1-NP	247	5.29	0.10	0.26	2.08	0.008
6-NC	273	5.41	ND	ND	ND	0.005

[a] log K_{ow}, from Environmental Health Criteria (2003) pp 31-34
ND: not determined

The highest assimilation efficiency was for 1-NN, comparatively, the assimilation for 2-NF, 3-NPh and 1-NP were lower than that of 1-NN. Due to the small number of data no significant relationship could be found between the assimilation efficiency (log α) and log K_{ow} ($r=0.8480$, $p<0.07$), but we obtained a negative correlation trend where NPAHs with low K_{ow} were highly assimilated compared to NPAHs with high K_{ow}. Elimination rates of NPAHs were rapid and half-lives were within 2 to 4 days for the target NPAHs. These results were also similar when the same fish were exposed to water-borne NPAHs (Bacolod et al., in press). In the present study dietary exposure, NPAHs concentration in fish were lower compared to their concentration in test diet, this suggests that dietary NPAHs cannot be accumulated by fish, but rather accumulated, followed by faster elimination (including faster biodegradation) in fish.

Induction of MN and NA

MN and NA were formed in the marbled flounder erythrocytes exposed to NPAHs. Fig. 5.2 exemplifies a normal cell (A), and cell with MN (B) and other NA (C-F) detected from the exposure groups. The MN frequency ranged between 0.33-9.50 per 1000 erythrocytes, while NA frequency ranged from 2.83 to 50.17 per 1000 erythrocytes.

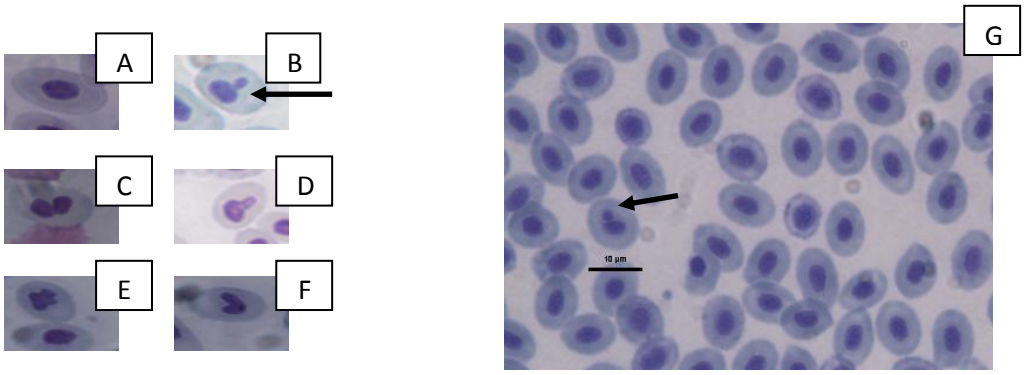

Fig. 5.2 Photomicrograph showing micronucleus (MN) and other nuclear abnormalities (NA) from erythrocytes in marbled flounder exposed to NPAHs (x1000 magnification). A- normal cell; B- cell with micronuclei; C to F – other nuclear abnormalities (C- binucleated cell; D- blebbed cell; E- lobbed cell; F- notched cell); G- cells observed in exposed fish (arrow shows MN location)

Our results are similar to some fish MN studies as reviewed by Al-Sabti and Metcalfe (1995), such as observed frequencies higher than 0.7 - 4.0 per 1000 erythrocytes in wild crucian carp, *Carassius auratus* exposed to Tomio river, Nara, Japan (Hayashi *et al*. 1998); 0.2 - 2.79 in *Pseudopleuronectes americanus* exposed to chlorinated hydrocarbons; 3.4 in *Genyonemus lineatus* exposed to a mixture of PAHs.

Fig. 5.3 (A) shows the frequencies of MN in exposed and control groups. MN frequencies of exposure groups were significantly different from control frequencies (p<0.05) on day 7 only, this can be due to the end of exposure and start of elimination period. MN frequencies between exposed groups were significantly different on days 9 and 11 only (p<0.05).

Figure 5.3 (B) shows that the frequencies of NA in exposed groups were significantly different from control (p<0.05) on days 7 and 14, while NA frequencies between exposed groups were significantly different on day 11 only (p<0.05). Both MN and NA frequencies were greater in high exposure compared to low, with more NA recorded than MN in both exposures. The highest MN and NA frequencies per 1000 erythrocytes at day 7 were 5.33 and 40.33 for high exposure and 4.83 and 37.5 for low exposure respectively, with control registering only 0.33 and 5.33 per 1000 erythrocytes.

The results suggest that even at low concentrations of NPAHs affected hematopoiesis in fish and showed their genotoxicity although NPAHs were not accumulated, but showed an increasing of NPAHs in fish body by dietary exposure while they were not biomagnified. In addition, a dose-dependent response between NPAHs concentration and MN and NA; and exposure-time dependent increase in MN and NA were observed. There appears to be significant differences of MN and NA between low and high exposure at days 9 and 11 (p<0.05), when ΣMN+NA frequencies in both exposure groups were compared. Furthermore, MN and NA formations continued to increase in both diets 2 to 3 days after exposure was terminated, similar to the findings of Cheikyula *et al.* (2009) in red sea bream exposed to PAHs. MN or NA erythrocytes persist after exposure and into the rest of the erythrocyte life span, estimated to be 127 days (Fischer *et al.* 1998), perhaps to be corrected only in the next cell cycle when new daughter cells are produced or when cells are destroyed either by necrosis or by programmed cell death (apoptosis). The after-exposure decreases in MN and NA frequencies in our results may be due to the genotoxic effect of NPAHs, which greatly affected the fish erythrocytes resulting to apoptosis.

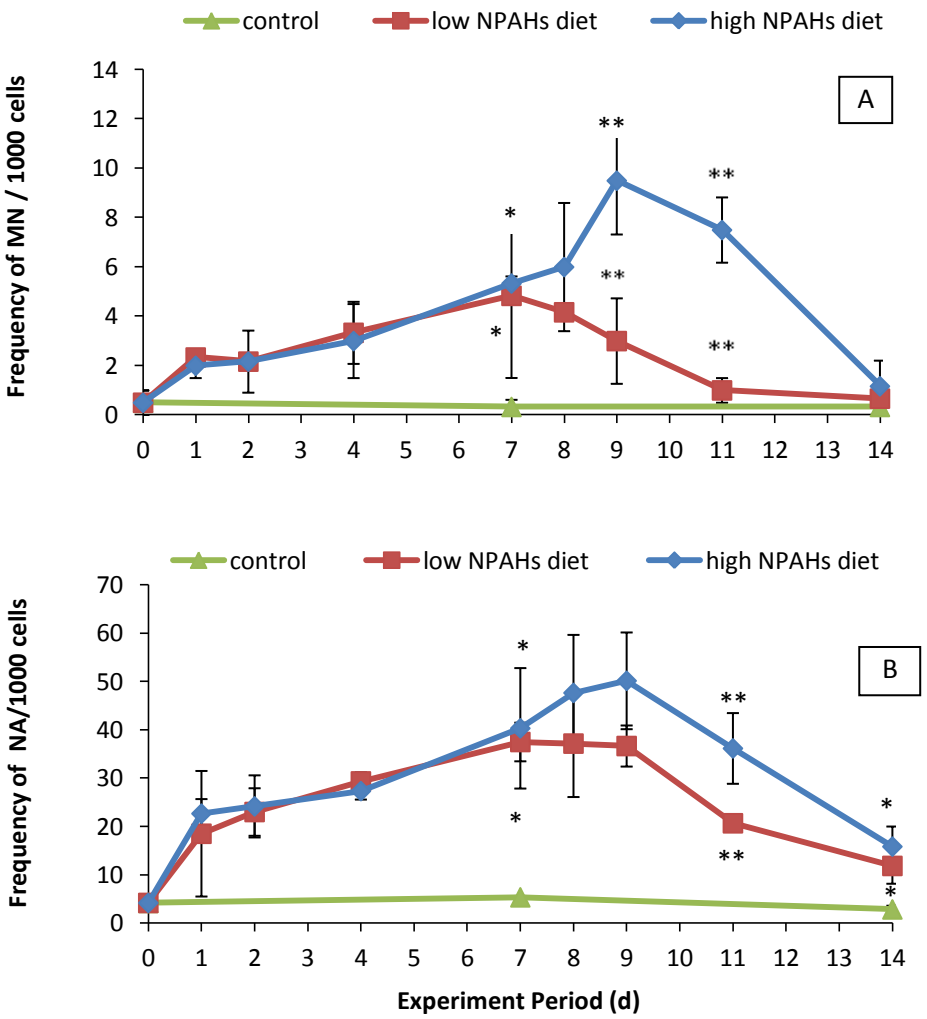

Fig. 5.3A - Frequency of erythrocytic MN per 1000 erythrocytes in marbled flounder exposed to high dietary NPAHs (n=3, $p<0.05$); **B** - Frequency of erythrocytic NA per 1000 erythrocytes in marbled flounder exposed to high dietary NPAHs (n=3, $p<0.05$); * - significantly different from control; ** - significantly different between exposed group.

This study shows that MN and NA can be induced even by low total NPAHs concentration in fish (57-250 pg/g) exposed to low NPAHs concentration in test diet. In addition, a dose-dependent response between NPAHs concentration and induction of MN and NA; and exposure-time dependent increase of MN and NA frequency were observed. These suggest that NPAHs induce MN and NA, and are genotoxic for marbled flounder even in low concentrations in fish.

Conclusions

Dietary NPAHs exposure induce genotoxic effects as manifested in MN and NA formations which are shown to be useful tools for the evaluation of genotoxic effects of NPAHs in marbled flounder. The presence of MN and NA despite the absence of accumulation shows the possible genotoxicity of NPAHs even at low concentrations.

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PART IV

FIELD EXPERIMENTS

Chapter 6

Genotoxicity of river water collected from Butuanon River in Cebu, Philippines and their NPAHs, PAHs and metal concentrations

Abstract

Fish has been a model for accumulation and genotoxicity studies of pollutants, and rivers are one of the ultimate sink for residential and industrial wastes including vehicular emissions. Polycyclic aromatic hydrocarbons (PAHs) and nitropolycylic aromatic hydrocarbons (NPAHs) are one of the pollutants from vehicle emissions known to be carcinogenic and mutagenic. This is a first report regarding genotoxicity and simultaneous accumulation of PAHs and NPAHs in freshwater fish exposed to polluted Butuanon River in Cebu, Philippines. In this experiment, fish were exposed to river water collected at two different sites for 14 days, followed by a 7 day elimination period. PAHs and NPAHs were detected in river water, and were accumulated in river water-exposed fish. In addition total PAHs and NPAHs concentration in the water was higher in lower stream. PAHs were detected in both sites, while most NPAHs were detected in lower stream only. Heavy metal analysis such as Al, Cr, Co, Ni, Cu, Zn, As, Ag, Cd, Sn and Pb from river water collected from both stream showed lower concentrations (µg/L) to cause genotoxicity. Genotoxicity evaluation using micronucleus test in fish erythrocytes showed induction of micronuclei (MN) and other nuclear abnormalities (NA) in fish erythrocytes. Induction of MN and NA were higher in fish erythrocytes exposed to lower stream. A strong correlation was obtained between total NPAHs and MN or NA induction ($p<0.01$) as compared with total PAHs and MN or NA induction. This study suggests that NPAHs is possibly one of the agents that caused genotoxic effects in fish.

Introduction

PAHs are produced during combustion of petroleum products, while NPAHs are produced directly or indirectly from PAHs, thus, PAHs are more abundant in the environment as compared to NPAHs. Several studies have reported that these compounds are carcinogenic and mutagenic to aquatic organisms and even to humans, and are found in all environmental matrices. Other PAHs and NPAHs, heavy metals are common pollutants and can result to adverse effects to aquatic organisms. These pollutants are persistent in the atmosphere and can be transported into the aquatic environment as fallouts, chiefly during rain and can finally accumulate in aquatic organisms, thereby causing genotoxic effects.

Fish has been a useful model for the evaluation of pollution in aquatic ecosystems including genotoxicity and accumulation. Genotoxicity parameters like micronuclei (MN) and nuclear abnormalities (NA) are one of many biomarkers used in environmental genotoxicology to detect chromosomal damage. MN`s are small fragments of chromosomes or whole chromosomes separated from the main nuclei during mitosis in red blood cells (Cavas and Ergene-Gozukara, 2005). Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Fenech et al. 1999). The micronucleus (MN) test has been used with different fish species to monitor an aquatic pollutant that displays mutagenic properties (De Flora et al., 1993) other than easy to perform comparing with other genotoxic biomarker. In addition, Kligerman (1982) showed that fish inhabiting polluted waters have greater frequencies of micronuclei and possibly other NA. The micronuclei frequencies may also vary according to the season, the kind of pollution involved, and the species of fish.

Several studies reported that tilapia, *Oreochromis niloticus* and *Tilapia rendalli* is susceptible to MN and NA formation when exposed to genotoxic substances (Palhares and Grisolia 2002) such as mitomycin C. In addition, MN and NA formation were also

detected in *O. niloticus* when exposed to a refinery effluents (Cavas and Ergene-Gozukara, 2005; Souza and Fontanetti 2006).

An investigation of PAHs and NPAHs accumulation together with genotoxicity will give valuable information regarding possible threat to fish and even to humans. Furthermore, this is the first to report on genotoxic effect of river water in Cebu, Philippines together with simultaneous accumulation of PAHs and NPAHs.

Materials and Methods

Chemicals

Chemicals such as standard NPAHs and others were purchased as described in Chapters 1 and 3.

Description of the Study Area

Butuanon River is located in Philippines, particularly in Cebu (Figure 1). The river is nearly ten kilometers in length, and ten meters wide, flowing from the deforested mountains of Barangay Pit-os, Cebu City passing through residential, commercial and industrial areas of both cities of Cebu and Mandaue, down to Mactan Channel. It is surrounded by potential contaminant such as industrial sources, including a steel foundry and a furniture manufacturer. It is also subject to essentially indiscriminate wastewater dumping by both domestic and industrial sources including vehicle emissions (EMB 2010). In addition, it has been reported that the river is highly polluted with heavy metals, and serve as sink from sanitary and solid wastes coming from rural and urban areas (Mendoza et al., 2000). There is limited information available as to the extent of pollution in the river, thus, the river is therefore a reasonable site to represent the extent of pollution in the area.

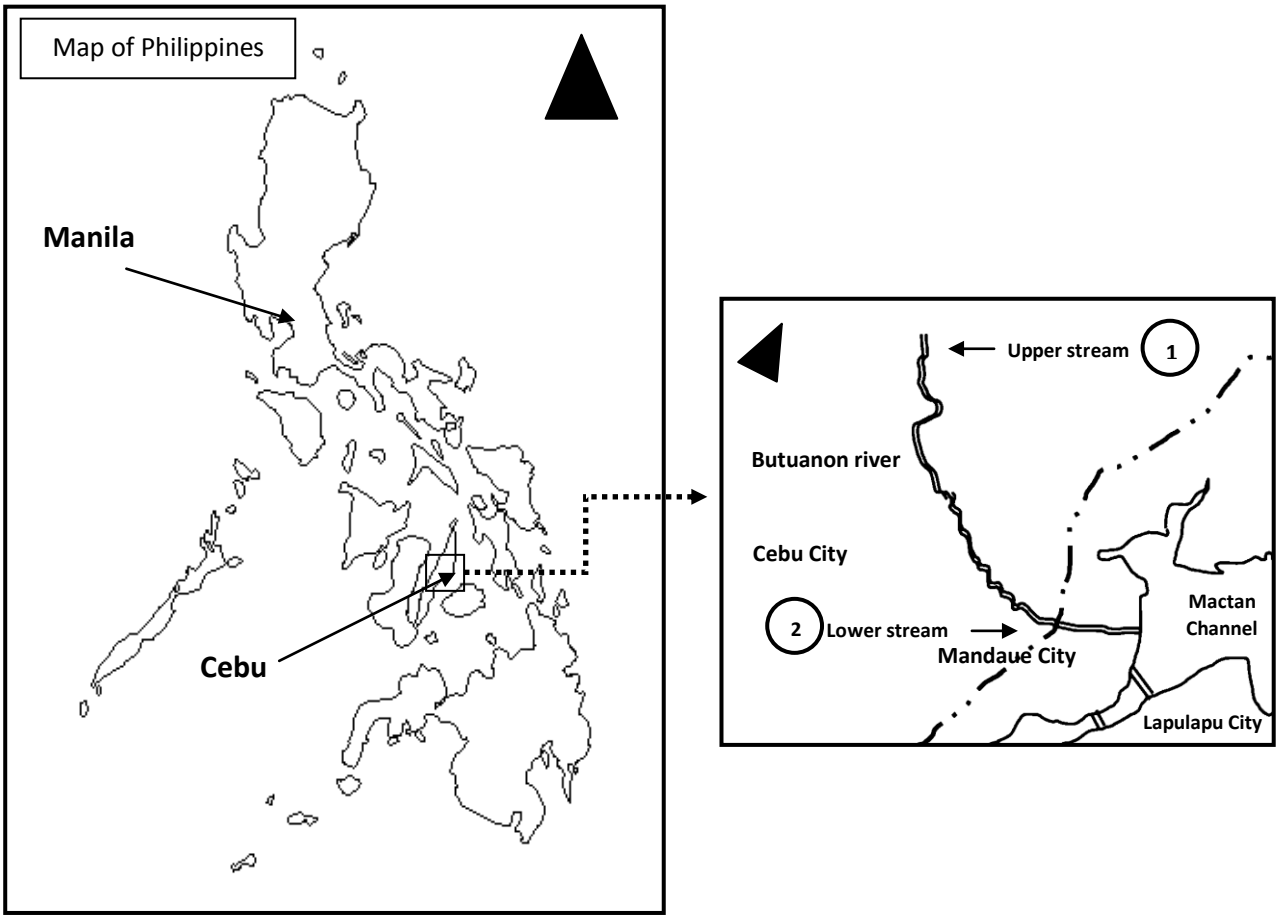

Figure 6.1 Location of the study area. The circled numbers (1-2) indicate water sampling stations.

Water Sampling

Water samples were taken every 2 days for a period of 14 days from the Butuanon River in upper and lower stream, representing the rural and urban areas, respectively (Fig. 6.1). For each site, approximately 60 L of water were collected in two 45-L plastic containers and brought to the laboratory the same day. Water samples were filtered and transferred to test tanks for bioassay experiment and the remaining water samples were used for extraction of NPAHs and PAHs using blue rayon method modified from Sakamoto et al., (1990) as described somewhere. Water samples were extracted on days 3, 7, and 13 for NPAHs and PAHs analyses.

Fish Exposure

Tilapia *O. niloticus* were purchased from a general aquaculture facility of Cebu Technological University in Cebu, Philippines and reared in a 500L flowthrough tank with aeration for 5 months in the laboratory with filtered and dechloronized tap water until individuals grew to a weight of about 8 g. The fish were regularly fed with a commercial fish diet, 6P pellet (Nippon Formula Feed Manufacturing, Tokyo) before exposure to the treatment media.

A total of 45 tilapia were randomly distributed, that is, 15 individuals for control, 15 for low stream and15 for downstream exposures. Their body weights and total body lengths were 8.27±0.30g and 80.7±1.41mm, respectively and maintained in glass aquaria, each containing 40 L of water and measuring 40x30x60 cm provided with mild aeration. The exposure periods lasted for 14 days, while elimination period lasted for 7days. The test water was renewed every 48 hours to minimize changes due to metabolization, complexation, volatilization of less stable substances, and organism catabolites. The aquaria were kept closed to minimize possible loss of volatile substances and under dark condition to prevent cannibalism. The fish in control and exposure group were fed with the same commercial fish diet at 1% of daily average body weight per day during the experiment period and were adjusted everyday depending on the number of fish remaining. Exposure period were based from previous bioconcentration experiment (Bacolod et al., 2013b). Water parameters such as pH, dissolved oxygen (DO), salinity and temperature were measured daily.

Fish Sampling

For accumulation and genotoxicity evaluation, three fish samples were taken on days 0, 3, 7, 14 and on day 21 during the exposure and elimination period in each control

and exposure group. Fish collected were anaesthetized by immersion in a 0.5 ml/L solution of 2-phenoxy ethanol for 2 minutes.

For MN and NA determination blood was collected from the caudal blood vessel using a heparinised syringe with 25 gauge needle (Terumo, Japan). Blood smears were prepared based on the process of Cheikyula et al. (2009) with slight modifications. Blood smear was air-dried for 24 hours, fixed in absolute methanol for 30 seconds, washed with distilled water, and air-dried for another 24 hours before staining. The dried smear was added with May-Grunwald solution and stained with 10% Giemsa stain in phosphate buffer. Duplicate smears were done for each fish.

MN and NA were identified based on Al-Sabti and Metcalfe (1995) and Fenech *et al.* (2003) using a Nikon Eclipse E600 microscope at 1000 magnification. A total of 1000 erythrocytes were observed per slide. The final frequency was obtained after getting the average MN & NA of two slides from one fish, and then finally getting the average MN and NA from three fishes, n=3.

The remaining fish were freeze-dried and homogenized for NPAHs and PAHs analyses. Digestive tract was removed before the fish were freeze-dried to remove undigested food in the fish.

Analyses of NPAHs and PAHs

River Water

River water was extracted with 0.5 g blue rayon per liter of water sample. The blue rayon was suspended in water with mild aeration to mix water and blue rayon for 24 hours, removed and completely washed with Milli-Q water, air-dried and stored until extraction and analyses. For extraction, 0.5 g blue rayon was extracted twice by shaking with a mixture of 80 ml methanol:ammonia (1:1) for 15 minutes. The extract was divided for PAHs and NPAHs measurement after subsequent clean-up and derivatization as described

previously. Recovery of PAHs and NPAHs was done using same method. Recovery of PAHs and NPAHs were within 53 to 112% suggesting that the method can be used in real samples.

Fish

The analysis of NPAHs and PAHs such as extraction, clean-up, derivatization and quantification were discussed previously.

Analysis of heavy metals

River water samples were collected in upper and lower stream for the analysis of heavy metals. Water samples were filtered using a disposable cartridge filter 0.4 µm. Heavy metals were analyzed by ICP-MS, Agilent Technologies 7500 series (USA).

Data analyses

Concentration data are presented as average of samples. MN and NA frequencies were reported as number of MN and or NA observed per 1000 cells. Analysis of variance （ANOVA） with post hoc Dunnett`s test at $p<0.05$ or the non-parametric Games-Howell test were carried out using the SPSS 11 statistical package (SPSS Inc, USA) to determine differences in control and exposure MN & NA. Relationship between NPAHs and MN and NA were determines using Pearson coefficient (r). MN and NA frequencies were reported as average number of MN and or NA observed per 1000 cells.

Results and Discussion

Many of the pollutants found in the aquatic environment are known genotoxic and carcinogenic substances. These substances may interact, directly or after metabolic activation, with DNA or DNA processing machinery and thereby induce the cytogenetic

alterations in the cells of organisms (Chen and White 2004). MN and NA are important biomarker of cytogenetic damages in the cells of organisms (Fenech 1998).

MN and NA were observed in tilapia erythrocytes exposed to river water and results were shown in Table 6.1. There were significant increases of MN and NA frequencies observed in fish exposed to lower stream, compared with the control and upper stream.

Table 6.1 Average frequencies of erythrocytic MN and NA per 1000 erythrocytes (mean±SD) in tilapia exposed to river water

	Micronuclei (MN)			Nuclear abnormalities (NA)		
Day	Control	Upper stream	Lower stream	Control	Upper stream	Lower stream
0	0.01±0.03	0.01±0.03	0.01±0.03	0.45±0.12	0.45±0.12	0.45±0.12
3	0.01±0.03	0.02±0.04	0.03±0.04	0.45±0.12	0.45±0.15	0.80±0.29*
7	0.02±0.04	0.01±0.03	0.03±0.05	0.47±0.17	0.43±0.12	1.42±0.29*
14	0.01±0.03	0.02±0.04	0.13±0.05*	0.49±0.19	0.46±0.14	2.04±0.49*
21	0.01±0.03	0.03±0.06	0.05±0.05	0.47±0.13	0.43±0.11	0.90±0.11*

* significantly different from control and upper stream ($p<0.05$)

Results indicate that the lower stream clearly had genotoxic effects to tilapia, and that the genotoxic substances in the river are available for exposure and accumulation to aquatic life and possibly to human beings.

It can be noted that the lower stream is located in the urban areas, which is greatly affected by the presence of heavy traffic congestion, residential and industrial areas. Fish exposed to lower stream showed significant induction of MN at day 14 of exposure as compared to upper stream and control ($p<0.05$) with MN frequency of 0.13, 0.02 and 0.01 in lower stream, upper stream stream and control respectively.

Other than MN, NAs were also observed in tilapia erythrocytes in control and river-exposed groups, however, the inductions of NA were observed to be higher than MN induction in all test groups. NAs are also indicators of genotoxicity and their presence is

an indication of genotoxicity. NAs frequencies were higher at day 14 in all test groups with 2.04, 0.49 and 0.46 in lower stream, control and upper stream. NAs induction in lower stream were significantly different with upper stream and lower stream at days 7 and 14 ($p<0.05$), however, NAs in river-exposed groups were significantly different from control at days 3, 7, 14 and 21 ($p<0.05$). This suggests that MN or NAs induction is dependent on exposure time and aquatic life may be affected by MN and NA as time passes. In this study, the MN and NA frequency falls within the range of 0.43-70.7 per 1000 erythrocytes reviewed by Al-Sabti and Metcalfe (1995) in some fish MN studies. Our previous study, also observed MN and NA formation in marbled flounder exposed to NPAHs (Bacolod et al., 2013a). In addition, MN and NA persisted even after exposure. This is possibly due to the erythrocyte life span, estimated to be 127 days (Fischer et al., 1998), perhaps to be corrected only in the next cell cycle when new daughter cells are produced or when cells are destroyed either by necrosis or by programmed cell death (apoptosis).

NPAHs are formed from parent PAHs and are seemed to be more toxic than PAHs (Albinet et al., 2006). Average NPAHs concentrations in river water and whole fish are presented in Table 6.2 and 6.3.

Table 6.2 Average NPAHs concentration in river water (ng/L)

NPAHs	Control	Upper stream	Lower stream
1-NN	0.130±0	0.147±0.02	0.218±0.040
2-NN	0.172±0.007	0.164±0.008	0.220±0.044
2-NF	<0.002	<0.002	0.100±0.007
3-NPh	<0.005	<0.005	0.134±0.031
1-NP	<0.002	<0.002	0.086±0.019
3-NFr	<0.001	<0.001	0.040±0.006
7-NBaA	<0.001	<0.001	0.067±0
6-NC	<0.001	<0.001	0.189±0.097
ΣNPAHs	0.302	0.311	1.05

Table 5.3 Average NPAHs concentration (ng/kg dry) in whole fish exposed to river water

	Day	Control				Upper stream				Lower stream			
NPAHs	0	3	7	14	21	3	7	14	21	3	7	14	21
1-NN	9.65	9.45	10.2	9.7	13.3	11.1	10.3	10.1	12.5	19.3	19.0	20.0	12.7
2-NN	6.91	5.90	5.80	6.20	7.35	6.15	8.40	5.80	3.89	13.8	11.0	10.5	7.3
2-NF	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
3-NPh	2.89	3.06	4.22	5.65	<5	4.15	3.54	7.05	<5	6.6	10.3	17.6	8.05
1-NP	<2	<2	<2	<2	<2	<2	<2	<2	<2	2.67	3.00	3.74	<2
3-NFr	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
7-NBaA	<1	<1	<1	<1	<1	<1	<1	<1	<1	0.43	2.70	2.93	<1
6-NC	4.01	3.66	6.20	7.30	4.98	4.88	7.15	7.75	7.35	8.4	19.2	23.6	17.1
ΣNPAHs	23.5	22.1	26.4	28.8	25.6	26.3	29.4	30.7	23.7	51.1	65.2	78.4	45.0

Most of the target NPAHs was detected in lower stream as compared to upper stream, suggesting NPAHs sources may be due to traffic conditions or urbanization in the lower stream. These findings are in agreement with reports that NPAHs are mostly formed during combustion of petroleum products (Paputa-Peck et al. 1983; IARC 1989). Total NPAHs concentration in lower stream was 1.00 ng/L with 1- and 2-NN having the highest concentrations of 0.218 and 0.220 ng/L, respectively. Interestingly, carcinogenic NPAHs such as 6-NC and 1-NP were detected in the water of lower stream but not upper stream.

Table 6.3 tabulates the NPAHs detected and their concentration in river water-exposed fish. NPAHs detected were 1- and 2-NNs, 9-NPh, 1-NP, 7-NBaA and 6-NC with concentration at day 14 of 20, 10.5, 17.6, 3.74, 2.93 and 23.6 ng/kg dry weight, respectively in lower stream and 1- and 2-NNs, 9-NPh and 6-NC with concentration at day 14 of 10.1, 5.8, 7.05 and 7.75 ng/kg dry weight respectively, in upper stream. Total NPAHs concentrations in fish exposed to lower stream were higher than in upper stream with highest total concentrations of 30.7 and 78.4 ng/kg dry in upper and lower stream, respectively. The findings were in agreement with the NPAHs concentration in river water. The high concentration can be due to sample site that is directly influence by traffic condition and the presence of industries in the area. For example, 1-NP was only detected in fish exposed to lower stream; 1-NP has been reported as a biomarker for diesel use. In

addition, 1-NP and 6-NC are reported to be carcinogenic among the NPAHs detected. NPAHs concentrations in fish were higher than their concentration in water, suggesting NPAHs were accumulated in fish. Furthermore, BCF of each NPAHs estimated from the ratio of individual NPAHs concentration in fish with individual NPAHs concentration in water were higher than 1. These results suggest bioconcentration of NPAHs and showed similar BCFs with our previous where NPAHs were bioconcentrated from water-borne sources by *P. yokohamae* (Bacolod et al. 2013b).

The 16 USEPA priority PAHs have been used to evaluate anthropogenic pollution levels in environment (Mai et al. 2002). The individual concentrations measured of the 16 priority PAHs in river water and in whole fish were listed in Table 6.4 and 6.5.

Table 6.4 Average PAHs concentration (ng/L) in river water

PAHs	Control	Upper stream	Lower stream
naphthalene	0.150±0.018	0.242±0.039	0.219±0.099
acenaphthylene	<0.001	0.019±0.021	0.036±0.039
acenaphthene	<0.001	0.021±0.018	0.011±0.008
fluorene	<0.001	0.07±0.009	0.098±0.065
phenanthrene	4.05±1.5	1.63±1.18	4.11±0.23
anthracene	0.185±0.055	0.095±0.099	0.31±0.02
fluoranthene	1.45±0.50	1.23±0.48	3.31±1.26
pyrene	0.922±0.4	1.26±0.65	2.63±0.72
benzo[a]anthracene	0.040±0.014	0.051±0.022	0.269±0.09
chrysene	0.056±0.021	0.246±0.027	0.613±0.433
benzo[b]fluoranthene	<0.001	0.064±0.007	0.147±0.09
benzo[k]fluoranthene	<0.001	0.054±0	0.064±0.025
benzo[a]pyrene	0.012±0.004	0.015±0.003	0.08±0.034
benzo[g.h.i]perylene	<0.001	<0.001	0.044±0.028
dibenzo[a.h]anthracene	<0.001	0.077±0.02	0.094±0.03
indeno[1.2.3-c.d]pyrene	<0.001	0.032±0.028	0.083±0.053
ΣPAHs	6.86	5.11	12.1

Most of the 16 USEPA priority PAHs were detected in lower stream suggesting that the lower stream is affected by heavy traffic congestion, and presence of residential and industrial establishments. The total PAHs concentrations in river water were higher in lower stream as compared to upper stream, however, total PAHs in whole fish were almost similar in both river-exposed fish.

Table 6.5 Average PAHs concentration (ng/kg dry) in whole fish exposed to river water

	Day	Control				Upper stream				Lower stream			
PAHs	0	3	7	14	21	3	7	14	21	3	7	14	21
naphthalene	113	107	118	106	124	121	142	190	141	173	146	197	178
acenaphthylene	34.6	25.8	21.5	19.7	22.9	27.9	26.4	27.7	23.8	30.3	33.1	44.0	24.4
acenaphthene	72.4	46.8	42.1	40.5	36.6	53.5	46.8	52.0	41.7	55.5	54.5	51.5	42.9
fluorene	68.5	40.3	43.4	47.0	43.3	57.5	47.1	56.5	44.8	64.0	43.7	60.0	51.5
phenanthrene	91.2	90.0	54.8	83.8	83.4	87.5	61.0	83.0	82.5	91.5	69.0	88.0	82.0
anthracene	4.91	12.4	14.4	13.9	12.8	12.4	15.8	13.2	15.4	15.6	15.6	14.9	13.7
fluoranthene	12.9	11.2	13.9	18.0	14.3	20.6	13.9	15.7	13.0	21.0	16.6	19.4	15.5
pyrene	10.6	13.6	14.3	14.5	12.5	15.4	12.3	14.4	12.4	21.1	14.9	16.7	13.3
benzo[a]anthracene	1.29	0.462	0.882	0.767	1.14	1.12	0.73	1.52	0.98	2.70	0.98	1.07	0.80
chrysene	3.36	4.27	3.52	2.47	4.96	5.40	3.95	7.20	4.39	7.20	5.65	8.65	6.00
benzo[b]fluoranthene	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
benzo[k]fluoranthene	<1	<1	<1	0.54	0.53	0.69	0.84	<1	<1	1.48	0.37	0.84	<1
benzo[a]pyrene	4.98	2.63	6.53	6.85	8.10	2.47	5.55	9.45	6.40	7.10	7.30	11.0	6.60
benzo[g.h.i]perylene	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	0.68	4.33	<1
dibenzo[a.h]anthracene	<1	<1	<1	<1	<1	<1	<1	<1	<1	1.47	0.35	0.49	<1
indeno[1.2.3-c.d]pyrene	<1	<1	<1	<1	<1	<1	<1	<1	<1	2.10	1.34	0.90	<1
ΣPAHs	418	355	334	354	364	405	376	470	386	494	410	520	435

Total PAHs concentrations in river water were 5.11 and 12.1 ng/L in upper and lower stream, respectively, while highest total PAHs concentration were 470 and 520 ng/kg in whole fish exposed to upper and lower stream, respectively, and were highest at day 14 of the exposure period. The 16 USEPA priority PAHs detected in river water were also accumulated by tilapia. MN and NA frequencies were compared with the total measured priority PAHs. Increasing of PAHs concentration in fish did not cause increasing of MN and NA. This suggested that the priority PAHs might only contribute to a portion of the genotoxicity of river water.

Heavy metals concentrations of for Al, Cr, Co, Ni, Cu, Zn, As, Ag, Cd, Sn and Pb in upper and lower stream of Butuanon river are shown in Table 6.6.

Table 6.6 Average heavy metal concentration in river water

	Concentration (µg/L)	
Heavy metals	Upper stream	Lower stream
Al	8.71	19.9
Cr	0.08	0.64
Co	0.06	0.28
Ni	0.56	1.41
Cu	<0.0475	<0.0476
Zn	0.66	5.06
As	0.45	1.89
Ag	<0.0435	<0.0436
Cd	<0.0009	0.004
Sn	<0.0046	0.030
Pb	<0.0007	0.122

Concentrations of most heavy metals were higher in lower stream than in upper stream. These results suggested that the lower stream is influenced by urbanization and presence of industries along the area. Mendoza et al., 2000 reported that the river is highly polluted with heavy metals, and serve as sink from sanitary and solid wastes coming from rural and urban areas. Among the heavy metals, Al has the highest concentration with 8.71 and 19.9 µg/L in upper and lower stream, respectively. Other heavy metals were of very low concentration such as Cr, Co, Ni, Cu, Zn, As, Ag, Cd, Sn and Pb.

Heavy metals such as Cu and Cd did not cause significant induction of micronuclei but cause a significant induction of nuclear abnormalities in *Gambusia affinis* exposed to concentrations of 0.1 amd 1 mg/L (Guner et al., 2011). Other authors also observed the same with Cu in European minnow (*Phoximus phoximus*) (Sanchez-Galan et al., 1999) and Cd (Ayllon et al., 2000). In this study, heavy metal concentrations are very low to cause genotoxic effects to tilapia.

Figure 6.2 shows the positive relationship between total NPAHs concentration with MN, NA and MN+NA frequency in lower and upper stream. However, total NPAHs is significantly correlated only with NA ($p<0.05$) and MN+NA ($p<0.05$) at lower stream, however there was no significant relationship between total NPAHs with MN, NA and

MN+NA in upper stream. This finding is in agreement with the detected NPAHs in lower stream, and also carcinogenic 1-NP and 6-NC which were detected in lower stream only might contribute to the genotoxic effects observe in the lower stream.

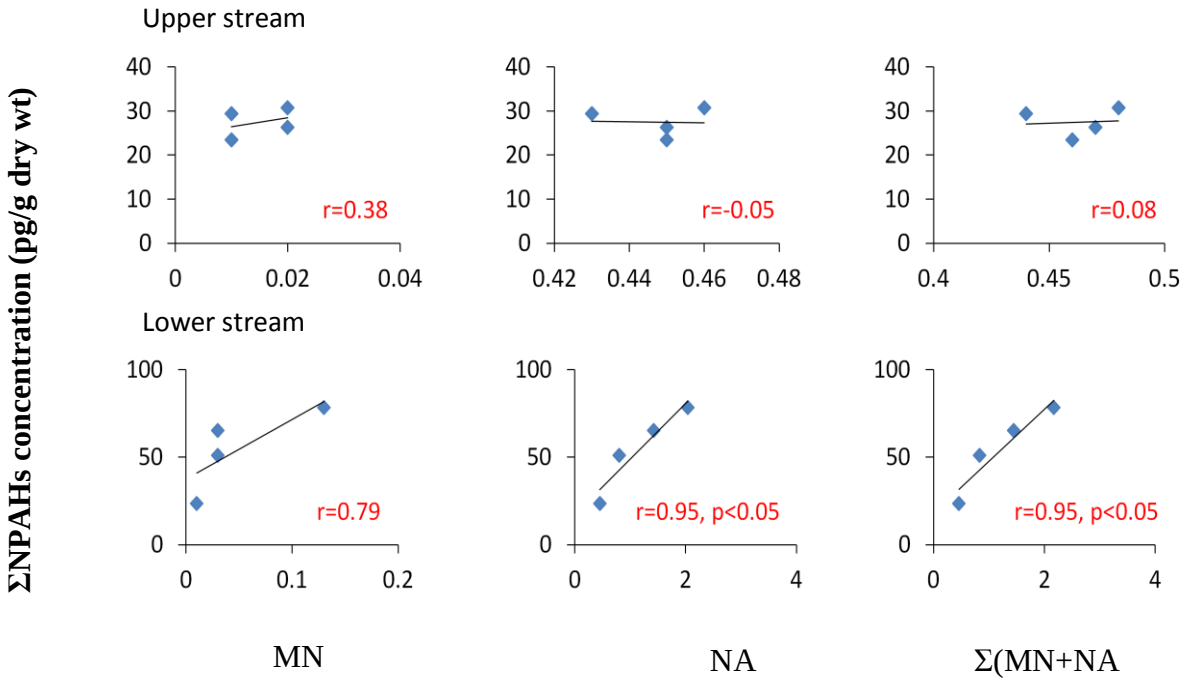

Figure 6.2 Relationship of total NPAHs concentration in fish with MN, NA and MN+NA in upper and lower stream

Furthermore, individual 1-NP and 6-NC concentration are significantly correlated with induction of MN ($p<0.05$). Finally, genotoxic effects may be attributed to NPAHs which are detected in lower stream, especially carcinogenic NPAHs such as 1-NP and 6-NC were detected.

Conclusions

The species *O. niloticus* demonstrated to be a sensitive organism to pollution and showed a pattern of genotoxicity for environmental testing, thereby proving to be an adequate bioindicator for genotoxicity of river waters. Our results revealed that the genotoxic effects of lower stream were much stronger than those of upper stream. In

conclusion, water from the lower stream station on the Butuanon River systems was genotoxic. The genotoxicity of the water samples generally correlated with their NPAHs concentration. These results highlight the genotoxicity of the pollution in this area, and suggest that industrial and domestic effluents discharged into the river and vehicle emissions may place local populations at risk of genotoxicity. Our results also indicate that caudal erythrocytes maybe suitable for the evaluation of micronuclei in fish. Finally, we recommend that Butuanon River water be further analyzed for additional classes of pollutants that maybe responsible for the genotoxicity of river other than NPAHs, PAHs and heavy metals.

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PART V

EVALUATION OF HAZARDS OF ENVIRONMENTALLY RELEVANT CONCENTRATION

Chapter 7

Oxidative stress and genotoxicity biomarker responses in tilapia (*Oreochromis niloticus*) exposed to waterborne 1-nitropyrene

Abstract

This study aimed to assess whether lower 1-nitropyrene (1-NP) concentration will induce genotoxicity and oxidative stress in tilapia *Oreochromis niloticus* exposed to waterborne 1-nitropyrene. Cellular antioxidant enzyme activity of glutathione peroxidase and oxidative damage, i.e., lipid peroxidation, protein and DNA oxidation were used as biomarkers of oxidative stress, while the micronucleus test was used for evaluation of chromosome damage and was used as an indication of genotoxicity. Antioxidant enzyme activity of glutathione peroxidase was increased in fish exposed to 1-NP as compared to control suggesting that oxidative stress was induced in fish. This was further supported by the increase in lipid peroxidation, protein and DNA oxidation in exposed groups as compared to control. Micronuclei and other nuclear abnormalities were also higher in exposed groups as compared to control, indicating genotoxic effects in fish due to exposure to 1-nitropyrene. Results suggest that lower 1-NP concentration detected in Butuanon River can induce genotoxicity and oxidative stress. The biomarkers used in this study are useful for the assessment of nitroarenes exposure or contamination in aquatic organisms.

Introduction

Oxidative stress occur when the critical balance between oxidants and antioxidants is disrupted due to the depletion of antioxidants or the excessive accumulation of the reactive oxygen species (ROS), or both leading to damage (Scandalios, 2005). The imbalance between production of ROS and antioxidant defenses may lead to oxidative stress manifested as oxidative damage of lipids, proteins, and DNA. Thus, changes in antioxidant defenses and oxidative damage are used as biomarkers of oxidative stress (Livingstone 2001). Biomarkers can be characterized as functional measures of exposure to stressors, which are usually expressed at the sub-organism level of biologic organization (Adams et al., 2001). Sub-organisms responses to environmental stressors occur before other disturbances, such as disease, mortality, or population changes, and thus may offer early warnings of pollution impacts (Depledge and Fossi 1994). Genotoxic pollutants induce changes in the genetic material of aquatic organisms, including DNA damage as well as gene and chromosomal alterations. Many types of environmental contaminants, such as metals, PAHs, polychlorinated biphenyls (PCBs), and pesticides, are known to exhibit genotoxic properties (De Flora et al.1991). Biomarkers indicating DNA and chromosomal damage are used to assess genotoxic effects in aquatic organisms (Ohe et al. 2004).

Fish has been a useful model for the evaluation of pollution in aquatic ecosystems including oxidative stress, genotoxicity and accumulation. Biomarkers of oxidative stress and genotoxicity have been applied in several fish species for pollution assessment in laboratory and field experiments (Tsangaris et al., 2011; Bacolod et al., 2013a, Bacolod et al., 2013b). In laboratory, a mixture of nitroarenes showed genotoxic effects through induction of micronuclei in marbled flounder after exposure (Bacolod et al., 2013a), while field experiments showed accumulation of nitroarenes and induction of genotoxicity in tilapia exposed to river water of Cebu, Philippines as shown in Chapter 5. Both results

showed accumulation and genotoxic effects due to nitroarenes exposure. In addition, 1-nitropyrene (1-NP) has been detected in aquatic environment with concentration ranging from 100 to 1000 pg/L in river water (Ohe et al., 1996), in sea water collected at Sea of Japan with concentration of 0.2 to 0.5 pg/L (Murahashi et al. 2001), in river and sea sediments (Ozaki et al., 2010) and in aquatic organisms (Uno et al., 2011). Furthermore, 1-NP has been used as a biomarker for diesel exposure and is classified as group 2B as a potential carcinogen (IPCS, 2003). In Chapter 5, a positive relationship was observed between 1-NP concentration in fish and MN, a biomarker of genotoxicity. The study intended to evaluate hazards of environmentally relevant NPAHs concentration and compare with field experiments in terms of genotoxicity and oxidative stress.

Materials and Methods

Reagents

Standard 1-nitropyrene (1-NP) was purchased from Sigma-Aldrich, USA and 1-nitropyrene-d_9 (as an internal standard) (Central Chemicals Inc., Canada). Other reagents were of laboratory grade or analytical grade and purchased from Wako Pure Chemical Industries (Japan), Merck (Germany), or Sigma Aldrich (Japan).

Exposure and Sampling

Tilapia, *Oreochromis niloticus* juveniles were purchased from a private aquaculture facility in Ibusuki, Japan and reared in a 200L tank with aeration for a month in the laboratory with dechlorinized tap water until individuals grew to a weight of about 50g. The fish were regularly fed with a commercial fish diet, 6P pellet (Nippon Formula Feed Manufacturing, Tokyo) before exposure to the treatment media.

A total of 60 *O. niloticus* having body weights of 55±7 g and total body lengths of 15±0.8 cm were used in the experiment, with 15 individuals for control, 15 for low

concentrations, 15 for medium concentrations and 15 for high concentrations. Nominal test exposure concentrations containing 1-NP were 250, 2500 and 25000 pg /L; and measured concentrations were 153±7, 1320±163 and 15000±5030 pg/L, for low, medium and high exposures, respectively. Concentration of 1-NP in Butuanon river in Cebu, Philippines was 86pg/L. Its measured concentration can be predicted a half from the nominal concentration. Thus, lowest concentration was set at 250pg/L. The measured concentration will be expected to be similar with Butuanon river. These were maintained in a 40 L (90 x 45 x 25 cm) glass aquarium maintained in flow-through system control water or test water concentration with mild aeration. The exposure set-up consisted of four different 40L tank containing each stock solution in acetone of 250 (low); 2,500 (medium); 25,000 pg/L 1-NP and dechlorinized tap water as control. In addition, another four different 40L tanks containing *O. niloticus* for low, medium, high and control group was also prepared. Stock solution was flowing into the test tank at a rate of 1.7L/hr for 24hrs. The stock solution was replaced every 24 hrs until the duration of the experiment.

Five fish from each exposure group and control were collected after 3, 7 and 14 d. Fish were anaesthetized by immersion in a 0.5 ml/L solution of 2-phenoxyethanol. Blood was collected for MN and NA analysis as previously discussed in Chapter 5. The remaining blood was centrifuge for 16,000 x *g* for 5 min and the supernantant was collected for DNA oxidation analysis. In addition, liver was collected and separated for oxidative stress biomarker analysis. The remaining fish was used for 1-NP analysis. Water samples for the measurement of 1-NP concentration were collected after 1, 5 and 12 d. During the experimental period, the mean and standard deviation of the water temperature, dissolved oxygen and pH in all test groups were 25.3±0.2^{o}C, 6.8±0.1 mg/L, and 7.60±0.1, respectively.

Biochemical Analyses

Oxidative biomarkers activity were measured in liver samples except for 8-hydroxydeoxyguanosine (8-OHdOG). Liver samples were homogenized in 0.1M phosphate buffer, pH 7.4 using 0.1g liver per mL buffer in a micro tube. The homogenates were centrifuged at 16000 x *g* for 10min at 4^{o}C and the supernatant was kept at 4^{o}C until analyses. All preparation procedures were performed at 4^{o}C, thus the biochemical analysis was finished within a few days. Oxidative biomarkers activities were assayed using the supernatant.

Glutathione peroxidase (GPx) activity was assayed using the procedure of Gallo et al. (2009). The procedure involved the addition of 0.5mL sample to 1mL mixed reagent containing 0.5M K_2HPO_4 (pH 7.0), 2.5 mM EDTA, 0.18U/mL glutathione reductase (GR), 100mM glutathione and 10mM reduced nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was initiated by the addition of 0.1 mL cumene hydroperoxide. The enzyme activity was then measured at 340nm every 30 s for 5 min using a UV-vis spectrophotometer (Shimadzu, Japan). GPx activity was calculated as follows (GPx Assay Kit, BioVision, USA): GPx Activity = (Absorbance $_{T1\&T2}$ – Absorbance $_{blank}$) / (T2-T1) x V_{sample} ; where, T1&T2, the change in absorbance at any time point; T2-T1, difference between initial and final time; and V $_{sample}$, sample volume. GPx activity was expressed as U/mg protein.

Lipid peroxidation was measured according to the procedure of Chauhan et al. (2004). About 1 mL of homogenate was added with 2mL 0.37% thiobarbituric acid (w/v) - 15% trichloroacetic acid (w/v) in 0.25M HCl. The mixture was heated in boiling water bath for 15 minutes and allowed to cool. The resulting solution was then centrifuged at 16,000 x *g* for 10 minutes. The thiobarbituric reactive substances (TBARS) were measured on the supernatant at 532nm using a UV-vis spectrophotometer (Shimadzu,

Japan). The results were expressed as nmol TBARS/mg protein based on the extinction coefficient for MDA 2.56 x 10^5 $M^{-1}cm^{-1}$.

Carbonyl protein was assayed by the procedure of Levine at el. (1990). About 0.5ml of homogenated sample was reacted with 4 mL of 10mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M hydrochloric acid for 1 h at room temperature with shaking at 15 minutes interval and precipitated with 20% trichloroacetic acid (TCA) after 1 h. The precipitated protein was washed thrice by resuspension in 4 ml ethanol:ethyl acetate (1:1). Proteins were then solubilized in 2 mL 6M guanidine in 20mM K_3PO_4 and centrifuged at 16,000 x *g* for 5 minutes. The carbonyl content was measured spectrophotometrically (Shimadzu UV-vis spectrophotometer, Japan) at 366 nm. The results were expressed as nanomoles of DNPH incorporated/mg protein based on the molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$.

Oxidized DNA level using 8-OHdG was assayed using serum samples. Blood samples were centrifuged at 16,000 x *g* for 10 min and 8-OHdG levels of the supernatants were determined using a competitive ELISA kit (New 8-OHdG Check, Japan Institute for the Control of Aging, Shizuoka, Japan). The ELISA assay was performed according to the manufacturer's instructions. Briefly, 50µL of blood serum or standard was used during the analysis. The absorbance was measured at 450nm using a microplate reader (Corona Electric, USA). Levels of 8-OHdG in sample were expressed as ng/mL serum calculated from the plot of absorbance and log (concentration of standards).

Genotoxicity Analyses

Genotoxicity analysis using induction of MN and NA were discussed previously in Chapter 5.

Chemical Analyses

The procedure for the analysis of 1-NP was previously discussed in Chapter 4.

Data analyses

Data are presented as mean ± SD of the mean. Analysis of variance (ANOVA) was applied to determine differences between test groups. Multicomparison were made to determine which values differ significantly when significant overall ANOVA was found. Correlation between biomarkers were examined by Pearson`s correlation coefficient. Statistical analysis was performed using the SPSS statistical package. The level of significance was set at $p < 0.001$ or $p < 0.05$.

Results and Discussion

1-NP concentration in whole fish

Measured mean concentration (mean±SD) of 1-NP in test water and whole fish is presented in Table 7.1. Target nitroarene, 1-NP was not detected in test water and fish of control group, including the diet food.

Table 7.1 Concentration (mean±SD) of 1-nitropyrene in test water (ng/L) and whole fish (ng/kg dry weight)

	Test water concentration (ng/L)		Whole fish concentration (ng/kg dry weight)		
Test group	Nominal	Measured	Day 3	Day 7	Day 14
Control	0	<0.002	<0.002	<0.002	<0.002
Low	0.25	0.15±0.004	3±0	54±9	98±10
Medium	2.5	1.32±0.094	189±15	368±53	420±26
High	25	15.6±2.91	484±7	623±12	1010±69

The concentration of 1-NP in whole fish was increasing after 3 d in exposed groups with highest concentration of 98, 420 and 1010 ng/kg dry weight for low, medium and high exposure group, respectively. Measured mean concentration of test water were one-half lower than the nominal concentration. This can be due to photolysis or adsorption to test tanks. In addition, 1-NP concentrations in fish were higher than 1-NP concentration in test water, suggesting that 1-NP was accumulated. This confirms our previous study, wherein 1-NP was bioconcentrated from water-borne sources but not from dietary sources

using marbled flounder, *P. yokohamae* (Bacolod et al., 2013b). In addition, BCF of 1-NP in *O. niloticus* (BCF, 64~653) seemed to be similar with *P. yokohamae* (BCF, 71~151). The possible BCF differences could be due to different species used and interspecies differences.

Antioxidant enzyme activity

Antioxidant enzymes can be induced by enhanced production of ROS as a protection mechanism against oxidative stress (Winston and Di Giulio 1991; Cossu et al., 1997). Antioxidant enzyme activity using glutathione peroxidase (GPx) is shown in Figure 7.1A. Results showed that GPx activity in exposed groups were significantly different from control, with values ranging from 3.9 to 4.6, 3.3 to 5.2 and 4.6 to 5.4 U/mg protein for low, medium and high exposure groups, respectively. In addition, significant differences between test groups were observed especially after 3 and 14 d of the exposure period. GPx activity continued to increase after 3d in all test groups, except for control group.

An increase in antioxidant activities with exposure to a variety of organic and metal contaminants has been observed in laboratory and field experiments (Livingstone, 1991; Livingstone et al., 1994; Livingstone and Nasci, 2000). GPx is responsible for the detoxification of hydrogen and lipid peroxides, thus its activity is governed by the amount of these reactive oxygen species (ROS). In the present study, GPx activity seems to be induced as a result of 1-NP exposure even at lowest exposure concentration. Furthermore, 1-NP may also contribute to the increase of hydrogen and lipid peroxides in fish. It has been reported that GPx activity is induced by a number of pollutants. Thus, 1-NP exposure can increase GPx activity resulting from oxidative stress.

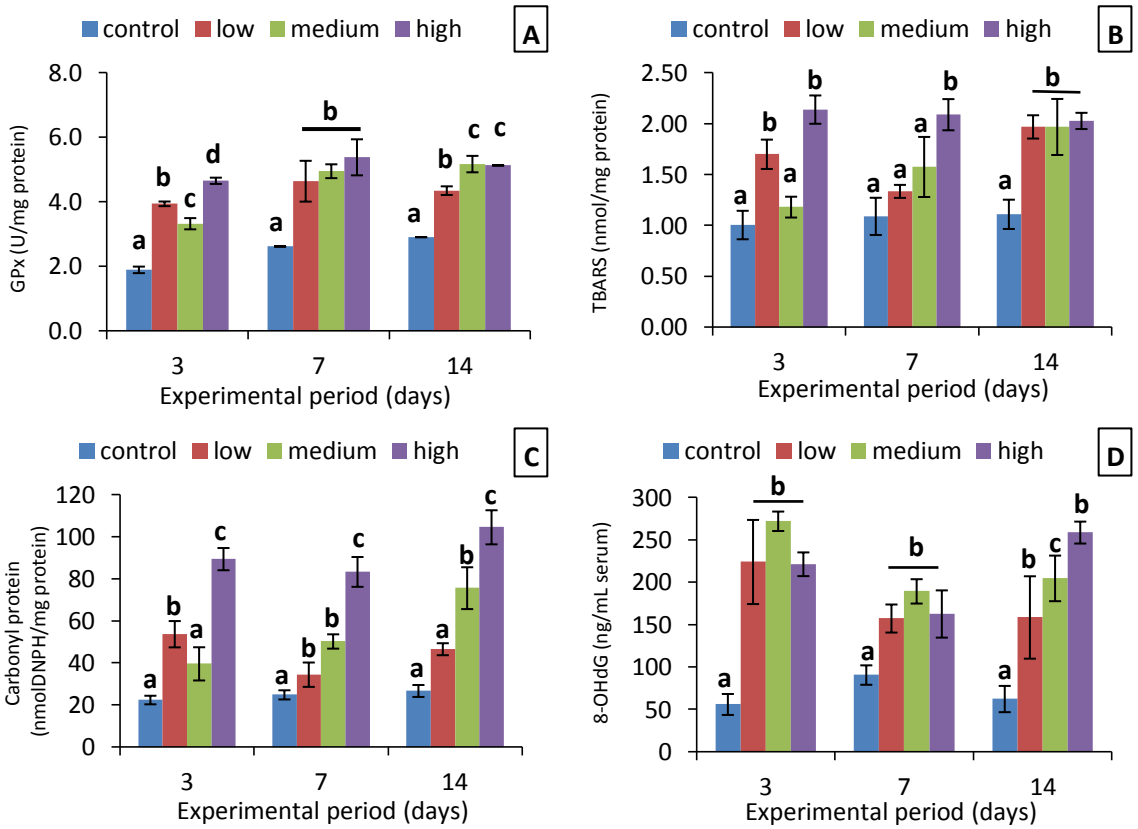

Figure 7.1 Biomarkers of oxidative stress measured in *O. niloticus* A– antioxidant enzyme activity (GPx); B – lipid peroxidation (TBARS); C – carbonyl protein (DNPH incorporated); and D – DNA oxidation (8-OHdG). *Similar letters* indicate not significant and *different letters* indicate significant among test groups ($p<0.05$)

Oxidative damage

Lipid peroxidation, protein and DNA oxidation are one of the oxidative damage resulting from oxidative stress due to the effect of ROS. Lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) is shown in Figure 7.1B with values ranging from 1.34 to 1.97, 1.18 to 1.97 and 2.03 to 2.14 nmol/mg protein in low, medium and high exposure groups, respectively. Lipid peroxidations in exposed groups were significantly different from control especially after 3 and 14 d ($p<0.05$). However, after 7 d, only high exposure group was significantly different from low and medium test concentration including control group ($p<0.05$). Lipid peroxidation generally increased after 3 d in exposed groups. These results suggest that, lipid peroxidation is dependent on

exposure time and concentration. Lipid peroxidation is caused by the reaction of ROS with macromolecules such as lipids in cell membrane, protein or DNA molecule. In the present study, the increase in lipid peroxidation in fish is further supported by the high antioxidant activity of GPx as discussed above. GPx activity generally increases during the high production of hydrogen peroxide and lipid peroxide. The increased lipid peroxidation in the present study suggests that ROS-induced oxidative damage can be one of the main toxic effects of 1-NP. It has been reported that lipid peroxidation maybe induced by a variety of environmental pollutants (Lemaire and Livingstone, 1993; Di Giulio et al., 1995; Halliwell and Gitteridge, 1999). Thus, 1-NP exposure can result to oxidation of lipids resulting from oxidative stress.

Protein oxidation, carbonyl protein, measured as DNPH incorporation (mean±SE) is shown in Figure 7.1C with values ranging from 34.5 to 53.7, 39.6 to 75.6 and 83.4 to 105 nmol/mg protein in low, medium and high exposure concentration, respectively. Protein oxidation was significantly different after 3, 7 and 14 d in high exposure group as compared to low and medium exposure group including control group ($p > 0.05$). The result of the present study suggests that lower 1-NP concentration is not capable of protein oxidation. In addition, protein oxidation is not usually observed at short exposure period especially at lower concentration and the highly reactive hydroxyl radical (OH*), which is one of the ROS generated in the process leading to oxidative stress, is considered to be responsible for the formation of carbonyl groups in proteins (Farber and Levine, 1986; Oliver, 1987). Protein oxidation can lead to loss of critical sulfhydryl groups in addition to modification of amino acids leading to the formation of carbonyl and other oxidized moieties (Stern, 1985; Bainey et al., 1996). In this study, 1-NP exposure can result to oxidation of amino acids resulting from oxidative stress.

One of the most abundant oxidative lesions caused by reactive oxygen species (ROS) in DNA is 8-hydroxydeoxyguanosine (8-OHdG), a potential mutagenic lesion

(Gajewski et al, 1990). 8-OHdG, one of the major DNA base-modified products, is induced by either hydroxyl radical, singlet oxygen, or photodynamic action (Kasai 1997) , and its pairing with adenine as well as cytosine is known to be mutagenic, leading to G:C to T:A transversion on DNA replication (Shibutani et al., 1991) .DNA oxidation measured as 8-hydroxy-deoxyguanosine, 8-OHdG (mean±SE) is shown in Figure 7.1D with concentrations ranging from 157 to 224, 189 to 272 and 162 to 259 ng/mL serum in low, medium and high exposure groups, respectively. A significantly higher 8-OHdG concentration was observed after 3, 7 and 14 d in exposed groups as compared to control ($p<0.05$). However, there was no significant difference among exposure groups after 3 and 7 d, except on after 14 d ($p<0.05$). In addition, it was observed that 8-OHdG concentration in fish serum decreased after 7 d, but increases on after 14 d. The increased 8-dOHG production in fish serum in the present study suggests that ROS-induced oxidative damage can also be one of the main toxic effects of 1-NP exposure. Genotoxicity of 1- nitropyrene was also reported when DNA damage was induced in mussels (Mitchelmore et al., 1998a) and in brown trout (Mitchelmore et al., 1998b). Thus, in this study exposure to 1-NP can result to DNA damage resulting from oxidative stress.

Genotoxicity of 1-NP

Genotoxicity assays, such as those evaluating MN and NA frequencies, have been employed as biomarkers of genotoxicity in several fish species exposed to genotoxic chemicals in laboratory and in field conditions (Al-Sabti and Metcalfe 1995; Ohe et al., 2004; Cavas et al., 2005; Ergene et al., 2007; Bacolod et al., 2013a; and our results in Chapter 5. In the present study, micronuclei (MN) and other nuclear abnormalities (NA) were confirmed in the tilapia erythrocytes in exposed and control groups, however there were more MN and NA in exposed group as compared to control group.

Table 7.2 shows the frequencies of MN (mean±SD) in exposed and control groups. MN frequencies of exposure groups were significantly different from control frequencies after 7 and 14 d (p<0.001). The highest MN frequencies per 1000 erythrocytes were 1.27, 2.93 and 4.87 in low, medium and high exposure groups, with control registering only 0.33 per 1000 erythrocytes. MN frequencies continued to increase after 3 d. The results suggest that accumulated concentrations of NPAHs affected hematopoiesis in fish and showed their genotoxicity.

Table 7.2 Frequencies (mean±SD) of erythrocytic micronuclei and nuclear abnormalities per 1000 erythrocytes in tilapia exposed to 1-NP

	Micronuclei			Nuclear abnormalities		
Test group	Day 3	Day 7	Day 14	Day 3	Day 7	Day 14
Control	0.26±0.15	0.33±0^{a}	0.28±0.13^{a}	2.93±0.43^{a}	3.80±0.60^{a}	3.62±1.59^{a}
Low	0.33±0	0.93±0.37^{b}	1.27±0.64$^{a, b}$	6.13±0.69^{b}	12.8±3.05^{b}	14.5±7.53^{b}
Medium	0.40±0.15	2.53±0.38^{c}	2.93±1.35^{b,c}	8.47±1.77^{b}	22.2±6.60^{c}	20.1±7.02^{b}
High	0.67±0.24	1.80±1.07^{d}	4.87±1.41^{c}	8.60±2.54^{b}	23.7±10.1^{c}	25.3±11.1^{b}

$^{a, b, c, d}$ *different letters* are significant (p<0.001), while *same letters* are not significant among test groups

NA has been used to evaluate genotoxicity along with MN. Our previous study also reported MN and NA frequencies in marbled flounder exposed to a mixture of nitroarenes (Bacolod et al., 2013b) and tilapia exposed to river water shown in Chapter 5. In the present study, NA were significantly higher in exposed groups as compared to control in all exposure period with values ranging from 6.13 to 14.5, 8.47 to 22.2 and 8.60 to 25.3 in low, medium and high exposure groups as compared to control with 2.93 to 3.80 per 1000 erythrocytes. In addition, frequencies of NAs were higher than MN frequencies in all test groups. The present study showed that NAs were induced by 1-NP exposure and can also be used to evaluate genotoxicity along with MNs. Furthermore, MN test in erythrocytes of *O. niloticus* proved to be the most responsive biomarker showing constantly higher frequencies in exposed groups as compared to control group.

1-NP was detected in *O. niloticus* exposed to lower stream of Butuanon river with concentration between 2.67 to 3.74 ng/kg. In this experiment, a similar concentration was measured in O. niloticus exposed to 1-NP with concentration between 3 to 98 ng/kg (low group). From these results, it can be concluded that 1-NP detected in Butuanon river can induce genotoxicity and oxidative stress. MN and NA observed in O. niloticus exposed to Butuanon river water were induced by oxidative stress.

Correlations between oxidative stress, genotoxicity and 1-NP fish body residue were evident; 1-NP fish body concentration was positively correlated to TBARS ($r = 0.57$, $p < 0.05$), GPx ($r = 0.64$, $p < 0.05$), carbonyl protein ($r = 0.81$, $p < 0.05$), 8-OHdOG ($r = 0.49$, $p < 0.05$), MN ($r = 0.77$, $p < 0.05$) and NA ($r = 0.64$, $p < 0.05$). These correlations suggest that oxidative stress and genotoxicity observed in fish is due to exposure to 1-NP. Our previous study also observed similar positive correlation with total NPAHs concentration when *O. niloticus* was exposed to lower stream river water in Cebu, Philippines. The river was affected by heavy traffic situation and industrialization as discussed in Chapter 5. Among biomarkers, a strong positive correlation was found between MN and NA ($r = 0.84$, $p < 0.01$). Tsangaris et al. (2011) also found similar positive correlation between MN and NA ($r = 0.81$, $p < 0.05$) when *Mugil cephalus* were exposed to a polluted environment in Saronikos Gulf, Greece. However, the present study intended to assess overall effects of 1-NP exposure and did not address relations among biomarkers of oxidative stress and genotoxicity. The correlations between biomarkers and genotoxicity suggest that observed effects were due to 1-NP exhibiting oxidative stress potential that can also induce genotoxicity.

Conclusion

The present study demonstrated that NPAHs, such as 1-NP induces oxidative stress and genotoxicity to *O. niloticus*. It is evident that nitroarenes produced from parent PAHs

during combustion can affect aquatic organisms, thus sources and decrease production of PAHs and NPAHs should be properly evaluated for environmental concern. The results of the present study also suggested that the parameters used in the study are suitable as biomarkers of exposure to oxidative stress and genotoxicity caused by 1-NP. However, the mechanism on how 1-NP lead to oxidative stress and genotoxicity should be further investigated. Finally, it can be that 1-NP concentration detected in Butuanon River may cause the induction of MN and NA resulting from oxidative stress.

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PART VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

NPAHs are persistent in the environment and are reported to be carcinogenic and mutagenic. These compounds adsorbed to particulate matter and found in all environmental compartments. To investigate possible risks of NPAHs pollution to aquatic organisms and determine possible biomarker for NPAHs pollution, laboratory and field experiments were done. Six different studies were conducted to answer these objectives: 1. determine the level of NPAHs contamination in the surface soil; 2. compare NPAHs concentration in road soil, river sediment and water; 3 and 4. investigate if NPAHs are bioaccumulated through dietary or waterborne sources; 5. investigate bioaccumulation of NPAHs and genotoxicity; exposed to waters from Butuanon River, Cebu, Philippines; and 6. assess the overall effects of NPAHs in terms of oxidative stress and genotoxicity.

NPAHs were detected in environmental compartments such as road soil, river sediment and river water. In road soils, NPAHs were detected and their concentration varies from region to region. Among the sampling regions, Thailand had the highest total NPAHs concentration, while India and Cebu (Philippines) have the highest carcinogenic 1-nitropyrene. Other carcinogenic NPAHs were also detected such as 2-nitroflourene, 4-nitropyrene and 6-nitrochrysene in most of the sampling regions. One of the main factors that resulted to varying nitroarene concentrations in road soils could be the type of fuel used in automobiles, traffic situation, urbanization and industrialization. In addition, diesel engine is probably used in area where higher NPAHs in road soil were detected.

NPAHs in road soil near the river bank, river sediment and river water were also investigated in three rivers in Cebu, Philippines and one in Kagoshima, Japan. Butuanon river is more polluted with NPAHs as compared to Guadalupe, Kotsuki (Japan) and Mahiga rivers. The presence of residential, commercial and industrial establishments, other than vehicular emissions could attribute to differences in concentration and

composition of NPAHs in these areas. In addition, a positive correlation was observed between NPAHs concentration in road and river sediment. However, NPAHs concentration in road soil was higher as compared to river sediment and river water. The most important factor for the behavior of NPAHs are their physical and chemical characteristics and the input into the aquatic system is controlled by the location and activation of particle sources. Furthermore, selective transport, temporary storage of the particles, and a succession of mobilization and immobilization of NPAHs during flooding leads to redistribution until the NPAHs enter the river.

Accumulation of NPAHs from water and dietary sources were investigated in marbled flounder (*Pleuroncetes yokohamae*). Results showed that NPAHs were bioconcentrated but not biomagnified. One of the main reasons for their difference is the different type of uptake. Toxicokinetic parameters of nitroarenes seemed to have similar trends with their parent PAHs, but can be influenced by the following factors, such as log K_{ow}, molecular structure and size, and biotransformation processes. In addition, genotoxicity was manifested in the significantly higher frequencies of micronuclei (MN) and other nuclear abnormalities (NA) formation in *P. yokohamae* exposed to dietary sources. NPAHs from water sources can also induce genotoxicity.

Field exposure and genotoxicity study was also conducted using tilapia *Oreochromis niloticus*. PAHs and NPAHs were detected in river water and were accumulated in *O. niloticus* exposed to river water. PAHs were detected in both sites, while most NPAHs were detected in lower stream only. Heavy metal analysis of river water showed lower concentrations to cause genotoxicity in both sites. Genotoxicity evaluation showed significant induction of MN and other NA in fish erythrocytes exposed to lower stream. This study suggests that NPAHs is possibly one of the agents that cause genotoxic effects in fish.

Evaluation of hazards using environmentally relevant concentration was finally conducted. *O. niloticus* was exposed to water borne 1-NP. Results showed that glutathione peroxidase, oxidative damage, and induction of MN and NA were significantly increased in exposed groups. These results suggest that test organism was under oxidative stress and biomarkers used in this study are useful for the assessment of nitroarenes exposure or contamination in aquatic organisms. In addition, it can be concluded that 1-NP concentration detected in Butuanon River may cause the induction of MN and NA resulting from oxidative stress.

Finally, NPAHs in the environment can accumulate in fish and cause genotoxicity and oxidative stress. Based on these results, it seems NPAHs pose a risk to aquatic organisms. However, continued monitoring of NPAHs in aquatic environment should be continued to determine further risks. In addition, further investigations should also be conducted on the biological effects of NPAHs such as teratogenic and multigenerational in order to completely understand their fate in aquatic organisms. It is evident that NPAHs produced from parent PAHs during combustion can affect aquatic organisms, thus sources and decrease production of PAHs and NPAHs should be properly evaluated for environmental concern.

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