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AN ASSESSMENTOF FISH HEALTH NEAR AN ALUMINUM SMELTER IN KITIMAT,B.C.,CANADA

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Abstract

From 2000 - 2004 we conducted a monitoring study to evaluate the impairment of fish health due to exposure to aluminum smelter-derived polycyclic aromatic hydrocarbons (PAHs) in the marine waters of Kitimat, British Columbia, Canada. These waters are part of the historical fishing grounds of the Haisla First Nation, and since the 1950s the Alcan Primary Metal Company has operated an aluminum smelter at the head of the Kitimat Arm embayment. As a result, adjacent marine and estuarine sediments have been severely contaminated with a mixture of smelter-associated PAHs in the range of 10,000–100,000 ng/g dry wt, concentrations above those shown to cause adverse effects in fish exposed to PAHs in urban estuaries. Because relatively low levels of other contaminants such as PCBs and other chlorinated compounds (e.g., DDTs, chlordanes) are found in this environment, we were provided a unique opportunity to study PAH contamination in the relative absence of these other compounds. Moreover, there was some question about the comparable toxicity of the PAHs at this site that are primarily soot-based and less bioavailable, to PAHs from other sources. Spatial as well as temporal trends in contamination were determined, in part to evaluate the efficacy of process changes at the smelter, to reduce PAH input to the surrounding environment.

Over the 5-year study we conducted biennial collections of adult flatfish [(English sole (*Parophrys vetulus)* and yellowfin sole (*Limanda aspera*)], annual collections of juvenile Chinook salmon (*Oncorhynchus tshawytscha)*, and collections of sediment samples at the corresponding capture sites. Additional sediments were also collected at over 200 sites at the head of Kitimat Arm. Various tissue samples (e.g. bile, liver, kidney, gonad, stomach contents, carcasses) were taken from each animal to determine levels of exposure, biological effects, and to determine the comparative biological effectiveness of smelter-derived PAHs compared to urban mixtures of PAHs. Results showed significant intersite differences in concentrations of PAHs, with salmon collected at sites nearest the smelter showing increased PAH exposure compared to sites more distant from the smelter. This spatial trend in exposure was paralleled in English sole, and in addition this species had significantly higher prevalences of PAH-associated liver disease and signs of PAH-associated DNA damage. We also saw distinct differences in the PAH profiles in the stomach contents of salmon and flatfish from different sites. However we did not see changes in biological effects such as reproductive dysfunction in either flatfish species. There was variability from year to year in the levels of exposure, but little evidence of temporal trends over the 5 years of the study. Our findings did suggest that PAH uptake and exposure in Kitimat fish may be relatively lower compared to fish collected from other sites that have comparable sediment PAH concentrations, but further study to characterize habitat utilization patterns of our target species will be needed to support that finding.

Finally this work was a unique collaboration, with support and funding for portions of the work provided by the Haisla First Nation of Kitamaat Village, BC and the Alcan Primary Metal Group, with the work conducted by our Agency in the United States.

Key Words: polycyclic aromatic hydrocarbons (PAHs), aluminum smelter, fish health

1. INTRODUCTION

Since the 1950s the Alcan Primary Metal Company has operated an aluminum smelter at the head of the Kitimat Arm, located in Kitimat, British Columbia, Canada. As a consequence, emissions generated by the production of aluminum at the smelter have been a major anthropogenic source of polycyclic aromatic hydrocarbons (PAHs). Adjacent marine and estuarine sediments have been severely contaminated with a mixture of smelter-associated PAHs in the range of 10,000–100,000 ng/g dry wt., concentrations above those shown to cause adverse effects in fish exposed to PAHs in urban estuaries (Horness et al., 1998, Johnson et al., 2002). Because these waters are also part of the historical fishing grounds of the Haisla First Nation they have been concerned for some time about the potential impact of PAHs on the fisheries resources. In an effort to try to address these concerns, a five-year study (2000-2004) was conducted at the request of the Haisla. This study involved a unique collaboration, with support provided by Alcan, the Kitamaat Village Council (Haisla) and our laboratory representing the US National Marine Fisheries Service (NMFS). The assessment was designed to provide characterization of contaminant distribution in sediment, contaminant exposure and effects in representative fish species and evaluation of contamination from non-PAH sources in fish and sediment.

An important characteristic of smelter-associated PAHs is that they are primarily soot-based rather than hydrocarbon-based such as those found at industrialized urban sites. Soot-based PAHs are known to be less bioavailable because they can be bound to the carbon in soot particles, limiting their uptake by organisms (Paine et al., 1996, Simpson et al., 1998, Naes et al., 1998). Therefore, an additional goal of this study was to assess how the biological impacts of smelter-derived PAHs might differ from the impacts of PAHs derived from other sources.

Kitimat Arm is an unusual site in that it is contaminated primarily by soot-based smelter-derived PAHs. Very low concentrations of other industrial contaminants are found there, due to the lack of potential source inputs. Highly industrialized-urban study sites typically tend to have a 'cocktail' of other contaminants, such as PCBs and heavy metals, that may exacerbate the biological effects of PAHs, acting, for example, as tumor promoters (Myers et al. 2003). Study of PAHs in the relative absence of other contaminants provided an opportunity to improve our understanding of their toxicity to aquatic organisms.

Our Environmental Assessment of Kitimat Arm included the following components:

Contaminant exposure of juvenile outmigrant Chinook salmon. Juvenile Chinook salmon from 6 sites in Kitimat Arm were studied to determine their degree of exposure to PAHs, and to compare these levels of exposure to fish from previous studies where exposure to PAHs, alone or in combination with other contaminants, have been linked to biological dysfunction. Juvenile salmon were chosen as a target species because of

the importance of salmon as a commercial and subsistence fishery resource, and because previous studies have shown that the health and survival of juvenile outmigrants could be affected by exposure to PAHs (Arkoosh et al. 1991, 1994, 1998, 2001; Varanasi et al. 1993; Stein et al. 1995; Casillas et al. 1991; Meador et al. 2006; Johnson et al. 2006).

Contaminant exposure and histopathology in English sole. English sole from sites around Kitimat Arm were studied to assess their levels of PAH exposure and biological injury by measurement of chemical concentrations, early biochemical responses and the prevalence of PAH-associated liver lesions. These levels were then compared with data from English sole collected from sites contaminated with PAHs from different sources.

Contaminant exposure and preliminary assessment of reproductive function in yellowfin sole. Reproductively maturing yellowfin sole in Kitimat Arm were studied for evidence of inhibited gonadal development and related types of dysfunction that have been observed in English sole from PAH contaminated sites in Puget Sound.

Our laboratory has a wide body of data on English sole showing the sensitivity of this species to PAH exposure in field surveys (Malins et al. 1982, 1984; Rhodes et al. 1987; Myers et al. 1987, 1992,1994; Stehr et al. 2004). The biological endpoints measured (liver lesions, DNA damage, enzyme induction, and altered reproductive development) have been established as effects of PAH exposure, in both field surveys and controlled laboratory studies (Malins et al. 1982, 1984; Krahn et al. 1986a; Johnson et al. 1988, 1999; Casillas et al. 1991; Schiewe et al. 1991; Stein et al. 1991, 1992, Collier et al. 1992, 1993a,b, 1995; Myers et al. 1998a,b, 2004; Sol et al. 1998, 2000). Causative relationships among PAH exposure, DNA damage, and liver cancer and related lesions in sole and other fish are especially well-documented (Myers et al. 2003). Additionally, statistical evaluation of this data was the basis for which a threshold PAH concentration in sediments that is associated with development of biological effects (liver lesions, DNA damage, reproductive impairment) were calculated (Horness et al., 1998, Johnson 2002). This large body of background data made this suite of endpoints especially useful and appropriate for assessing PAH-related injury to fish from sites potentially impacted by the ALCAN smelter. Yellowfin sole were primarily chosen because the timing of their reproductive cycle was optimal for a field assessment of reproductive function.

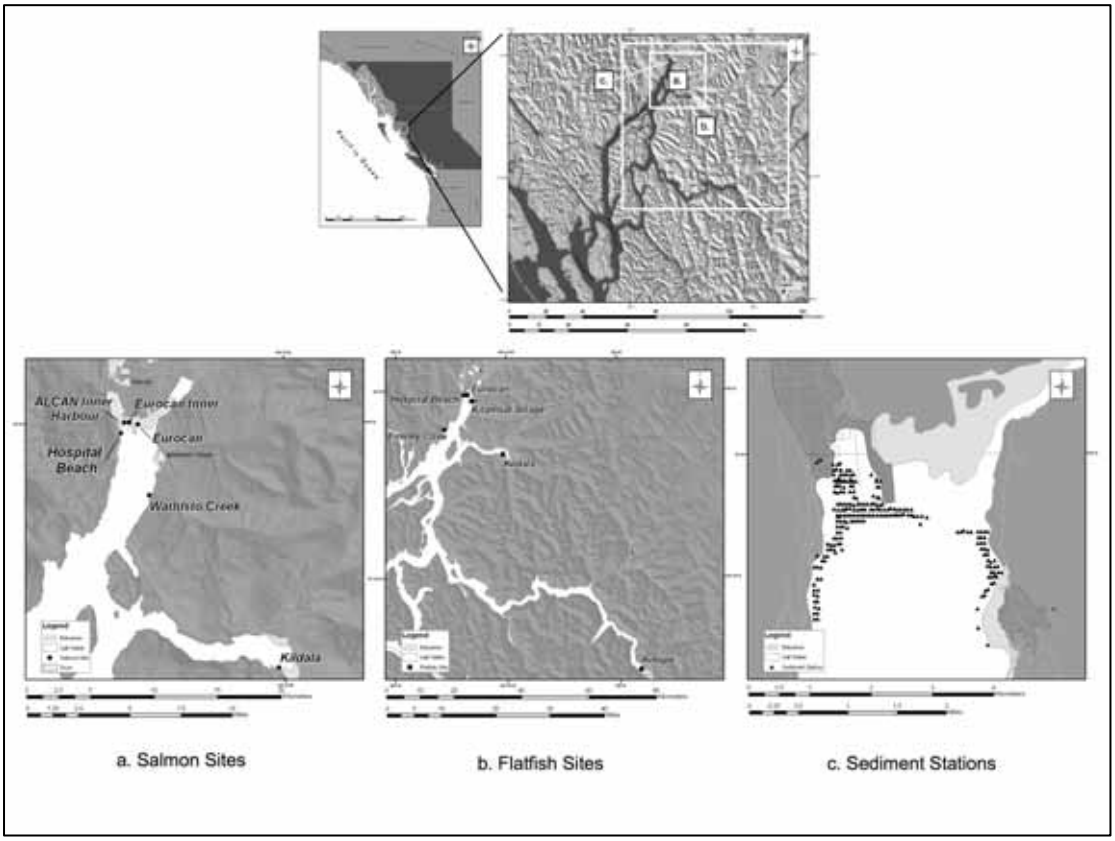
Sediment contamination characterization study. A detailed assessment of sediment throughout Kitimat Arm, was conducted to better characterize the spatial extent of contamination in and around this area. In addition data on the level of sediment contamination would provide information to establish links between PAH exposure and effects in resident biota, and identify sources of exposure.

Measurement of concentrations of selected organochlorines (OCs) and metals and PAHs in edible flatfish muscle tissue. English sole were examined to establish baseline levels of OCs and metals in Kitimat fish, and to assess the potential for impacts on fish health or seafood safety.

2. METHODS

The study area and sampling locations are shown in Figure 1. Table 1 provides a summary of samples collected, analyses conducted and analytical methods used. Field sample collection protocols and sample analytical methods used are described in detail in Collier et al., 1998, and in Johnson et al., (in prep).

Figure 1. Locations of sampling sites.



3. RESULTS AND DISCUSSION

Key Findings from the study were:

- Concentrations of PAHs, especially high molecular weight PAHs are elevated in sediments within Kitimat Arm, especially at sites closest to the Alcan plant. The types of individual PAHs in sediments at sites within Kitimat Arm, including Kitamaat Village, are similar to those in Alcan smelter sources. At the references sites outside Douglas Channel, PAHs from natural products (wood or decomposing matter) are more prominent. These wood-derived PAHs are also common in sediments near the Eurocan pulp mill.

- Both salmon and flatfish are exposed to PAHs through consumption of prey organisms residing in Kitimat Arm. Stomach contents of salmon and flatfish from sites within Kitimat Arm, especially those closest to the smelter, contained significant concentrations of PAHs similar to those produced by the smelter.

- PAH exposure levels in juvenile salmon from most Kitimat sites are below the levels typically associated with health effects such as immunosuppression and reduced growth. In juvenile salmon from Alcan Inner Harbour and Hospital Beach PAH concentrations in bile and stomach contents are comparable to concentrations found in juvenile salmon in Puget Sound sites where reduced disease resistance has been observed in wild populations. In addition to PAHs, however, the Puget Sound fish were also exposed to high concentrations of other immunosuppressive contaminants such as PCBs present only in very low or undetectable levels in Kitimat Arm. Therefore, it is uncertain whether PAHs alone would produce the same health impact.

- PAHs are having some effects on the health of flatfish in Kitimat Arm. English sole from sites within Kitimat Arm showed increases in DNA damage, typically caused by mutagenic PAHs, as compared to sole from pristine reference sites outside Douglas Channel. (Salmon did not show significant DNA damage, probably because of their short residence time in Kitimat Arm). Also, from 10-20% of English sole and 5-10% of yellowfin sole from sites within Kitimat Arm had some type of PAH-associated liver disease. These conditions were not generally found in sole from reference sites outside Douglas Channel where the natural background prevalence of toxicopathic liver disease was in the 0-2% range.

- Exposure levels (concentrations of PAHs in stomach contents and bile) are lower in Kitimat fish than fish from sites contaminated with similar concentrations of PAHs from non-smelter sources, suggesting reduced biological availability of smelter-derived PAHs.

- Biological effects are less severe in Kitimat flatfish than in flatfish from sites contaminated with PAHs from other sources, such as in Puget Sound. In English sole collected from sites closest to the smelter liver lesion prevalences were lower than expected for sediment PAH levels, in comparison with sole from other sites along the West Coast of the U.S. Moreover, neither yellowfin sole nor English sole showed significant evidence of PAH-related reproductive dysfunction commonly observed in other contaminated industrial area.

- Concentrations of PCBs, DDTs, and other chlorinated pesticides are very low in English sole and salmon stomach contents, and would be unlikely to impact fish health or increase PAH toxicity.

- Concentrations of PAHs, PCBs, chlorinated pesticides, and heavy metals were also quite low in fillets of of English sole from all sampling sites, and well below levels considered to be a human health risk by Canadian regulatory agencies.

The process changes introduced by Alcan appear to be effective at reducing inputs of PAHs into the environment and biota of Kitimat Arm, as PAH concentrations in sediments and fish, and fish disease prevalences, have remained stable or declined over the past five years of sampling.

Overall, the Kitimat Marine Assessment Study was successful in collecting substantial baseline data on sediment chemistry, as well as multi-year sampling of juvenile salmon and marine flatfish.

We determined that PAHs similar to those produced by the smelter were present at high concentrations in sediments nearest the Alcan plant, and at more moderate levels at other sites within Kitimat Arm (e.g., Kitamaat Village, Emsley Cove). These contaminants were being absorbed by fish through their diet, and their breakdown products were found in bile of flatfish and salmon.

As expected, given that fish metabolize PAHs and do not accumulate them in their tissues (Varanasi et al. 1989), we found that concentrations of PAHs in muscle tissue of flatfish were very low. Concentrations were well below levels that would constitute a health risk to humans consuming the fish.

Other than PAHs, we found very little contamination in marine organisms from Kitimat Arm. Organochlorine compounds and metals were not present in high concentrations in fish stomach contents, and level in filets of English sole fell well below the action levels recommended by Canada Health and Welfare (Ahmed 1991).

Even though fish do not accumulate PAHs, their breakdown products can be quite toxic, and we found that the PAHs released from smelter operations were having some effects on the health of flatfish from Kitimat Arm. English sole from this area had PAH-related DNA damage and liver lesions rarely found in sole from the reference sites outside Douglas Channel. However, biliary levels of PAH metabolites in salmon and flatfish, as well as prevalences of liver disease and DNA damage in flatfish, were lower than would be expected considering the concentrations of high molecular PAHs in sediments at sites near the smelter, where PAHs have been discharged in the past. Furthermore, neither yellowfin sole nor English sole showed significant evidence of PAH-related reproductive dysfunction as is commonly observed in other contaminated industrial areas such as Puget Sound (Johnson et al. 1998, 1999). These findings support earlier studies indicating the limited bioavailability of soot-associated, smelter-derived PAHs.

Because we did not directly measure health effects of PAHs in juvenile salmon, we cannot be certain how PAHs may be affecting them. We did find, however, that concentrations of PAHs in stomach contents and bile of salmon from Kitimat sites were below the levels typically associated with health effects such as immunosuppression and reduced growth except in salmon from the site nearest the smelter (Alcan Inner Harbour),. For the Alcan Inner Harbour salmon, exposure levels were comparable to those causing changes in growth and metabolism characteristic of starving fish in feeding studies with PAHs (Meador et al. 2006). Immunosuppressive effects have also been found in salmon exposed to similar concentrations of PAHs in combination with other immunosuppressive contaminants such as PCBs (e.g., Arkoosh et al. 2001). Since these compounds are present only at very low or undetectable concentrations in Alcan's receiving environment, it is uncertain whether PAHs alone would produce the same health impacts.

Our monitoring also yielded some confounding data on PAHs at the Kildala and Kitlope reference sites. English sole from Kitlope, and to a lesser extent, from Kildala, showed exposure to high molecular weight PAHs, based on concentrations of metabolites of these compounds in bile. However, PAH concentrations in both sediments and stomach contents of English sole from this site were low, and yellowfin sole collected from this site showed no signs of exposure. A laboratory exposure study with English

sole indicated that compounds in Kitlope sediments, including perylene, a naturally-occurring compound that is one of the main high molecular weight PAHs at Kitlope, are not responsible for high PAH metabolite levels in bile of Kitlope English sole (Appendix B. Johnson et al., in prep). Fish were injected with both extract solutions from sediment collected at Kitlope and Hospital Beach as well perylene. The source of PAH exposure in Kitlope English sole remains unclear, but could be from natural product PAHs in the water column. The fish from this site do not appear to be affected, as they show no signs of liver disease or DNA damage.

Finally, our monitoring results suggest that the process changes introduced by over the last decade have been effective at reducing inputs of PAHs into the environment and biota of Kitimat Arm, as PAH concentrations in sediments and fish, as well as fish liver lesion prevalences, have remained stable or declined over the past five years of sampling. Given the additional process improvements and clean-up efforts to come, as indicated in Lachance et al. 2006, we would expect continuing improvement over time, which could be confirmed by monitoring on a periodic (e.g., 3-5 year) basis. The studies conducted thus far serve as a benchmark from which the efficacy of process changes intended to reduce PAH contamination and remediation efforts in Kitimat Arm can be assessed.

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LIPOFUSCIN-LIKE PIGMENT IN THE GONADS OF THE SEA URCHIN STRONGYLOCENTROTUS INTERMEDIUS INHABITING POLLUTED COASTAL WATERS (PETER THE GREAT BAY, SEA OF JAPAN)

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Abstract

During the past two to three decades, numerous attempts have been made to use accumulation of lipofuscin-like pigments (LLP) as an integral index of both age and stress in aquatic invertebtates. The most data obtained concern crustaceans and bivalve molluscs. In this study, the first description of morphological features and quantification of autofluorescence of LLP in the nutritive phagocytes (NPs) of female and male gonads of the sea urchin *Strongylocentrotus intermedius* inhabiting polluted areas of Peter the Great Bay (Sea of Japan) are presented.

In August 2002, sea urchins and sediment samples were collected at several stations from coastal zone of Peter the Great Bay (Sea of Japan): st. 1 and 2 located near Vladivostok city ("near city" zone), st. 3, 4 and 5 in the "island" zone of the bay, and reference station (6) remote far from the sources of pollution. In the histological sections from the sea urchin gonads, LLP was visible as yellow-brown globules and granules located mainly in the cytoplasm of NPs; accumulations of LLP granules occurred in haemal sinuses and coelom. Lipofuscin-like pigment in the sea urchin NPs is evidently a product of secondary lysosomes performing intracellular digesting of phagocytosed material of abnormal/dead gametes. By sight, the gonads of the sea urchins from "near city" zone contained much more LLP than the gonads of the animals from "island" zone and st. 6. For fluorescence quantification, epiofluorescence video microscopy and image analysis were used. Within each animal sample, 3–13 males and females with immature gonads were selected. Fluorescence intensity of 25 images of the gonad section occupied by NPs was measured at λ_{ex}=450 nm, and granule concentration (area fraction, %) of lipofuscin was determined. Each animal sample contained from 1 (st. 6) to 7 (st. 1) sea urchins with high means (>1%) of lipofuscin area fraction. High area fraction means were attributed to NP containing a bulk of lipofuscin granules. Sediment contamination with DDT, oil hydrocarbons and heavy metals appears to intensify the LLP formation in the sea urchin gonads. Quantity of LLP in NPs may apparently be used as one of the indices of the sea urchin gonad condition.

Key words: Sea urchin gonads, Lipofuscin, Heavy metals, Organochlorine pesticides, Pollution.

1. Introduction

The terms 'lipofuscin', 'ceroid', lipofuscin-like pigments' refer to a group of lipopigments with similar chemical composition, physical and chemical properties. These brown-yellow, autofluorescent, electron-dense pigments have been found in different cells of all taxa studied so far including mammals and other vertebrates, protozoans, fungi, nematodes, annelids, molluscs, crustaceans and insects. Lipofuscin was described as an age pigment accumulating in human nerve cells with senescence over a century ago, and thereafter, the rapid accumulation of similar pigments named 'ceroid' was observed in a wide variety of animal tissues under pathological conditions (see for review: [1, 2]). To present day, the nature and genesis of the lipopigments as well as the differences in their composition and properties are not totally understood, and these questions were intensively debated during the past two decades (see for review: [1–5]). The vast majority of data has been obtained for mammalian lipopigments and concerned with the problems of both aging and pathology known as ceroid-lipofuscinosis (Batten's disease). Most of the authors believe that the term 'lipofuscin' may be referred only to autofluorescent pigment consisting of intracellular secondary lysosomes and progressively accumulated by cells during normal senescence. Molecular composition of lipofuscin and the mechanisms of its formation are likely to vary between different animal species and even from tissue to tissue, therefore Katz and Robison [5] recommend to use the plural form, 'lipofuscins', for generic references to age pigment. As to the term 'ceroid', these authors believe that different ceroids may have very little in common, so they suggested avoiding the term 'ceroid' altogether and using the alternative term, 'lipofuscin-like pigments', in order to refer to lysosomal storage bodies with lipofuscin-like autofluorescence properties that accumulate as a consequence of anything other than normal senescence. This suggestion is all the more reasonable when we deal with autofluorescent lipopigments of the invertebrates which display great taxonomic diversity, inhabit large number of ecological niches and experience the influence of different environmental factors.

During the past two to three decades, numerous attempts have been made to use accumulation of lipofuscin-like pigments (LLP) as an integral index of both age and stress in aquatic invertebtates. The most data obtained concern crustaceans and bivalve molluscs. It was shown in many studies on crustaceans that the amount of LLP in the brain can be suitable as a marker of individual age [e.g. 6–8]. Studies of LLP in molluscs have focused primarily on the effect of stress such as exposure to pollutants or anoxic conditions [e.g. 9–13]. LLP content in the digestive cells of bivalve molluscs has been suggested as one of the biomarkers to assess the effects of marine pollution on the animal health status [14].

Today practically no information is available on LLP occurrence in cells and tissues of echinorderms. Study of the state of reproductive function in the sea urchin *Strongylocentrotus intermedius* inhabiting polluted coastal waters of Peter the Great Bay (Sea of Japan) gave the first observation of the presence of LLP in the sea urchin gonads

[15, 16]. In this paper, morphological and quantitative fluorescent characteristics of lipofuscin-like pigment in the gonads of the sea urchin *S. intermedius* from different areas of Peter the Great Bay are presented.

2. Materials and Methods

2.1. Field sampling. Sampling was performed in August 2002 at several stations in coastal zone of Peter the Great Bay (Fig. 1). Stations 1 and 2 were located near Vladivostok city ("near city" zone), stations 3, 4 and 5 were in the open "island" zone of the bay. Reference station (6) was situated in the nothern part of coastal zone of Primorsky Territory (near Rassypnoi Cape, Japan Sea), a remote area far away from the sources of pollution.

At each station, sea urchins *S. intermedius* were sampled (n=30). At stations 1–3 and 5, surface sediment samples (2 cm layer) were taken, frozen and stored at –25ºC before chemical analysis.

2.2. Histological analysis. Pieces of the sea urchin gonads were fixed in Boin's fluid, then washed with 70% ethanol, dehydrated in a graded athanol series, and embedded in paraffin wax. Histological sections (6 μm thickness) were stained with haematoxylin and eosin and examined under light microscope in order to determine the gonad maturity state. Only the gonad samples which state corresponded to the early stage of reproductive cycle, when the gonad acini are occupied mostly by the nutritive phagocytes (NPs), were taken for LLP quantification. Whithin each animal sample, from 3 to 13 females and males with immature gonads were found.

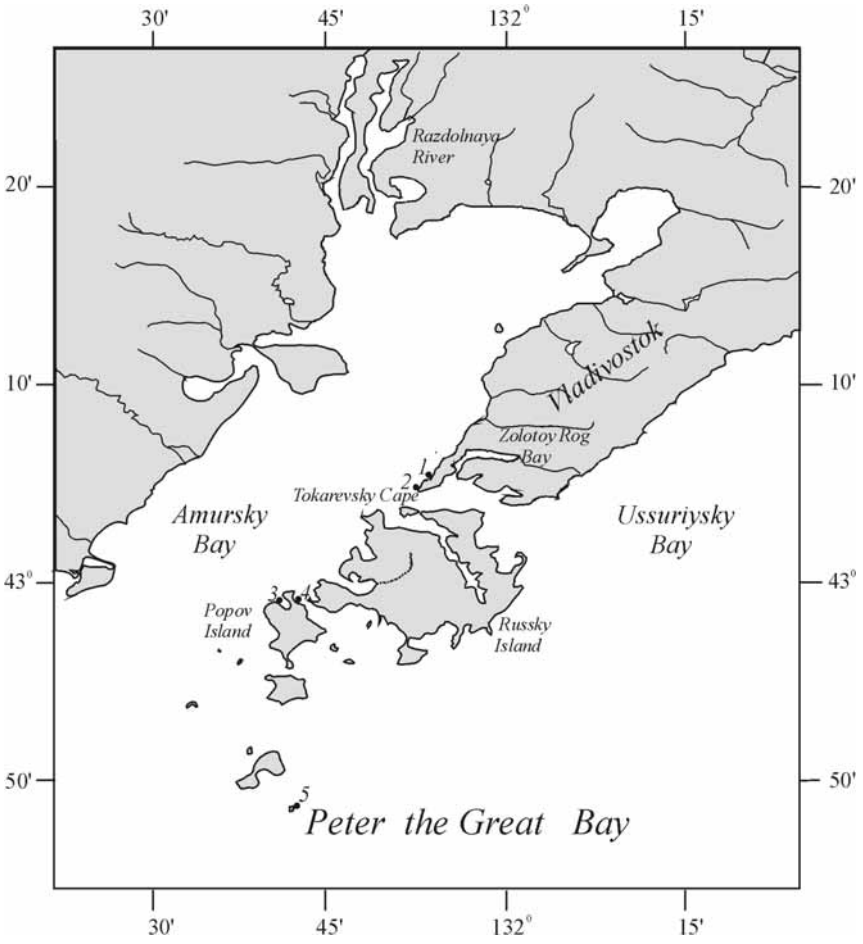

Fig. 1. Sampling sites (1–5) in Peter the Great Bay (Sea of Japan). 1 – Sport Harbour, 2 – Tokarevsky Cape, 3 – Alekseev Bight, 4 – Stark Strait, 5 – Verkhovsky Islands.

2.3. Lipofuscin-like pigment quantification. An estimation of LLP fluorescence in the unstained 6 μm sections was conducted by fluorescence image analysis. For this purpose, microscopic glasses with samples were mounted on a cell chamber of fluorescent imaging system based on inverted microscope Axiovert 200 (Zeiss, Germany). The 75W Optosource xenon arc lamp and DAC-controlled Optoscan monochromator (Cairn Research Ltd., UK) were used as a light source to excite fluorescence at λ=450 nm; HQ FITC filter-block (Chroma Technology Corp., USA) and Fluar 40×/1.30 Oil objective (Zeiss, Germany) were set for visualisation of LLP fluorescence. The 12-bit images of fluorescent LLP granules were acquired using digital CCD videocamera Hamamatsu Orca-ER C4742-95 (Hamamatsu Photonics K.K., Japan), captured and transferred to an IBM-compatible computer P-IV with Firewire data interface card. The fluorescence intensity of randomly selected 25 images of the gonad section occupied by NPs was measured with AQM Advance 6 software (Kinetic Imaging Ltd., UK), expressed as an average pixel intensity of grey level for each image, and LLP granule concentration (area fraction, %) was calculated by dividing the area of LLP granules by the total area of analyzed tissue and multiplying by 100. The emission spectrum of LPP granules in the samples was determined using AQM Advance 6 software options.

2.4. Chemical analysis. Sediments were dried at 80ºC and analysed for such contents of heavy metals, oil hydrocarbons and chlorinated hydrocarbons (*p,p'*- DDT, *p,p'*- DDD and *p,p'*- DDE, α- and γ-isomers of hexachlorocyclohexane – HCH). Concentrations of heavy metals (Fe, Mn, Zn, Cu, Ni, Co, Pb, and Cr) were determined by flame atomic-absorption spectrophotometry according to the method 3050 (U.S. EPA); oil hydrocarbons and chlorinated hydrocarbons contents were determined by the routine method of IR-spectrophotometry and the method of gas chromatography, respectively, according to the standard techniques of the Russian Hydrometeorological Service (for more detailed description, see: [17]).

2.5. Statistical analysis. The means and standard errors for each sample were calculated and plotted using a computer program GrafPadPrizm 4.0 for Windows (GrafPadSoftware, Inc.).

3. Results

3.1. Sediment analysis. The highest concentration of oil hydrocarbons was found in the sediments from site 1 (table 1), this value was about 2.5 higher than the maximal normal environmental background level of 100 μg/g dry weight [18]. In sediments from other sites, the concentrations of oil hydrocarbons were very close to the background level.

Total content of α- and γ-isomers of HCH (ΣHCH) in the sediment samples varied from 0.0 to 7.4 ng/g dry weight, and the highest HCH concentration was registered at site 1 (table 1). At this station, maximal concentration of DDT and its metabolites (ΣDDT) was found as well. The least ΣDDT content was measured in the sediments from station 4, while concenrations of this pesticide in the sediments from stations 2 and 5 were rather high, about 11 ng/g dry weight (table 1).

Table 1. Content of oil hydrocarbons (OH, μg/g dry weight), hexachlorocyclohexane (∑HCH, ng/g dry weight) and DDT (ng/g dry weight) in bottom sediments from Peter the Great Bay (Sea of Japan), August 2002

Site	OH	∑HCH	DDT	DDD	DDE	∑DDT
1	240	7.4	8.3	29.5	9.3	47.1
2	130	0.2	9.1	0.4	1.9	11.4
3	100	0.3	4.5	0	0.5	5.0
4	90	0.3	1.5	0.4	0.4	2.1
5	70	0.0	9.6	0.5	1.4	11.5

In the sediments from sites 1 and 2 located in the "near city" zone and from the nearest to the city site 3, concentrations of Fe, Mn, Zn, Cu, and Cu were higer than in the sediments from station 5 (table 2). Besides, the sediments from sites 1 and 2 contained more Pb than the sediments from two "island" stations, 3 and 5. In the sediments from all sites, heavy metal concentrations were above the background level. Station 1 was the most heavily polluted by Zn and Cu.

Table 2. Content of iron (mg/g dry weight) and other heavy metals (μg/g dry weight) in bottom sediments from Peter the Great Bay (Sea of Japan), August 2002

Site	Fe	Mn	Zn	Cu	Co	Cr	Pb
1	43.0	168.0	210.0	58.0	28.0	31.0	27.0
2	38.0	134.0	152.0	42.0	25.0	35.0	32.0
3	37.5	155.0	83.0	22.0	26.0	33.0	12.0
4	20.0	127.0	75.0	12.0	18.5	20.0	11.0
5	22.0	116.0	67.0	13.0	20.0	28.0	12.0
Background concentration*	15–20	–	35–40	8–9	7.5	7.5	8–9

*Data from Shulkin et al., 2002.

3.2. Lipofuscin-like pigment characteristics. Light microscopy examination of histological sections stained with hematoxylin and eosin allowed discriminating morphological features and location of LLP in the gonads of the sea urchin *S. intermedius* (Fig. 2). In the gonad acini, LLP was revealed mainly as intracellular inclusions in the nutritive phagocytes (NPs). These inclusions were of different sizes, from about 1 to 20 μm. Colour of large inclusions named 'globules' varied from gold-yellow to brown-yellow (Fig. 2 A), while the smallest inclusions named 'granules' were dark brown (almost black) (Fig. 2 B). Single LLP granules were also present in the cytoplasm of oocytes and coelomocytes, the cells of coelomic fluid which could be found in the gonad acini.

Aggregations of LLP globules and granules were found in the haemal sinuses of the sea urchin gonad (Fig. 2 C) as well as outside the gonad acini, in the gonadal coelom (Fig. 2 D).

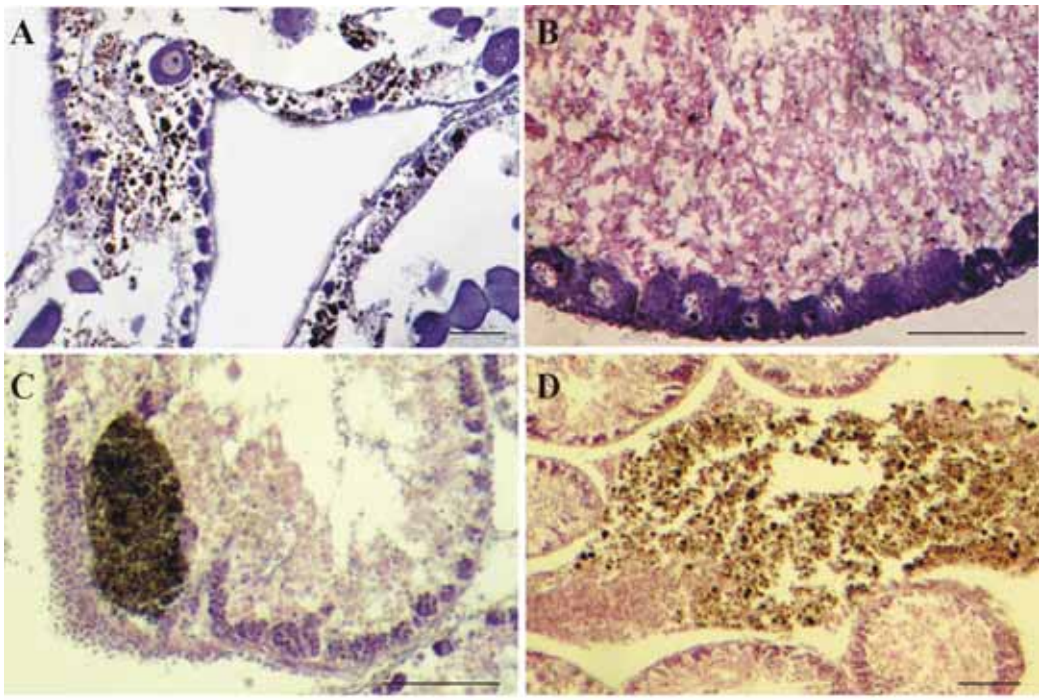

Fig. 2. Lipofiscin-like pigment (LLP) in the gonads of the sea urchin *Strongylocentrotus intermedius*: A – large brown-yellow globules in cytoplasm of nutritive phagocytes, B – small dark granules in cytoplasm of nutritive phagocytes, C – large aggregation of LLP in the haemal sinus, D – large aggregation of LLP in the gonadal coelom. Hematoxylin and eosin. Scale bars = 100 μm.

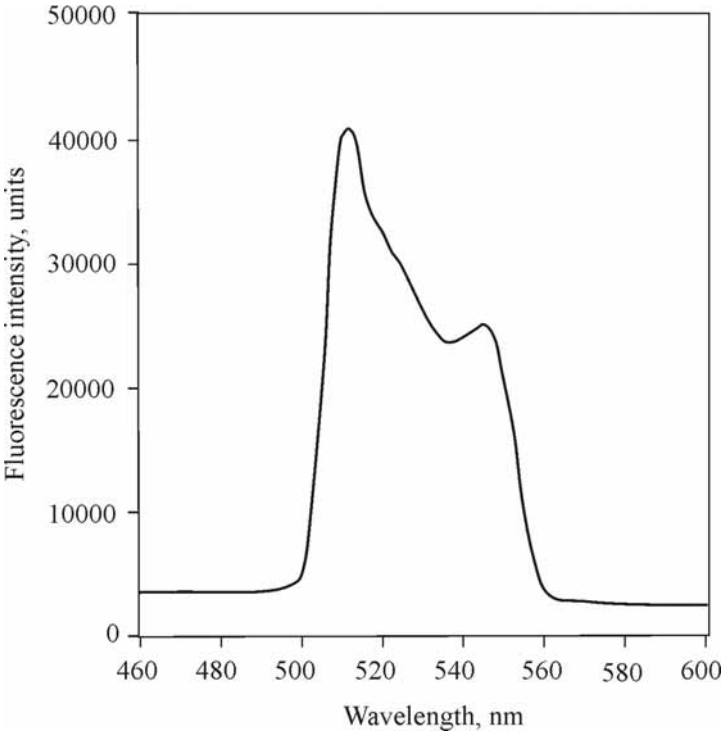

Fig. 3. Emission spectrum of autoflurescence of lipofuscin-like pigment in the nutritive phagocytes of the gonad of the sea urchin *Strongylocentrotus intermedius*.

LLP globules and granules contained in NPs of the gonad of the sea urchin *S. intermedius* exhibited strong autofluorescence with excitation maximum of 450 nm and emission maximum of 512 nm (Fig. 3). In Figure 4, micrographs of LLP autofluorescence in NPs of female gonad are presented. Aggregations of different sizes containing numerous LLP granules are visible in the cytoplasm of NPS (Fig. 4 B); another NPs contained, along with LLP granules, gomogenous globules of round shape exhibiting fluorescence (Fig. 4 C).

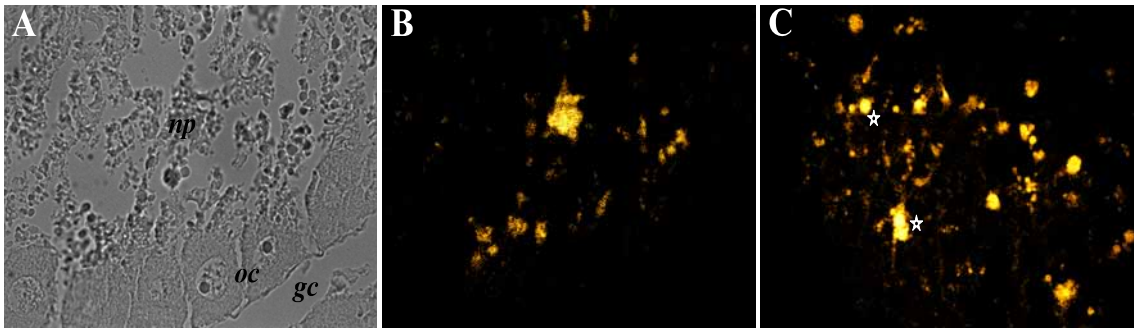

Fig. 4. Autofluorescence of lipofuscin-like pigment (LLP) in the gonad of the sea urchin *Strongylocentrotus intermedius*. A – unstained section of the female gonad; B – autofluorescence of the same part of the section showing fluorescence of aggregations of LLP granules in the nutritive phagocytes; C – autofluorescence of another part of the gonad section showing fluorescence of both LLP granules and LLP globules (white asterisks). *gc* – gonadal coelom, *np* – nutritive phagocytes, *oc* – oocytes. Magnification, ×400.

3.3.Lipofuscin-like pigment quantification. Gonads of 39 females and 36 males of *S. intermedius* were analysed for their LLP concentration as described above. LLP concentrations ranged from 0.0 to 4.57±0.53% area fraction (AF) in females and from 0.0 to 4.61±0.35% AF in males (Fig. 5). In the sea urchin samples from all stations, individuals with extremely high LLP concentrations in the gonads (≥1.5%) were found: 1 male at station 1, 1 male and 1 female at stations 2 and 3, 2 females and 1 male at stations 4 and 5, and 1 female at the reference station 6 (Fig. 5). Three individuals with the highest LLP concentrations in the gonads (>4%) were found in the sea urchin samples from two 'island' stations (1 female at station 3 and 1 female and 1 male at station 4).

Student *t*-test analysis performed on the average values of LLP concentrations in the sea urchin gonads and calculated taking into account all individual data has shown no significant differences between the samples from different stations, both for females and males (Fig. 6 A). When average values of LLP concentrations in the sea urchin gonads were calculated excluding the extremal values (≥1.5%), *t*-test analysis has revealed significant differences between the sample from station 1 and other samples in a number of cases (Fig. 6 B).

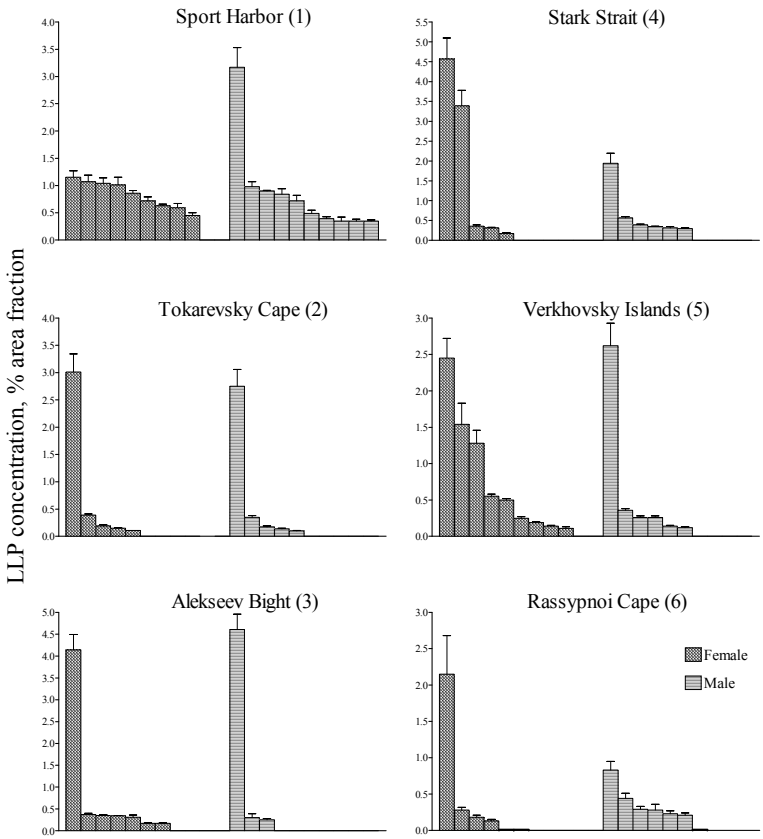

Fig. 5. Lipofuscin-like pigment (LLP) concentrations in the gonads of female and male individuals of the sea urchin *Strongylocentrotus intermedius* from different areas of Peter the Great Bay and the reference station 6 (Sea of Japan).

Lineal regression analysis has been performed to reveal relationship between concentrations of different pollutants (OH, ΣDDT, and heavy metals) in the sediments and LLP concentrations in the sea urchin gonads. Only one statistically significant positive relationship (p<0.05) was found, between the content of ΣDDT in the sediments and the concentration of LLP in female sea urchin gonads (Fig. 7 A); other relationships were not statistically significant. Nevertheless, a tendency for positive relationshipe was also revealed for ΣDDT and LLP concentration in male sea urchin gonads (Fig. 7 A) and for content of OH in the sediments and LLP concentrations in the gonads (Fig. 7 B).

4. Discussion

The LLP-containing granules and globules found in NPs of the gonads of the sea urchin *S. intermedius* show morpholofical and autofluorescent properties similar to those described for lipofuscins/ceroids in various tissues of other invertebrate and vertebrate species [e.g. 4, 6, 8, 20]. Shape and sizes (1–20 μm) of LLP-containing granules and globules are close to those of common intracellular inclusions of NPs which represent the nutrient storage. In norm, they stained by hematoxylin and eosin, and basophilic, eosinophilic and heterochromophilic inclusions (so-called ‘globules’) could be distinguished ([21]; our own observations).

Nutritive phagocytes (called also ‘accessory cells’) are somatic cells of the sea urchin gonads which carry out several functions and play an important role in the

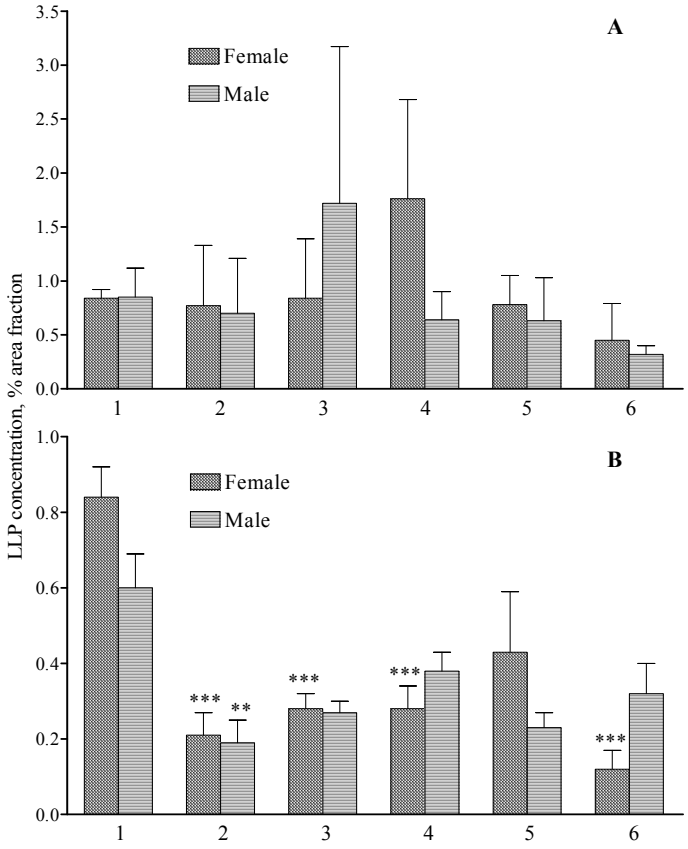

Fig. 6. Average values (± standard error) of lipofuscin-like pigment (LLP) concentrations in the gonads of female and male individuals of the sea urchin *Strongylocentrotus intermedius* from different areas of Peter the Great Bay and the reference station 6 (Sea of Japan): A – average values calculated from all individual data, B – average values (± standard error) calculated excluding the extremal values (LLP concentration ≥ 1.5). Differences are significant (compared to station 1) at $^{**}p<0.01$, $^{***}p<0.001$.

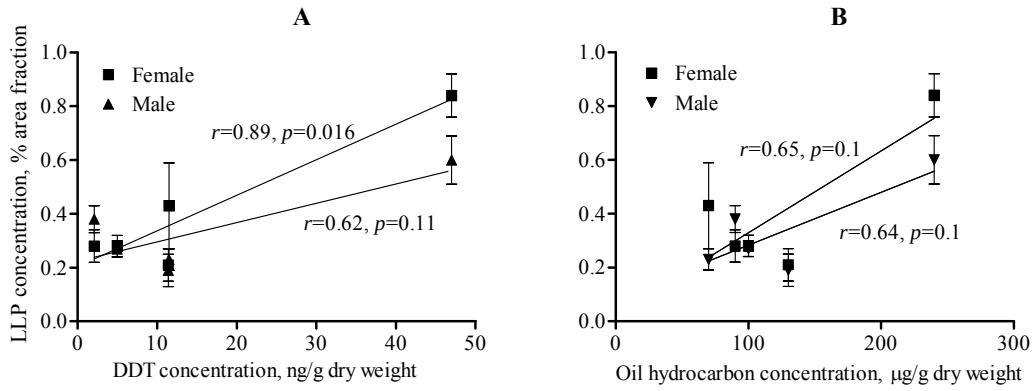

Fig. 7. Lipofuscin-like pigment (LLP) concentration in the gonads of the sea urchin *Strongylocentrotus intermedius* from different areas of Peter the Great Bay (Sea of Japan) in relation to (A) concentration of ΣDDT in the sediments and (B) concentration of oil hydrocarbons in the sediments.

processes of gametogenesis in sea urchins. The most important functions of NPs are phagocytosis of relict/abnormal gametes, restoration of nutritive substances, and participation in nutrition of growing and differentiating gametes [21–25]. Phagocytosis of sexual cells in the sea urchin gonads followed by utilization and restoration of nutritive substances in the cytoplasm of NPs is commonly called ‘resorption’.

During reproductive cycle of the sea urchin, significant changes in size and number of NPs as well as in size, number and composition of their cytoplasmic inclusions take place [21, 23, 24]. The largest quantitative characteristics of NPs were registered at the early stage of the sea urchin reproductive cycle, ‘the beginning of gametogenesis’. In the course of gametogenesis, along with increase in the number of gametes and level of their differentiation the amount of NP globules as well as NP number and size gradually decrease. At the late stage of the sea urchin reproductive cycle (‘pre-spawning’), when the gonad acini are filled with mature gametes, NPs become exhausted, and the globules almost completely dissappear from their cytoplasm.

Active processes of the resorption which take place in the sea urchin gonad at the post-spawning stage of the reproductive cycle or under unfavorable ecological conditions such as very low/very high water temperature and pollution lead to accumulation of the globules in NPs cytoplasm [15, 16, 21, 26]. It was noted that such ‘cleaning’ processes in the sea urchin gonad were accompanied by appearance of yellow pigment in NPs cytoplasm and accumulation of yellow-brown granules in different compartments of the gonad – NPs, haemal sinuses, gonadal coelom and gonad support mesentery; besides, LLP granules were found in coelomocytes located around and inside damaged gonad acini [15, 16, 21, 26, 27].

Despite NPs have long been recognized as unique phagocytes playing a pivotal role in the sea urchin gametogenesis, the cellular and molecular mechanisms involved in their phagocytic and nutritional functions are not fully understood. Recently it was shown that NPs have well developed lysosomal-vacuolar system which is involved in both digesting of phagocytosed remnant sperm and autophagocytosis of cellular compartments; such an autophagy results in NPs shrinkage in post-spawn gonad of the sea urchin *Anthocidaris crassispina* [25]. According to the authors, normal functioning of NP lysosomal-vacuolar system leads to complete degradation of phagocytosed/autophagocytosed material. It is very attractive to propose that dysfunction of NP lysosomal-vacuolar system caused by some unfavorable factor, pathological or environmental, can lead to LLP formation and accumulation in NPs. Today there is no doubt that lipopigments accumulate in the lysosome (termed secondary lysosomes, cytosomes, residual bodies, or dense bodies based on their ultrastructural features), and possible mechanisms of lysosomal dysfunction that could result in accumulation of autofluorescent lysosomal storage material in a pathological condition are intensively debated (see for review: [2, 28]). Seehafer and Pearce [6] have suggested a model explaining possible mechanisms of lipopigment (lipofuscin and ceroid) accumulation in mammalian cells. This model considers three ways leading to lipopigment accumulation during the processes of intracellular digesting: 1) disruptions in autophagy, 2) altered lysosomal function (for example, altered pH resulting in altered activity of hydrolases), and 3) recycling of macromolecules (proteins, lipids) and

cellular organelles damaged because of oxidation caused by reactive oxygen species.

Taking into consideration evolutionary conservatism of the processes of intracellular digesting [1, 28] one can suppose similar mechanisms of lipopigment formation in cells of invertebrate animals. Based on the observation that increased resorption processes in the sea urchin gonad lead to accumulation of LLP in NPs we supposed that enhancement of endocytosis (phagocytosis of spermatogenic cells and the material of dead oocytes) may result in overloading of lysosomal-vacuolar system of NPs and, as a consequence, in LLP formation. An inadequate intralysosomal digestion may be another reason, or both these events may take place. Recently, Jolly and coauthors [29] based on the data of chemical analysis of lipofuscin from different mamallian tissues have proposed as a working hypothesis, that lipofuscinogenesis is initiated in secondary lysosomes due to delayed proteolysis, perhaps associated with an initial overloading.

One more interesting question is connected with a fate of LLP in the sea urchin gonad. Our observations provide evidence of elimination of LLP from the gonad acini into the heamal sinuses (Fig. 2 C), and then into the gonadal coelom (Fig. 2 D). It is very likely that LLP granules and globules are exocytosed from NPs into the acinus lumen and phagocytosed by coelomocytes migrating from coelomic fluid into the gonad acini and participating in cleaning processes. Recently, it was demostrated that even lipofuscin, accrued through normal ageing, can be lost from neural tissue of decapod crustaceans [30]. The mechanism of loss probably involves exocytosis and possibly blood transport.

Data on seasonal changes in the number and sizes of NPs in the gonad connected with the sea urchin reproductive cycle [21, 24, 25] supports the idea that NPs represent a renewal cell population. This is a very important idea from a point of view that LLP accumulation in NPs is evidently not dependent on the age of the sea urchin. Taking into consideration a body of evidence concerning the sea urchin nutritive phagocytes we have assumed that quantitative analysis of LLP in NPS may be used as an index of gonad condition in the sea urchins inhabiting polluted coastal waters.

In the present study, some unexpected results have been obtained. In the sea urchin sample from each area studied, even from the station located far away from the sources of pollution (reference station 6) we have found from one to four individuals with high LLP concentration (≥1.5%) in NPs. These extremely high values stipulated a great variance in data, large values of the standard error and, as a conseaquence, the absence of statistically significant differences between the samples (Fig. 6 A). Exclusion of extremely high values from data set permits us to show that general level of LLP in the female gonads of the sea urchins from station 1 was significantly higher than in the female sea urchins from other stations (with the exception of station 5); such a tendency was also revealed for males but it was less prominent (Fig. 6 B).

The data of chemical analysis exhibit higher level of sediment contamination with oil hydrocarbons, organochlorine pesticides and heavy metals at station 1 compared to other stations. At the same time, concentrations of a number of heavy metals (Zn, Cu, Co, Cr, and Pb) in the sediments from all stations in Peter the Great Bay (Table 2) were higher than background concentrations for the bay sediments [19]. This

fact provides evidence of moderate contamination with heavy metals of both 'near city' and 'island' zones of Peter the Great Bay. Besides, rather high concentrations of DDT and its metabolites, DDD and DDE, were revealed at the 'near city' stations 1 and 2 and at the 'island' station 5 (Table 1). It is noteworthy that "fresh" (unmetabolised) DDT comprised 18, 80 and 84% of ΣDDT, respectively. In our previous research of 2001, we have found high proportions in ΣDDT in the sediments of inner, middle and open parts of Amursky Bay as well as in the liver of the plaice *Pleuronectes pinnifasciatus* and made conclusion about recent input of "fresh" DDT from an unknown source into the bay ecosystem [17]. In the present study, the total content of DDT and its metabolites in the upper layer of bottom sediments at the stations 1, 2 and 5 was higher than that observed in the sediments of Amursky Bay in 2001 and exceeded the values that, according to the results of numerous studies reviewed by Long et al. [31], caused adverse biological effects in 30–50% of cases.

In our study, as the results of lineal regression analysis showed, the only significant relationship was between LLP concentration in the sea urchin female gonads and the content of ΣDDT in the sediments (Fig. 7 A). In available literature, we did not find information about the influence of DDT on the LLP formation in any kind of cells. At the same time, enhanced lipopigment deposition has been consistently observed in digestive cells of mussels exposed to polycyclic aromatic hydrocarbons (PAHs) or heavy metals under both laboratory and field conditions [e.g. 9–11, 32–34].

Thus, in this study the first description of morphological features and quantification of autofluorescence of LLP in NPs of female and male gonads of the sea urchin *S. intermedius* inhabiting polluted areas of Peter the Great Bay (Sea of Japan) are presented. Lipofuscin-like pigment in the sea urchin NPs is evidently a product of secondary lysosomes performing intracellular digesting of phagocytosed material of abnormal/dead gametes. Quantity of LLP in NPs may apparently be used as one of the indices of the sea urchin gonad condition. Sediment contamination with DDT, oil hydrocarbons and heavy metals appears to intensify the LLP formation in the sea urchin gonads.

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USE OF CONSTRUCTED WETLANDS TO REMOVE TOXICANTS FROM TREATED SEWAGE EFFLUENT

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Abstract
One way of improving the quality of the water discharged to the sea, and thereby reducing the pollutant impacts on marine seafood resources is the inclusion of constructed wetlands to 'polish' the effluent prior to discharge to the sea. This paper reports on an investigation of the removal from sewage effluent of selected chemicals including suspected environmental endocrine disruptors (EEDs) at a free water surface constructed wetland at Cooroy in Queensland and a subsurface flow wetland at Oxley, Brisbane, Australia. Compounds detected in the raw effluent from the STPs included pesticides, herbicides, some heavy metals and the human hormones 17β estradiol and estrone. Individual patterns of removal for individual pesticides (including herbicides) were found in the constructed wetlands depending on the chemical nature of the contaminants.
Keywords :Constructed wetlands, chemicals, ocean discharge, marine ecosystem, seafood contamination

1 BACKGROUND

Coastal, bay and inlet waters support a variety of commercial activities: shipping, oil and natural gas fields, fishing and aquaculture. In Australia 85% of the countries population live close to the coastline, and therefore the majority of sewage effluent disposal is to near shore coastal environments. Several important recreational activities also focus on these coastal waters: recreational fishing, boating, sightseeing and related tourism - all of which are of great significance to coastal economies. Careful impact monitoring, assessment and planning are essential to ensure that the benefits derived from these activities do not occur at the cost of the marine resources and environments on which they depend. Trace levels of toxicants are released in treated sewage and other effluents which are discharged directly into bays and coastal waters.

PCBs and chlorinated pesticides such as DDT and dieldrin are persistent compounds which show high degrees of bio-accumulation in marine food chains. Both of these classes of organic toxicants are present (at levels below the maximum permitted concentrations) in flathead flesh from all sites sampled in Port Phillip Bay, Victoria, Australia (Nicholson et al. 1991). Flathead livers showed elevated levels of heptachlor in the Geelong Arm and PCB in Corio Bay and the Geelong Arm. An unpublished 1990 report indicated that levels of PCDD and PCDF (toxic equivalents) in mussels and fish represented only a low human health risk, on the basis of international standards. Congener profiles show a different pattern on each side of the Bay, suggesting different processes affecting contamination; the Werribee Treatment Complex is important to the western side, and diffuse run-off to the eastern side of the Bay. The lethal and

sublethal effects of toxicants on marine species and communities present in Victoria's bays, inlets and coastal waters are presently unknown.

2 METHODS

2.1 Constructed wetlands

In Queensland, several surface flow constructed wetlands were established in the 1990's to further polish secondary treated municipal wastewater effluent (Greenway, 1997; Greenway and Woolley, 1999). The tropical-subtropical climate is ideal to promote almost year around macrophyte growth, thereby providing improved water quality discharge. Increasingly, management options for improved receiving water quality, water reclamation and reuse, involve the application of constructed wetland technology (Bavor *et al.*, 1995). The constructed wetlands reported on here are a free water surface wetland (FWS) located at Cooroy in south-east Queensland and a subsurface wetland at Oxley, Brisbane. The Cooroy wetland established in 1995 has two sets of three cells in parallel with the water following a sinusoidal path. Oxley has four adjacent cells with a linear flow of water through each cell. The cells differ in the size of the gravel and the age since establishment of the individual cells.

2.2 Sample collection

Water for chemical analyses was collected directly into solvent rinsed amber glass bottles with Teflon lid liners and immediately placed on ice. The water for heavy metal analysis was stored in plastic containers with nitric acid preservation. Sets of five sediments samples from each location in the wetland were sampled using a stainless steel corer and mixed using all steel apparatus. A aliquot of the sediment composite (~200g) was taken, stored in solvent rinsed glass jars and placed on ice. Influent from the sewage treatment plant (STP) was sampled by dipper and immediately transferred to the solvent rinsed amber glass bottles. Samples for heavy metal analysis were also collected by dipper and stored in plastic containers with nitric acid until analysis. Following collection, all samples were stored on ice and immediately transported to the analytical laboratory.

2.3 Chemical analyses

The compounds screened were selected for analysis subsequent to a literature search on which ones were most likely to be present in domestic sewage effluent and could be detected by available analytical methods. This study has focussed on sewage treatment systems suitable for small or regional communities, and therefore some of the industrial chemicals suspected or known to be EDCs were not included in this study as the likelihood of detection was low. Effluent from the STPs was screened for herbicides, pesticides, PCBs, lead, mercury and cadmium and the estrogen suite including 17βestradiol, 17αethinylestradiol and estrone. Effluent and sediments from the Cooroy wetland were sampled for herbicides, pesticides and PCBS from Cooroy and the same analyses were conducted for effluent only from Oxley.

Quantitative determination of the pesticides was accomplished using a combination of gas-liquid chromatography (GLC) and gas-liquid chromatography/mass spectrometry (GCMS). Organochlorine pesticides (OCs) and polychlorobiphenyls (PCBs) were analysed by GLC with electron capture detection and two analytical columns. Identity was established by comparison of retention times with standards on two different columns. Organophosphate pesticides were determined by GC with nitrogen/phosphorus detectors in dual column mode. All samples with positive results were run on GCMS for confirmation of identity of the pesticides. Heavy metals were analysed using graphite furnace atomic absorption spectrometry.

Hormone analyses were carried out by the Australian Government Analytical Laboratory (AGAL), Pymble, NSW. Methods for extraction of the estrogenic hormones 17βestradiol, estrone and 17αethinylestradiol from the sewage effluent were developed by AGAL. This involved solid phase extraction (SPE) using a 6mL reversed phase SPE cartridge containing 1g C18 sorbent (ENV1-18, Supleco). Recoveries were carried out with replicate effluent water samples (volume 1 litre) which were spiked with 10µl and 100 µl of a 1 mg/L estrogen mix, extracted by SPE, derivitised with PFPA followed by analysis by GCMS-SIM. The limit of reporting was set at 5ng/L for most samples.

3 RESULTS

3.1 Cooroy Constructed Wetland

3.1.1 Pesticides

Concentrations of pesticides found in the wetland inlet water at the Cooroy FWS wetland were reduced (eg. carbaryl, diazinon and piperonyl butoxide) or removed to below the limit of detection (eg. chlorpyrifos, dimethoate, fipronyl and DEET) in the outlet water from the wetland. Only diazinon and carbaryl were detected in the final effluent at 0.02 and 1.6ug/L respectively at the first sampling occasion (Table 1) and DEET and Diuron on the second sampling (Table 2, Figs 1-2). Piperonyl butoxide was detected in the inlet but was below detection at the outlet end of the wetland. There were no pesticides detected in sediments sampled at the same time and place as the water samples were taken, with the exception of diuron and piperonyl butoxide which were detected, but only in the sediments of the inlet pond and first stage of the constructed wetland (Table 3) but absent at the end. PCBs, synthetic pyrethroids and organochlorine pesticides were not detected in water or sediments.

Table 1 Changes in the concentrations of pesticides in **wetland water** between inlet, middle and ou tlet of the Cooroy Wetland. No pesticides were detected in sediments sampled at the same location and at the same time as the water samples were taken.

Location in wetland	units	Carbaryl	Chlorpyrifos	DEET	Diazinon	Dimethoate	Fipronyl	Piperonyl butoxide
Cell 3.1	ug/L	2.80	0.02	2.00	0.13	0.16	0.29	1.60
Cell 6.2	ug/L	2.00	<lor	0.57	0.02	0.12	<lor	3.30
Cell 9.3	ug/L	1.60	<lor	<lor	0.02	<lor	<lor	0.20

Table 2 Changes in the concentrations of selected pesticides and herbicides in **wetland water** at increasing distances from the inlet pond (Cell 1) to the outlet (cell 9) Cooroy Wetland. Carbaryl was detected in all samples but not quantified in this set of analyses.

Location in wetland	units	DEET	Diuron	piperonyl butoxide
Cell 1 (inlet pond)	ug/L	0.87	0.08	3.00
Cell 3.1	ug/L	0.43	0.07	1.71
Cell 3.2	ug/L	0.17	0.07	0.60
Cell 6.1	ug/L	0.12	0.06	0.15
Cell 6.2	ug/L	0.12	0.06	<lor
Cell 9.1	ug/L	0.10	0.07	<lor
Cell 9.2 (outlet)	ug/L	0.10	0.07	<lor

Table 3 Concentrations of selected pesticides and herbicides in **sediments** from the same location as water samples in Table 2.

Location in wetland	units	DEET	Diuron	piperonyl butoxide
Cell 1 (inlet pond)	ug/kg	<lor	<lor	**7**
Cell 3.1	ug/kg	<lor	**7**	**6**
Cell 3.2	ug/kg	<lor	<lor	<lor
Cell 6.1	ug/kg	<lor	<lor	<lor
Cell 6.2	ug/kg	<lor	<lor	<lor
Cell 9.1	ug/kg	<lor	<lor	<lor
Cell 9.2 (outlet)	ug/kg	<lor	<lor	<lor

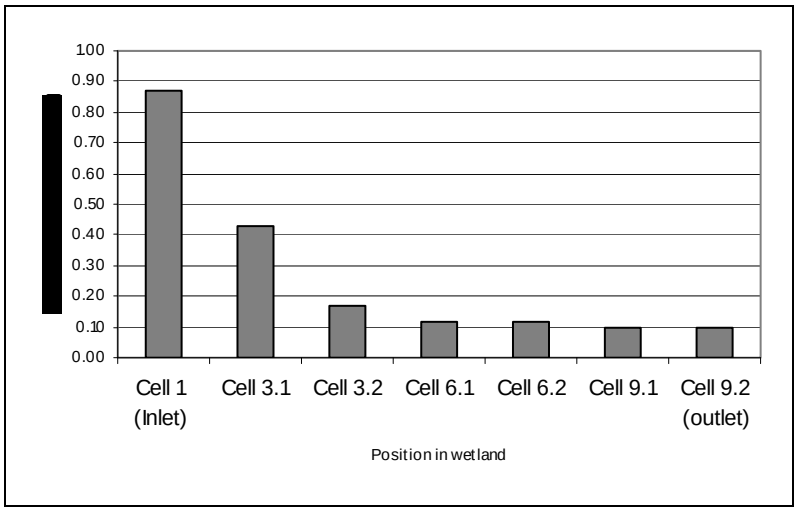

Figure 1. Decreasing concentrations of DEET from the inlet to the outlet of the Cooroy constructed wetland.

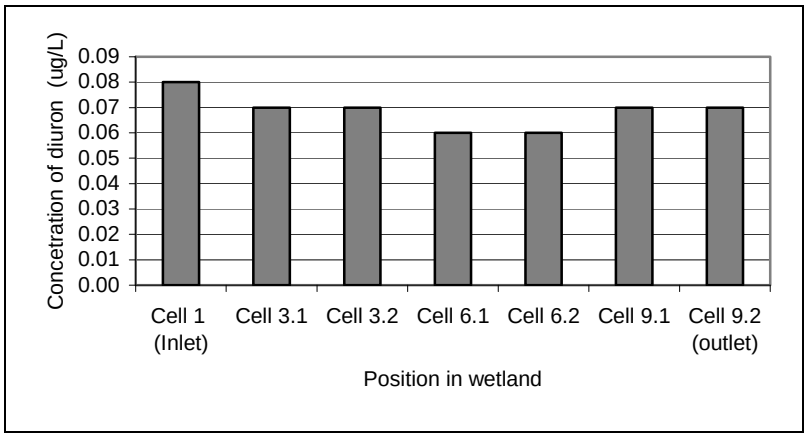

Figure 2. Concentrations of diuron from the inlet to the outlet of the Cooroy constructed wetland. The concentrations were relatively unchanged throughout the wetland.

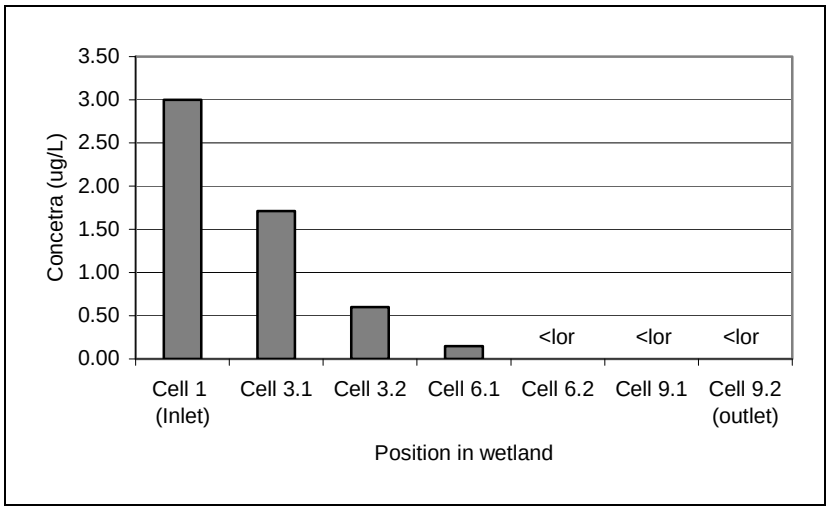

Figure 3. Decreasing concentrations of piperonyl butoxide from the inlet to the outlet of the Cooroy constructed wetland.

3.2 Oxley constructed wetland

3.2.1 Pesticides

Sampling and analyses was carried out for pesticides and herbicides from each of 4 parallel cells in the subsurface wetland. This was to assess if concentrations in the influent into each cell was consistent across cells. Samples were also taken from the outlet of these wetlands to assess if there were differences in removal efficiency with cell type. For many of the pesticides, the concentrations in the outlet water was less than in the inlet water as expected (for example chlorpyrifos, diazinon and tebutryn), however some (DEET, dimethoate) were present in higher concentrations in the outlet water. Diomethoate, in particular was below detection in all inlet water samples but present in all 4 outlet samples. The reason for this will require further investigation. The herbicides were similar between inlet and outlet (Table 4; Figure 4).

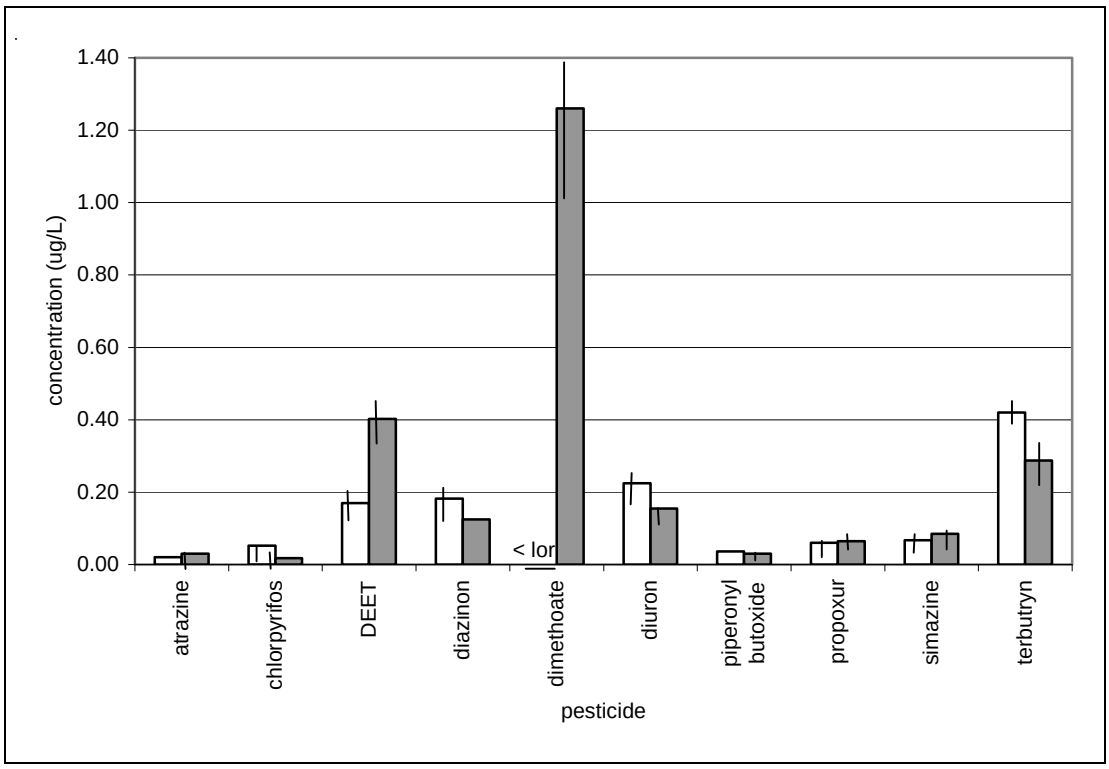

Figure 4 Mean concentration (+ SD) of pesticides from the Oxley subsurface wetland. White bars are water taken at the inlet and grey bars are the water taken at the outlet.

4 RESULTS DISCUSSION

4.1 Cooroy Free Water Surface Wetland

Substances identified in the water from the Cooroy wetland included a range of insecticides and herbicides. In general the Cooroy wetland reduced the concentration of most of the insecticides, but there was little evidence of the same occurring for the triazine or urea herbicides. The herbicides in particular require further investigation, as there appears to be support in the literature for impacts to wildlife. DEET, the active ingredient in insect repellents such as RID™ appears to be present in most samples analysed, but little information about DEET as an environmental contaminant is available.

Table 5 lists pesticides and herbicides found at one or both of the wetlands including information on the status of these as endocrine disruptors. Many of them have been identified as "potential" endocrine disruptors but the amount of evidence for each varies considerably.

4.2 Oxley Subsurface Wetland

Only preliminary data is available for the Oxley subsurface wetland. Dimethoate and DEET, were both present at higher concentrations in the outlet water, than in the inlet.

Table 4 Pesticides in effluent from the Oxley constructed wetland.

	Cell A	Cell A	Cell B	Cell B	Cell C	Cell C	Cell D	Cell D outflow TB
sample type	inflow	outflow	inflow	outflow	inflow	outflow	inflow	outflow
units	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L
atrazine	0.02	0.03	0.02	0.04	0.02	0.02	0.02	0.03
Chlorpyrifos	0.06	0.03	0.06	0.01	0.05	0.02	0.04	0.01
DEET	0.17	0.37	0.18	0.4	0.17	0.48	0.16	0.36
diazinon	0.2	0.14	0.2	0.11	0.18	0.15	0.15	0.1
Dimethoate	<lor	1.83	<lor	1.47	<lor	0.78	<lor	0.96
Diuron	0.21	0.17	0.22	0.14	0.23	0.16	0.24	0.15
phospho tri-n-butyl	0.03	0.02	0.04	0.02	0.03	0.02	0.03	0.02
piperonyl butoxide	<lor	<lor	0.04	0.04	0.04	0.02	0.03	0.03
Propoxur	0.06	0.06	0.07	0.07	0.06	0.07	0.05	0.06
Simazine	0.06	0.07	0.06	0.07	0.06	0.06	0.09	0.14
Terbutryn	0.44	0.27	0.46	0.31	0.42	0.26	0.36	0.31

Table 5. Substances detected in one or both of Cooroy and Oxley constructed wetlands.

Pesticide	Class	Known mode of action	EDC status	Reference
DEET	insect repellent	repellent	unknown	-
Diuron	urea herbicide	herbicide	unknown	-
fipronyl	organophosphorous pesticide	cholinesterase inhibitor	unknown	-
piperonyl butoxide	synergist	synergist for synthetic pyrethroids	unknown	-
proxopur	carbamate insecticide	cholinesterase inhibitor	unknown	-
carbaryl	carbamate insecticide	contact insecticide	Suspected EDC	Keith, 1997
chlorpyrifos	organophosphorous pesticide	cholinesterase inhibitor	Suspected EDC	Keith, 1997
dimethoate	organophosphorous pesticide	cholinesterase inhibitor	Suspected EDC	Rawlings et al, 1998
diazinon	organophosphorous pesticide	cholinesterase inhibitor	unknown	-
atrazine	s-triazine herbicide	selective herbicide of broadleaf and grassy weeds	estrogen receptor antagonist	Colborn et al, 1996
simazine	triazine herbicide	selective herbicide of broadleaf and grassy weeds	estrogen receptor agonist	Keith, 1997
terbutryn	triazine herbicide	inhibitor of photosynthesis	estrogen receptor antagonist	EDSTAC, 1998
17aethinylestradiol	synthetic estrogen	estrogen receptor agonist	estrogen receptor agonist	EDSTAC, 1998
17bestradiol	natural estrogen	estrogen hormone	estrogen receptor agonist	EDSTAC, 1998
dieldrin	organochlorine	insecticide	estrogen receptor agonist	EDSTAC, 1998
estrone	natural estrogen	estrogen receptor agonist	estrogen receptor agonist	EDSTAC, 1998

Others such as the herbicides diuron and terbutryn, and organophosphates appeared to decrease and others did not appear to change significantly (fipronyl). More data is required to see if these trends are typical.

There are a number of constraints on the capability to predict effects based on chemical measurements alone because some EDC chemicals are known to be biologically active at concentrations less than the limit of reporting, and there are differences in estrogenic potency of the substances themselves. For example, DDT is reported to have 0.000001 times the potency of 17βestradiol. The estrogenic potency of nonyl phenol has been reported as 0.000003 (Soto *et al.,* 1992). Exceptions to this are diethylstilboestrol (10 times the potency of 17βestradiol) and the synthetic estrogen 17αethinylestradiol (2-10 times the estrogenic potency of 17βestradiol). For many compounds this information is unknown.

5 CONCLUSIONS

Among the constraints in using direct chemical measurement of a suite of compounds in a complex mixture, is to be able to identify the full range of components and how to account for interactions between them. They may interact as synergists (one chemical may increase the effect of another), antagonists (one chemical may reduce the effect of another) or in an additive manner (the effect of the mixture is the sum of the individual effects). Even if individual responses are known the likely interactions both on a temporal and spatial scale are rarely understood. It has also been hypothesised that either the sewage system or sewage treatments may reactivate some of the EDCs that are being found in the final effluent.

An approach currently being used world-wide is the use biomarkers of exposure and biomarkers of effect in response to whole effluent exposure. These are physiological responses in a test animal (eg. fish), or a cell line, which can provide a measure of the combined chemical/hormonal activity of the mixture. If there is negligible or no response, then the risk is also negligible. This approach can be used to provide information on whether to proceed with further investigation or risk reduction.

Endocrine disruption to wildlife or humans depends on a number of factors including concentration in the environment or in food and water, duration of the exposure, and average daily intake. It is well recognised that while causality has been difficult to demonstrate there are significant data gaps and a need to establish validated *in vitro* and *in vivo* screening assays for estrogenic and androgenic chemicals. Investigations should include environmental, economic, health and social considerations with science based risk assessments that are relevant to environmental and human health, are economically responsible and useful in a resource management setting. While this is valid in terms of current knowledge it should be recognised that our knowledge on the behaviour and effects of EDCs is limited.

6 Acknowledgements

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USING SPECIFIC BIOMARKERS TO INDICATE PAHS POLLUTION IN MARINE COASTAL ENVIRONMENT

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Abstract

With increasing emphasis on the assessment and monitoring of marine coastal environment, appropriate and useful biomarkers for these locations need to be developed. Our study has been demonstrated that parent PAH residues and some biochemical biomarkers such as catalase, superoxidase and lipid peroxide level could response to Polycyclic aromatic hydrocarbons (PAHs) exposure and be the early warning singnals to indicate PAHs pollution. We also found that PAHs Metabolites in fish bile could provide useful information regarding contaminant exposure and adverse effects in populations in situ. In this study, the analysis methods of 1-hydroxy pyrene (1-OH Py) and 3-hydroxy benzo (a) pyrene (3-OH BaP), and metabolites of pyrene and benzo (a) pyrene in fish bile were established using fluorescence and HPLC, respectively. The fishes (*Lateolabrax japonicus* and *Sparus macrocephalus)* were exposed at different concentrations of Py and BaP (0.1-10 μg/L) for 7 days. The results showed that the fish could be induced to metabolize and eliminate their metabolites in vivo with the increasing of Py and BaP concentrations in seawater. The higher concentrations of Py and BaP were, the higher metabolites concentrations in fish bile were found. A significant dose-related increase of the metabolites concentrations in bile was observed at exposure 1, 3 and 7 days for pyrene and 2, 4 and 7 days for benzo(a)yrene respectively. These results provide the bases to further study of the ecotoxical effects of organic pollutants as to establish the early warning signals in the marine environment.

Keywords: PAHs, metabolites, bile, pollution monitoring, marine fish

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the marine environment as a result of uncontrolled spills, river transport, surface runoff and atmospheric deposition (Clark, 1992). Both laboratory experiments and field surveys unequivocally demonstrated that PAHs adversely affect estuarine and marine ecosystems. PAHs are implicated in the development of lesions and tumors in fish，they produce biochemical disruptions and cell damage that lead to mutations, developmental malformations and cancers (Phillips, 1983; Autrup, 2000; Pavanello, et al, 2000; Schoket, et al, 2001; Bols, et al, 2001; Biró, et al, 2002; Viganò, et al, 2002).

During the last decade, many studies had been conducted on monitoring the inputs, fluxes and fate of PAHs in the marine environment. Now, the relationships between PAHs exposure and the biological effects in aquatic organisms are in great concern. In

order to assess the state of the marine ecosystem, it is important to develop some appropriate and useful biomarkers for these locations. Our study has demonstrated that parent PAH residues and some biochemical biomarkers such as catalase, superoxidase and lipid peroxide level could response to PAHs exposure and be used as early warning singnals to indicate the PAHs pollution. Instead of measurement parent PAH residues, the determination of PAH metabolites in fish can avoid underestimation of true uptake (McElroy et al., 1989). The measurement of PAHs metabolites in fish is an easy-to-use, accurate and cost-effecient technique.

In the present study, the analysis methods of metabolites, such as 1-hydroxy naphathene, 1-hydroxy pyrene (1-OH Py) and 3-hydroxy benzo (a) pyrene (3-OH BaP) in fish bile were established using fluorescence and HPLC, respectively and evaluated as a means of assessing exposure to PAHs.

2. **Using the metabolites of naphthalene and pyrene in marine fish to indicate PAHs pollution**

Naphthalene and Pyrene are the basic components of PAHs, and the metabolites of pyrene adequately reflects the exposure level to total PAHs, and they are co-carcinogenic (Haddads, et al, 1997; Rao, et al, 1989; Strickland, et al, 1999). Thus, they are useful to study their metabolic activity in fish.

2.1 Fixed wavelength fluorescence analysis for 1-naphthol and 1-pyrenol

Fish bile samples were diluted 1:1600 in 48% ethanol. The fluorescence spectra of 1-naphthol and 1-pyrenol were scanned (Fig1). Fixed wavelength fluorescence was then measured at the excitation/emission wavelength pairs 297/465 nm for naphthol and 345/386 nm for pyrenol. Measurements were performed in quatz cuvettes through fluorescence spectrometry (Cary Eclipse, Varian, USA), and slit widths were set at 2.5 nm for both excitation and emission wavelengths. The standard curves were drawn on 1-naphthol and 1-pyrenol for the calculation of their concentrations in fish bile (Fig2).

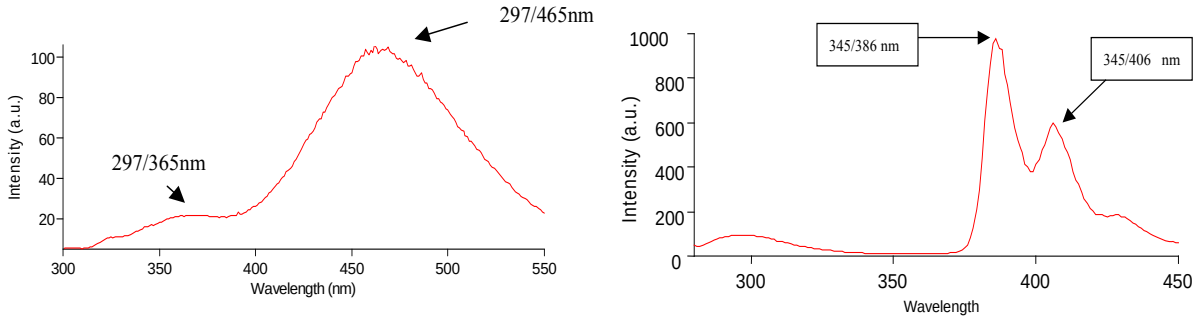


Fig. 1 The fluorescence scan of 1-naphthol (1μg/ml) and 1-pyrenol (10 ng/ml)

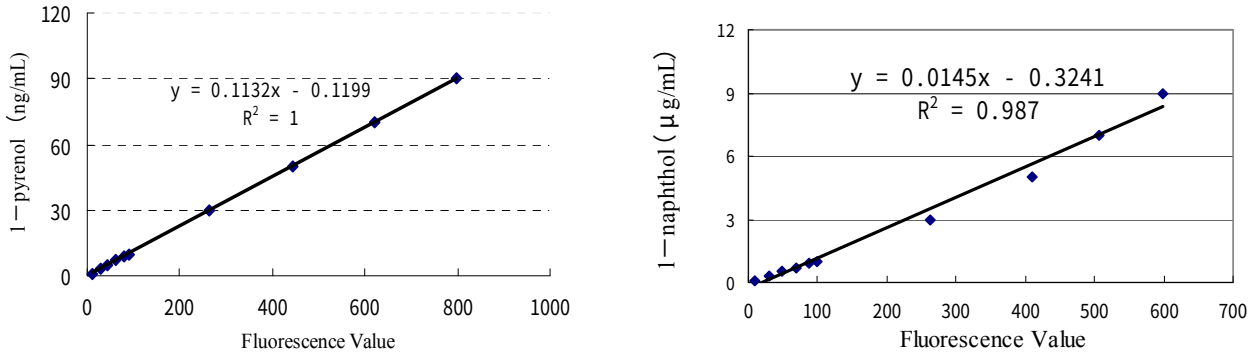


Fig. 2 Standard curve of 1-naphthol (297/465 nm) and 1-pyrenol (345/386 nm)

2.2 Pyrene metabolism in the liver of Lateolabrax japonicus

Weever *Lateolabrax japonicus* (210±30 g in weight, 24.5±3.5 cm in length) were exposed at different concentrations of pyrene (0.1, 1.0, 10.0 ug/L). Fish bile was collected at 2, 4, 5, 7 and 11 day respectively. Pyrene and pyrenol concentrations in bile were analyzed by GC-MS and Fixed wavelength fluorescence. In liver, pyrene and naphthalene were metabolized into pyrenol and naphthol respectively. They were easily depurated from liver and passed into gall bladder. Therefore, pyrene and pyrenol concentrations in gall, and the ratio of $C_{(1\text{-pyrenol, bile})}/C_{(\text{pyrene, bile})}=K$ could be used to indicate its metabolism in liver. In reference group, K values were stable with the passage of time; but in all exposure groups, K values increased greatly with exposure time (Tab1). These data showed that *Lateolabrax japonicus* could be stimulated to metabolize pyrene with the increase of pyrene concentrations in seawater. In fact, a significant relationship was found between pyrene concentration in seawater and the concentration of 1-pyrenol in fish bile (Fig3), pyrenol concentration in fish bile could be used to indicate pyrene concentration in seawater.

Table 1 Comparison of pyrenol and pyrene concentration in bile when fish were exposed to pyrene in different concentrations

day	Group	$C_{pyrenol}$ (μg/ml)	C_{pyrene}(ng/ml)	$K=C_{1\text{-pyrenol}}/C_{pyrene}$
1	Control	0.737±0.226	4.4	168
	Acetone Control	0.799±0.358	4.3	186
	0.1 ug/L	1.069±0.501	3.9	274
	1.0 ug/L	1.217±0.547	17.5	70
	10.0 ug/L	18.81±1.72	207.6	91
3	Control	0.638±0.124	4.4	145
	Acetone Control	1.199±0.311	4.1	292
	0.1 ug/L	2..321±0.801	4	580
	1.0 ug/L	6.598±2.892	14.1	468
	10.0 ug/L	65.17±13.31	35.5	1836
7	Control	0.768±0.210	4.4	175
	Acetone Control	2.524±0.402	3.8	664
	0.1 ug/L	5.632±0.792	3.7	1522
	1.0 ug/L	22.9±8.106	17.5	1309
	10.0 ug/L	212.9±2.928	93.8	2270

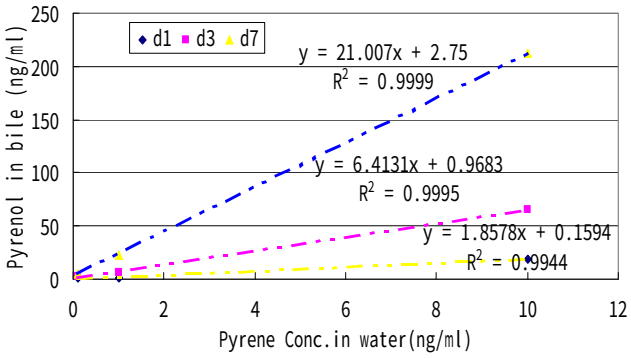


Fig. 3 The relationship between pyrenol concentration in bile and pyrene concentration in seawater at different exposure time

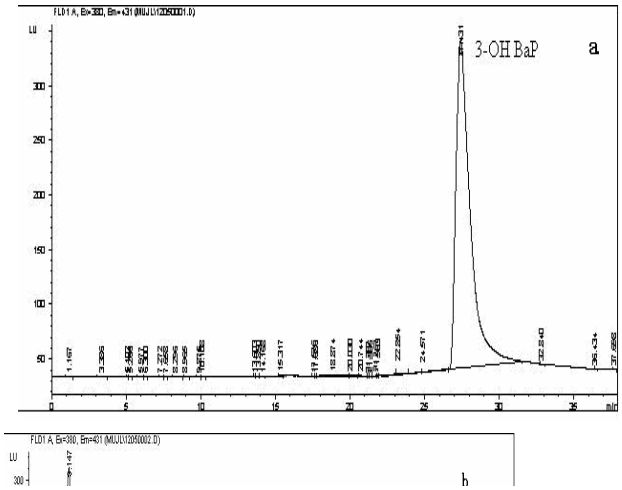
3 Using the metabolites of benzo(a)pyrene in marine fish to indicate PAHs pollution

Benzo(a)pyene(BaP), one of the most carcinogenic PAHs occurring in the marine environment, can be bioactivated by hepatic CYP1A to many metabolites in fish. Metabolite levels are indicative of a short-term BaP exposure, and provide some information on the metabolism of marine fish.

3.1 HPLC analysis for 3-benzo(a)pyrene in fish bile

The hydrolyzed bile was analyzed using a modification of the method of Van Schanke et al (2001). 20μl of fish bile was added to 460μl of HPLC grade water in a microcentrifuge tube with 20μl β-glucuronidase and arylsulfatase enzyme solution, containing 30 and 60 U/ml activity, respectively. The mixture was incubated in a shaking-water bath at 37℃. After 1h, the reaction was stopped by the addition of 1500μl of chilled methanol to give a final 4 fold dilution. After centrifugation (16000g for 20 min) supernatants containing deconjugated metabolites were transferred to the individual amber vials and stored at 4℃ before HPLC analysis.

Bile metabolites were separated by HPLC with a fluorescence detector (λex/λem=380/430) using a reversed-phase Alltech Platinum C18 analytical column (250 ×4.6mm ID，5μm). The column assembly temperature was held at 40□. Samples (20μl) were separated using methanol-water (50/50, v/v) for 5 min followed by a linear gradient to 100% methanol over 30 min and final 5 min at 100% methanol. The flow rate was 1ml/min. The Representative HPLC traces obtained for 3-OH BaP standard and deconjugated bile were shown as Fig 4.



3.2 Benzo(a)Pyrene metabolism in the liver of Black porgy

Black porgy *Sparus macrocephalus* (11.2±1.5g in weight and 9.12±1.3cm in length), were exposed to the different concentrations of BaP (0.5, 1.0, 2.0 and 5.0μg·L-1) for 14days. Fish were sampled at exposure 2h, 4h, 12h, 1d, 2d, 4d, 7d and 14d. The gall bladder was removed from each sacrificed fish and snap frozen in liquid nitrogen and analyzed using the HPLC method mentioned above.

The results showed that the fish could be induced to metabolize and eliminate their metabolites in vivo with increasing BaP concentrations in seawater. The dominant compound detected was always 3-OH BaP, which contributed 75 to 82% of all total BaP metabolites. Concentrations of 3-OH BaP reached maximum after 4d of exposure with a plateau for the 7days and then decrease (Fig 5, a). A significant dose-related increase of the metabolites concentrations in bile was observed at exposure 2, 4 and 7 days (P=0.998, 0.93 and 0.94) (Fig 5,b).

The rapid metabolism and elimination of polycyclic aromatic hydrocarbons (PAHs) by vertebrates, such as fish, result in low residual concentrations of PAHs in muscle and liver tissues (Van der Oost et al., 1994). Chemical analysis of fish tissues therefore has limited usefulness as an indicator of environmental exposure to PAHs. Several fish species living in habitats contaminated by PAHs have been shown to contain high concentrations of biliary PAHs metabolites (Krahn et al., 1987), which may therefore provide an alternative indicator of exposure. In the environment, fish may be exposed to BaP, and BaP may be bio-transformed to numerous metabolites including 1-OH-BaP, 3-OH-BaP, 4,5-dihydrodiol-BaP, 7,8-dihydrodiol-BaP, 9,10-dihydrodiol-BaP, 7-OH BaP and 9-OH BaP. Both 3- and 9-OH-BaP are of toxicological interest because they are precursors of mutagenic metabolites. 3-OH-BaP is a typical metabolite in fish because it is more chemically stable than others metabolites and is a major metabolite in all total BaP metabolites.

Generally, PAHs is rapidly metabolized by fish liver. The metabolite concentrations in fish bile indicate the recent PAHs exposure levels. While the accumulation of PAHs metabolites in the gall bladder will depend on the levels of exposure, their concentration

in bile is influenced by feeding status. Richardson et al.(2004) found PAHs metabolite concentrations in bile of plaice (Pleuronectes platessa) increasing with the prolong of exposure in starvation condition, but no significant increase was observed in feeding condition, and concluded the bile production and release was dependent on the feeding regime, the metabolite concentrations in bile could be affected. In this study, black porgy was feed during the experimental period. The gall bladder samples were collected about 8-10 o' clock in the morning to reduce variations associated with the sample time. Therefore, it can be thought that the 3-OH BaP concentrations in bile have a certain association with the changing of CYP1A.

In this study, an obvious time- related increase and then decrease of the 3-OH BaP concentrations were observed, which indicated a high level of metabolic rate of BaP in fish. The 3-OH BaP concentrations began to drop gradually after exposure for 7d. We considered that the competing conjugation and the change on the capability of detoxification system in liver of black porgy may affect on them. In phase II reaction, four emzymes have somewhat competing roles in BPDE formation, glutathione-S-transferase (GST), glucuronyl transferase (GT), sulfotransferase (ST) and expoxide hydrolase (EH). GT and GST can conjugate BaP metabolites, facilitating excretion before adducts form. In a similar way, Beyer (1997) and Aas et al (2000) found that the BaP metabolites concentrations in bile of flounder (*Platichthys flesus*) and English Sole (*Parophrys vetulus*) with exposed to BaP slowly increased from the exposure with a plateau for the 14d and 30d and a following decrease. Similar time responses have been observed in Carp (Cyprinus carpio) exposed to PAHs. Aas et al (2000) suggested the long time-accumulation and CYP1A response lead to the decrease of BaP concentrations.

A large amount of lab and field investigations indicated there was a co-relativity between BaP metabolites and exposure concentrations in seawater, which not only to evaluate the BaP current pollution but also to response the bioavailability of BaP in liver. In this study, the results showed a significant dose-related increase of the

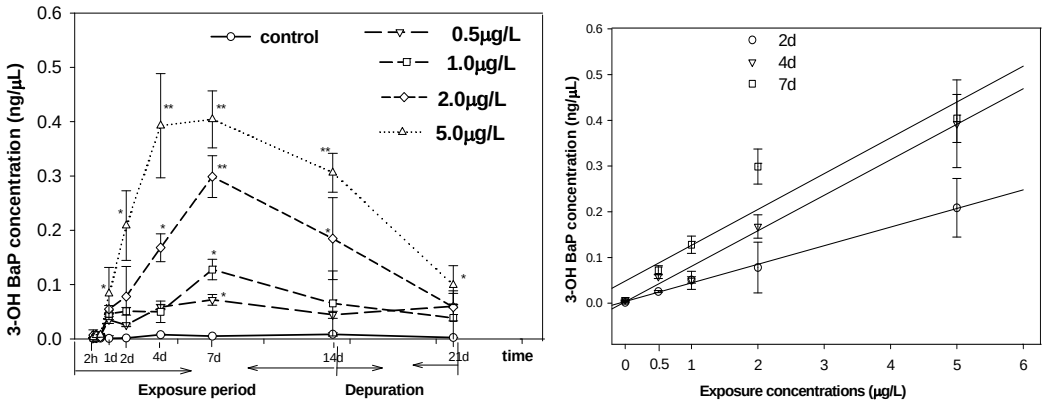


Fig5. The variation of 3-OH-BaP concentration in bile of black porgy at 2h, 6h, 12h, 1d, 2d, 4d, 7d and 14d exposed to BaP and depurated for 7 d. respectively.
Statistical differences: *P<0.05; **P<0.01.

metabolites concentrations in bile at exposure 2, 4 and 7 days (P=0.998, 0.93 and 0.94). This was consistent with Hellou and Upshall et al (1995) whose study showed that PAHs metabolites in bile has a positive correlation with the PAHs levels in sediment, and conclude that PAHs metabolites in bile may better be as a biomarkers to indicate the PAHs level than hepatic EROD activities. Boleas et al (1998) reported that the BaP metabolites in bile reflected the parent BaP concentrations in seawater, and could be used as a suitable tool for monitoring BaP pollution. For the 2.0 and 5.0μg/L groups, the 3-OH BaP concentrations were strongly reduced after 1 week exposure, but were still significantly higher than the control group. These results demonstrated that 3-OH BaP concentration in fish bile is a sensitive biomarker to assess recent exposure to BaP, but it cannot fully reflect a long-term BaP exposure.

Conclusion

It is well recognized that metabolites play an important role in the metabolism and clearance of PAHs. The significant dose-related increase of the metabolites concentrations in bile indicated that PAHs Metabolites can be used as useful and specific biomarkers to indicate PAHs pollution in the marine coastal environment. However, when using the metabolism as biomarkers, several factors need to be considered such as exposure time, sensitivity, variability and methodology.

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EFFECT OF POLLUTANT ON ENZYMATIC BIOMARKER WITH SEASONAL VARIATION – A CASE STUDY IN MASAN BAY, KOREA

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Abstract

Most biological indicators can be expected to vary with natural factors such as age, sex, maturity, season and reproductive status (all of which themselves co-vary) and enzymatic biomarkers are no exception. For example, the dependence of EROD (ethoxyresorufin *O*-de-ethylase) activity in fish liver, which is used widely as a biomarker of organic contaminant exposure, on sex and season has been shown explicitly. In this study, the variation of ChE in marbled sole from the Haegeumgang "reference" site was investigated. Masan Bay is a large harbour in the southern part of Korea which receives a wide range of industrial and sewage discharges and wastes from shipping activity. Previous studies have shown that there are high concentrations of PCBs and TBT (among other chemicals) in water and in sediments. We choose the flatfish for monitoring fish. Since most contaminants of concern are sediment-bound, the selection criteria were that the fish should be preferably a benthic species (thus maximizing its exposure to contaminated sediment). Fish from Masan Bay have higher CYP1A1 mRNA induction and EROD activity than those from the Haegeumgang in spring and but Brain-AChE were inhibited in Masan fish in spring but not later in the year. The results from this study suggest that *Limanda yokahamae* in Masan Bay may affect by other environmental factor.

Introduction

Masan Bay is a large harbour in the southern part of Korea which receives a wide range of industrial and sewage discharges and wastes from shipping activity. It is a semi-enclosed embayment and with Changwon and Chinhae cities surrounded. High concentrations of PAHs (207-2067 ng/g dry wt. in sediment) and TBT (60ng Sn/g in the flounder liver) in Masan Bay were recorded . Total concentrations of PCDDs/DFs in mussel and clam were 750 pg, and 3418 pg per lipid weight, respectively (Im et al., 2004). These results show a clear need to study the physiological effects on the native fish in the Masan bay. In addition, most of the studies have focused on the level of pollutant there are few studies to biological risk assessment.

Polyaromatic hydrocarbons(PAHs), Polychlorinate biphenyls(PCBs) and other organic compounds are known to be marine environmental pollutants(Goksoyr and Forlin, 1992). Cytochrome P-450 1A as measured by 7-ethoxyresorufin-*O*-deethylase (EROD) activity can be induced by organic compounds such as PAHs and PCBs (Celander et al., 1993; Van der Weiden et al., 1994). The induction of CYP1A system in different fish species has been used in many studies as a monitoring tool in field studies (Van der Weiden et al., 1991; Eggens et al., 1995; Stein et al., 1998; Watanabe et al.,

2004).

In the present study, we carried out biochemical and physiological experiments on a native fish from spring 2004 to 2005 to assess the toxic response simultaneously caught in two sites of the south sea in Korea. We described the ChE activity in marbled sole from an industrial harbour, the inner reaches of Masan Bay with that in sole from a reference site, Haegeumgang, in Geoje Island. We also describe seasonal variation (or its absence) in ChE activity from the reference site. For comparison with ChE we have also measured indices of cytochrome P-450 1A (CYP1A) activity in these fish. The CYP450-linked mixed-function oxygenase (MFO) enzyme system has been extensively studied in the field. There were been broad scale studies in association with a variety of contamination sources including PAH, pulp (Payne et al., 1988; Miller et al., 2003; Lee and Anderson, 2005). Therefore, the use of these two measurements, each responding to different contaminants, provides a more comprehensive picture of the effects of contamination in Masan Bay.

Materials and Methods

Marbled sole were caught by gill-netting on six occasions between March 2004 and March 2005 from the reference site at Haegeumgang. Sole were gill-netted on three occasions at Masan Bay, on dates roughly coinciding with some of the reference site collections. Reference site fish were transported live to the laboratory for processing and Masan Bay fish were processed on the dockside in Masan. Fish were killed by a blow on the head, and length, weight and sex were recorded; and gonad weights were recorded in mature individuals. Samples of brain tissue, muscle (minus skin) and gall bladders from the mid-lateral region (dorsal side) and liver were frozen in cryovials in liquid nitrogen until analysed were frozen at -80°C until analysis. Brain and muscle tissue samples were thawed on ice, and were homogenised and analysed for ChE activity using a microplate reader adaptation of the Ellman procedure (Ellman, 1961) as described by Jung and Addison (submitted). Liver samples were homogenised and microsomes prepared and suspended in 0.1 M phosphate buffer pH 7.6 essentially as described by Addison et al., 1994. Ethoxyresorufin *O*-de-ethylase activity in microsomes was determined using reagent concentrations described by Addison and Payne (1986) in a fluorescence microplate reader with excitation and emission filter set at 544 nm and 590 nm respectively. CYP 1A was estimated by western blotting using a probe raised against a 22 amino-acid oligopeptide as described by Lin et al (1997). Liver microsom separated by SDS-PAGE. Separated proteins were transferred onto PVDF nitrocellulose membrane. Non-specific binding sites on the membrane were blocked with 0.5% goat IgG in TBST (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.05% Tween 20) for 2 hr at 37□. Antibody of CYP1A was diluted 1: 5000 in TBST and the membrane was incubated for 1h at room temperature. After washing, membrane was incubated with secondary antibody (goat anti-mouse IgG biotin conjugate) for 30 min at room temperature. The blots were developed using ABC kit (Vector, Laboratories, Burilingame, CA) for 30 min. Substrate solution containing 0.02% 3,3'-Diaminobenzidine tablets (Sigma) and reaction was stopped with distilled water. Templates for each probe were made from the cloned VTG fragments. cDNA probes were generated by transcription with 5.0 uL of [□-^{32}P]dATP (Boston, MA NEN) using

Random Primer Labeling kit (Takara, Japan). Total RNA of the individual liver tissues was extracted with ISOGEN mRNA purification kit (Wako, Japan). It was separated on 1% formaldehyde agarose gel, transferred to Hybond N^+ (Amersham, UK). RNA was pre-hybridized for 3h in 50% formamide, 5 × SSC (Sodium chloride-Sodium Citrate), 5 × Denhard's sol. 0.1% SDS(Sodium Dodecyl Sulfate) and 10% blocking reagent (Roche, USA). Hybridization proceeded in a solution containing 50% formamide, 5× SSC, 5 × Denhard's solution. 0.1% SDS and 10% blocking reagent, radio labeled probes at 42 □ for 16h. Following hybridization, blots were washed 15 min. each with low (2× SSC, 0.5 % SDS) and high (0.5 × and 0.5% SDS) washed at 55 □. The membranes were analyzed using a BAS 2000 Bio - Image Analyzer (Fujix, Japan).

Protein concentration in the sample were assayed using the commercial BCA kit (Pierce Chemicals) with the product being read in a microplate reader at 550nm, using bovine serum albumin (BSA) as a standard.

Results and Discussion

Fish weight increased fairly steadily from a mean of about 100g at the first collection (mid-May 2004) until the following winter when they reached over 300g (January 2005). Fish length was highly correlated with weight ($r = 0.94$, $n = 41$, $P < 0.001$) and so variables will be considered in terms of fish weight, rather than length, as an indicator of body size. Sex could not be determined easily until about September when the gonads began to mature. Gonad weight increased throughout the winter and presumably these fish are early spring spawners. Considering the environmental concentrations of a wide range of contaminants in the Masan Bay flounder metabolism and thereby growth being affected by this pollutant.

In late spring 2004, sole were approximately the same size at both size, but those at Masan had higher HSI level than Haegeumgang. There are clear difference in ChE activity in Masan bay, with brain AChE being reduced by about 30% (Fig 1), muscle AChE 50% (Fig 2). A similar high level of enzyme inhibition was observed in muscle (BChE by 50%, Fig 3) compared to those from Haegeumgang. A marked induction of MFO activity was also observed in liver tissue of sole Masan fish, in comparison with fish from reference site. EROD activity of Masan fish was induced approximately 5-6 fold higher (Fig. 4). Higher level of CYP1A induction 6-8 fold difference in fish from Haegeumgang from Masan fish was shown (Fig 5, 6). In contrast, Masan bay marbled sole didn't differ from Haegeumgang fish on ChEs activity (Fig.1,2,3) in fall 2004. However, Masan bay fish showed significant induction of EROD and CYP1A level compared to those from reference site (Fig. 4, 5, 6). The activity of ChEs was similar in Masan fish from the reference site, Haegeumgang in winter 2004. A marked induction of MFO activity was also observed in Masan fish, in comparison with fish from the reference site. EROD activity of Masan fish was induced approximately 3-5 fold higher. Our results also support that EROD activity and CYP1A levels can be induced by environmental contaminants (Kirby et al., 1999; Miller et al., 2003; Shim et al., 2003; Myers et al., 1998). Hepatic microsomal EROD activities correlated with CYP1A protein level. This is similar to an earlier field study in which EROD activity was correlated with CYP1A1 and CYP1A2 activities in English sole (Miller et al., 2003). Between spring 2004 and winter 2005, Masan fish had abnormally elevated EROD

activity and CYP1A level than Haegeumgang fish.

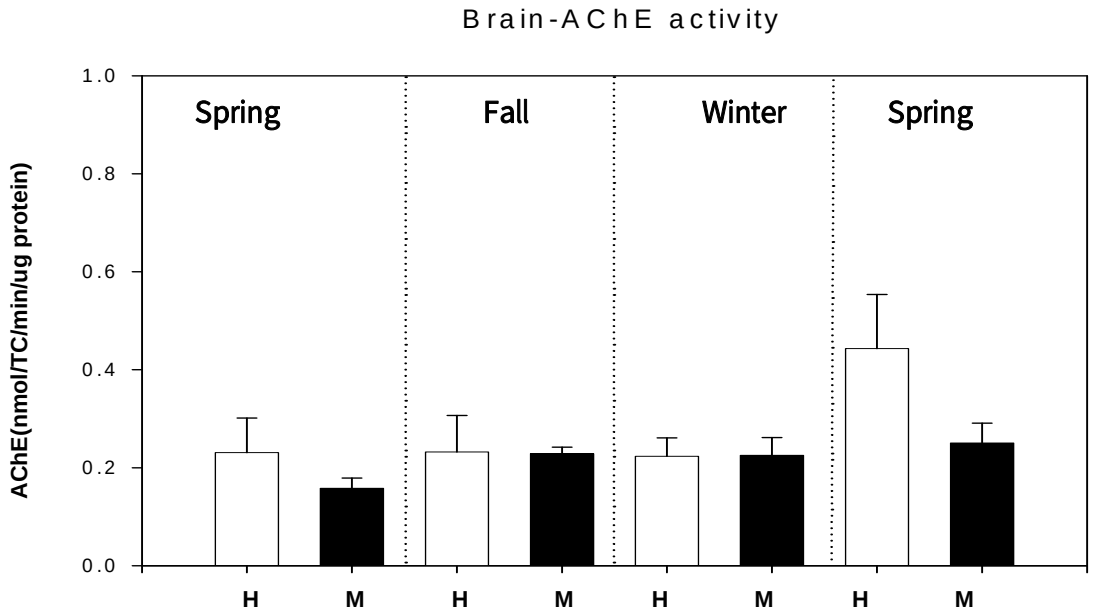

Fig. 1. Seasonal variation of Brain-AChE activity in between Haegeumgang fish and Masan bay fish (H: Marbled sole in Haegeumgang , M: Marbled sole in Masan bay)

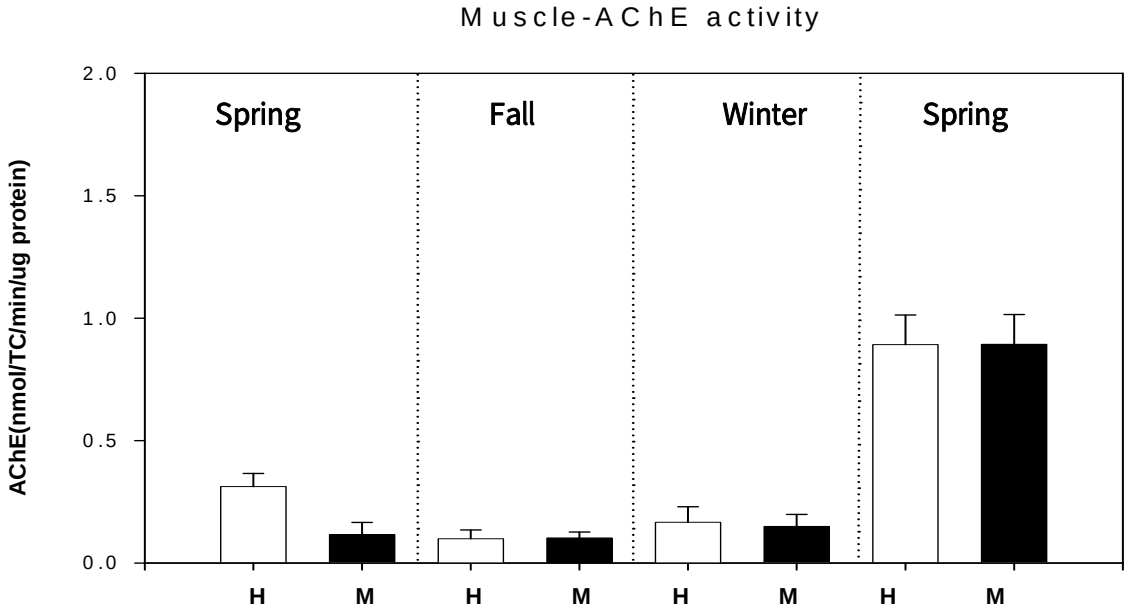

Fig. 2 Seasonal variation of muscle -AChE activity in between Haegeumgang fish and Masan bay fish (H: Marbled sole in Haegeumgang , M: Marbled sole in Masan bay

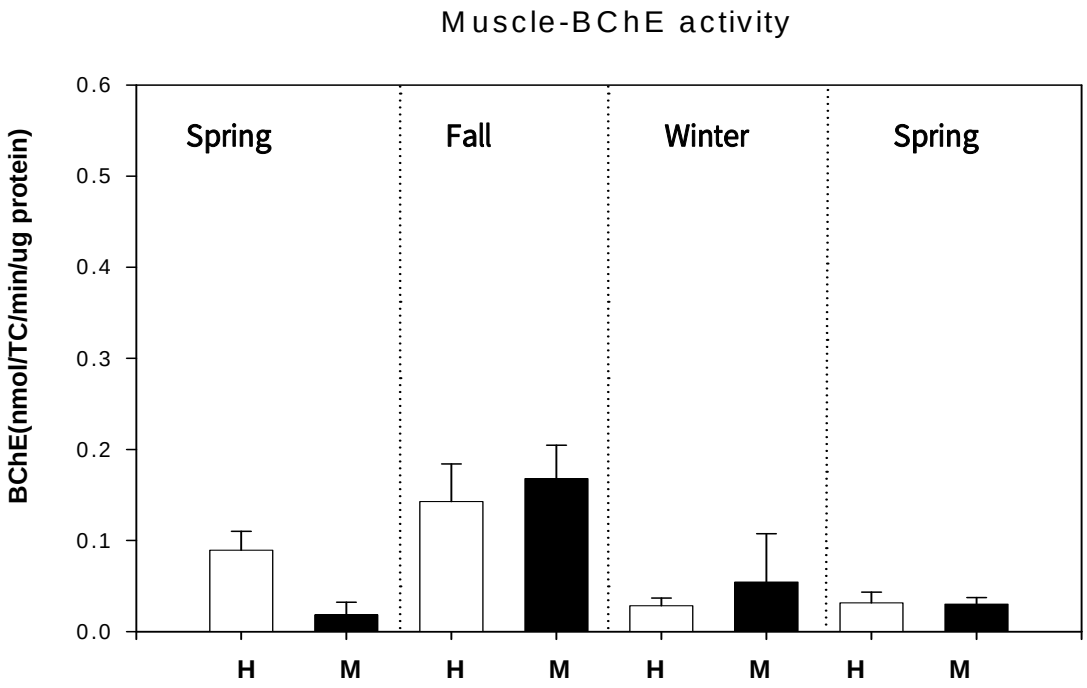

Fig. 3. Seasonal variation of muscle -BChE activity in between Haegeumgang fish and Masan bay fish (H: Marbled sole in Haegeumgang , M: Marbled sole in Masan bay

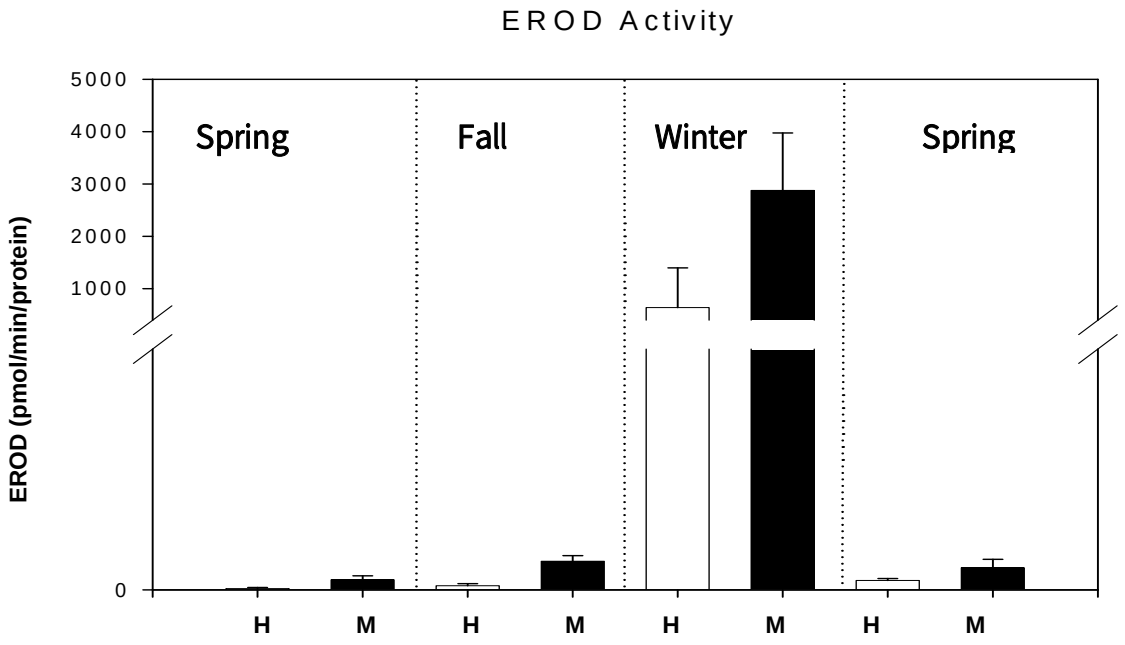

Fig. 4. Seasonal variation of EROD activity in between Haegeumgang fish and Masan bay fish (H: Marbled sole in Haegeumgang , M: Marbled sole in Masan bay)

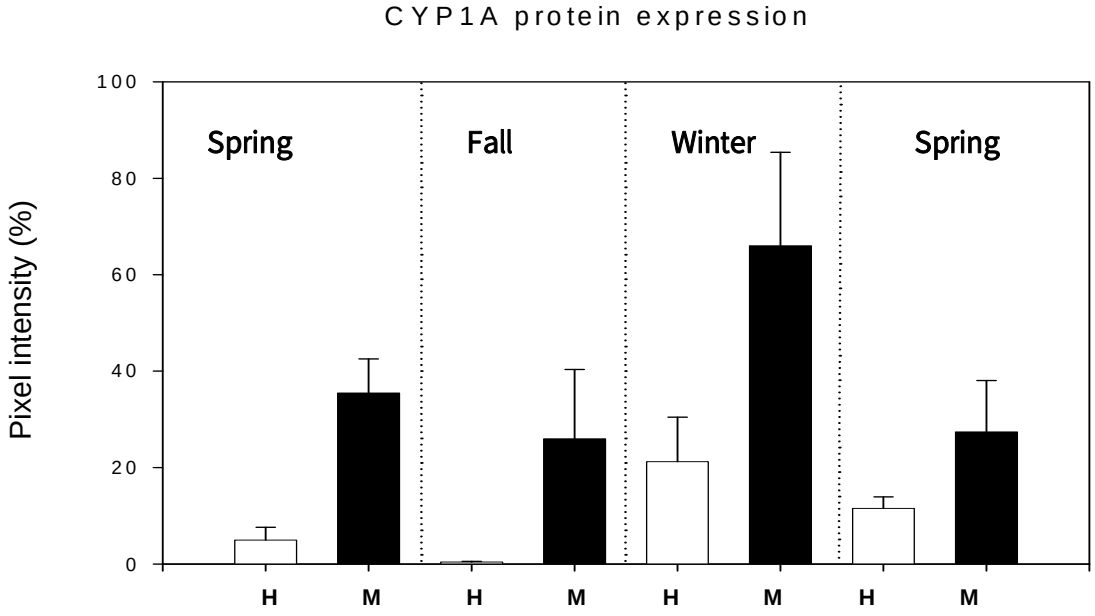

Fig. 5. Seasonal variation of CYP1A protein expression in between Haegeumgang fish and Masan bay fish (H: Marbled sole in Haegeumgang , M: Marbled sole in Masan bay)

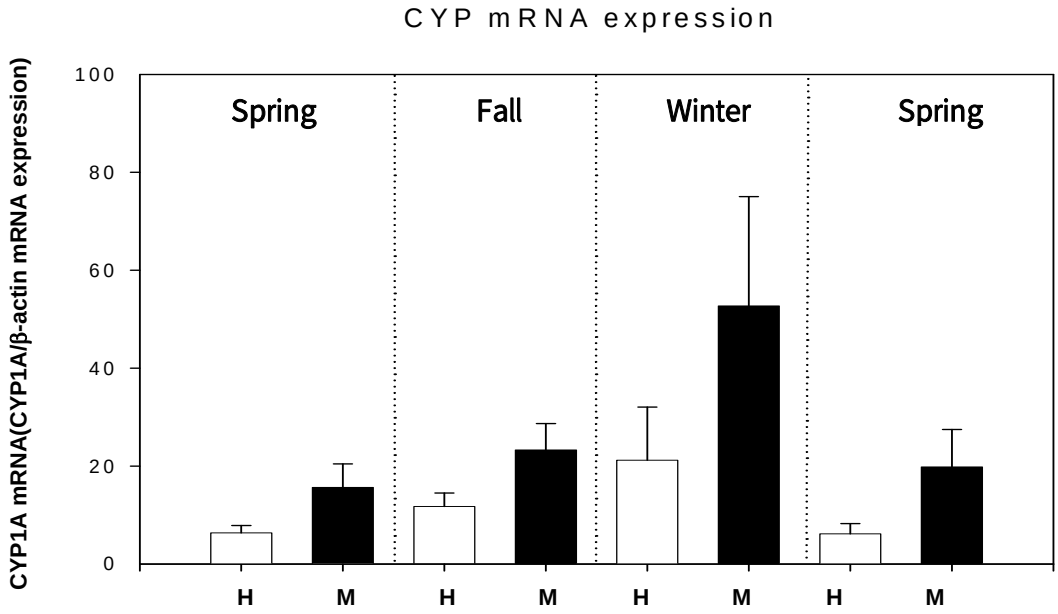

Fig. 6. Seasonal variation of CYP1A mRNA expression in between Haegeumgang fish and Masan bay fish (H: Marbled sole in Haegeumgang , M: Marbled sole in Masan bay)

Temperature is probably one of the most important factors for EROD between the two sites. Sleiderink (et al., 1995) have suggested that negative compensation take place and fish increase their heptic enzymetic MFO activity at low water temperature. But, mean temperatures of sea water in Masan bay was 21°C in June but those of Haegeumgang was 19.8°C. Hence results of induced hepatic EROD in Marbled sole have different from water temperature. Other factors, such as age of the organism, sex and nutrition can also affect the detoxification activity (Bucheli and Fent, 1995). Fish

were approximately the same size at both sites, but those from Masan had significantly larger livers and highly induced hepatic MFO activity. Although the Masan fish were smaller two sampling periods. There spawning might be show earlier than those in Haegeumgang. But they had significantly highly induced EROD and CYP1A expression over one year.

The lower AChE activity at Masan Bay in spring compared with Haegeumgang seem to indicate the presence of organophosphorus compound and carbamates which are known inhibitors of AChE (Galgani, and Bocquené, 2000). In summary, our results showed that exposure to PAHs, as measured by 1-OH pyrene in bile, was higher for Masan than Haegeumgang flounder. The elevated MFO activity and inhibition of AChE in spring in marbled sole from Masan can be suggested an effect from chemical pollutant.

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DEVELOPMENT OF MOLECULARLY IMPRINTED SOL-GEL SPME DEVICES FOR THE DETERMINATION OF POLYBROMINATED DIPHENYL ETHERS

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Abstract

Polybrominated diphenyl ethers (PBDEs) are undoubtedly the most important group of organohalogenated compounds that captures the attention of environmental scientists in recent years. PBDEs are widely used flame retardant additives in polymers and textiles and are present in nearly all of our daily appliances. There are growing evidences that these brominated flame retardants are entering the global ecosystem at a significant rate. In order to assess their risk to the environment and public health, their levels in the various environmental compartments have to be closely monitored. This relies on the development of reliable, rapid and sensitive analytical techniques for polybrominated organic compounds. Solid-phase microextraction (SPME) is one of the advanced sampling and sample preparation techniques that are useful in the determination of trace organics in environmental samples. There are already numerous reports on the use of SPME for PBDE determination in water and solid samples. However, there are still problems to be solved before the technique can be applied to more demanding sample matrices, such as blood plasma. We have examined the applicability of most commercially available SPME coatings in the direct sampling of PBDEs in aqueous media and found them to show rather poor analyte affinity and repeatability, especially towards heavier PBDE congeners. There is a call for the development of new and more robust SPME coatings that possess selective affinity for PBDE congeners. In this context, we explored the feasibility of using molecularly imprinted sol-gel silica films as SPME coatings. A series of ORMOSIL films of various compositions were fabricated and examined for stability under the SPME sampling and GC desorption conditions. The sol-gel silica coating from phenyltrimethoxysilane and tetraethoxysilane (TEOS) is found to be the most suitable candidate. Molecular imprinting of BDE209, the heaviest PBDE congener, into the sol-gel SPME coating can be easily accomplished by dissolving the congener into the sol prior to gelation. The resulting sol-gel SPME coating is found to possess much higher stability and affinity towards PBDE congeners. In this work, we present the detail fabrication procedures for the molecularly imprinted SPME coating and the evaluation of its performance in the determination of BDE209 and other lighter PBDE congeners in various environmental sample matrices.

Solid-phase microextraction of polybrominated diphenyl ethers (PBDEs)

Polybrominated diphenyl ethers (PBDEs) are one of the most important groups of persistent organic pollutants that have aroused much attention in recent years because of their widespread use as flame-retardant additives in polymers and textiles and the fact that they are rapidly accumulating in the global ecosystem. PBDEs are widely used flame retardant additives in polymers and textiles and are present in nearly all of our daily appliances (WHO, 1994). For example, decabromodiphenyl ethers are used in high-impact polystyrene for electronic enclosures and upholstery textiles; octabromodiphenyl ethers are used in business equipment housings made of acrylonitrile-butadiene-styrene resins; pentabromodiphenyl ethers are used in flexible polyurethane foams (de Boer *et al*., 2000; Hardy, 2002a & 2002b; Kalantzi *et al*., 2004). In 2001, over 56,000 tonnes of PBDEs were produced globally and about 43 % were consumed in Asia (BSEF, 2004). This production/consumption rate is expected to have increased even further in recent years. As flame retardant additives, PBDEs are dissolved in polymer materials rather than being covalently bonded. Thus, it is probable that they can be leached out from these materials into the surrounding environment. In fact, there are growing evidences that PBDEs are entering the global ecosystem at a significant rate (de Boer *et al*., 2000; Ikonomou *et al*., 2002; Norstrom *et al*., 2002; Kierkegaard *et al*., 2004). Like other persistent organohalogenated contaminants, PBDEs are able to bio-accumulation (Boon, *et al*., 2002). Numerous recent studies have revealed the presence of PBDEs in human blood and breast milk (Sjödin *et al*., 1999 & 2001; Kalantzi *et al*., 2004).

Because of the environmental and potential human health risks posed by PBDEs, there is a great demand for their convenient, cost-effective and sensitive determination in the various environmental compartments, such as waters, sediments and biota tissues, and in commodity goods. While traditional liquid-liquid extraction is still the most commonly adopted sample preparation method in PBDE determination, more advanced, and environmental-friendly, techniques such as solid-phase extraction (SPE) and solid-phase microextraction (SPME) are beginning to gain more attentions (Polo *et al*., 2004; Salgado-Petinal *et al.* 2006). SPME is a modern extraction technology utilizing a thin layer of stationary phase coating on a fused-silica fibre for analyte extraction (Pawlyszin, 1999). It is a true solvent-less extraction technique and is particularly suitable for applications in aquatic samples as it is free from the plugging problem frequently occurred in solid-phase extraction. It also combines sample extraction and introduction in a single step as analytes sampled by a SPME device can be directly analyzed by gas- and liquid-chromatography without further preparation. Sensitivity of SPME determinations depends on the affinity of the targeted analytes for the SPME stationary phase. Such an analyte-affinity can be expressed in terms of the partitioning coefficient, K_{SPME}, of the analyte between the sample media and the SPME stationary phase:

$$K_{SPME} = \left(\frac{n_S}{V_{SPME}}\right) \Big/ \left(\frac{n_V}{V_V}\right)$$

where n_S and n_V are amount of analyte distributed in the SPME stationary phase and the sample media respectively; V_{SPME} and V_V are volume of the SPME stationary phase and the sample media respectively.

In the determination of non-polar organic contaminants, such as PAHs, in water using a non-polar SPME stationary phase, such as PDMS, K_{SPME} can be as high as 10^6. The resulting high pre-concentration of analytes can lead to outstandingly low method detection limits and relatively small interference. On the other hand, although there are already literature reports on the headspace determination of selected PBDE congeners by commercially available SPME devices, it remains unclear whether common SPME stationary phases are suitable for measuring the broad spectrum of PBDE compounds. Physical strength of commercially available SPME devices is usually insufficient to withstand frequent and repetitive uses. It has been demonstrated that the polyacrylate (PA) SPME stationary phase, which is considered the most appropriate for PBDE determination, has preference towards the partitioning of small to medium size BDE compounds (tetra- and pentabromodiphenyl ethers) (Polo *et al*., 2004; Salgado-Petinal *et al.* 2006). Its affinity for heavier BDE compounds is significantly smaller. We used the heaviest BDE congener, BDE209 (decabromodiphenyl ether), to check the extraction efficiency of two commonly adopted commercially available SPME stationary phases, PDMS and PA, for heavy BDE compounds in water. Table 1 shows that the two stationary phases have rather poor affinity for BDE209.

Table 1 Partitioning coefficients of BDE209 for selected SPME stationary phases

SPME stationary phase	**Partitioning coefficient, K_{SPME}***	
	No NaCl added	**15% NaCl added**
PDMS	2×10^{-2}	17×10^{-2}
PA	7×10^{-2}	21×10^{-2}

* Spike level of BDE209 was 1.0 μg/ml. Sampling temperature and duration were 25 °C and 2 hr respectively.

Although BDE209 is not as frequently monitored as other smaller BDE compounds in the literature, we pick this BDE compound as our model analyte because: (a) it is one of the mostly used flame retardant additives; (b) preliminary monitoring data show that its level in the coastal aquatic environment is high (Liu *et al*., 2005; Thomas *et al*., 2005; Law *et al*., 2006), and (c) it is known to degrade into other smaller BDE congeners (McDonald, 2002).

In view of the apparent deficiencies of commercially available SPME stationary phases in PBDEs determination, we developed an organically modified silicate (ORMOSIL) based SPME stationary phase containing special molecularly imprinted receptor sites for PBDE compounds. Molecular imprinting is a template-directed polymerization process that enables the fabrication of rigid and inert polymeric materials with analyte-specific receptor sites without tedious molecular design and synthesis (Bartsch & Maeda, 1998; Komiyama *et al*., 2003). In fact, there are already

few literature examples on the use of molecularly imprinted polymers (MIPs) as SPME stationary phases (Mullett *et al*., 2001; Fan, *et al*., 2005). Most of the SPME devices reported in the literature were in-tube SPME instead of conventional fibre-type SPME devices. For PBDE determinations, a fibre-type MIP-SPME is more appropriate as it can directly transfer the sampled analytes into the GC injector port. We chose ORMOSIL as the matrix of the MIP-SPME stationary phase as it is sufficiently thermally stable for GC applications (Zheng *et al*., 2001). Such a stationary phase coating is formed on a fused-silica optical fibre and the resultant SPME fibres resemble conventional, commercially available SPME devices.

In this work, we adopted BDE209 as the molecular template for the molecular imprinting. Besides the justifications given above, we proposed that with the imprinting of binding cavities for the largest BDE compound in the ORMOSIL-MIP-SPME stationary phase should also show considerable affinity for other, smaller, BDE compounds. We hope that by using BDE209 as the imprinting template, an ORMOSIL-MIP-SPME stationary phase that is able to sample a wide spectrum of PBDE compounds can be obtained.

Fabrication of the ORMOSIL-based molecularly imprinted polymer SPME stationary phase for BDE209 congener

After studying the properties of various ORMOSIL materials formed from different alkoxylsilianes and sol-gel conditions, the ORMOSIL matrix from tetraethoxylsilane and phenyltrimethoxylsilane (1:3 w/w) is found to be able to produce an even silicate coating of thickness > 5 μm on fused-silica optical fibres with sufficient mechanical strength and thermal stability to withstand repetitive baking at 280 °C. Fig. 1 shows the SEM images of the optical fibre after surface treatment (Fig. 1a) and after the formation of the ORMOSIL coating (Fig. 1b). No surface cracking is observed after more than three rounds of one-hour conditioning at 280 °C in the GC injector port. The coating remains tightly adhered to the fused-silica optical fibre substrate after repeated heating and cooling.

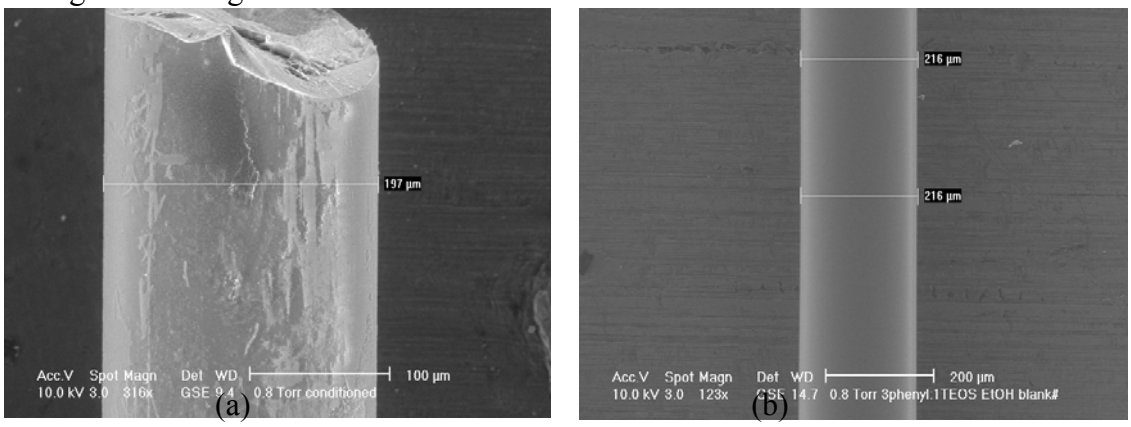

Fig. 1 SEM images of the SPME devices with the ORMOSIL coating: (a) fused-silica optical fibre after surface treatment; (b) an even layer (ca. 9.5 μm thick) of ORMOSIL coating on the fused-silica optical fibre substrate after curing at 120 °C (2 hr) and 200 °C (4 hr) and conditioning at 280 °C for 1 hr.

Imprinting of BDE209 into the ORMOSIL coating is very easily achieved by dissolving the BDE compound into the sol. After the ORMOSIL coating is cured, the BDE templates are removed from the silicate matrix by immersing in a stirring DMSO solution for 12 hr followed by baking at 280 °C in a GC injector port. The latter step also serves to verify the complete stripping of the BDE compound from the ORMOSIL-MIP coating. In all subsequent SPME analysis, ORMOSIL-MIP-SPME devices are conditioned at 280 °C for 1 hr in the GC injector port prior to each sample extraction. Control ORMOSIL-SPME stationary phase coatings were fabricated in a similar way except that the sol did not contain any dissolved BDE209. The control SPME coatings were also cured, stripped and conditioned in a similar way as the ORMOSIL-MIP-SPME coatings.

Performance of the ORMOSIL-MIP-SPME stationary phase in PBDE determination

All the optimizations and evaluations of the ORMOSIL-SPME stationary phase were performed in aqueous media. Fig. 2 shows the effect of ionic strength of the sample matrix on the extraction efficiency of the SPME process. The ORMOSIL-MIP-SPME stationary phase is able to take up much more analyte from the aqueous sample solution than the control stationary phase. A clear salt-out effect is demonstrated and the optimal ionic strength for BDE209 extraction is at 15% (w/w) of NaCl. We expect that such an optimal ionic strength also applies to the SPME sampling of other lighter BDE compounds. At the optimal ionic strength, the partitioning coefficients of BDE209 between the aqueous media and the SPME stationary phases are found to be 7.9×10^3 (MIP-SPME) and 1.7×10^3 (control SPME). Thus, the affinity of the ORMOSIL-MIP-SPME stationary phase for BDE209 is 38,000-folds greater than the commercially available PA-SPME stationary phase.

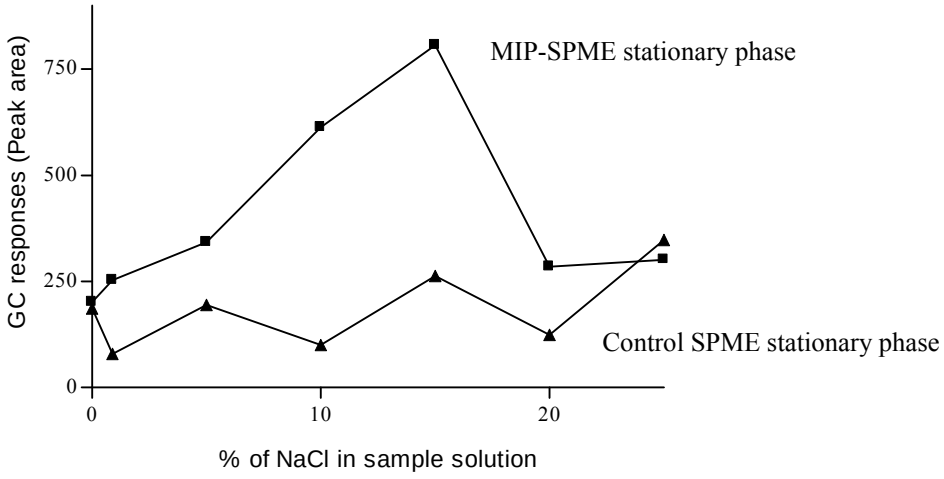

Fig. 2 Effect of sample ionic strength on the extraction efficiency of the ORMOSIL-MIP-SPME process. The sample matrix was Milli-Q water. Spike level of BDE209 in samples was 10 ng/ml. Volume of sample was 5.0 ml. SPME sampling duration was 2 hr.

Fig. 3 shows the time profile of the SPME process using the

ORMOSIL-MIP-SPME stationary phase. Under the optimal ionic strength condition, partitioning equilibrium is achieved in 2 hr. We expect that for other lighter PBDE compounds, the equilibration time will be even shorter.

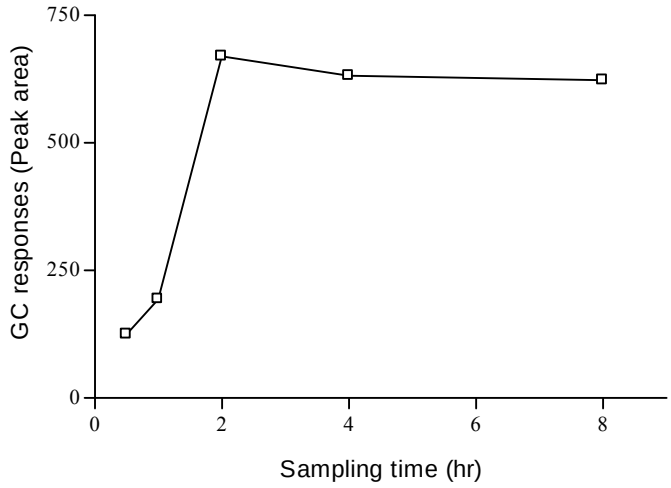

Fig. 3 Time profile of the SPME process using the ORMOSIL-MIP-SPME stationary phase. Sample matrix was Milli-Q water with 15% (w/w) NaCl. Volume of sample was 5.0 ml and spike level of BDE209 was 10 ng/ml.

Fig. 4 shows the repeatability of the ORMOSIL-MIP-SPME process in six consecutive determinations. A repeatability of 12.8% and a detection limit of 1.7 ng/ml of BDE209 (n = 6, $P < 0.05$) were achieved. We expect that the sensitivity of the SPME process will be much higher for lighter PBDE compounds. For information, detection limits of 7.5 to 190 pg/L were achieved for small to medium size PBDE compounds (di- to hexabromodiphenyl ethers) using PA-SPME stationary phase in headspace sampling mode (Polo *et al*., 2004). With the much improved affinity for BDE compounds, the present ORMOSIL-MIP-SPME stationary phase is likely to have even greater sensitivity than the PA-SPME stationary phase. Work on the evaluation of the affinity of the ORMOSIL-MIP-SPME stationary phase for other lighter and more commonly determined BDE compounds such as BDE47, BDE49, BDE100, BDE153, BDE154 and BDE183 are in progress. We expect that with the inevitable binding site heterogenicity induced during the removal of molecular templates from the imprinted binding cavities, a wide spectrum of binding sites of different sizes are present in the ORMOSIL-MIP coating. While these sites may not be available for the binding of BDE209 anymore, they may become suitable for the binding of other, smaller BDE compounds.

Closing remarks

In this work, we demonstrated a convenient method for the fabrication of an ORMOSIL-based SPME stationary phase molecularly imprinted with a brominated diphenyl ether template. Using the largest, and probably the most difficult to analysed, BDE209 compound as the molecular template, we are able to fabricate an ORMOSIL-MIP-SPME stationary phase that shows much greater affinity for the heavy BDE compound (ca. 38,000-folds) than commercially available SPME stationary phases. The ORMOSIL SPME coating also possesses much better physical strength and thermal stability than commercial SPME products. We hope that such an

ORMOSIL-MIP-SPME approach can contribute to the more sensitive determination of PBDEs in more complex sample matrices, such as human blood plasma and other biological fluids.

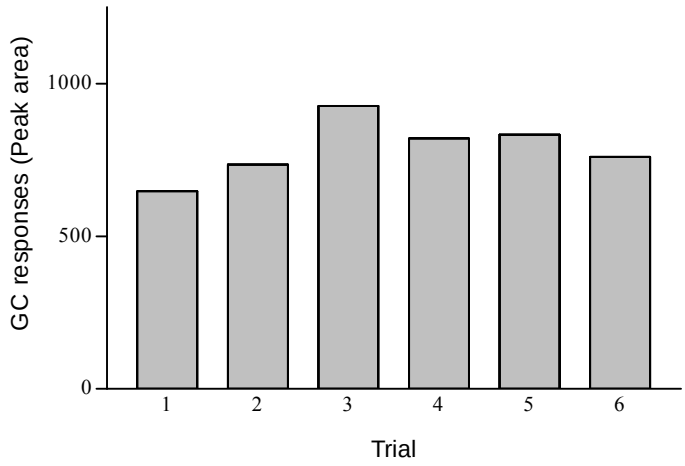

Fig. 4 GC-ECD responses of six consecutive SPME determinations of aqueous samples spiked with 10 ng/ml of BDE209. Ionic strength of the sample solutions was 15 % NaCl. Sampling duration was 2 hr.

Acknowledgement
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Experimental
The protective PDMS coating on a 1-cm segment of a fused-silica optical fibre was removed by burning with a butane torch. The exposed fused-silica surface was treated with a mixture of aqueous NH_3 / H_2O_2 / H_2O (1:1:5 v/v/v) for 1 hr, rinsed with Milli-Q water, followed by another treatment with a mixture of HCl / H_2O_2 / H_2O (1:1:5 v/v/v) for 30 min. The resultant fused-silica surface was rinsed with Milli-Q and air-dried.
The ORMOSIL sol containing BDE209 templates was prepared from 269 μl of phenyltrimethoxysilane, 107.5 μl of tetraethoxysilane, 112 μl of absolute ethanol, 138 μl of Milli-Q water, 2 μl of trifluoroacetic acid and 20 mg of BDE209 in 80 μl of DMSO. The mixture was vigorously mixed and sonicated for 1 min. and warmed to 70 °C in a water bath for 120 min. before another 1 μl of trifluoroacetic acid was added. The fused-silica optical fibre was dipped into the resultant jelly-like sol for 5 min. followed by air-drying for 7 days. The air-dried ORMOSIL coating on the fused-silica optical fibre was then cured by heating to 120 °C for 2 hr. then 200 °C for 4 hr in a furnace. After cooling to room temperature, the fibre was immersed into a stirring DMSO solution for 12 hr followed by inserting into the GC injector port at 280 °C for 3 hr. No GC peak of BDE209 was observed throughout the baking period.
All SPME sampling studies were carried out at room temperature. BDE compound sampled by the SPME device were analysed by an Agilent 6890 GC with a 10 m DB-5MS column and an μECD.

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EXPOSURE OF CATFISH IN MEKONG RIVER DELTA TO POLYBROMINATED DIPHENYL ETHERS AND PERSISTENT ORGANOCHLORINES: ASSESSMENT ON POLLUTION SOURCES AND RISK TO HUMAN CONSUMPTION

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Abstract

Commercial feeds for aquaculture and catfish samples were collected from Mekong River Delta, Vietnam for determination of polybrominated diphenyl ethers (PBDEs) and selected persistent organochlorines including polychlorinated biphenyls (PCBs) dichlorodiphenyltrichloroethane and its metabolites (DDTs), chlordane related compounds (CHLs), hexachlorocyclohexane isomers (HCHs) and hexachlorobenzene (HCB). The most abundant contaminants was DDTs with concentrations ranging from 10 to 700 ng/g lipid wt., followed by PCBs (1.0-80 ng/g); CHLs (<0.01-8.2 ng/g); PBDEs (0.12-3.7 ng/g); HCHs (<0.03-5.1 ng/g) and HCB (<0.07-3.2 ng/g). PBDEs were detected in all samples, suggesting their widespread contamination in the region. However, PBDEs contamination levels in the present specimens of catfishes were low in worldwide comparison. Interestingly, residue levels of all the contaminants were significantly higher in catfishes collected near a municipal dumping site compared to farmed catfishes. This fact suggests that runoffs from the dumping site during floods and rains may have brought pollutants to the surrounding areas. Contamination pattern in aquaculture feeds revealed elevated levels of PCBs and PBDEs in samples from foreign companies, perhaps implying their higher residues in some imported ingredients. Congener profiles of PBDEs and PCBs demonstrated similarity between the farmed catfishes and the aquaculture feeds, suggesting these feeds as major pollution source to the farmed catfishes. On the other hand, the PBDEs and PCBs profiles in the dumpsite catfishes are clearly different with those of the farmed catfishes, revealing their exposure to different sources. Risk assessment showed significantly higher intake of the contaminants by people who eat catfishes cultured near the dumping areas. Further investigation toward fate and occurrences of the contaminants in dumping sites would be necessary.

Keywords: Polybrominated diphenyl ethers; Organochlorines; Catfish; Aquaculture feed; Dumping site

1. Introduction

The Mekong River Delta (MRD) in South Vietnam is one of the most densely populated areas in the world. Approximately, 20 million people live in municipal areas and industrial zones along Mekong River. In this region, most of the sewage is directly discharged into the environment [1]. Besides, household solid wastes and electronic appliances are abandoned in open dumping sites with very poor management. Several studies suggested such discharges as potential pollution sources of various anthropogenic pollutants including Organochlorines (OCs) and Polybrominated diphenyl ethers (PBDEs) to environment [2-4]. Lack of proper waste management in many open dumping sites may redistribute such contaminants into the environment. Thus investigation toward evaluating possible influence of such open dumping sites to the surrounding environment would be necessary.

Catfish aquaculture is a very common practice in MRD, which has rapidly developed, and become an important economic sector. Production of catfish doubled every two years from 1995 and reached 120 thousand tons in 2001 [5]. Understanding contamination status in the catfish is thus important in order to assess possible health risk to the catfish consumers. Besides, due to wide distribution of catfish in the region, examining their contamination profile could provide information regarding pollution sources and accumulation characteristics in aquatic biota. In the present study, we collected catfishes from MRD for analysis of PBDEs as well as some OCs such as PCBs, DDTs, CHLs, HCB and HCHs. The catfish samples included farmed catfish as well as catfish from ponds located near an open dumping site of Can Tho city, Vietnam. We anticipated that runoffs from that site might have brought contaminants to the surrounding environment and thus analyzing catfishes near the dumping site would provide information for further assessments. Our primary objectives were to elucidate contamination status and sources of PBDEs and selected OCs in fishes as well as to assess their potential risk to aquatic biota and humans.

2. Materials and methods

2.1. Sample collection

Twenty farmed catfishes (*Pangasius sp.*) were collected from Can Tho and Cao Lanh provinces, Vietnam, in May 2004. The farmed catfishes are reared in large cages submerged in the river or in ponds near the river and fed with formulated diets. We also collected five catfishes in ponds located near a municipal dumping site (named as dumpsite catfishes, hereafter) in Can Tho city. These ponds were suspected to receive leachate and runoffs from the dumping site during floods and rain events. In general, the dumpsite catfishes were slightly smaller than the farmed catfishes.

Five samples of commercial feeds were also collected from the local markets for the present study. Of these, three are produced by domestic companies (Feed A, B and C) and two by foreign companies (Feed D from The Netherlands and Feed E from US). More details of the feed samples were given elsewhere in results and discussion. All the catfish and feed samples were kept in polyethylene bags and preserved in dry ice during transport to our laboratory where they were stored at -20^{o}C until chemical analysis.

2.2. Analytical methods

OCs were analyzed following the procedure described by Kajiwara et al. [6]. Briefly, 15 g of sample (skinned muscle or homogenized feed) was ground with Na_2SO_4 and extracted using Soxhlet apparatus with a mixture of diethyl ether and hexane (3:1). The aliquot of extract was concentrated to 10 mL and a portion of 2 mL was used for fat content determination by a gravimetric method. the sample was subjected to gel permeation chromatography (GPC) for fat removal. A procedural blank was run for every batch of five samples to verify cross-contamination. PCBs, DDTs, HCHs, CHLs and HCB were quantified by GC-ECD (Agilent 6890N) using DB-1 fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness. The PCB standard used for quantification was a mixture of sixty-two PCB congeners obtained from Wellington Laboratories Inc., Ontario, Canada. Concentrations of individually resolved peaks of PCB isomers and congeners were summed to obtain total PCB concentrations. Recovery rates of the target chemicals through this analytical method were between 80 - 110%. Concentrations were not corrected for recovery rates and expressed as ng/g on lipid wt basis, unless otherwise specified.

PBDEs were analyzed following the method described by Ueno et al. [7]. Fish muscle and feed (15 g) were extracted by Soxhlet apparatus and determined for lipid content as explained above. The aliquot (5 mL) before being subjected to GPC was spiked with 5 ng of ^{13}C-Brominated diphenyl ether congeners (BDEs, including ^{13}C-BDE-3, BDE-15, BDE-28, BDE-47, BDE-99, BDE-153, BDE-154, BDE-183, BDE-197, BDE-207 and BDE-209 as surrogates. The GPC fraction containing organohalogens was concentrated and passed through a column packed with 1.5 g of activated wako gel S-1 (Wako Pure Chemicals Industry Ltd., Japan) for cleanup and fractionation. PBDEs and PCBs were eluted with 80 mL of 5% dichloromethane in hexane. ^{13}C BDE-139 was added to the final solution prior to GC-MSD analysis as an internal standard. Quantification was performed using a gas chromatograph (Agilent 6890N) equipped with a mass-selective detector (Agilent 5973) for mono- to hepta-BDEs, and a gas chromatograph (Agilent 6890N) coupled with a mass-selective detector (JEOL GC-Mate II) for deca-BDE. Recovery of ^{13}C-labled BDEs ranged between 60 and 120%. Concentrations of major PBDE congeners including BDE-3, BDE-15, BDE-28, BDE-47, BDE-99, BDE-100, BDE-138, BDE-153, BDE-154, BDE-183, BDE-196, BDE-197, BDE-206, BDE-207 and BDE-209 were summed to obtain total concentration of PBDEs. The detection limit was calculated as 3 times the procedural blank (0.02 ng/g for mono- to di-BDEs, 0.1 ng/g for tetra-BDE, 0.05 ng/g for tri- and penta- to hepta-BDEs, 0.06 ng/g for octa- to nona-BDEs, and 4 ng/g for deca-BDE).

2.3. Statistical analysis

Statistical analysis was performed with the StatView software (SAS Institute Inc., V.5, 1998). The Mann-Whitney *U* test was used to examine statistical differences between groups ($p < 0.05$). Spearman's rank correlation test was used to examine significance of correlations between residue levels of the contaminants.

3. Results and discussion

3.1. Contamination by PBDEs in catfishes and aquaculture feeds

In the present study, residue levels of all contaminants did not significantly correlate with gender and body size of fishes (data not shown). Therefore, data of all the male and female fishes were pooled for the interpretation. PBDEs were found in most of the catfish and feed samples suggesting their widespread contamination in the aquatic environment. Total concentration of PBDEs was the sum of six major congeners including BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-183. Other congeners from mono- to tri-BDEs and octa- to deca-BDEs could not be quantified in most of the samples (see analytical methods for details of detection limits). Mean concentrations of PBDEs in the farmed catfishes and the dumpsite catfishes were 0.77 and 2.7 ng/g (**Table 1**). Interestingly, concentrations of PBDEs in the dumpsite catfishes were statistically higher compared to the farmed catfishes, suggesting additional exposure of the dumpsite catfishes to PBDEs. It is noteworthy that the dumpsite catfishes were collected from ponds located in vicinity of the Can Tho dumping site. In this dumping site, municipal wastes including household goods and small electric appliances, which may contain PBDEs as flame retardants were dumped. Under ambient conditions, PBDEs may be emitted from such materials and contaminate dumping site soil. Therefore, it is anticipated that runoff and leachate from the dumping site during flood and rains in turn, may have carried PBDEs to the vicinity, causing higher contamination in the catfish.

Table 1: Concentrations (ng/g lipid wt.) of PBDEs and persistent organochlorines in catfish and aquaculture feeds from Vietnam

	Body length	**Lipid (%)**	**ΣPBDEs**	**ΣPCBs**	**ΣDDTs**	**ΣCHLs**	**ΣHCHs**	**HCB**
Catfish								
Farmed catfish	30	3.8	0.77**	7.2**	59**	0.62**	0.47*	0.73**
(n = 20)	(29-32)	(0.6 -7.2)	(0.12-1.4)	(0.91-27)	(7.9-150)	(<0.01-2.6)	(<0.03-1.5)	(<0.07-1.8)
Dumpsite catfish	28	3.6	2.7**	50**	390**	5.7**	2.2*	2.6**
(n = 5)	(25-30)	(3.2-4.1)	(1.4-3.7)	(37-77)	(330-700)	(4.2-8.2)	(0.86-5.1)	(2.4-3.2)
Aquaculture feed								
Feed A (Vietnam)	-	3.4	0.35	6.3	22	1.7	0.46	0.38
Feed B (Vietnam)	-	3.7	0.94	3.3	6.9	0.27	5.7	1.0
Feed C (Vietnam)	-	3.3	1.5	12	47	2.3	3.5	1.3
Feed D (Netherlands)	-	3.4	3.7	20	40	5.2	25	2.4
Feed E (US)	-	3.3	7.0	25	36	2.6	7.7	1.2
Mean (all feeds)	-	3.4	2.7	13	30	2.4	8.5	1.3

ΣDDTs = *p,p'*-DDE + *p,p'*-DDD + *p,p'*-DDT; ΣCHLs = *trans*-chlordane + *cis*-chlordane + *trans*-nonachlor + *cis*-nonachlor; ΣHCHs = α-HCH + β-HCH + γ-HCH

** $p < 0.01$ and * $p < 0.05$ indicate significant difference levels between two fish categories; figures in parentheses indicate the range

PBDEs residue levels in the feed samples were relatively variable. For example, three feeds of Vietnam companies (Feed A, B and C) contained residues of PBDEs below 1.5 ng/g, while feed D and feed E from foreign companies contained 3.7 and 7.0 ng/g PBDEs, respectively. Worldwide data on contamination by PBDEs seemingly demonstrates that PBDEs levels in North America are one to two orders of magnitude higher compared to Japan and Europe [8]. Some ingredients used for feed D and E might be imported from Europe and North America and hence contained more PBDEs residues and potentially caused higher PBDEs concentration in the ultimate products, the feeds.

3.2. Contamination by OCs in catfishes and aquaculture feeds

OCs were detected in all the samples including the farmed catfishes, the dumpsite catfishes and the commercial feeds. Contamination pattern consistently followed DDTs

> PCBs > CHLs > HCB > HCHs. However, OCs concentrations were generally higher in the dumpsite catfish compared to the farmed catfish (Table 1). The pattern in this study clearly demonstrates DDTs and PCBs as two abundant contaminant groups in the environment. In fact, this observation agrees with previous studies of water, sediments, mussels, birds and human breast milk collected from Vietnam [9-11].

Five commercial feeds in this study show relatively similar levels of OCs. The levels are comparable to those in the farmed catfishes, however, much lower than those in the dumpsite catfishes. This result perhaps supports our earlier finding that the dumpsite catfishes may be exposed to additional pollution sources beside aquaculture feeds. Interestingly, feeds with different origins show somewhat different residue levels of OCs. For instance, feed D and E from foreign companies contained higher level of PCBs than those from domestic companies (Table 1). Perhaps higher PCBs residues in the ingredients imported from Europe and USA for production of these feeds have caused the phenomenon.

3.3. Composition of the contaminants

Congener profiles of six major PBDEs found in catfishes and feeds of this study are shown in **Fig. 1**. Generally, in the dumpsite catfishes, BDE-99 was the most abundant congener, accounting for 29%, followed by BDE-47, BDE-153 and BDE-183. On the other hand, BDE-47 had the highest contribution (46%) in the farmed catfishes, followed by BDE-99, BDE-100 and BDE-154. Some congeners such as BDE-153, BDE-154 and BDE-183 were slightly lower in the farmed catfishes compared to the dumpsite catfishes. In order to clarify the usage pattern of PBDEs in Vietnam, composition of PBDEs in all the catfishes from Vietnam were compared with those in commercial products such *penta*-, *octa*- and *deca*-BDE products. The result showed presence of all representative congeners for *penta*-product (BDE-47, BDE-99 and BDE-100), as well as those for *octa*-product (BDE-183) [8], hence suggesting usage of these products in Vietnam. Alternatively, no quantifiable level of BDE-209, the representative congener for *deca*-product was found and therefore it is not yet clear to what extent *deca*-product has been used in Vietnam. Nevertheless, it should be noted that due to low bioaccumulative ability, BDE-209 is often not found in biological samples [12, 13]. In this context, other environmental matrices such as soil and sediment should be investigated to elucidate the presence of *deca*-product in Vietnam.

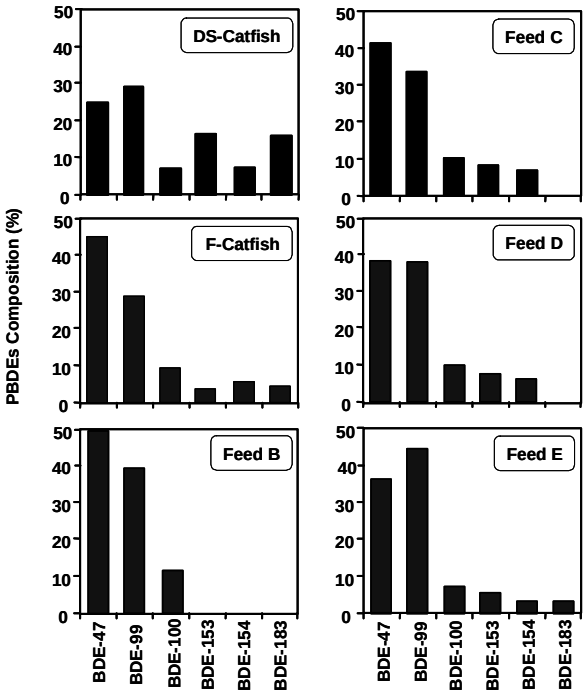

Fig. 1: PBDEs congener profiles in dumpsite catfish (DS-Catfish), farmed catfish (F-Catfish) and

Fig. 2 demonstrated congener profiles of PCBs in three sample groups. In these profiles, relative abundance of each congener was normalized to those of PCB-153 for comparison. The profile of the farmed catfishes is similar to the feeds, except it shows less accumulation of tetra-CBs and penta-CBs in the farmed catfishes. In contrast, the PCBs profile in the dumpsite catfishes was different compared to the feeds and the farmed catfishes. These are important evidences that the aquaculture feed is the major pollution source of PCBs to the farmed catfishes while other sources have strong influence on the PCBs contamination in the dumpsite catfishes. Relative lower abundance of tetra-CBs and penta-CBs in the farmed catfish compared to the feeds may be due to stronger bioaccumulative ability of higher chlorinated congeners such as CB-138 and CB-153 in fish [26]. On the other hand, specific profile in the dumpsite catfishes with low contributions of tetra-CBs and penta-CBs could be due to characteristics of pollution sources [27], which were suspected in this study to be runoffs from the nearby dumping site as well as from human habitat.

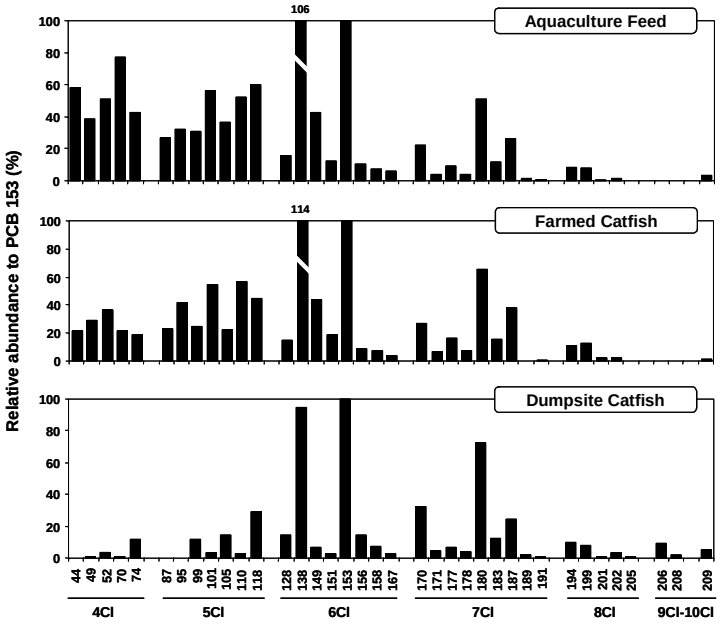

Fig. 2: PCBs congener profiles in commercial feeds, farmed catfish and dumpsite catfish (Number 4Cl – 10Cl indicate degrees of chlorination from *tetra*- to *deca*-PCBs; Numbers

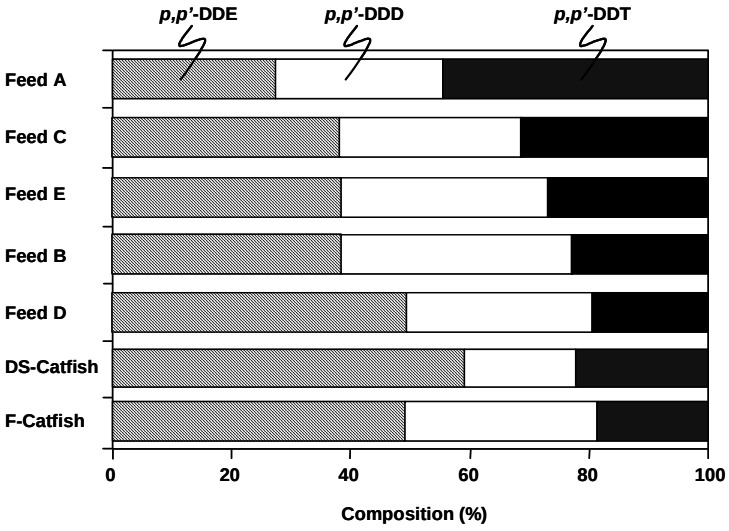

Fig. 3: Composition of DDTs in commercial feeds (Feed A, B & C from Vietnam; Feed D from The Netherlands; Feed E from US,)dumpsite catfish (DS-Catfish) and farmed catfish

Patterns of DDTs in the farmed catfishes, the dumpsite catfishes and feeds are shown in **Fig. 3**. The composition of DDTs appear to be slightly different in the two categories of catfishes, showing *p,p'*-DDT slightly higher in the dumpsite catfishes. On the other hand, the composition in feeds is somewhat different, showing the proportion

of *p,p'*-DDT up to 40% in one sample from Vietnam. This result thus indicates that some feeds might contain relatively high residues of DDT, making them as pollution source to the aquaculture fishes. Besides, Minh et al. [14] reported proportion of *p,p'*-DDT ranging from 15 to 40% in dumping site soils collected from cities of Vietnam. This range is only comparable to those in the commercial feeds. These facts thus may explain the relatively comparable proportion of *p,p'*-DDT between the farmed and the dumpsite catfishes. Composition of DDTs in catfishes of the present study is somewhat similar to those in catfishes collected from Bangladesh in 1997 [15] and Mexico in 1996 [16].

3.4. Toxicological risk assessment

Production of the farmed catfish from large-scale culture accounts for major part in the total catfish production in Vietnam. Alternatively, the production from ponds located near municipal dumping sites is only very minor and entirely consumed by the local people. Nevertheless, the present results demonstrated significant higher levels of POPs in these "dumpsite" pond-cultured catfishes and thus may raise concern on possible health risk for the local people who consume these fishes.

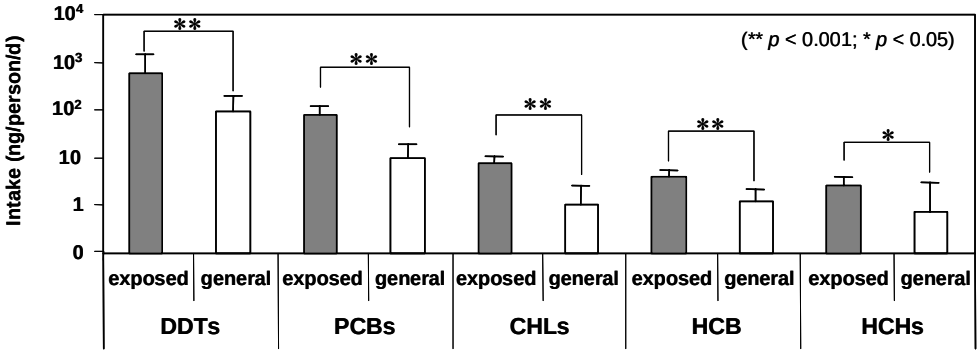

Fig. 4: Comparison for intake of the contaminants via catfish consumption in two groups of people eating dumpsite catfishes (exposed) and farmed catfishes (general).

Concentration of DDTs on wet weight basis ranged from 1.0 – 5.1 ng/g in the farmed catfishes and 3.2 – 29 ng/g in the dumpsite catfishes. Canadian guidelines for protection of consumers of aquatic biota recommended the tolerance limit of 14 ng/g wet wt for total DDTs [17]. In comparison to this guideline, only one sample among the five dumpsite catfishes exceeded the tolerance limit, whereas all the farmed catfish samples had DDTs levels below this limit. This fact suggests possible higher risk for consumers of the dumpsite catfishes but not for those who eat the farmed ones. Recently, Food and Agriculture Organization (FAO)[18] estimated total fish consumption of the Vietnamese is about 50 g/person/day for all kinds of fishes (more than three times higher than those in the early 1990s [19]). Using the recent consumption data with similar approach previously described by Kannan et al. [19], POPs intake by the Vietnamese via fish consumption was assessed. In general, the intake via dumpsite catfish consumption was one order higher than via the farmed catfishes (**Fig. 4**). However, OCs intake via these catfishes was one to two orders lower compared to the estimated intake in the early 1990s [19]. This result revealed decreasing OCs intake in

Vietnam during the last decade. However, consumption of the dumpsite catfishes may cause additional exposure to various other contaminant groups such as heavy metals and dioxin-related compounds [3,4]. These results suggest that assessment of human health risk caused by exposure to various pollutants from open dumpsite should be considered with more attention.

4. Conclusions

This study demonstrated DDTs and PCBs as two major groups of OCs in catfishes cultured in Mekong River Delta. The other contaminants such as PBDEs, CHLs, HCHs and HCB had relatively low contamination levels, suggesting their insignificant contamination. Intake of OCs by Vietnamese humans via fish consumption decreased during the last decade, probably by one to two orders of magnitude. Interestingly, the contamination pattern in fishes also suggested existence of local sources of PBDEs and OCs such as municipal dumping sites in the surrounding environment. This is probably the first comprehensive study reporting contamination by PBDEs in the environment of Vietnam. Municipal dumping sites seem to act as pollution sources of these chemicals to the ambient environment and therefore it is important to pay more attention on the ecological impacts of enormous numbers of such dumping sites in Vietnam as well as in other Asian developing countries. Our investigation on several commercial feeds suggested that some of them may contain higher residues of PBDEs depending upon the country of origin. This may be another source of PBDEs to aquaculture.

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WIDESPREAD CONTAMINATION BY PERSISTENT TOXIC SUBSTANCES IN VIETNAM AND THEIR IMPLICATIONS ON ENVIRONMENTAL QUALITY AND HUMAN HEALTH

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Abstract

Vietnam is a developing country located in the central part of the Southeast Asian tropical region. The country comprises the Red River and Mekong River Delta, inhabiting more than 30 million people, which is one of the most populous areas in the world. These deltas have become one of the most productive agricultural regions in Southeast Asia. Agrochemicals have been used extensively in the past and until very recently for agricultural purposes and malaria eradication program. The present paper provides a comprehensive overview of the environmental distribution, patterns and trends of contamination of toxic substances including persistent organochlorines (OCs) and endocrine active compounds (EACs) in different environmental compartments from Vietnam. Monitoring data reported during the 1990s demonstrated widespread and elevated contamination of DDTs in air, water, sediments and soils from in Vietnam. Recent studies in frame of the Asia-Pacific Mussel Watch Program have also revealed that fish, mussels and resident birds from Vietnam contained higher concentrations of DDTs as compared to other countries in region, suggesting the role of Vietnamese environment as a significant emission source of DDT in the Southeast Asian region. Subsequent surveys on coastal lines from north and middle part of Vietnam likewise demonstrated that contamination of some endocrine active compounds such as alkylphenols and phthalates are ubiquitous. In particular, relatively high concentrations of bis-phenol A were found in some locations in Red River delta, comparable or higher than those reported for several locations in developed nations in Western Europe and North America. A case study on seasonal variation of alkylphenols and phthalates in surface water of river delta and estuary of north and central Vietnam indicated the differences in distribution of these compounds between dry and rainy seasons. Higher concentrations of alkylphenols and phthalates were found in dry season in estuary; while the contrasting pattern was observed in the river delta, showing elevated residues in rainy season. This result suggests the different behavior of alkylphenols and phthalates in river delta and in coastal environment. The temperature dependence in tropical ecosystem and the influence of the specific local sources may be reasons for the observed results in the seasonal variations. To our knowledge, this is the first extensive study on the widespread contamination of EACs in Vietnam. Regarding the trends of contamination by OCs, preliminary survey conducted in Read River delta water and

sediments indicated a rapid decline trend in water and a slow decrease in sediments during 1995-2001.

From ecotoxicological and human health perspectives, concentrations of bis-phenol A and di(2-ethylhexyl)phthalates in surface water from some locations in Vietnam exceeded the guideline values for Ecotoxicological Effects and the Environmental Risk Limit, respectively, suggesting potential for toxic implications on aquatic wildlife. Human exposure to persistent organic pollutants indicated that DDT residues levels in human breast milk from both Hanoi and Hochiminh city were among the highest values reported for Asian developing countries as well as developed nations. Daily intakes estimating based on the exposure through sea-foods indicates that intakes of DDTs by Vietnamese populations were among the highest rank in Asia-Pacific countries, suggesting potential risk for human exposure to elevated DDT pollution. Future studies should be focused on the time trends of POPs and EACs in biota of Vietnam with view of predicting the future trend of contamination and to reveal new clues for understanding possible toxic impacts on aquatic organisms.

1 Introduction

Global contamination and toxic effects of persistent organic pollutants (POPs) has been an emerging environmental issue and has received considerable attention during the past decades. Although the extent of contamination by POPs has been predominant in industrialized nations, an increasing number of recent investigations highlighted the role of Asia-Pacific as a potential source of emission for POPs, particularly to pristine areas such as the Arctic and the Antarctic (e.g. see review by Tanabe *et al*., 1994; Tanabe, 2002).

Vietnam is a developing country located in the central part of the Southeast Asian tropical region. The country comprises the Red River and Mekong River Delta, inhabiting more than 30 million people, which is one of the most densely populated areas in the world. These two deltas have become one of the most productive agricultural regions in the Southeast Asia. Agrochemicals have been used extensively in the past and until very recently for agricultural purposes and malaria eradication program. During the recent decade, in the context of the Asia-Pacific Mussels Watch Program and the JSPS Core University Program, extensive studies on the contamination, fate and human health implications of POPs in Vietnam has been conducted. This paper review of the results of the monitoring surveys in Vietnam, an agriculture-based country located at the center of the South East Asian region. The extent of contamination, fate and human health implications of POPs and EDCs in Vietnam are discussed.

2 Widespread contamination in different environmental compartments

On the basis of Asia-Pacific Mussel Watch Program conducted in 1990s, widespread and relatively high contamination of DDTs in various environmental compartments such as air, water, sediment and soils and also fish from both north and south Vietnam were reported (Thao *et al*., 1993, Iwata *et al*., 1994, Kannan *et al*., 1995). This result suggests the widespread usage of DDTs in Vietnam in the past for both agricultural purposes and malaria control. In addition, relatively higher concentrations of PCBs were observed in municipal wastewater from Hochiminh City, Vietnam (Iwata *et al*., 1994). Interestingly,

survey during 1989-1993 showed that Vietnamese fish also contained higher PCB residues as compared to fish from other Asian countries. The high PCB contamination in Vietnam observed during survey in early 1990s could be derived from both the electrical equipments imported from industrialized nations like former Soviet Union and Australia and the army weapons extensively used in Indochina War during 1961-71.

Interestingly, monitoring studies in recent years (1997-2003) have continued to show elevated contamination of DDTs in different environmental media. For example, one of the recent data in the Mussel Watch Program showed that DDT residue concentrations in mussels from Vietnam are among the highest reported for the countries surveyed in South East Asian region (Monirith *et al*., 2000; 2003) (Figure 1). Resident birds collected from Red river delta estuary, North Vietnam, contained elevated concentrations of DDTs (Minh *et al*., 2002). DDT residue levels in resident birds from North Vietnam were also higher than those in other countries in the region (Figure 1). It is worthy to note that though the recent amounts of DDTs used in Vietnam were lower than those of other countries in the region, the extent of DDT contamination in environmental samples in Vietnam is higher. This observation suggests that the application of DDTs in Vietnam has continued until very recently, resulting in elevated contamination of these compounds in different species occupying low to high trophic levels in food chain.

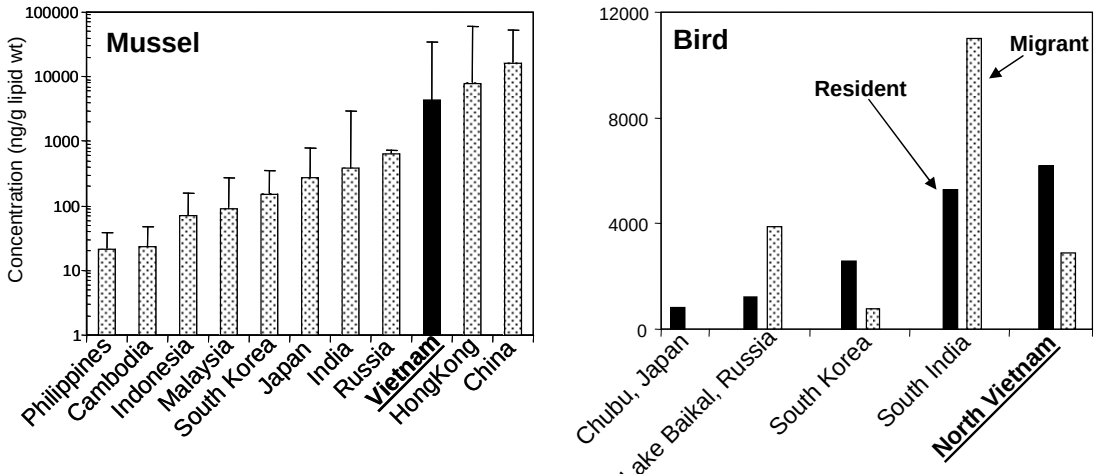

Figure 1. Comparison of DDT residue levels in mussels and birds from Vietnam and other countries in Asia-Pacific region. Data cited from Monirith *et al*., 2003 and Minh *et al*., 2002.

As far as the EDCs contamination are concerned, extensive investigations have been made to examine distribution of alkyphenols and phthalates in Red River delta and Huong River delta, the two larges rivers in the north and middle Vietnam. Again, alkylphenolic compounds have been detected at almost all sampling locations examined, supporting the concept of ubiquitous contamination of EDCs in both river delta and estuary ecosystems.

In particular, relatively high concentrations of bis-phenol A were observed in surface water collected from Red River delta, which were comparable, and in higher at some sites, than those reported in industrialized nations in western Europe and North America, suggesting the presence of significant source of this compound in the Red River delta.

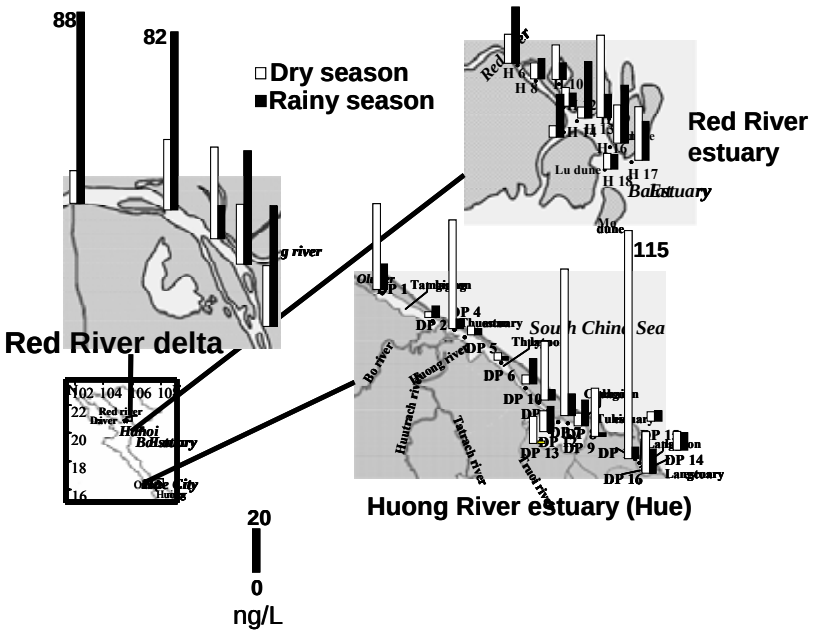

Figure 2. Distribution of alkylphenols (ng/L) in water from the two biggest rivers in north and middle Vietnam: Red River and Huong River

3 Implications for environmental quality and human health

Widespread contamination by OC insecticides, particularly DDTs in different environmental samples of Vietnam has been apparent as indicated in the survey in early 1990s. Estuarine sediments collected from various locations from the northern to southern part of the country, contained relatively high concentrations of DDTs (Iwata *et al*., 1994). The Environment Canada has recently updated the sediment quality guidelines for protection of the aquatic life. The Interim Fresh water Sediment Quality Guidelines (ISQGs) and the Probable Effect Levels (PELs) for *p,p'*-DDE are 1.42 and 6.75 ng/g dry wt, respectively, while these values for *p,p'*-DDT are 1.19 and 4.77 ng/g dry wt (Canadian Environmental Quality Guideline, 2002). Among the 18 locations examined throughout Vietnam, about half of the sediment samples contained *p,p'*-DDE and *p,p'*-DDT greater than the ISQG values. Several samples collected from the municipal sewage canal contained elevated levels of DDTs, far beyond the probable effect levels (PELs). Similarly, residue concentrations of DDTs in soils collected from some locations from north, middle and south Vietnam (Thao *et al*., 1993) approached or exceeded the guideline level of 700 ng/g dry wt proposed by Environment Canada and the level of 1000 ng/g dry wt recommended by Japanese Government. Given these facts, it is important to note that the magnitude of contamination by DDTs in Vietnam is of concern and warrant further studies. From the environmental health and global contamination point of view, the role of Vietnam as potential source of DDTs for other countries in the region as well as in higher latitudes should be considered as the priority research focus in future.

In a recent survey examined endocrine active compounds in surface water along various locations in Vietnam, elevated levels of bis-phenol A were found in several sites in the Red River delta. The maximum concentrations in Balat estuary and in the river

delta were 1200 and 1300 ng/l, respectively. These levels were exceeding the predicted no-effective concentration ($PNEC_{water}$) of 1000 ng/L (United States and European Standard) (Figure 3). The magnitude of contamination by DDTs and bis-phenol A in Vietnam has been of concern and deserves continued monitoring studies.

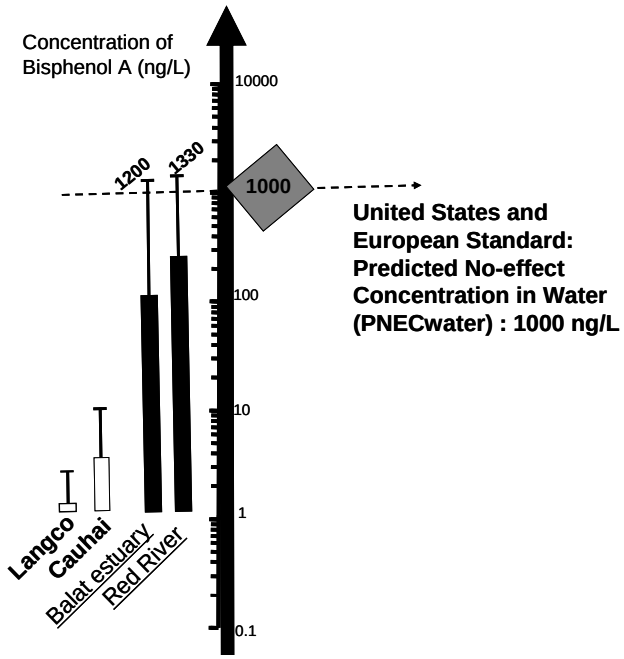

Figure 3. Magnitude of contamination of bis-phenol A in surface water from river delta and estuary of north and middle areas in Vietnam in comparison with the guideline value for ecotoxicological effects [United States and European Standard: Predicted No-effect Concentration in Water – $PNEC_{water}$ (1000 ng/L)]

In the perspective of human health implication, surveys conducted in early 1990s on OCs in foodstuffs provided useful information regarding the dietary intake of these compounds by Vietnamese population (Kannan *et al*., 1992). Interestingly, the estimated average daily intakes based on the exposure through foodstuff to PCBs in Vietnam were higher than India, Thailand and comparable to those reported for developed nations like USA and Germany. Particularly, average daily intake of DDTs by Vietnamese was estimated to be 19 □g/person/day; and this value was the highest as compared to the countries in the region and in developed nations (Kannan *et al*., 1992). Although the data used for estimation has been reported a decade before, this fact clearly suggests elevated exposure to DDTs and PCBs by Vietnamese population and that the usage of DDTs has been extensive during the past 10 year.

Surveys in the framework of recent Asia-Pacific Mussel Watch Program indicated that dietary intake of DDTs and PCBs from fish in Vietnam were higher than those in Cambodia and Thailand, but still lower than those in industrialized nations such as Australia, Japan and Hong Kong (Monirith *et al*., 2000). On the basis of the recent data of average seafood consumption reported by Food and Agriculture Organization of the United Nations, the average daily intake of PCBs and DDTs from seafood for different countries in Asia-Pacific region were estimated (Monirith *et al*., 2003) (Table 1). Interestingly, results again showed that intakes of DDTs by Vietnamese population were apparently higher than those reported in other countries examined (Table 1).

Table 1. Estimated daily intakes of PCBs and DDTs from seafood in countries in Asia-Pacific region

Country	Seafood consumption (g/person/day)	Intake of PCBs (ng/person/day)	Intake of DDTs (ng/person/day)
Cambodia	54.2	40	18
China	68.5	170	16440
Hong Kong	68.5	250	8200
India	12.3	47	52
Indonesia	53.4	69	53
Japan	173	5200	610
South Korea	140	520	490
Malaysia	158	160	220
Philippines	81.1	460	32
Thailand	78.6	240	440
Vietnam	**51.8**	**72**	**2100**
Russia	53.2	3400	640

Intakes of PCBs and DDTs were estimated based on the mean concentrations in mussels reported by Monirith et al , 2002 and 2003.
Seafood consumption data cited from WHO (2000)
(http://apps.fao.org/lim500/wrap.pl?FoodBalanceSheet&Domain=FoodBalanceSheet)

4 Tolerable daily intake by breast fed children

A recent study extensively examined persistent organochlorines (OCs) in human breast milk from Hanoi and Hochiminh city, the two largest cities in Vietnam showed interesting results (Minh *et al.*, 2004). Similar to those observed in environmental samples, break milk of mothers living in suburb of Hanoi and Hochiminh city contained elevated levels of DDTs, which were among the highest levels reported for Asian developing countries as well as developed nations (Minh *et al*., 2004). In order to assess the risk for breast-fed infants due to OC exposure via breast milk, daily intake of OCs by infants was calculated based on the assumption that the average milk consumption of a 5 kg infant is 700 g/day (Oostdam *et al*., 1999). The mean values of daily intake of OCs were estimated by using the following equation:

$$DI = \frac{C_{milk} \times 700g \times C_{Lipid}}{5}$$

where DI is daily intake (μg/kg body wt./day); C_{milk}: concentration of the chemical in milk (μg/g lipid wt); C_{lipid}: lipid content in milk (%).

The estimated daily intakes are shown in Figure 4. It was recognized that although intake of DDTs by most infants is below the guideline proposed by Health Canada (Oostdam *et al*., 1999) in average, intake by some individuals is close to or exceeds this guideline. This fact may raise greater concerns on infant health because children are highly susceptible to effects from environmental contaminants.

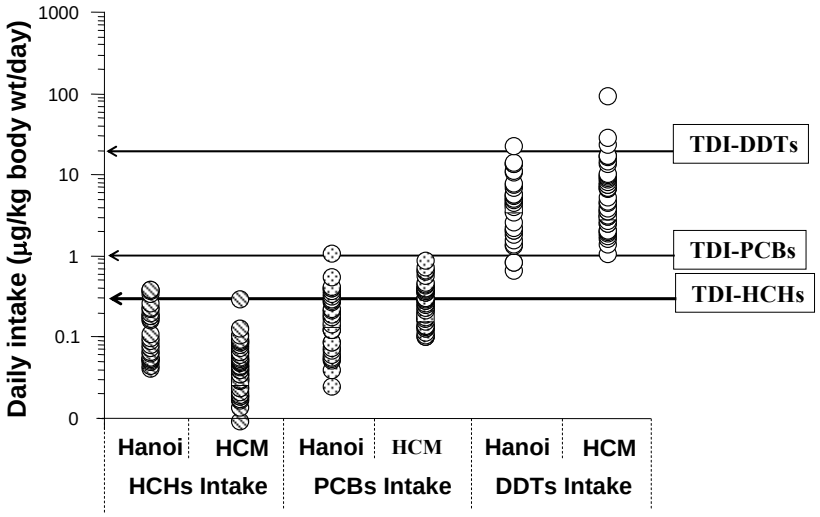

Figure 4. Estimated daily intake (ng/kg body wt/day) of HCHs, PCBs and DDTs by breast-fed children living near dumping sites in suburb areas of Hanoi and Hochiminh city, Vietnam in comparison with the Tolerable Daily Intake (TDI) proposed by Health Canada

5 Conclusions and recommendations

Multi-media monitoring studies conducted during the last decade on POPs in Asia-Pacific region including Vietnam has elucidated that contamination by OC insecticides, particularly DDTs, has been apparent. As a consequence, high degree of exposure of general populations to DDTs via foodstuff, particularly fish and other seafood has been of concern over the last many years. In addition, a certain group of people living near the dumping sites of municipal wastes are exposed to elevated concentrations of organochlorine insecticides and PCBs. Particularly, the first children are exposed at greater levels of contaminants via breast milk and thus are at higher risk. It is important to note that despite the decrease in global contamination by POPs in the future; developing countries in Asia-Pacific region may continue to be a potential source for certain contaminants such as DDTs. Temporal trend studies are therefore needed for developing countries. Possible toxic effects on human health and wildlife should also be thoroughly investigated.

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THE EVOLUTION OF APPROACHES TO MARINE POLLUTION MONITORING --- A RETROSPECTIVE VIEW OVER FORTY YEARS

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Abstract

During the last forty years or so, our approaches to monitoring pollution and its impacts on the marine environment have evolved considerably. When pollution first became a concern during the 1950's - 1960's, most approaches to dealing with it were based on chemical analyses, whose sensitivity and specificity were fairly limited: "parts per thousand" (i.e., $mg \cdot g^{-1}$) was the normal range of detection at that time. Chemical analyses were supplemented with biological assessments based mainly on acute toxicity measurements to a few selected species, and to community analyses based on univariate statistical approaches. Since then, analytical chemistry techniques have advanced both in terms of specificity and sensitivity; it has been estimated that as a rough rule of thumb, sensitivity increases by about three orders of magnitude per decade, with the result that now some chemical residues can be measured at $fg \cdot g^{-1}$ concentrations (implying instrument detection limits of down to 10^{-17} g, or few hundred thousand molecules). Biological measurements to assess pollution impacts have also evolved, and a range of biochemical, "whole-organism" physiological and community structure measurements (these last based on multi-variate statistical approaches) are now available to complement chemical analyses. These biological effects monitoring approaches may be less sensitive and specific than the most modern chemical analyses, but they are still useful in assessing the impacts of marine pollution. Examples of these trends in pollution assessment will be presented and discussed.

Introduction

One advantage --- perhaps the only one --- of having worked in a subject area over a long period is that it provides the opportunity to develop a perspective on the evolution of that speciality. In this paper, I will review the development, over the last forty years or so, of our approaches to assessing the impact of marine pollution. Such a review is inevitably subjective, since I have worked mainly on one aspect of marine pollution (the distribution and impact of some organic contaminants) and much of my experience has been developed within the environment of a western government scientist. Nevertheless I hope that this personal (and probably prejudiced) view will stimulate some ideas on how our approaches to marine pollution assessment might evolve in the future.

Historical Perspective.

Marine pollution began to become of general public concern during the 1950's and 1960's. Interpretive science writing such as Rachel Carson's "The Sea Around Us"

(Carson, 1951) stimulated general public interest in the marine environment, and a succession of major oil spills in Europe (e.g., that of the *Torrey Canyon* off southwest England in 1967: Smith, 1968) raised public awareness about the scale (in both spatial and temporal terms) of the impact of such "catastrophic" events. At roughly the same time, descriptions were appearing of the "chronic" effects of such persistent chemicals as DDT and related organochlorine pesticides, and this accumulation of evidence that marine pollution could have widespread, long-lasting and often unpredictable impacts had, by the early 1970's, persuaded government and other funding agencies --- at least in the west --- to stimulate research into the approaches to assessing pollution impacts.

The 1970's therefore saw a considerable expansion of the development of methods to assess the impact of pollution. Before this period, such assessments were based mainly on chemical analyses of the distribution of contaminants, measurements of the acute toxicity (i.e., lethality) of contaminants to some selected marine species, and observations of community structure changes in response to pollution. By the end of the 1970's, a wide range of sub-lethal biological effects measurements and the organism and sub-organism level had been proposed to complement community analyses; community analyses themselves had become more sophisticated though the application of multi-variate statistical approaches, and chemical analyses had advanced in terms of both sensitivity and specificity.

Some sense of the rate of development of biological effects measurements during the 1970's emerges from reading a report of a workshop conducted by ICES (International Council for the Exploration of the Sea) in 1979 (McIntyre and Pearce (Eds.), 1980). A wide range of potentially useful measurements was described: these included measurement of blood chemistry variables in fish, tissue "adenylate energy charge" in various organisms, tissue taurine : glycine ratios, steroid hormone metabolism disruption, and induction of metallothionein (MT) and of "mixed function oxidases")now known as Cytochrome P4501A [CYP1A] induction). Perhaps not surprisingly, most of these *potential* sub-lethal effects measurements did not become useful in practice, for various reasons, including lack of specificity towards identifiable contaminants (Addison 1996). Those potentially useful approaches (such as CYP1A and MT induction) which have proved generally useful in practice are specific to a limited range of organic compounds and of metals, respectively. And although it was not discussed at the ICES Beaufort Workshop, another relatively specific "biomarker" --- that of cholinesterase (ChE) inhibition --- is also growing in popularity as an indicator of the impact of various organophosphorus compounds (among others).

The ICES Beaufort Workshop represented one "milestone" in the development of biological effects measurements to assess pollution impacts, and a second milestone was set in 1986, when the Intergovernmental Oceanographic Commission's (IOC) Group of Experts on the Effects of Pollution (GEEP) organised a workshop at the University of Oslo. Its objectives were to test several of the approaches suggested at the ICES Beaufort Workshop and elsewhere, by requiring their proponents to analyse "blind" samples (i.e., whose origins were unknown to the proponents) and to identify whether the samples had come from "reference" or contaminated sites. The outcome of this workshop (Bayne et al. (Eds.) , 1988) showed that only two general approaches,

measurement of "scope for growth" (physiological condition) of molluscs, and CYP1A induction in fish, could provide reliable information about whether organisms had been sampled from "reference" or contaminated habitats. Since then, these measurements, and a handful of others such as cholinesterase inhibition, have continued to be assessed, and have reached the stage of being generally recognised as fairly "standard" approaches to assessing pollution impact, even to the extent of having something approaching standard operating procedures (SOP) written for them (e.g., ICES "TIMES" [Techniques in Marine Environmental Science]; see http://www.ices.dk/products/techniques.asp), or of being the subject of inter-calibration exercises (e.g., Stagg and Addison, 1995).

Since the mid 1980's, there seems to have been relatively little development of now approaches to assessing the impact of pollution. Existing approaches have certainly been refined, usually by application of computer-based technology; thus, CYP 1A induction (which is usually measured as induction of activity of EROD (ethoxyresorufin O-de-ethylase) was formerly measured, sample by sample, in a conventional spectrofluorimeter, with a throughput of about 50 samples per day. Now, with the use of fluorescence microplate readers, that throughput can be increased 10-fold. Nevertheless, the principle of assessing the impact of some organic compounds by measuring CYP1A induction has *not* changed, or even evolved.

It is interesting to ask why no significant evolution of the principles of assessing pollution impact (by biological measurements) has taken place over the last twenty or so years. One answer, to which I alluded above, may be that funding agencies have not (at least in the west) supported exploratory research into this field to the extent that they did in the 1970's. However, this is not a completely satisfactory explanation. Although there was a burst of activity during the 1970's in developing approaches to measuring biological impacts, development of analytical chemical approaches to detecting and measuring pollutants has proceeded fairly steadily over the last forty years. (There is a rule-of-thumb among analytical chemists that the sensitivity of analyses increases by about three order of magnitude per decade, as a result of technical improvements in instrumentation. This seems plausible: in the early 1970's, residues of the insecticide DDT and related compounds could be detected by electron capture detection in 10^{-9} g quantities whereas now, 35 years later, detection limits are in the 10^{-18} g range using high resolution GC-MS procedures.) The development of analytical chemistry depends also on support from research funding agencies, so why did analytical chemistry continue to evolve, whereas "analytical biology" has remained relatively stagnant over the last twenty years or so? One answer to that question may lie in our approaches to contaminant regulation (at least in the west). Regulatory agencies find it easier to express limits or tolerances in terms of analytical chemistry data than in terms of biological effects: it is simply easier for the regulatory agencies (and for the entities which they regulate) to state (or monitor, or accept) a limit of (say) 10 µg/g PAH (polynuclear aromatic hydrocarbon) in sediments than it is for them to state that (say) EROD in fish from a contaminated site should no be induced more than 3-fold over that in fish from a reference site, or that the abundance/diversity index in meiofaunal community from a contaminated site should not deviate more than 25%

from that in a reference site. One consequence of this is that relatively few discharges or "receiving" bodies (water or sediment) are regulated or monitored using biological criteria alone; however, there seems to be a growing recognition (at least in some western agencies) that biological measurement should be made to complement analytical chemistry data.

The Future.

What evolution can we foresee in approaches to assessing pollution impacts?

First, analytical chemistry approaches will probably continue to evolve towards improving both sensitivity and specificity, and this development will be "driven" by the (perceived) needs of regulatory agencies (and, of course, by the intellectual satisfaction which analytical chemists derive from being able to detect less and less of something ...). However, it is worth asking how much more improvement in the sensitivity of analytical procedures is really necessary. If modern instruments can detect contaminants in the 10^{-17} g range, this means that they can detect a few hundred thousand molecules in a sample. If this sample is derived from (say) a gram of liver tissue, the detection range is around a few molecules per cell (or maybe less, depending on the compound and species considered. Is it possible to assess the *biological* significance of these chemical concentrations? Obviously, the answer depends on the compounds in question, but I would guess that this is well below the expected threshold for detection of biological effects of most contaminants of current interest.

Does it follow that there is a need for more effort to be put into research on biological effects so that the biologists can "keep up" with the chemists? (Otherwise the chemists will be producing more and more data of unknown biological relevance.) I find this more difficult to answer. On the one hand, it is certainly attractive to hope that the biologists and toxicologists *could* at some stage interpret what 10^{-18} g of contaminant per g liver really means. On the other hand, I suspect that by focusing on improving the sensitivity of effects measurements, we run the risk of "not seeing the forest for the trees" --- in other words, we lose sight of the "big picture". The pursuit of increased sensitivity in biological effects measurements should not distract us from addressing other important pollution-related questions, and I will raise three of these (selected quite randomly, and just to stimulate some thought and discussion):

1. In an earlier review (Addison, 1996) I observed that our approaches to measuring pollution impacts could be scaled according to the complexity of biological organisation of the measurement; thus, "simple" biochemical measurements (such as CYP1A or MT induction) tended to be specific to a limited suite of contaminants, and rapid, but were of low ecological relevance. At the other end of the scale of biological complexity, measurements of changes in community structure were of high ecological relevance, but were non-specific and tended to occur over relatively long time scales. There have been very few studies of the links between measurements at different scales of biological organisation (see Stein et al. 1990 for one example) and this remains an area which deserves further investigation. For example, what does EROD induction in an individual fish mean (if anything) to its population, species or community?

2. On a related theme, most of the sub-cellular biological effects measurements that we use fairly routinely now (such as CYP1A or MT induction, or ChE inhibition)

appear to be essentially phenotypic --- they occur only in individuals that are exposed to contaminants. Yet we know from community studies that *populations* adapt to contamination (e.g., Chambers and Yarborough 1979). Can the phenotypic response that we measure in individuals be "translated" to a genotypic change, and if so, how? (Given the range of techniques now available to molecular biologists, I find it surprising that relatively little work is being done in this field, and most of that work focuses on micro-organisms.)

3. Finally, how do we establish a scientific basis for accepting or rejecting some level of environmental degradation? Intensive coastal net-pen aquaculture, for example, leads usually to some degradation of the underlying benthos. Most scientists (and regulators) accept this degradation, provided it is on a relatively small scale. But what scientific criteria do we use to accept degradation of (say) 1 km^2 under a fish farm, yet reject degradation of (say) 10 or 100 km^2 ?

I do not want to suggest that these are the only questions that need to be addressed in the general area of assessing pollution effects, but they do illustrate some of the general issues that should keep marine environmental scientists occupied for some time to come.

Summary and Conclusion.

I have presented a very personal view of the evolution of our approaches to assessing the significance of marine pollution. I do not claim that it is objective, or balanced (more probably it reflects a set of prejudices that I have accumulated over the last forty years or so!) but I hope that it will stimulate some thought about what marine environmental scientists do, and perhaps more importantly, why we do it.

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EFFECTS OF CONTAMINANT EXPOSURE ON REPRODUCTIVE ENDPOINTS IN ENGLISH SOLE (PAROPHRYS VETULUS) FROM URBAN AREAS OF PUGET SOUND, WASHINGTON

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Abstract

The Environmental Conservation Division of NOAA/NMFS/NWFSC has been conducting a series of field and laboratory studies to examine the effects of contaminants on reproductive parameters in Puget Sound English sole *(Parophrys vetulus)*. Female English sole from urban and industrially contaminated sites show various reproductive effects including reduced plasma steroid levels, altered gonadal development, and inhibited spawning, which are highly correlated with concentrations of polycyclic aromatic hydrocarbons (PAHs) and organochlorines (OCs) in fish tissue or bile. Male English sole tend to be less sensitive to these industrial pollutants than female English sole. In our studies, no significant correlation was observed between PAH exposure and reproductive endpoints measured in male English sole; however, concentrations of certain OCs in liver were correlated with reductions in gonad size and plasma testosterone levels, suggesting possible inhibition of normal gonadal development. Abnormal reproductive timing in females, and induction of the female-specific yolk protein, vitellogenin, in males were detected in fish from urban sites with high inputs of contaminants from industrial discharges, surface runoff, and combined sewer outfalls, suggesting xenoestrogen exposure and possible endocrine disruption.

Introduction

Field and laboratory studies have shown that exposure to industrial and urban associated pollutants can have detrimental effects on reproduction in several marine and fresh water species. In females, exposure to common industrial and agricultural pollutants (compounds such as chlorinated pesticides, polychlorinated biphenyls (PCBs), dioxins, and polycyclic aromatic hydrocarbons PAHs) has been shown to inhibit oocyte development and maturation, increase follicular atresia of both yolked and previtellogenic oocytes, and cause abnormal yolk deposition and yolk formation within oocytes and abnormal egg maturation and egg production (see Lam, 1983; Susani, 1986; and Donaldson, 1990 for reviews). Similarly, alterations in reproductive steroids have also been reported in male fish exposed to oil and other pollutants (Burton et al. 1995; Idler et al. 1995). Also, recent studies have noted that endocrine disrupting chemicals such as synthetic and natural steroids, nonylphenols, and bisphenol-A, discharged from industries and municipal sewage treatment plants ultimately reach coastal areas and embayments. Exposure to these chemicals can lead to abnormal physiological responses and cause adverse effects on the reproductive systems of fish (Desbrow et al. 1998; Giesy et al. 2000; Lee and Peart 2000; Eganhouse and Sherblom 2001) including

reduced testicular growth and sperm production in male teleosts (Jobling et al. 1996; Nimrod and Benson, 1996).

English sole *(Parophrys vetulus)* is a commercially and ecologically important flatfish species indigenous to the west coast of North America. English sole has been a primary sentinel species for a number of environmental monitoring programs on the west coast of the United States, including the National Benthic Surveillance Project (Myers et al. 1994; 2003) and the Puget Sound Ambient Monitoring Program (PSAT 2004). Previous studies have shown that English sole from urban areas of Puget Sound, Washington, USA, exposed to sediments containing PAHs and PCBs, have higher prevalences of liver disease (Myers et al. 1994), elevated levels of cytochrome p4501A in liver (Johnson et al. 1999; Collier et al. 1998a, b), and DNA damage (Reichert et al. 1998; French et al. 1996).

The Environmental Conservation Division of NOAA/NMFS/NWFSC has been conducting a series of field and laboratory studies to examine the effects of contaminants on reproductive parameters in Puget Sound English sole. This paper summarizes the results of ongoing field and laboratory studies that examine the effects of contaminants on reproductive parameters.

Industrial, and urban associated pollutants and female reproduction

The first of these studies (Johnson et al. 1988, 1991, 1993), started in 1985, investigated ovarian development in prespawning English sole from four sites in Puget Sound WA with differing in levels of contaminants in sediments (Fig. 1). Two sampling sites, the Duwamish Waterway and Eagle Harbor, had high concentrations of xenobiotic compounds in sediment. The Eagle Harbor site was the former location of a creosote plant where extremely high levels of polcyclic aromatic hydrocarbons (PAHs) in sediment were measured, whereas sediments from the Duwamish Waterway contained a variety of industrial pollutants including PCBs and PAHs. The other sites, Port Susan and Sinclair Inlet, had lower contaminant concentrations in sediments. A number of factors associated with ovarian maturation were measured including plasma concentrations of estradiol, gonadosomatic index (GSI), oocyte maturational stage, and ovarian atresia. Results showed that female English sole from these contaminated sites had lower plasma estradiol levels and lower in vitro production of estradiol by ovarian tissue than fish from minimally to moderately contaminated sites, and were less likely to enter vietellogenesis and undergo normal ovarian development (Table 1). At the minimally to moderately contaminated sites, approximately 80-90% of adult females underwent gonadal development, while at the highly contaminated sites, the percentage declined to 57-64% (Table 1). In addition, significant correlations were found between the probability of ovarian development and concentrations of fluorescent aromatic compounds (FACs) in bile, an indicator of recent exposure to PAHs (Krahn et al. (1986, 1992). Additional studies suggested that a significant proportion of English sole from contaminated areas that did successfully enter vitellogenesis experienced inhibited spawning ability and reduced viability of eggs and larvae (Casillas et al. 1991). When gravid English sole from the field were brought into the laboratory and artificially induced to spawn with LHRHa (luteinizing hormone releasing hormone analogue), spawning success was significantly lower in fish from Eagle Harbor and the Duwamish

Waterway compared to fish from non-urban sites. In addition, when naturally spawning populations of English sole were examined, animals with elevated levels of contaminants in tissue were rare, even at spawning sites adjacent to industrial waterways, suggesting that fewer animals from industrial sites migrate to spawning areas (Collier et al. 1992).

Table 1. Indicators of reproductive success in English sole from four sites in Puget Sound, Washington. Data compiled from Johnson et al. 1998; Casillas et al 1991; Landahl and Johnson, 1993; Johnson and Landahl, 1994.

	Port Susan	Sinclair Inlet	Duwamish Waterway	Eagle Harbor
Sediment	least polluted	moderately polluted	high OCs and PAHs	high PAHs
% maturing	80	90	64	57
% spawning	90	75	54	35
% fertilization	52	35	44	24
%normal larvae	74	54	59	68
%overall reproductive success	28	13	9	3.2

In 1994, reproductive function in English sole from the Hylebos Waterway and Colvos Passage (Fig. 1) was evaluated, as part of a natural resource damage assessment conducted by NOAA (Johnson et al. 1999; Collier et al. 1998a, b). Hylebos Waterway is one of the most polluted areas on the west coast of the United States (Brown et al. 1988; Collier et al. 1998a, b), and Colvos Passage is a nearby non-urban area, that is minimally to moderately contaminated by industrial pollutants. Sediments from Hylebos Waterway contain a variety contaminants, including PAHs and PCBs and other organochlorines (OCs) including hexachlorobutadiene (HCBD) and hexachlorobenzene (HCB). Female English sole from the Hylebos Waterway had significant alterations in their pattern of reproductive development when compared to female English sole from Colvos Passage. Hylebos Waterway female English sole entered vitellogenesis at an earlier age than Colvos Passage female English sole, with about 50% of sole below 5 years of age maturing in the Hylebos Waterway, as compared to 20% of Colvos Passage sole in this age range. However, while proportions of maturing Colvos Passage females increased with age to 70% for fish 5 years of age or greater, the proportions of maturing females in the Hylebos Waterway remained at about 50%. Enhanced growth, as well as exposure to both PAHs and OCs, were associated with precocious maturation in sub-adult Hylebos Waterway sole. When the results from this study were combined with the data from previous studies on fecundity and spawning success of female English sole (Johnson et al. 1997; Casillas et al. 1991), sole from Hylebos Waterway

were also estimated to have lower egg production as compared to sole from Colvos Passage (Fig. 2).

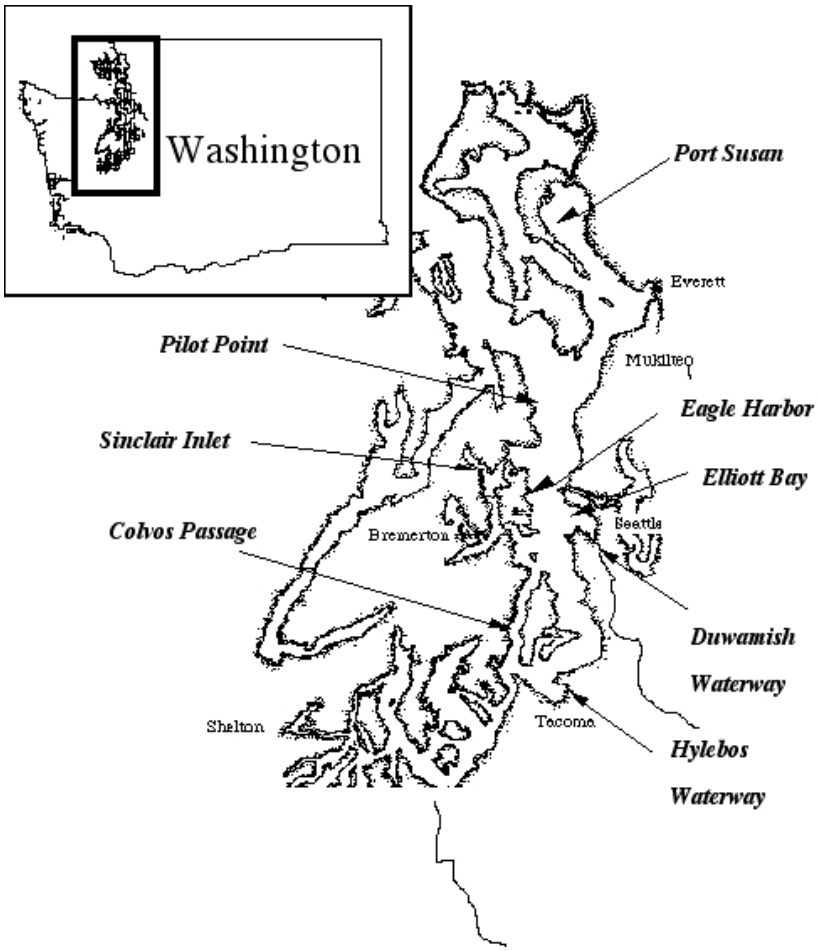

Figure 1. Map of Puget Sound, Washington showing areas of sample collection

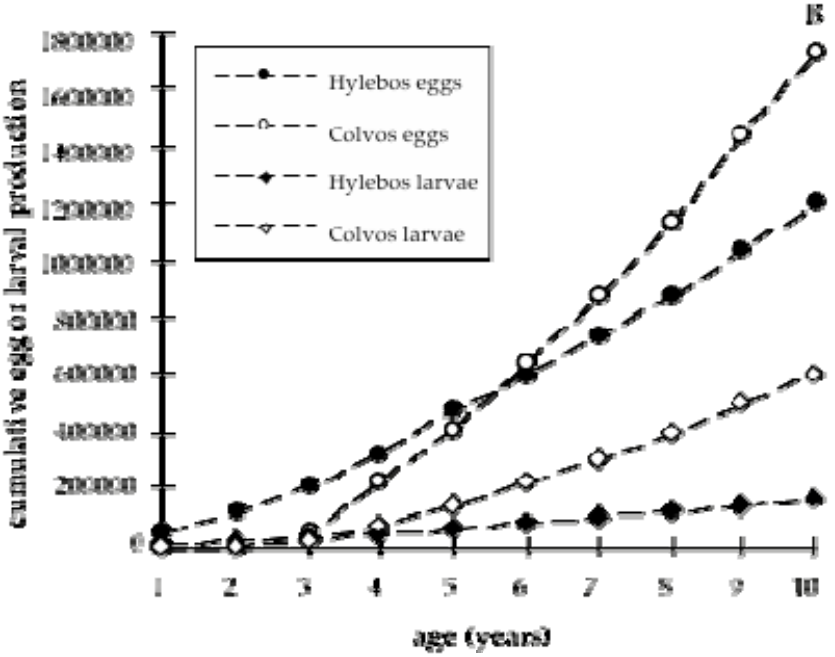

Figure 2. Estimated cumulative egg and larval production by age in female English sole from the Hylebos Waterway and Colvos Passage.

Egg production estimates are based data from this study, and fecundity estimates for Puget Sound English sole (Johnson et al. 1997). Larval production estimates for Hylebos Waterway and Colvos Passage sole are based on reproductive success data for English sole from the Duwamish Waterway, near Seattle, Washington, an urban waterway with sediment contaminant concentrations similar to those in the Hylebos Waterway, and Port Susan, a nonurban reference site in northern Puget Sound (Casillas et al. 1991).

Industrial, and urban associated pollutants and male reproduction

In 1993, we began to examine the effects of industrial and urban associated pollutants on reproductive processes in male English sole. Eagle Harbor and Hylebos Waterway were chosen as the urban sites representing primarily PAH exposure and combined PAH and PCB exposure, respectively, and nearby non-urban sites Pilot Point and Colvos Passage were chosen as reference sites (Fig. 1). Results showed that male English sole tended to be less sensitive to these pollutants than female English sole from the same areas. Although plasma steroid levels and GSI tended to be lower in sole from urban sites compared to sole from non-urban sites, no significant differences were observed (Fig. 3), and the proportion of maturing (spermiogenic) fish at each site were not different (Fig. 4). Additionally, no significant relationship was observed between PAH exposure and reproductive endpoints measured; however, the concentration of certain chlorinated compounds in liver was found to be correlated with reduction in reproductive parameters, suggesting possible inhibition of gonadal development (Table 2).

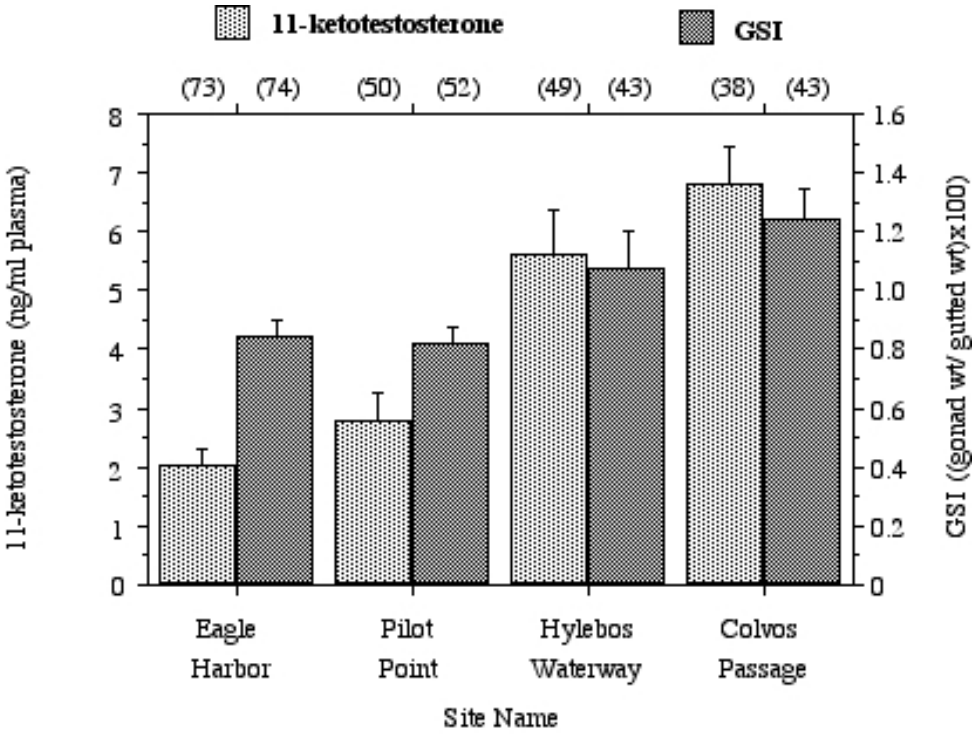

Figure 3. Mean (±SE) plasma 11ketotesterone and gonadosomatic index in male English sole separated by area of capture.

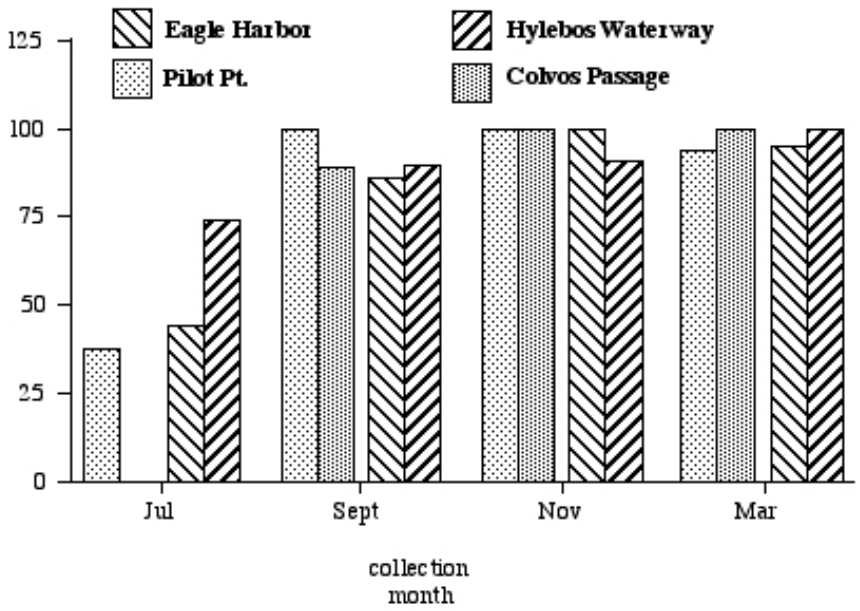

Figure 4. Proportions of maturing male English sole separated by month and area of capture. Maturity of the animals was determined by histological analyses of the gonads.

Table 2. Spearman-rank correlations between reproductive parameters and selected indicators of contaminant exposure. Associations which are statistically significant at p≤ 0.05 are indicated in bold.

	GSI	Plasma 11ketotestosterone
FAC-BAP	rho = -0.093	rho = 0.105
	n = 54	n = 52
	p = 0.5	*p* = 0.4550
FAC-PHN	rho = -0.132	rho = -0.0014
	n = 54	n = 52
	p = 0.3371	*p* = 0.9922
FAC-NPH	rho = -0.09	rho = 0.090
	n = 54	n = 52
	p = 0.5129	*p* = 0.5195
∑PCBs	**rho = -0.386**	rho = -0.255
	n = 44	n = 51
	***p* = 0.0114**	*p* = 0.0717
DDTs	rho = -0.298	rho = -0.129
	n = 42	n = 49
	p = 0.0567	*p* = 0.3711
HCB	**rho = -0.480**	**rho = -0.361**
	n = 40	**n = 45**
	***p* = 0.0027**	***p* = 0.0165**
∑PCB-TEQs	**rho = -0.384**	**rho = -0.284**
	n = 44	**n = 51**
	***p* = 0.0118**	***p* = 0.0446**

Estrogenic compounds and effects on reproduction

In the past few years, numerous effects of environmental estrogens on wildlife have emerged (Depledge et al. 1999) including changes in the sex of riverine fish (Allen et al. 1997). In 1997-2001, as part of the Washington State's Puget Sound Ambient Monitoring Program, we surveyed English sole from a number of Puget Sound, Washington sites for evidence of xenoestrogen exposure, using vitellogenin production in males as an indicator (Lomax et al. *in prep*; Johnson et al. 2006). Vitellogenin, a yolk protein produced in the liver of oviparous animals in response to estrogens, normally occurs only in sexually mature females with developing eggs. However, males can synthesize vitellogenin when exposed to environmental estrogens, making the abnormal production of vitellogenin in male animals a useful biomarker for xenoestrogen exposure. Significant levels of vitellogenin were found in male fish from several urban sites, with especially high numbers of fish affected in three Elliott Bay sites along the Seattle Waterfront (Fig. 5). Additionally, the spawning cycle was altered in English sole from the Seattle Waterfront sites. Gonadal recrudescence in Puget Sound English sole typically begins in early fall (September- October), vitellogenesis peaks in early winter (December-January), and spawning takes place during late winter months (February- March) (Johnson et al. 1988; Sol et al. 1998). In both male and female sole from the Seattle Waterfront sites, spawning appeared to be delayed, with many ripe fish still present in April and May, and female sole were maturing at a younger age than at other reference sites in Puget Sound. Subsequent monitoring revealed the presence of female English sole with developing ovaries at the Seattle Waterfront sites in July, a phenomenon not observed in any other areas in Puget Sound during the summer months. Sources of xenoestrogens and types of xenoestrogens present in Elliott Bay are unknown, but may be associated with industrial discharges, surface runoff, or combined sewer outfalls.

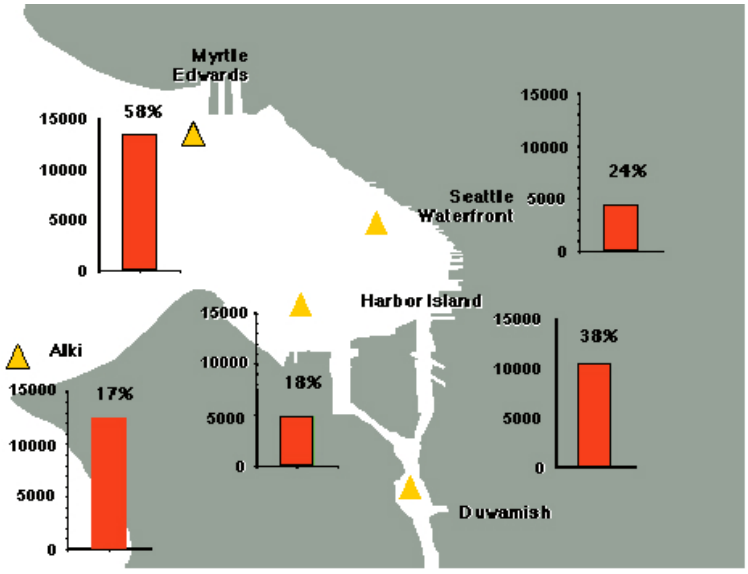

Figure 5. Vitellogenin concentration (ng/ml plasma) in male English sole from sites in Elliott Bay, WA, and the percentage of males from each area that showed evidence of detectable levels of vitellogenin in plasma.

Conclusion

The quality of the marine environment in Puget Sound and its impact on commercially and recreationally important fish stocks in this region is a major concern to fisheries managers. On the basis of research conducted to date, there is considerable evidence that industrial and urban associated pollutants are impairing the health and reproduction of female English sole (Casillas et al. 1991; Johnson et al. 1988, 1991, 1993, 1994, 1998), at various levels of the endocrine system. More subtle effects on endocrine function have been observed in male English sole, primarily associated with exposure to OCs. Exposure to estrogenic compounds in Puget Sound may alter the reproductive cycle and normal development of English sole, but this area of research is fairly new, and there are many gaps in our knowledge concerning the identity, sources, and pathways of uptake of the chemicals involved. We are working with other research agencies to better understand this process.

In summary, as the human population and inputs of contaminants continue to increase in nearshore marine areas, these impacts can pose a threat to the health of fish stocks residing in these reigons. On the basis of research to date, there is considerable evidence that industrial contaminants, as well as estrogenic compounds that could be associated with sewage outfalls and wastewater discharges, are impairing the health and reproduction of Puget Sound English sole, and may have similar impacts on other fish species in other urbanized areas.

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STUDY ON HEAVY METALS ACCUMULATION IN NILE TILAPIA (TILAPIA NILOTICA) FROM NAKHON RATCHASIMA MUNICIPAL WASTEWATER TREATMENT PLANT

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Abstract

The average accumulation of cadmium lead copper and zinc in NILE TILAPIA (Tilapia nilotica) which collected from wastewater treatment plant of Nakorn Ratchasima municipal were 0.049, 0.398, 1.541 and 13.924 mg/kg wet weight, respectively. It was found that the average accumulation in flesh was less than the average accumulation in visceral. The average accumulation in flesh were 0.043, 0.327, 0.545 and 8.260 mg/kg wet weight, respectively and the average accumulation in visceral were 0.055, 0.469, 2.537 and 19.587 mg/kg wet weight, respectively. Although, the average accumulation of all metals in flesh and visceral were remained in the limitation for accumulation of metal in the food that recommend by the Ministry of Public Health of Thailand and many agency, but the average accumulation of lead was higher than the international food standard of Codex Alimentarius Commission (CODEX). Further more, lead has very effected to learning development in child.

Keywords : heavy metal; wastewater treatment plant; oxidation pond

Introduction

Population increasing is the main cause of many environment problem, especially in the large-sized municipal community. In order violence environment problem is wastewater, and many large-sized municipal usually treat community wastewater by using the Oxidation Pond. The prominent quality of Oxidation Pond is the cure by natural system with large-sized pond. This system can treat wastewater with organic substance well, but get not well as expected with inorganic wastewater compound. Normally, there are many aquatic animal residence in these treatment plant especially fish.

Fig 1 Oxidation Pond

In the past, the majority and quantity character of wastewater from these municipality will not different very extremely, by the character of wastewater that participate to assemble of the organic substance. In nowadays, the format of community activity has difference and various of activity, such as a metal coat factory, electrical equipment repair, garage, etc. All of these activity has caused of the wastewater that is poisonous, especially the toxic metal accumulations in the treatment system and transfer to aquatic animals residence and probability contaminated to food chain.

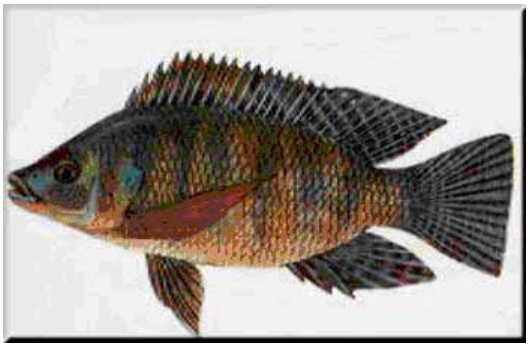

Fig 2 NILE TILAPIA (Tilapia nilotica)

NILE TILAPIA (*Tilapia nilotica*) reach to Thailand for the first time by 25 March 1965. The figure has black stripes and white dot alternate. Fin back area, buttocks fin and the body have green mix brown. There is black stripes leans to obstruct follow the body. The length about 10 – 30 centimeter. Consumes chicken mite water kind , moss , the caterpillar of an insect. In now can feed for economy fish of Thailand.[1] Many of them always found in the Oxidation Pond of the municipal wastewater treatment plant, and nearby wastewater treatment plant people often catch these fish to consume by did not know about dangerous that may happen for them life.

Fig 3 Nearby people catch fish from Oxidation Pond

The metal in the environment

Property of cadmium lead copper and zinc of each element is valuable different as follows.

Cadmium has majority source from the industry that abandons waste matter by did not appropriate treat and other part from the fertilizer, because of phosphate fertilizer has will the cadmium is mixing. Thus, fertilizer insertion appeared the farm field is cadmium addition to the farm field and contaminated can come to the food chain. In Thailand is still have no the specification standard of cadmium in food. But the data from some survey in Thailand was shown cadmium contaminated in the aquatic animals about 1 mg/kg which equal to the standard of the cadmium in the food of the New Zealand, while Australia specify the Maximum Permitted Concentration (MPCs) for the cadmium in the fish and the products equal to 0.2 mg/kg. World Health Organization (WHO) advise in each week should not receive the cadmium reaches 8.3 exceed microgram per 1 kilogram bodies

weight. Intake more cadmiums will effect to kidney and are the disease called ITI-ITI. [9]

Lead, there is the difference in each a kind of the food. Lead does not be regarded as the element that has necessity to bodies. World Health Organization (WHO) advise in each week should not receive the lead reach 50 exceed microgram per 1 kilogram bodies weight. Ministry of Public Health of Thailand has limitation lead in food not more than 1.0 mg/kg. Food international standard (Codex) have value specification of the lead in the fish keeps not exceed 0.2 mg/kg. Lead has strong affect for learning development in child.[10]

Copper, data shown the poison for some kind of fish (*Pimephalis promelas*) there is the poisonous rank arranges from much to less the copper has the poisonous most and the arrange as follows Cu > Cd > Be > Sb > Ni > V > Pb > Ti > U > Sr > Mo , while the poisonous for egg and the caterpillar of porcupine shellfish that Hg > Cu > Zn > Pb > Cd.[7] Ministry of Public Health of Thailand notice 98 **(**1986**)** has limitation of copper in food not exceed 20 mg/kg [4] and Adequate Daily Dietary Intakes (ADI) advise for an adult about 2 – 3 mg/day.

Zinc, be regarded as the element that have the necessity to bodies by affect protein and carbohydrate metabolism. Australia agency advises that in each day should receive zinc 12 – 16 milligram. Ministry of Public Health of Thailand has limitation of zinc contaminated in food not exceed 100 mg/kg

The Australian, New Zealand Food Authority (ANZFA) has appraise condensed intensity of metal in food as follows.[3]

Table 1 ANZFA metal standard in food

Metal	maximum permitted concentration (mg/kg)
Cadmium (Cd)	**0.2 ***
Lead (Pb)	**1.5 ****
Copper (Cu)	**10.0 ****
Zinc (Zn)	**150.0 ****

Remark : *** indicates maximum permissible levels in fish only**

**** indicates maximum permissible levels in food, where fish values not given**

National Health and Medical Research Council, Australian Government (NHMRC) advise the value of cadmium lead copper and zinc in aquatic that animals be supposed to 0.03 – 2.0, 0.05 – 2.0, 0.03 – 30 and 3.6 – 100 mg/kg wet weight.

Fig 4 Samples digestion

Material and Method

Nakorn Ratchasima municipal wastewater treatment plant using Oxidation Pond. The system digested organic substance by microorganism in natural, received air by the wind and algae which in the pond. System sized about 5 km^2, divided to 3 part and the part vacates to 3 pond, so there are 9 pond for treatment wastewater. Now, this wastewater treatment plant can treat wastewater about 32,000 m^3/day. By this studies, fish (Tilapia nilotica) samples collected at the same size which peoples collected for consume. 90 samples collected from 9 pond by vacate to 10 samples per a pond. All samples digested with acid follow to FAO/SIDA (1983) method.[6] Completely digested samples as follow analyzed cadmium, lead and copper by AAS of the Perkin Elmer model 4100ZL furnace type, while zinc was analyzed by AAS of the Perkin Elmer model 2100 flame type. The data continually for analyzed and summarized by using instant software such as SPSS, @Risk, Microsoft, etc.

Result and Discussion

The average weight of 90 fish samples from 10 ponds was 69.10 grams, by the size and the total of samples as follow the figure 5.

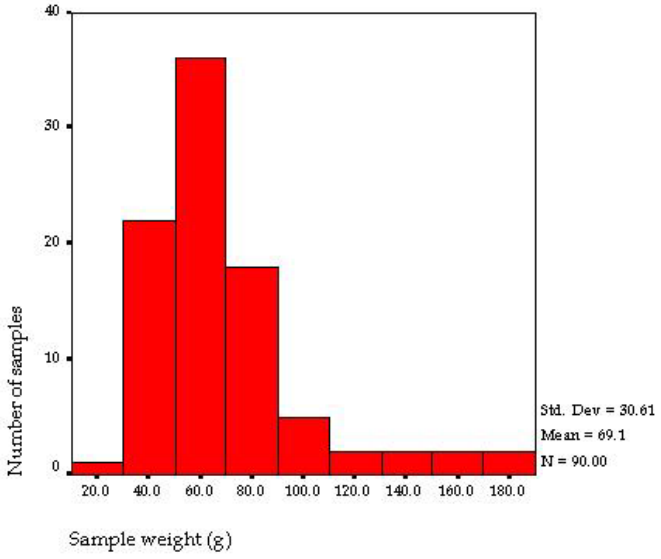

Fig 5 Average weight of all samples

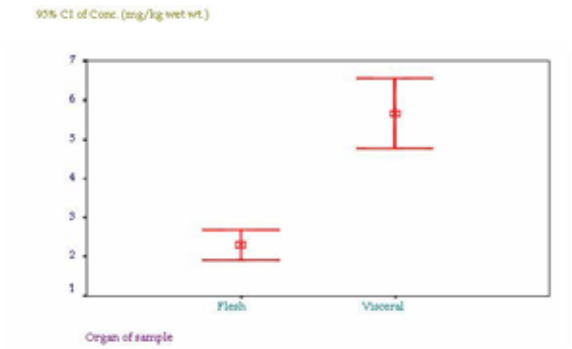

Fig 6 Average accumulation rang in flesh and visceral

Average accumulation of 4 kind of metals (cadmium, lead, copper and zinc) in flesh was found at 2.294 mg/kg wet weight, while the average accumulation of these metals in visceral was found at 5.662 mg/kg wet weight. The data was shown that the average accumulation of all metals in visceral was higher than flesh differently significant. ($\alpha = 0.05$)

Average accumulation of each metal, cadmium, lead, copper and zinc was equal to 0.043, 0.327, 0.545 and 8.260 mg/kg wet weight respectively. Average accumulation in visceral were found equal to 0.055, 0.469, 2.537 and 19.587 mg/kg wet weight respectively. Average accumulation of cadmium and lead in visceral were higher than flesh about 1.25 times, zinc was higher than 2.5 times and copper was higher than 5 times.

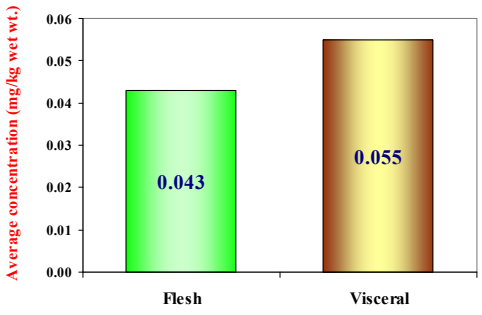

Fig 7.1 Cadmium

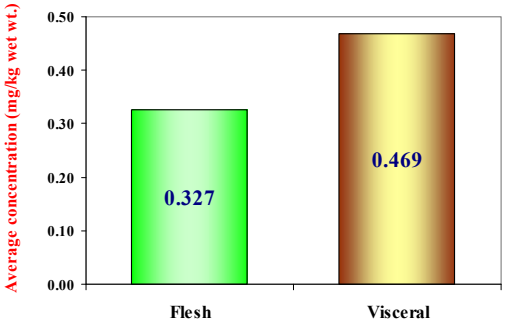

Fig 7.2 Lead

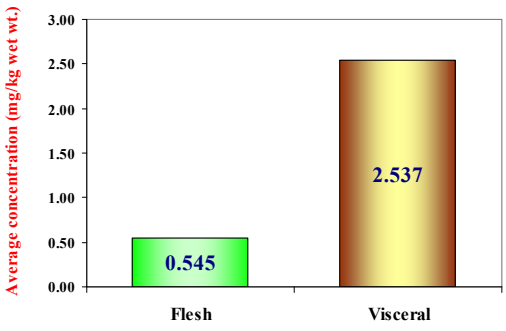

Fig 7.3 Copper

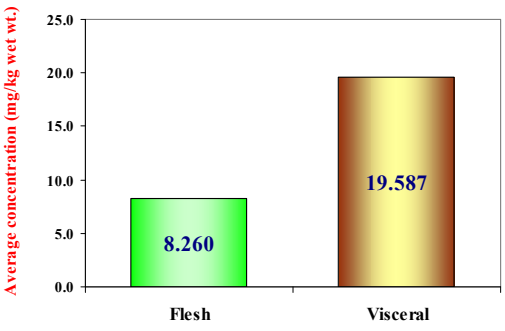

Fig 7.4 Zinc

Fig 7.1 – 7.4 Compare of average accumulation in flesh and visceral

The data of the average accumulation of all metals in flesh and visceral of fish samples collected from the wastewater of Nakorn Ratchasima municipal were not found higher than the food accumulation standard of Thailand, and the accumulation found remain recommend of the National Health and Medical Research Council, Australian Government (NHMRC). However, if compared the average accumulation with the international of food standard (CODEX), the result found that the average accumulation of lead in flesh and visceral were higher than the CODEX standard which recommended the lead accumulation in food not higher than 0.2 mg/kg.

Studied of some metal accumulation in economical sea animal in the east coast area of the gulf of Thailand by the Burapa University, found that the accumulation of metals as follow table. (11)

Table 2 the metals accumulation in some economical sea animal

Type	Average accumulation (mg/kg wet wt.)			
	Cadmium	Lead	Copper	Zinc
Fish	0.017	2.631	0.734	5.404
Squid	0.124	2.535	2.404	12.938
Crap	0.246	1.610	9.866	25.201
Mantis	1.225	3.442	17.234	22.531
Oyster	0.849	5.296	47.831	160.221
Shrimp	0.074	0.960	1.963	13.464
Standard	0.2	1.0	20	100

Follow by this study, almost of sample were found lead accumulation higher than the standard, especially oyster sample was found accumulation of copper and zinc higher than the standard too. Addition studied, the data of Department of Fisheries in 1999 were found cadmium accumulation in squid higher than 1.0 mg/kg about 25% of 1250 samples,(8) while the report of Ministry of Public Health after Tsunami cause in 2004, do not found metal accumulation in sea animal in the effected area.(2) The Department of Medical Science surveyed for mercury cadmium and lead accumulation in seafood such as shrimp shellfish fish and squid during 1992 – 1994, found 0.8% of 1368 samples has metal accumulation higher than the standard. Mohamed Ali ZYADAH, study of Accumulation of Some Heavy Metals in Tilapia Zilli Organs From Lake Manzalah, Egypt, the result found that the accumulation of cadmium lead copper and zinc about 0.05 – 0.64, 0.06 – 0.52, 0.23 – 2.10 and 7.15 – 49.60 mg/kg wet wt., respectively. (5)

Conclusion

Average of all metal accumulation in visceral were found higher than in flesh. Average accumulation of cadmium and lead in visceral were higher than flesh about 1.25 times, zinc was higher than 2.5 times and average accumulation of copper in visceral was higher than in flesh about 5 times. Accumulation average of all metal has

remained in acceptable standard of food majority agency, except accumulation average of lead was found higher than the standard of CODEX. Which lead has strong effect for child, especially for learning development. Thus, the relation agency for this case especial the health care agency should give the knowledge to people know about dangerous that may is born from consuming fish that has contaminated of the metal.

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(2) **Disease Control Department.** *Ministry of Public Health of Thailand guarantee have no the contagious disease after Tsunami back.* **http://www-ddc.moph.go.th/no_disease.html , 25/07/2005**

(3) **George E. RAYMENT. n.d.** *Australian and Some International Food Standard for Heavy metals.* **Queensland Department of Primary Industries.**

(4) **Ministry of Public Health. 1986.** *Notice of Ministry of Public Health 98 (1986). The food standard contamination.* **Ministry of Public Health , Bangkok , Thailand.**

(5) **Mohamed Ali ZYADAH. 1998.** *Accumulation of Some Heavy Metals in Tilapia Zillii Organs From Lake Manzalah, Egypt.* **Environmental Sciences Department, Faculty of Science at Damietta, (Mansoura University), Damietta, Egypt. Tr. J. Zoology, 23: 365-372, 1999.**

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ENDOCRINE DISRUPTION CAUSED BY ORGANOTIN COMPOUNDS IN GASTROPOD MOLLUSKS

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Abstract

Imposex, the superimposition of male type genital organs (penis and vas deferens) on female gastropods, is cause-specific and induced by low concentrations of certain organotins such as tributyltin (TBT) and triphenyltin (TPT) from antifouling paints. Reproductive failure may be observed at severely affected stages. The history of imposex study and the legislation of organotins are summarized. Fundamental knowledge of the endocrinology of gastropod mollusks is briefly described. Four hypotheses, such as aromatase-inhibition, regarding the induction mechanism of imposex induced by organotins in gastropods are reviewed. Finally, a new hypothesis that states that RXR plays an important role in inducing the development of imposex, the differentiation and growth of male type genital organs in female gastropods, is reviewed and discussed toward the elucidation of the entire mode of action of TBT or TPT in the development of imposex in gastropods.

Key words: imposex, ovarian spermatogenesis, endocrinology, retinoid X receptor (RXR), gastropod mollusks, organotin compounds

1. Introduction: gastropod imposex and organotin compounds

Certain environmental chemicals could cause feminization of males and/or masculinization of females in organisms, and such phenomena are generally called endocrine disruption [(1)]. The current status of studies of endocrine disruption both in wildlife and humans is reviewed by the International Programme on Chemical Safety (IPCS) under the joint work of the United Nations Environment Programme (UNEP), the International Labour Organization (ILO) and the World Health Organization (WHO) [(2)]. Here, the author will review the masculinization of female gastropod mollusks, called imposex, in terms of the basic biology and induction mechanism of imposex as well as its current status in gastropods.

The first report on masculinized female gastropod mollusks was made by Blaber [(3)], describing a penis-like outgrowth behind the right tentacle in spent females of the dog-whelk, *Nucella lapillus* around Plymouth, U.K. The term *imposex*, however, was coined by Smith [(4)] to describe the syndrome of a superimposition of male type genital organs, such as the penis and vas deferens, on female gastropods. Imposex is thought to be irreversible [(5)]. Reproductive failure may occur in females with severe imposex, resulting in population decline or even mass extinction [(6,7)]. In some species, imposex is typically induced by tributyltin (TBT) and triphenyltin (TPT), chemicals released from antifouling paints used on ships and fishing nets [(8-12)].

As of 2004, approximately 150 gastropod species have been reported to be

affected by imposex worldwide [13-20]; many of these gastropod species belong to the families Muricidae (e.g., *N. lapillus*, *Ocenebra erinacea*, *Thais clavigera,* and *Urosalpinx cinerea*), Buccinidae (e.g., *Babylonia japonica*, *Buccinum undatum,* and *Neptunea arthritica arthritica*), Conidae (e.g., *Conus marmoreus bandanus* and *Virroconus ebraeus*), and Nassariidae (e.g., *Ilyanassa obsoleta* and *Nassarius reticulatus*) of the Neogastropoda. [15,16].

Regarding Japanese gastropods, at least 39 species (seven mesogastropods and 32 neogastropods) have been found to be affected by imposex among 69 species examined [16,21]. Although imposex has been observed mostly in shallow-water species in previous surveys, the detailed studies of species living at a depth of 200 m or more must be conducted, because of the latest finding of imposex in Alabaster False Tun (*Galeoocorys leucodoma*) trawled from the depths of 200-250 m off the Atsumi Peninsula in 1999 [21].

In previous studies, the incidence or severity of imposex has been examined, the use of certain gastropod species as biological indicators of TBT contamination has been investigated, and TBT contamination using gastropods has been surveyed. However, only a few studies, however, have shown evidence for population-level effects of reproductive failure due to imposex, on the basis of either morphological or histological methods [5,6,21-29].

TBT and TPT compounds (TBTs and TPTs) have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s, although lower amounts of TPTs have been used [25,30]. In Japan, the production, importation, and use of TBTs and TPTs have been strictly regulated by legislation and government administrative guidance since 1990. These activities were reported to have been completely stopped by 1997, although evidence suggests illegal TBT use in antifouling paints in some areas [21,25]. The International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention) was adopted to enforce a worldwide ban on TBT and TPT during the International Maritime Organization (IMO) Convention in October 2001 [31,32], although the ban has not come into effect yet.

Endocrine disruption in abalone has also been reported in Japanese species as well as Australian one [33-36].

2. Mode of action of organotin compounds on development of imposex in gastropods

2.1 *Endocrinology of gastropod mollusks*

Because of lack of information on the basic biology of mollusks, knowledge of reproductive physiology and/or endocrinology of gastropods has been very limited. Knowledge has been mainly obtained from certain species of Opisthobranchia (e.g., *Aplysia californica*) and Pulmonata (e.g., *Lymnaea stagnalis*); that is several neuropeptides released from the visceral ganglia, cerebral ganglia, or the prostate gland of gastropods (e.g., *A. californica* and *L. stagnalis*) are egg-laying, ovulation, or egg-releasing hormones [37-39]. Little knowledge of the reproductive physiology and/or endocrinology of Prosobranchia (including Archaeo-, Meso- and Neogastropoda), however, has been obtained.

Although LeBlanc et al. [40] reviewed many studies and described that gastropods have both peptide and steroid hormones, it remains unclear what type of sex hormone gastropods have (see below).

Because sex steroid hormones, such as testosterone and 17β(beta)-estradiol, play physiologically important roles in the development of sex organs and the maturation of gonads (i.e., oogenesis and spermatogenesis) in vertebrates, similar sex steroid hormones might also regulate the reproduction of invertebrates, such as gastropods [40]. After the removal of the hermaphroditic organ, oogenesis and spermatogenesis were observed respectively in the gonads of 17β(beta)-estradiol-treated females and testosterone-treated males of the slug *Limax marginatus*; egg-laying was also induced by 17β(beta)-estradiol in female slugs, implying the existence of vertebrate-type sex steroid hormones in this species [41,42]. The *in vitro* metabolism of androstenedione and the identification of endogenous steroids (androsterone, dehydroepiandrosterone, androstenedione, 3α(alpha)-androstanediol, estrone, 17β(beta)-estradiol and estriol) by gas chromatography with mass spectrometry (GC-MS) were reported for *Helix aspersa* [43]. Several vertebrate-type sex steroids (androsterone, estrone, 17β(beta)-estradiol and testosterone) and the synthetic estrogen (ethynylestradiol) were also identified by high resolution GC-MS in the gonads of *T. clavigera* and *B. japonica*. The detection of the synthetic estrogen, ethynylestradiol, in the gonads indicated that contamination of the habitat of *B. japonica* had occurred [44]. Similarly, contamination with other vertebrate-type sex steroids of the habitats of *T. clavigera* and *B. japonica* may have occurred.

Evidence for steroid-producing cells and synthetic/metabolic enzymes for steroid biosynthesis must be completely obtained to clarify the existence of vertebrate-type sex steroid hormones in gastropods. Although aromatase-like activity has been measured and reported in several gastropod species [45,46], the measured aromatase-like activity does not necessarily mean the existence of vertebrate-type aromatase in gastropods. To the best of our knowledge, there has been no scientific report that has elucidated the successful isolation of aromatase protein from invertebrates.

Based on a study of fully sequenced invertebrate genomes, homologues of estrogen receptor (ER) and androgen receptor (AR) have not been found in invertebrates [47]. Thus, it remains unclear whether gastropods have AR and ER. Although ER-like cDNA was isolated from *A. californica* (Gastropoda: Opisthobranchia), it could not bind to estrogen and it was found that it was a constitutively activated transcription factor [48]. ER-like protein was also isolated from the rock shell (*T. clavigera*), but it could not bind to estrogen either, and it was also a constitutively activated transcription factor, similar to *A. californica* (Katsu, Iguchi and Horiguchi, unpublished data).Therefore, further studies are necessary to identify steroid receptors and clarify their functions in gastropods.

2.2 *Mode of action of organotin compounds on the development of imposex*

Regarding the induction mechanism of imposex, several hypotheses have been proposed and they can be summarized as follows: 1) increased androgen levels, such as testosterone, due to aromatase inhibition by TBT [49-51]; 2) inhibition of the excretion of

sulfate conjugates of androgens by TBT [52]; 3) disturbance of the release of penis morphogenetic/retrogressive factor from pedal/cerebropleural ganglia by TBT [53]; and 4) increase in a neuropeptide, APGWamide level caused by TBT [54,55].

Experimental evidence, however, is weak for these 4 hypotheses. There is a lack of correlation between the time course of the increase in testosterone titres and penis growth in females in the aromatase inhibition hypothesis [49]. It is unknown whether aromatase-like activity is actually inhibited by TBT concentrations in tissues of gastropods collected at natural sites slightly contaminated by TBT. There is also contradictory evidence of the relationship between reduced aromatase-like activity and advance imposex symptoms in the gastropod, *Bolinus brandaris* [45]. Santos et al. [50] suggested the involvement of AR, besides aromatase inhibition, in the development of imposex in *N. lapillus*, although gastropods may not inherently have AR [47]. If gastropods also have AR similar to vertebrates, it may be profitable to consider the possible activation of androgen receptor–mediated responses caused by TBT or TPT in gastropods, as the enhancements of androgen-dependent transcription and cell proliferation by TBT and TPT have been reported in human prostate cancer cells [56].

There is a possibility that the results given in support of the testosterone excretion-inhibition hypothesis [52] may reflect a phenomenon that is at least partly short-term and/or associated with acutely toxic TBT concentrations [57].

Several neuropeptides released from the visceral ganglia, cerebral ganglia, or the prostate gland of gastropods (e.g., *A. californica* and *L. stagnalis*) are egg-laying, ovulation, or egg-releasing hormones [37,38]. Féral and Le Gall [53] suggested that TBT-induced imposex in *O. erinacea* might be related to the release of neural morphogenetic controlling factors. Their study used *in vitro* tissue cultures derived from a presumed penis-forming area of the immature slipper limpet, *Crepidula fornicata*, and the isolated nervous systems of male or female *O. erinacea* in the presence/absence of TBT (0.2 μg/L) [53]. The accumulation of TBT or TPT in the central nervous systems of *H. gigantea* [35], *N. lapillus* [58], and *T. clavigera* [59] indicates the potential for the toxic effects of TBT and TPT on neuroendocrine systems. Oberdörster and McClellan-Green [54,55] reported that APGWamide, a neuropeptide released from the cerebral ganglia of gastropods such as *L. stagnalis,* markedly induced the development of imposex in female *I. obsoleta*. The effect of APGWamide in the induction and/or promotion of the development of imposex, however, appears weak based on the experimental results of the incidences of imposex and penis growth [54,55].

Thus, at present, four hypotheses regarding the induction mechanism of imposex in gastropods cannot be fully supported, because of the reasons mentioned above.

There are several characteristics in the development of imposex induced by organotin compounds, such as TBT and TPT in gastropods. At the initial stage of imposex development, the differentiation and growth of male type genital organs (i.e., penis and vas deferens) occur and lead to ovarian spermatogenesis at the severely affected stage, involving oviduct blockage due to the proliferation of epidermal tissues surrounding the vas deferens [6,21-25,27-29,33-36]. Therefore, the author considers that the true mechanism of action of TBT or TPT in the development of imposex in gastropods must encompass an explanation of each of the characteristics mentioned above [21].

Nishikawa et al. [60] proposed a unique mechanism of action of TBT or TPT on the development of imposex in gastropods, which was completely different from other hypotheses already proposed as the imposex induction mechanism. Nishikawa et al. [60] showed that organotins (both TBT and TPT) bound to the human retinoid X receptors (hRXRs) with high affinity and the injection of 9-*cis* retinoic acid (9-*cis* RA), the natural ligand of hRXRs, into female rock shells (*T. clavigera*) induced the development of imposex. The cloning of an RXR homologue from *T. clavigera* revealed that the ligand-binding domain of the rock shell RXR was very similar to that of the vertebrate RXR and bound to both 9-*cis* RA and organotins [60]. These findings suggest that RXR plays an important role in inducing the development of imposex, namely the differentiation and growth of male type genital organs in female gastropods.

Preliminary experimental results on RXR gene expression, RXR protein content, immunohistochemical staining with an anti-RXR antibody, and time-course RXR gene expression after exposure to organotins with the rock shell (*T. clavigera*) further support the hypothesis that RXR plays an important role in inducing the development of imposex caused by organotins in female gastropods (Nishikawa, Ohta and Horiguchi, manuscript in preparation). Further studies of histological, immunohistochemical, biochemical and molecular biological techniques are needed to elucidate the complete mechanism of action of TBT or TPT on the development of imposex in gastropods. A certain morphogenetic factor could be involved in the formation of the curved penis and vas deferens. It is also possible that other factors, such as certain neuropeptides, might be associated with the development of imposex through RXR gene expression induced in the head ganglia by exposure to organotins if these factors are induced in the downstream of the RXR cascade [61].

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EFFECTS OF HEAVY METAL IONS ON INTERACTION BETWEEN PY, AN AND HUMIC ACID BY FLUOROMETRY

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Polynuclear aromatic hydrocarbons (PAHs) are widespread and important pollutants found in environment, and are potential health hazardous because of their carcinogenic, mutagenic, toxic and genotoxic properties due to their low volatility, low water solubility, low reactivity and high octanol-water partition coefficient [1]. In real environment, it is well known that the mentioned characteristics of PAHs were greatly influenced within the presence of humic substances, such as humic acid (HA), as well as co-existence components, such as heavy metal ions and so on, because the interactions of HA with these co-existence changed the chemical structure of HA evidently. On the other hand, many studies have shown that the fraction of PAHs to cause ecotoxicological effects is not the total amount of PAHs, but only the bioavailable fraction of PAHs. Recently, it has been reported that bioavailability of PAHs has a very close relationship with the presence of HAs, especially to their chemical structure in real aquatic environment [2]. However, the characteristics and structure of HA varied greatly depended on their sources and their preparation methods from lab to lab [3]. In order to comprehensively understand the environmental behavior of PAHs, such as their remobilization, absorption on particles, evaporation, bioadsorption, complex with heavy metals, sediment treatment, water treatment, ecotoxicity, photolysis, biodegradation and so on, especially their ecotoxicological effects on marine ecosytem, the interactions between HA and PAHs in the presence of heavy metal ions should be studied.

In the passed decades, interactions between HA and PAHs were widely studied, and the interaction mechanism of HA and PAHs were also explored. Several models for describing the interaction mechanism, such as binding site and so on, were developed [4]. Among of them several models to describe the interaction between HA and PAHs, For example, membrane-micelle model (Micelle separation hydrophobic interaction), bit electrolytes model (Negative charge of HA) and Schnitzer & Khan model (Supramolecular interaction) were accepted [5-9]. Based on the proposed models, great progress had been made and most of the efforts were focused on the effects of pH value, ion strength, temperature, and salinity on structure of HA. Unfortunately, conflicted results were reported, because different sources and separation methods led to different structure of HA, which were used in different labs, resulted in conflicted explanation and different interaction mechanism of HA with PAHs.

Fluorescence quenching method was established and widely used because of no separation, simple and easy operation, which had been became a typical method for measuring the interactions between PAHs and other components [10]. The principle of

this method obeyed Stern－Volmer Equation as following:

$$\frac{F_0}{F} = 1 + K_{oc}[OC] \quad ----(1)$$

where F_0 is the initial fluorescence intensity from fluorophores, F is fluorescence intensity with in the presence of HA. K_{oc} is binding constant between HA and fluorophoes and [OC] means the concentration of HA. With this method K_{oc} values between HA and fluorophoes can be determined and the effects of chemical conditions on interaction mechanism of HA and fluorophoes can also be studied.

In the present work, An and Py have been selected as fluorescent model compounds with lower and higher molecular weight to investigate the effects of heavy metal ions on interaction between dissolved An, Py and HA by a newly established fluorescence quenching method for determination of dissolved PAHs. It was believed that dissolved fraction of PAHs has a very close relationship with their biovalaibility. Furthermore, it might be provide us a new way to try to understand more on the concept of molecular cluster of bioactivity and effects of bioactive components on the structure of HA. Satisfactory results were obtained with the established method.

1. Experiments

1.1Appaurators and regents

HA (C.P., The second Chemical factory of Shanghai) pretreatment procedures and preparation of An, Py (purity>99%, Aldrich) aqueous solution were described in reference [11], concentration of HA was 10mg/L. Milli-Q water was used throughout the experiment. $Ni(NO_3)_2$ (A.R.，Shanghai Hengxin Co.), $FeSO_4$, $ZnCl_2$ (A.R.，Shanghai Medical and Chemical Co.), $BaCl_2$ (A.R., SiLian Chemical Co.), $CuCl_2$ (A.R.，Shanghai KeChang Chemical Co.), NaOH (A.R.，Guangzhou Chemical reagent Co.) were used without any further purification.

Cary Eclipse 100 Fluorescence spectrophotometer (Varian, USA). The excitation and emission slits were set at 5nm, respectively. Scan speed is 20nm/min. λ_{ex}= 238nm, λ_{em}=373nm, Voltage of PMT was set at medium. Nicolet740 FT Infrared (IR) spectrometer (USA)。

1.2 Procedures

Sample preparation for infrared spectra (IR) measurement. A certain amount of different heavy metal ions solutions were pipetted into HA solution, mixed them up and kept the solution still for enough time. The precipitate came from the formation of complex between HA and heavy metal ions were obtained by high-speed centrifugation. Potassium bromide bill contains proper the precipitate samples were prepared for IR measurement.

Determination of K_{oc} HA-Py. Proper different amount of heavy metal ions solution were pipetted into a series HA-Py solution contain, where the concentration of Py was 1.0×10^{-7}mol/L. Kept all of the solution still for more than 12h, and then the

fluorescence spectra of Py was measured. Upper clear solution was used for measuring fluorescence spectra when the concentration of HA was higher than 20mg/L or precipitation appeared.

2. Results and discussion

2.1 Determination Koc of HA-An, HA-Py by Fluorescence quenching method

2.1.1 Characteristic of HA used in the Lab

Experimental results of element analysis of purified HA, which were used in the lab, were showed in Table one. It was found that purified HA contains relatively less carbon and hydrogen compared with the unpurified HA, it means that purified HA was the low-weight molecular fraction and with higher solubility in water, and this is very important for carrying on following experiments.

Tab.1.1 Element analysis of HA

Elements	C %	H %	N %
SHA(unpurified)	59.57	4.81	N.D
SHA (purified)	35.46	2.81	N.D
AHA(unpurified)	29.1	3.36	0.52
AHA(purified)	23.8	1.89	0.51

*SHA, AHA were two kinds of HAs

2.1.2 Determination of K_{oc} HA-An, HA-Py by fluorescence quenching method

Effects of concentration of HA on the fluorescence spectra of dissolved An, Py were investigated. The experimental results demonstrated that both of the fluorescence intensity of An and Py were quenched gradually with the increment of HA's concentration. However, either the shape of fluorescent spectra or the wavelength location of the fingerprinting peaks for both of An and Py seemed did not changed [11]. It was implied that fluorescence quenching method was an acceptable way to investigate the interaction of HA between An, Py and other co-existences.

2.1.3. Effects of pH on K_{oc} HA-An, HA-Py

It is well known that pH value has a great effect on the structure of HA because

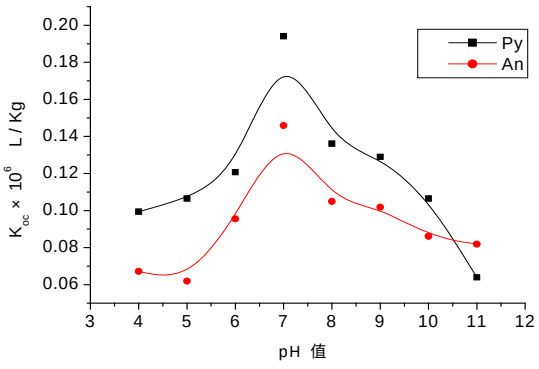

Fig. 1. Effects of pH on K_{OC} between Py, An and HA

($C_{Py}=1.0\times10^{-7}$mol/L，$C_{An}=1.0\times10^{-7}$mol/L)

HA contains hydroxide, carboxyl and other C-O bindings. Effects of pH on K_{oc} of HA-An, HA-Py were investigated and the experimental results showed in Fig.1. It was found that for both of An and Py had their own highest K_{oc} value when pH =7, respectively. These results can be interpreted quiet well with the application of membrane-micelle model.

2.1.4. Effects of heavy metal ions on the structure of HA

HA and HA-heavy metal ions complexes have their own characteristic infrared absorption spectra. Purified HA and its complexes with copper, iron and nickel ions, were investigated by IR spectra, respectively. The experimental results were showed in Fig. 2,3,4. From these experimental results, it is evident that the interaction points between HA and heavy metal ions were located in the substitutes of carboxyl and C-O bindings. At the same time, it can be found that the structure of HA, especially to the structure based on membrane-micelle model, were changed greatly in the presence of heavy metals.

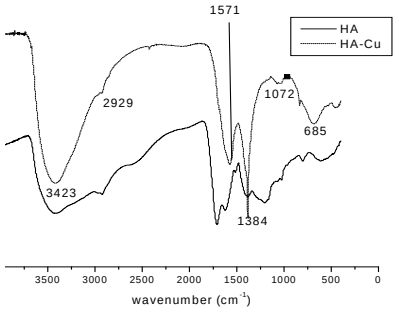

Fig.2 Comparable Infrared spectra of HA after complexation with Cu^{2+}
(C_{HA}=200mg/L, C_{Cu}^{2+}=0.1mol/L)

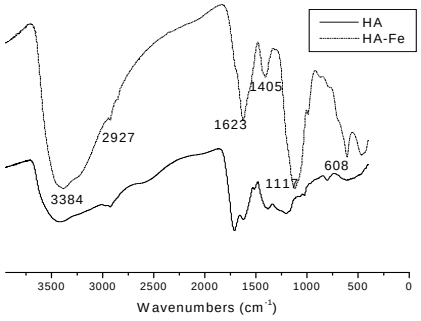

Fig.3 Comparable Infrared spectra of HA after complexation with Fe^{2+}
(C_{HA}=200mg/L, C_{Fe}^{2+}=0.1mol/L)

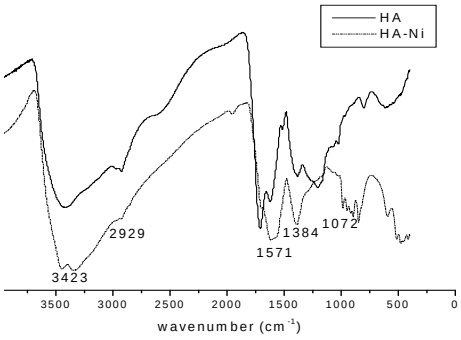

Fig.4 Comparable infrared spectra of HA after complexation with Ni^{2+}
(C_{HA}=200mg/L, C_{Ni}^{2+}=0.1mol/L)

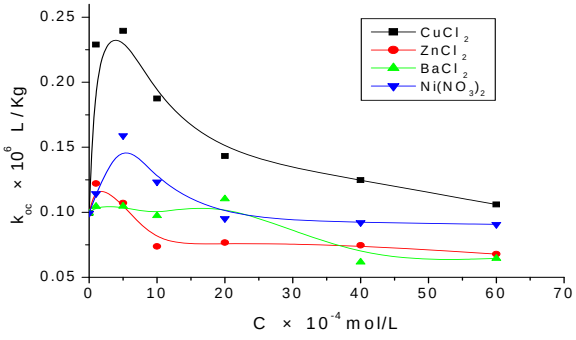

Fig.5 Effects of different heavy metal ions on K_{oc} of HA and Py （$C_{Py}=1.0\times10^{-7}$mol/L）

2.1.5. Effects of different heavy metal ions on the K_{oc} HA-Py

Copper, zinc, barium and nickel ions are some of heavy metal ions wide spread in the aquatic environment. They were selected to investigate the effects of different heavy metal ions on K_{oc} HA-An, HA-Py. The experimental results were showed in Fig.5. From Fig.5 it can be found that K_{oc} value were increased very quickly with the increment of heavy metal ions' concentration at the beginning stage. These experimental results can be explained that the effects of charge neutralization between HA and heavy metal ions were much stronger than that of hydrophobic interaction between Py and HA, especially for the nonpolar cavity formed from the membrane-micelle model. After that, K_{oc} values were decreased with the increment of heavy metals concentration at the second stage. Because complexation of heavy metal ions and HA were dominated processes and that destroyed the hydrophobic cavity from membrane-micelle, as well as electrostatic repulsion of heavy metal ions compressed molecule of HA. However, with the further increment of heavy metal concentrations, complexation between heavy metal ions and HA were completed, newly stabilized HA molecular structure were reconstructed, that resulted in the K_{oc} values toward constant at last. These experimental results were identical to some reported results[12] .

2.2. Study on the interaction between HA and Py by fluorescent probe

2.2.1. Validation of fluorescent probe method

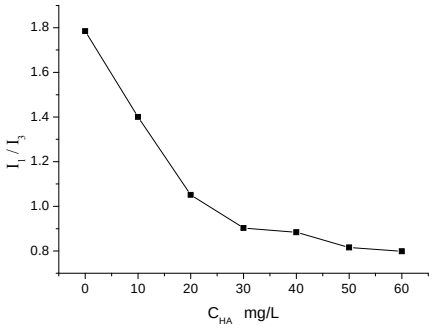

Fig. 6. Effects of Concentration of HA on I_1/I_3 of Py(C_{Py}=1.0×10^{-7}mol/L)

As mentioned elsewhere, Py was widely used as a nonpolar fluorescent probe in supermolecular chemistry, because the strength ratio of its third vibrational band to the first one (I_1/I_3) varied very sensitively to its microenvironmental polarity. This property of Py might be here firstly to be employed to investigate the effects of heavy metal ions on the interaction between Py and HA. Fig. 6 showed us the experimental results that the variation of I_1/I_3 of Py with the increment of HA's concentration, it is clearly that the hydrophobicity the nonpolar cavity formed from the membrane- micelle model probed by Py were increased. It is implied that I_1/I_3 of Py could be useful tool to study on the environmental behavior of Py in aqueous solution.

2.2.2. Determination of K_{oc} HA-Py

With the novel validated fluorescence probe method, Effects of pH on K_{oc} HA- Py

were also investigated. The experimental results showed in Fig.9. It was found that K_{oc} HA-Py had its highest K_{oc} value too when pH =7. It is further demonstrated that the interaction between Py and HA can be interpreted quiet well with the application of membrane-micelle model.

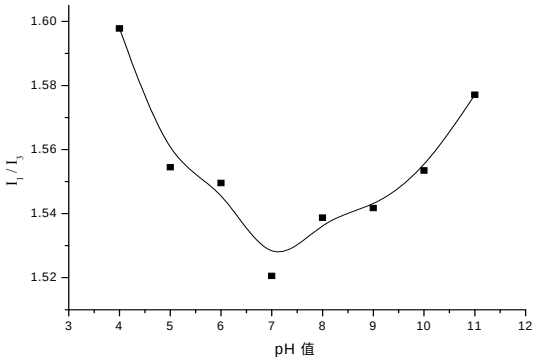

Fig.7 Effect of pH on I_1/I_3 of Py in the present of HA ($C_{Py}=1.0\times10^{-7}$mol/L)

2.2.3 Effects of salinity on K_{oc} HA-Py

In real aquatic environment, especially in estuary area, the environmental behavior of PAHs and their ecotoxicological effects were greatly influenced by the variation of water salinity. It is necessary to know the effects of salinity on the interaction between Py and HA. With the fluorescence probe method, effects of salinity (expressed as NaCl concentration in the lab) on K_{oc} HA-Py were investigated by I_1/I_3 of Py and the experimental results were showed in Fig.8. From Fig.8, it can be found that HA solution has a certain capacity to buffer the variation of salinity if the concentration of NaCl were not so high. This results was very agreeable to the experimental results obtained in 2.1.3 and further demonstrated that charge neutralization had a positive effects on formation membrane-micelle. However, I_1/I_3 of Py increased gradually when the concentration of NaCl was higher than 0.003 mol/L. this experimental results might be used to explain that the reasons why bioavailability of PAHs changed greatly in estuary area.

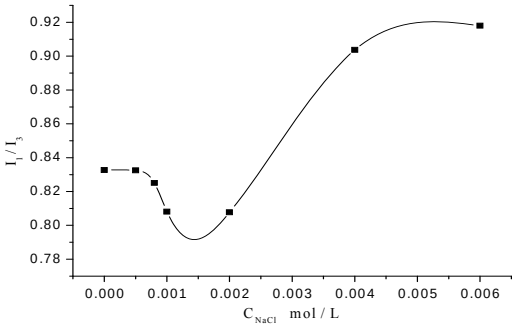

Fig.8. Dependence of I_1/I_3 of Py on the concentration of NaCl in the presence of HA (C_{HA}=40mg/L)

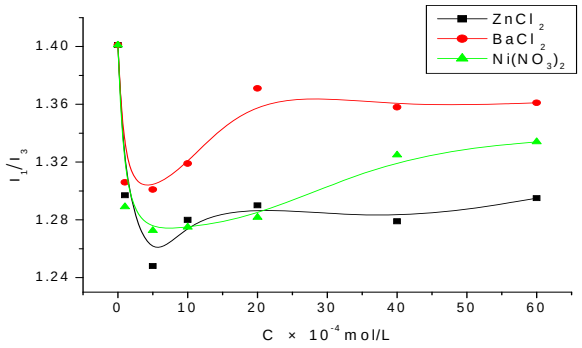

Fig. 9. Effects of concentration of heavy ions on I_1/I_3 of Py in the presence of HA (C_{Py}＝1.0×10^{-7}mol/L, C_{HA}＝10mg/L)

2.2.4. Effects of different heavy metal ions on the K_{oc} HA-Py by I_1/I_3 of Py

As mentioned above, copper, zinc, barium and nickel ions were selected to investigate the effects of different heavy metal ions on K_{oc} between Py and HA. To further demonstrated the effects of heavy metal ions on the K_{oc} HA-Py, zinc, barium and nickel ions were selected to investigate the effects of different heavy metal ions on K_{oc} HA-Py by I_1/I_3 of Py. The experimental results were showed in Fig.9. Compared Fig.5 with Fig.9, it can be found that a same variation tendency of K_{oc} values were also obtained by I_1/I_3 of Py. With the increment of heavy metal ion concentration, the variation tendency of K_{oc} are quite similar to that of shown in Fig.5. The explanation are as same as that of in 2.1.5. At the same time, these experimental results were identical to the references [12].

2.3 ^{1}H NMR spectra of HA in the presence of PAHs

2.3.1 Effects of pH on ^{1}H NMR spectra of HA

In order to understand the interaction between Py and HA more, the effects of pH on ^{1}H NMR spectra of HA were investigated. The experiments were showed in Fig.10. From the Fig.10, it was shown that ^{1}H NMR spectra of HA were greatly influenced by

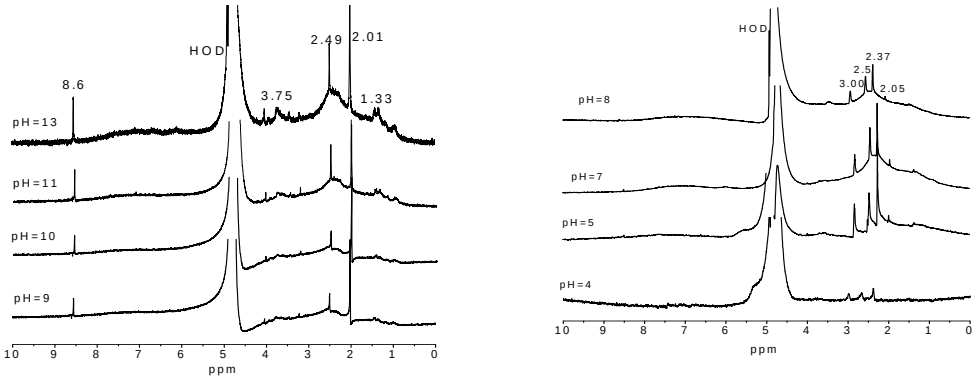

Fig.10 ^{1}H NMR spectra of HA with different pH

the variation of pH. It was quiet agreeable with the experimental results obtained by the two methods mentioned above. In order meet the requirement for applying this method to real samples, pH =7 was selected to study on where the interaction points were located for HA and PAHs.

2.3.2. ^{1}H NMR spectra of HA in the presence of PAHs

Under the selected experimental conditions, ^{1}H NMR spectra of HA in the presence of benzene, naphthalene and Py were measured and the experimental results were showed in Fig.11. From Fig.11, it can be found that great changes of ^{1}H NMR spectra of HA had been taken place in the aliphatic chain area when benzene, naphthalene or Py were added. That means the interactions of HA with Py were located in the aliphatic part of HA. The reason might be explained as the purified HA contains less aromatic rings.

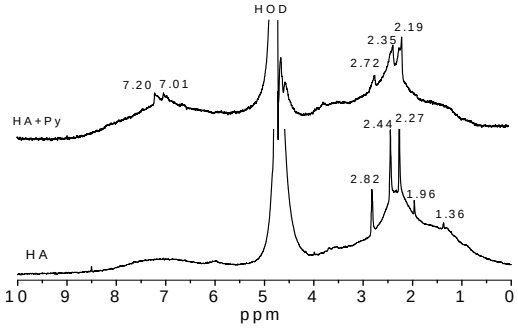

Fig. 11 ^{1}H NMR spectra of HA before/after Py added, pH=7

3. Conclusion

From the above experimental results, it can be concluded that the fluorescence quenching method for study on the interaction between HA and dissolved Py was acceptable. The effects of heavy metal ions on the interaction between HA and Py were depended on the binding sites of HA and heavy metal ions, they were located in the carboxyl and hydroxyl of HA and the interactions between HA and Py were located at aliphatic chain of HA. In the presence of heavy metal ions, charge neutralization and complexation changed the HA structure, the binding constant (K_{oc}) between HA and Py obeyed a same trend in which with the increment of heavy metal ions, K_{oc} increased firstly, and then decreased, finally reached a stable value. In the near future, molecular cluster of bioactivity and effects of bioactive components on the structure of HA were to be concerned.

Acknowledgment:

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DEVELOPMENT OF POLAR ORGANIC POLLUTANT INTEGRATIVE SAMPLER FOR ENDOCRINE DISRUPTING CHEMICALS IN WATER

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Abstract
A performance optimization of polar organic pollutant integrative sampler (POPIS) for endocrine disrupting chemicals (EDCs) in water was developed in this study. Four highly potent and polar EDCs including bisphenol A (BPA), estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2) were selected as the target compounds. The prototype POPIS consists of three major components, including a sorbent phase, membrane and deployment device. Laboratory experiments were performed in a flow through tank allowing the continuous mixing of target analytes and water. The kinetics of EDC uptake by POPIS was studied for a duration of 10 days. The effects on POPIS performance of environmental conditions including different initial EDC concentrations, pH and salinity were studied. In addition, the effects of the size of the prototype sampler and the type of membrane on POPIS performance were evaluated. Estimation of the ambient water concentration of target chemicals was achieved by using appropriate uptake models and the determination of POPIS sampling rates for appropriate exposure conditions. Use of POPIS in field validation studies targeting the selected EDCs in the River Ouse resulted in the detection of the chemicals at estimated concentration of 44.2-106 ng/L. These values are in agreement with the levels found in traditional grab samples taken concurrently.

***Keywords*:** Polar organic pollutant integrative sampler; EDCs, passive sampler;

1 Introduction

Water pollution is a worldwide problem. Of the various pollutants, EDCs are the focus of current environmental research and regulations. [1-3].A variety of adverse effects have been observed in the environment that have been attributed to EDCs, including mimicking and blocking hormonal action at receptor molecules, or interfering with synthesis, transport and feedback mechanisms. Considering the potential impacts of EDCs, it is urgent to routinely monitor the levels of such compounds in aquatic systems [4-6]. The most widely used technique for performing such monitoring is spot sampling followed by laboratory-based extraction and analysis of target compounds [7-9]. This approach, however, yields only an instantaneous measurement of pollutant levels and suffers from the uncertainty of short- and long-term concentration variations. An increase in sampling frequency or the use of flow- and time-weighted automatic samplers may reduce these errors and give a more accurate picture of time-integrated pollutant levels; however, the associated increase in costs may prove prohibitive [10-11].

There has been rapid development in the use of passive sampling devices that allow continuous monitoring of aqueous pollutants without the disadvantages of using organisms [10-12]. Of the various passive sampling devices, the most widely used is the so-called semi-permeable membrane device (SPMD) consisting of a tubular lay-flat low-density polyethylene membrane containing a thin film of a high molecular weight lipid such as triolein [13-14]. When placed in an aquatic environment, SPMD passively accumulates organic compounds. In comparison to traditional methods of water sampling, SPMD is easy to use, can be standardized, can be deployed over a long period, can detect extremely low levels of organic contaminants, and most important of all, mimics the bioconcentration of pollutants in aquatic organisms. In addition, SPMD does not suffer from adverse effects as organisms; hence offer the best approach for bioavailability studies [15-16]. However, SPMD is only suitable for hydrophobic organic pollutants (e.g. organochlorine pesticides), as either the membrane is impermeable to polar compounds or accumulation is thermodynamically unfavourable due to the low affinity of the receiving phase for such analytes [17]. Recently there has been development in the passive sampling of polar organic compounds including POCIS (polar organic chemical integrative sampler) [18] and Chemcatcher [19]. POCIS comprises a solid receiving phase material (sorbent) sandwiched between two microporous polyethersulfone (PES) diffusion limiting membranes. The sorbent used can be changed to target specific compounds or chemical classes. POCIS samples from the dissolved phase and thereby enables the chemicals to be estimated [12,18]. Chemcatcher uses a diffusion-limiting membrane and a bound, solid phase receiving phase. Accumulation rates and selectivity are regulated by the choice of both the diffusion-limiting membrane and the solid-phase receiving material; both are supported and sealed in place by an inert plastic housing [12,19]. Both of these samplers have advantages over traditional methods of sampling; however, they have limitations in their performance, for example, the POCIS compression holder was made of stainless steel which may be prone to corrosion when applied to the marine environment. Their calibration experiments were only carried out in very small systems (e.g. 1 L microcosms) [18]. Certain factors which may affect the sampling rate were not investigated, e.g. pollutant concentration, exposure size, pH and salinity [18-19].

This work will focus on the development and validation of a polar organic pollutant integrative sampler (POPIS). The POPIS consists of a PTFE material holder, membrane and sorbent. Laboratory calibration will be performed to evaluate the sampling rate for the four target EDCs including bisphenol A (BPA), E1, E2 and EE2. The effects of different initial EDC concentrations, pH, salinity and size of sampler on POPIS sampling rates, will be evaluated. Estimation of the ambient aqueous concentration of target chemicals will be achieved by using appropriate uptake models and sampling rates derived from laboratory experiments. POPIS will also be applied in the field to demonstrate its applicability for aquatic monitoring.

2 Experimental

2.1 Sampler Design

The POPIS device was made of three parts: the holder (component 1), membrane (component 3) and the sorbent (component 4), which were joined by a watertight screw

thread (component 2), as shown in Fig. 1. The device was suspended in the water by a nylon string attached to the fastening lug (component 5).

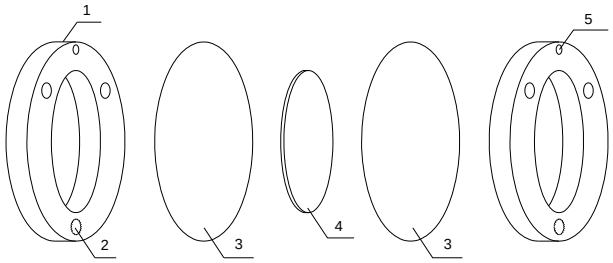

Fig. 1. The schematic diagram of the POPIS device (1-PTFE Holder, 2-screw, 3-membrane, 4-sorbent, 5-hole)

2.2 Reagents and Materials

The solvents used including methanol, ethyl acetate, acetone and dichloromethane (DCM), purchased from Rathburns, were of distilled-in-glass grade. Compounds E1, E2, EE2, and E2-d_2 were purchased from Sigma, UK, and BPA, BPA-d_{16} and BSTFA containing 1% of trimethylchlorosilane (TMCS) were supplied by Aldrich (Dorset, UK). Oasis HLB sorbent (poly[divinylbenzene]-co-N-vinylpyrrolidone) was supplied by Waters Ltd, UK. The PES membrane (0.1 μm pore size) and polysulfone (PS) membrane (0.2 μm pore size) were provided by Pall Gelman Sciences (VMR International, UK). PTFE used to construct the sampler was from Aquarius Plastics Ltd., UK. A two-channel peristaltic pump (Watson Marlow 401U/DM2) and tubes (Bore × Wall: 0.5mm×1.6mm and 3.2mm×1.6mm) for controlling the flow-through system were provided from Fisher, UK. Glass tanks (50cm×40cm×30cm) were from Norwood Aquarium LTD, UK.

2.3 Solvent Extraction and Solid-phase Extraction (SPE) and GC-MS

The target compounds in the sorbent were extracted by methanol. The recovery (with % RSD, relative standard deviation) of these four target analytes were from 83.1% (7.1%) for E1 to 114% (16.1%) for BPA. The target compounds were extracted from water samples by SPE technique, derivatization was necessary for these compound and then determined by GC-MS, following the previously developed method [20-21]. The recovery (with %RSD) of target compounds was from 81% (0.8%) for EE2 to 93% (3.8%) for BPA. The limit of detection (LOD) of target analytes was from 0.03 to 0.7 ng/L, with the limit of quantification (LOQ) being 0.1-2.2 ng/L.

2.4 Controlled Concentration in a Flow-through System

A flow-through tank allowing the continuous mixing of EDCs with water was used throughout the calibration and optimization studies. The sampling devices were exposed to an aqueous solution of EDCs under controlled conditions. Distilled water, and a water solution of the four analytes at 30 ng/mL were pumped into the exposure tank by the peristaltic pumps. The tank was filled with water to half of its volume throughout the exposure experiments. The rates of addition of distilled water and EDC solution were controlled at approximately 20 and 1 mL/min, respectively. This resulted in the target compound concentration in the exposure tank of about 1.5 ng/mL and a replacement

time for the system of 23.8 h. The concentration of analytes in the exposure tank was checked daily for their stability by analysis of water samples (100 mL) taken from the effluent.

2.5 Sampler Deployment in the Field

Three trial sites in the River Ouse, West Sussex, England, were chosen for field testing of POPIS, including the outfall of Scaynes Hill Sewage Treatment Works, and its upstream and downstream. An initial assessment of water quality was carried out by the extraction and GC-MS analysis of three spot samples (1 L). Three POPIS fitted with a 0.1 μm pore size PES membrane and HLB Oasis sorbent (100 mg for each POPIS) as receiving phase with the exposure diameter of 27 mm, were deployed in close proximity at each site. A nylon line was used to suspend the samplers vertically at a water depth of 50-100 cm. The samplers remained in situ for 1 week, after which 3 samplers from each site were removed for analysis. Three water samples (1 L) was also taken approximately weekly from each site to determine the levels of target compounds. Water properties such as temperature, pH and dissolved oxygen at each site was also recorded at each visit.

3 Results and Discussion

3.1 Principles

Models describing the uptake kinetics of organic contaminants in water by passive sampling devices constructed from a receiving phase and a diffusion-limiting membrane [13,18,22-24] have been developed. The principles of analyte uptake described for SPMD and other passive sampler are applicable to the sampler described in this study. For practical purposes, uptake in the linear phase could be described as follows [18,24-25]:

$$M_s = M_0 + C_w R_s t \qquad (1)$$

where M_s is the mass of analyte in the receiving phase, C_w represents EDC concentration in water during the deployment period. R_s is the sampling rate of the system. M_0 is the analyte mass in the receiving phase at the start of exposure. As M_0 is always equal to 0 at the start of exposure, so eq 1 could be simplified as:

$$M_s = C_w R_s t \qquad (2)$$

When R_s is known, C_w (time-weighted average concentration of a pollutant in water) may be calculated from the sampling rate (R_s), exposure time (t) and the amount (M_s) of the analyte trapped by the receiving phase.

R_s can be derived from eq 3:

$$R_s = K_{ov} A \qquad (3)$$

where K_{ov} [m/s] is the overall mass transfer coefficient and A [m^2] is the surface area of the membrane. The sampling rate of an individual chemical can be determined experimentally under fixed conditions at constant analyte concentration [25].

3.2 Membrane Evaluation

The microporous POPIS membrane acts as a semi-permeable barrier between the sorbent and the surrounding aquatic environment. It allows polar organic chemicals to pass through to the sorbent, while particulate matter, colloids, and biota (including microorganisms) with cross-sectional diameters greater than the membrane pore diameter will be excluded selectively. Direct contact of

these excluded materials with the sorbent may result in a site-specific bias of apparent contaminant concentrations in the sorbent, reduce uptake due to greater biofouling of the sorbent than the membrane, and potentially interfere with sample processing and analysis. Kingston et al. [19] suggested that PS membrane was suitable for polar organic compounds. Alvarez et al. [18] concluded that PES membrane was preferred to sample polar organic contaminants, after studying many types of membrane and considering factors such as the biodegradability, pore size, heat protection, strength and durability.

In this study, the performance of POPIS made by PES and PS membrane was compared. After 48 h exposure at EDC concentration of about 1.5 ng/mL in the flow-through system, the sampling rate of POPIS were calculated by eq 2. As shown in Fig.2, the sampling rate of the four target compounds for PES was from 0.033 to 0.048 L/d, with a RSD of 12.5-19.1%; while for PS the R_s was 0.004-0.017 L/d, with a RSD of 6.8-13.4%. The R_s for POPIS by PES was much higher than that by PS, so PES was selected as passive sampler membrane for further experiments.

3.3 Sampler Size Optimization

The kinetics of EDC trapping in POPIS are expected to depend on the size, or perhaps more importantly, the exposure surface area. Three different POPIS exposure sizes were selected for investigations, the diameter of which were 27 mm, 38 mm and 54 mm respectively. These three different samplers had an exposure area ($A = \pi r^2$) of 572, 1133 and 2289 mm^2 respectively. The relationship between the sampling rate (R_s) and sampler exposure surface area (A) was shown in Fig.3. The results show that R_s had a linear relationship with the exposure surface area of sampler, with the correlation coefficient (R^2) values of the regression from 0.82 (EE2) to 1.0 (BPA). The trend is similar to what was described in eq 3 [25].

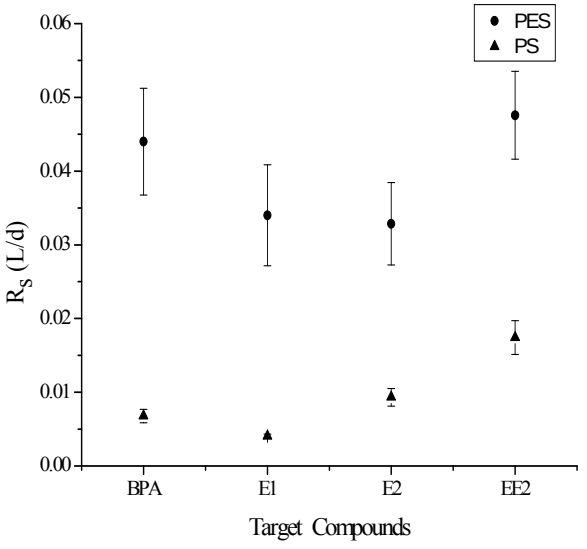

Fig 2. Comparison of the sampling rate for POPIS sampling rate through PES and PS membrane

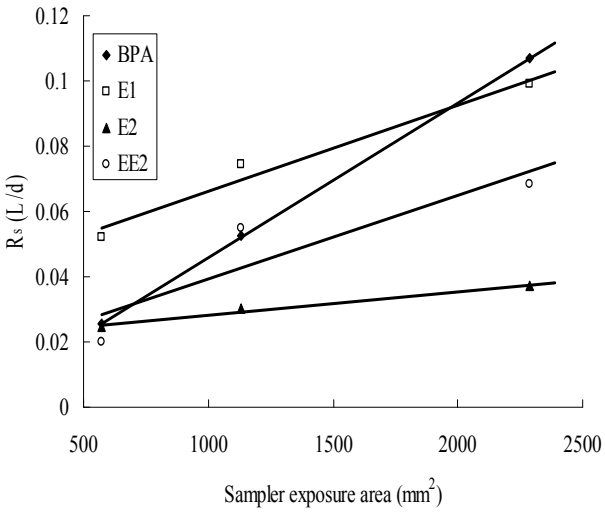

Fig 3. Relationship between the and the exposure surface area of POPIS

Huckins et al. [16] also suggested that the integrative sampling rate (R_s) was proportional to the surface area of the sampling device for SPMD.When investigating the amount of target compound absorbed per exposure surface area by POPIS, it was interesting to find that the amount of target compounds absorbed in sampler decreased with the exposure area (Fig. 4). This suggests that under identical conditions (same

amount of sorbent mass, same exposure time and same EDC concentration), the smaller the exposure area, the greater amount of the target compounds absorbed per unit surface area was for the sampler. This could be due to the higher diffusion gradient for the smaller exposure surface area. When the other exposure conditions are the same, especially for the same amount of sorbent mass of POPIS, the diffusion gradient will enhance the speed of sorbent absorbing target compounds from the ambient waters. It is therefore beneficial to save PTFE material when designing the sampler for field applications, by using smaller sized POPIS. As a result, POPIS with a sorbent diameter of 27 mm and a sampling surface area to sorbent mass ratio of about 57.2 cm^2/g was chosen as the optimum for further exposure experiments.

3.4 Sampling Rate

The sampling rate (R_s) is essential for the application of POPIS in field monitoring. R_s of target compounds can be determined experimentally under controlled conditions at constant analyte concentration. The sampler was fitted with a 0.1 μm pore size PES membrane and HLB Oasis sorbent (100 mg for each POPIS) as receiving phase with the exposure diameter of 27 mm. Passive samplers were exposed in a constant concentration flow-through exposure system for 10 days, where EDC concentrations remained at about 1.5 ng/ml. Satisfactory linear regression for the uptake data of analytes from water to the sampler sorbents were obtained for all compounds.

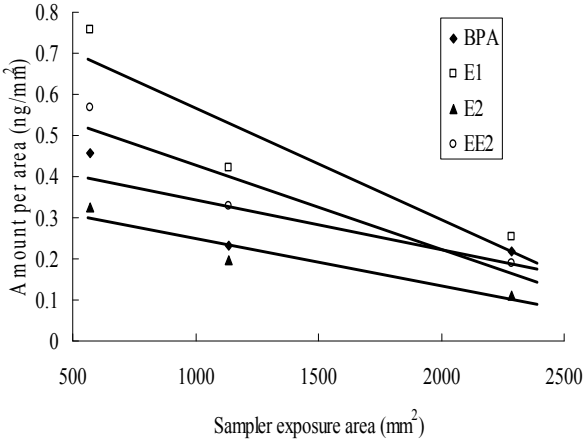

Fig.4 The absorbed amounts of target compound per unit exposure area for POPIS

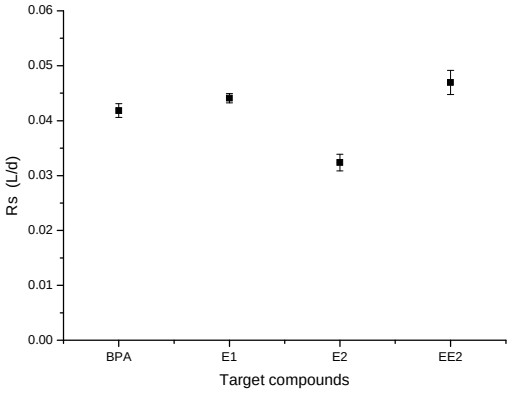

Fig.5 The effect of pH values on sampling rate

On the uptake pattern of analytes by passive samplers, previous studies [13,26] have divided the accumulation process into three possible stages of uptake: linear, curvilinear and finally steady state. During the initial deployment of samplers, the rate of analyte uptake is approximately linear. Under these conditions, the concentration of analyte in the receiving phase is dependent on the concentration to which the system has been exposed (eq 2). The results in this study showed that the uptake kinetics of POPIS was linear in the first 10 days, which supported the theory and confirmed its potential practicability. The R_s values were calculated by eq 2 and the values varied from 0.037 L/d for E2 to 0.051 L/d for EE2 (Table 1). In addition, various factors that may affect the sampling rate, such as pH values, salinity and EDC concentrations, were examined.

TABLE 1. Effect of EDC concentrations on the Sampling rates (R_s) of POPIS (L/d)

Conentration (ng/L)	BPA	E1	E2	EE2
10	0.047	0.034	0.041	0.047
20	0.040	0.039	0.037	0.065
50	0.053	0.040	0.028	0.049
100	0.028	0.026	0.029	0.046
250	0.038	0.040	0.039	0.053
500	0.042	0.039	0.042	0.051
1000	0.033	0.064	0.045	0.046
1500	0.044	0.034	0.033	0.048
Average	0.041	0.039	0.037	0.051
SD	0.008	0.011	0.006	0.006
RSD (%)	19.3	27.8	16.9	12.4

Effect of pH on Sampling Rate

The effect of pH on sampling fate was studied by adjusting the pH value of water sample with diluted solutions of sodium hydroxide and hydrochloric acid. Generally, the speciation of weakly acidic compounds in aqueous solutions depends on the solution properties, e.g. pH value. A series of experiments for investigation of pH effect on sampling rate were performed, over 3 days in the flow-through system with constant target compound concentration. The results (Fig. 8) show that the sampling rate (R_s) for all target compounds remains relatively similar among different pH values (4, 6, 8, 10). Coefficients of variation of the calculated sampling rate for each target compound did not exceed 5% among different pH values. The pK_a values of the target compound are all higher than 10, varying from 10.2 for BPA to 10.5 for EE2. At pH value less than 10, the test chemicals will stay as neutral molecules. So the solution pH value has little effect on the sampling rate.

Effect of Salinity on Sampling Rate

Natural waters can have different salinity. It is well known that the aqueous solubility of many organic compounds decreases with increasing salt concentration, thus their absorption efficiency in the sorbent of POPIS may increase. Therefore, the effect of water salinity on POPIS sampling rate was studied by varying salinity from 0 to 35‰. After 3 days exposure in the flow-through system, it was found that the sampling rate did not vary much with the value of salinity (Fig. 6). Coefficients of variation of the calculated sampling rate for each target compound did not exceed 12% in these three salinity values (0, 18‰, 35‰). Hence the sampler could be applied to many types of natural waters, such as fresh water, estuarine water or seawater.

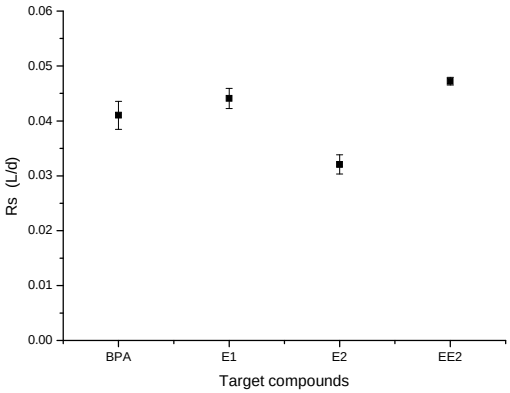

Fig.6 The effect of salinity values on sampling rate

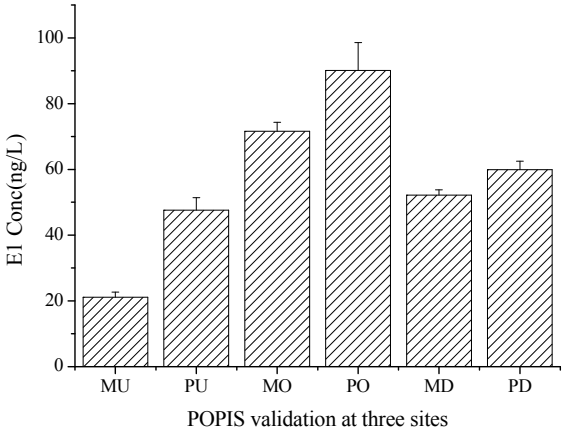

Fig.7 Comparison of instantaneous EDC concentrations by spot sampling and POPIS

Effect of EDC Concentrations on Sampling Rate

The concentrations of organic pollutants are highly variable in different aquatic environments. To investigate the effect of various EDC concentrations on the sampling rate of POPIS, the experiments were carried with EDC concentrations varying from 10 to 1500 ng/L. The exposure experiments lasted up to 3 days in the flow-through system with the concentration of target compounds remaining constant at each value. The sampling rates were calculated and shown in Table 1. The mean R_s value for four target compounds in the various water concentrations ranged from 0.037 to 0.051 L/d. The R_s nearly remained constant in the varying EDC concentrations with RSD from 12% for EE2 to 28% for E1. The results here were consistent with those described in the literature which indicated that the sampling rates for SPMD are independent of environmental concentration [16]. The findings suggest that the sampler can be applied for monitoring EDCs of different concentrations in different environments.

Validation by Field Deployment

The POPIS were deployed at three sites in River Ouse catchment, West Sussex, England, for a period of 1 week. These three sites included the outfall of Scaynes Hill Sewage Works, and its upstream and downstream. Three spot water samples (1 L) were taken adjacent to where the samplers were deployed at each site. The target compounds in POPIS and water were extracted and analysed by the methods described earlier. The laboratory derived sampling rates of 4 target chemicals for POPIS were used in the calculation of predicted ambient water concentrations by eq 2. As shown in Fig. 7 for E1(the other compounds not showed), the measured aqueous concentrations of EDCs by spot sampling were between 15.8-35.4 ng/L, 21.1-71.6 ng/L, 32.6-34.1 ng/L and 49.8-69.9 ng/L for BPA, E1, E2 and EE2, respectively. Such levels are consistent with earlier reports of these compounds in the region. The EDC concentrations predicted by POPIS were between 44.2-71.2 ng/L, 47.6-90.1 ng/L, 56.2-63.6 ng/L and 60.0-106 ng/L for BPA, E1, E2 and EE2, respectively. It is apparent that the predicted EDC concentrations are slightly higher than those by spot sampling, although the two sets of values from the two sampling methods show a very good agreement in the magnitude of

the analyte concentrations. In addition, the EDC concentrations close to the sewage outfall were always higher than those from upstream or downstream of the outfall, again consistent with other findings [20-21].

The passive sampling device such as POPIS has advantages over spot sampling: high reproducibility, low cost, robustness, and savings in manpower and travel costs. More importantly, POPIS has the potential to detect variability and peaks of EDC concentrations in aquatic systems. Spot sampling necessitates many more field visits during the trial period, and is generally more labor-intensive, than passive sampling. This prototype POPIS sampler may be preferable to standard sampling regimes as it provides time-averaged concentrations for analytes of interest, primarily samples the bioavailable fraction of chemicals from the water column, and requires no power, maintenance, or supervision during deployment. The development and subsequent field evaluation of POPIS suggests that this passive in situ device is a viable option for the integrative sampling of hydrophilic organic contaminants in natural waters.

Acknowledgements

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PETROLEUM HYDROCARBONS CONTAMINATION IN THE EASTERN COAST OF THE GULF OF THAILAND

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Abstract

In order to monitoring contamination of petroleum hydrocarbon in marine environment off the eastern coast of the Gulf of Thailand, water samples were collected at 1 meter depth by drop-bottle technique during September 2003 and October 2005. The total petroleum hydrocarbons (TPH) were determined by UV-fluorescence spectrophotometer after liquid-liquid extraction with *n*-hexane and using a chrysene as the standard. Concentrations of TPH were generally low with varied from <0.01 to 4.31 μg/l in industrial areas and varied from <0.01 to 6.72 μg/l in river mount areas water column, while those in the remote areas were varied from <0.01 to 5.34 μg/l. Elevated TPH concentrations were generally found near fishing ports at river mounts and areas adjacent to intensive shipping activity. Oil spills and leaks represented great concern sources of petroleum hydrocarbon pollution in the Gulf of Thailand. Although oil spills in Thailand's territorial waters have been frequently occurred, but the sudden change in living resources such as corals, seagrass and endangered species (marine turtles and marine mammals) were not shown. However TPH concentrations were generally low, but can have a severe detrimental impact on the living resources in marine environment. The contemporary information is required concerning the distribution and impact of TPH in the marine environment off eastern coast of the Gulf of Thailand. The utility of specialized monitoring tools such as biomarkers for Thai marine environments urgent needs to be examined. With this information, appropriate risk assessment and monitoring can be implemented and effective management strategies developed to protect the marine ecosystems.

Key words; petroleum hydrocarbon; Gulf of Thailand; oil spill

1. Introduction

Petroleum is a complex mixture of different organic compounds formed from a variety of organic materials that are chemically converted under differing geological conditions over long period of time. Crude oils contain primarily carbon and hydrogen, but also contain smaller amounts of sulfur, oxygen and nitrogen as well as metals such as nickel, vanadium and iron [1]. Large oil spill and vast area of oil slick were transported to coastal water and caused a great deal of mass mortality of marine organisms such as *Torrey Canyon* spilled in Britain during 1967, *Amoco Cadiz* was wrecked on the Brittany coast in 1978 [2] and *Exxon Valdez* spilled in coastal area of the Gulf of Alaska 1989 [3]. In Thailand, *Eastern Fortitude* spilled in coastal area off Map Ta Phud, Rayong. A historical of reported oil spills in Thailand shows that during

1973-2003, nearly $1.05 \cdot 10^3$ m^3 of oil have spilled into Thai marine waters which occurred in 44 incidents involving tankers, oil stations, pipeline and vessels [4,5].

Petroleum hydrocarbon pollution of sea water has become a big problem with the development of the petrochemical industry and installation of numerous petrol stations and pipeline on sea bottom along the coast of Thailand. Eastern coastal areas, particularly Chonburi and Rayong provinces, have been classified as the most risk area of oil contamination in the Gulf of Thailand [4]. Numerous studies of petroleum hydrocarbon contamination in sea water, sediment and biota in eastern coast of the Gulf of Thailand have been reported [6-9]. The aim of this study was to clarify the background level and the present status of petroleum hydrocarbon in marine environment along the east coast of the Gulf of Thailand and considerable great concerned to public health.

2. Materials and Methods

2.1 Study area

Eastern coast off the Gulf of Thailand extends over about 513 kilometers, from Chonburi province to Trat province. It was consisting of several activities, particularly industrial estates and heavy traffic of ships. Samples were collected from four areas namely Laem Chabang industrial estate, Chonburi province (13 stations), Map Ta Phud industrial estate, Rayong province (11 stations), Chang and Kut Islands, Trat province (14 stations) and river mouths in Rayong, Chanthaburi and Trat province (14 stations) as shown in Figure 1. There are three large industrial estates were located in this area, namely Laem Chabang in Chonburi province, Map Ta Phud in Rayong province and Thai Petrochemical Industry, Rayong province. All of them consisted of own deep sea port for loading and unloading. These caused increasing of ship traffic along the coastal areas off eastern part of the Gulf of Thailand.

2.2 Sample collection

Three liters of seawater were collected at 1 meter depth from sea surface with pre-cleaned amber glass bottle by drop-bottle technique [10] during October 2003 and August 2005. The fifty milliliters of re-distilled hexane were immediately added to the sample bottles and shook for five minutes on board. Shaken samples were stored in dark and cool place during the cruise. The samples were transported to laboratory, stored in dark and cool until chemical analysis.

2.3 Chemical analytical

The analytical method of total petroleum hydrocarbon was conducted following the method described elsewhere [4] with slight modification. The method consists of three extractions with 50 ml of re-distilled hexane. The residue moisture in pooled extracts was removed by anhydrous sodium sulfate ($NaSO_4$) and concentrated to finally volume about 10 ml by a rotary evaporator at 40ºC. Petroleum hydrocarbon quantification was made on UV-fluorescence spectrophotometer (Perkin-Elmer, model LB50) at constant excitation wavelength of 310 nm, emission wavelength of 360 nm and using a chrysene as the standard. The procedure blank was performed within every analytical batch (five samples and one procedure blank) and using subtracted for presented impurity values in

reagents. Concentrations were expressed as µg as chrysene equivalent per liter (µg/l).

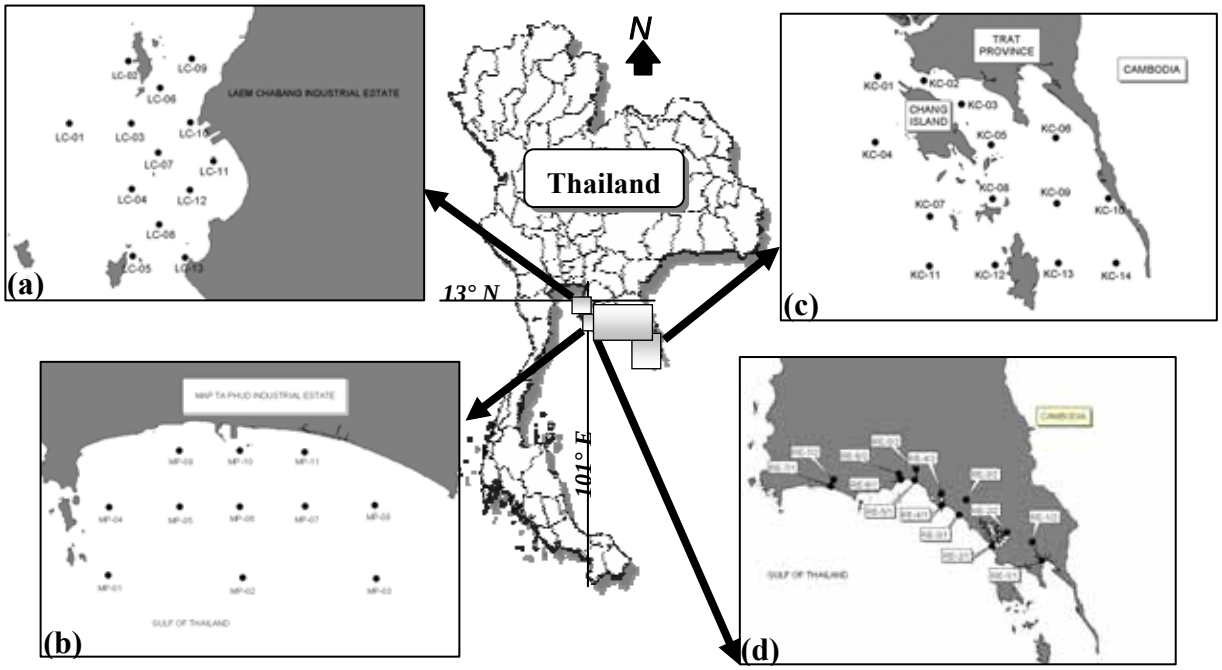

Figure 1. Map of study area off Eastern coast of the Gulf of Thailand. Samples were collected at Laem Chabang industrial estate, Chonburi province (a), Map Ta Phud industrial estate, Rayong province (b), Chang and Kut Islands, Trat province (c) and river mouths in Rayong, Chanthaburi and Trat province (d).

3. Results and Discussion

Concentrations of total petroleum hydrocarbon in sea water collected from east coast of the Gulf of Thailand between September 2003 and October 2005 were showed generally low with varied from <0.01 to 6.72 µg/l and averaging about 0.70±0.97 µg/l (Table 1). The similar levels of contamination were demonstrated in different activity areas such as varied from <0.01 to 4.31 µg/l in industrial areas and varied from <0.01 to 6.72 µg/l in river mount areas water column, while those in the remote areas were varied from <0.01 to 5.34 µg/l (Figure 2). The wide range of contamination of total petroleum hydrocarbon was founded in river mouths. Moreover, elevated total petroleum hydrocarbon concentrations were generally found near fishing ports at river mounts and areas adjacent to intensive shipping activity. These results indicated that sources of petroleum hydrocarbon in sea water were generated from activities of fishing boats which located at the river mouths. Although contamination of petroleum hydrocarbon in sea water adjacent to industrial areas were showed lower level than river mouth and remote area such as Chang and Kut Islands, however, these areas will be considerable to great concerned with high risk of oil contamination. These areas have been also classified as the most risk area of oil contamination in the Gulf of Thailand [4]. Because of the high activities of oil tankers, deep sea vessels, ship yards as well as oil

refineries and transportation.

During our studying, the large incident by oil tanker name “Dragon 1” spilled about 150,000 liters of fuel oil into the coastal water off Pattaya bay, Chonburi province, Thailand on 26 December 2003. This incident was elevated petroleum hydrocarbon concentration in sea water adjacent those area ranging from 0.16 to 0.35 µg/l. The low level of petroleum hydrocarbon in sea water seemed that the spilled oil from tanker did not the contamination sources. Recently, about five tons of used oil from the shipyard was leaked and elevated the petroleum hydrocarbon in adjacent coastal water up to 48.07 µg/l. However, petroleum hydrocarbon levels were decreased to normal level (0.60 µg/l) after two weeks. These may indicated that oil spills and leaks represented great concern sources of petroleum hydrocarbon pollution in the Gulf of Thailand. Although oil spills in Thailand's territorial waters have been frequently occurred, but the sudden change in living resources such as corals, seagrass and endangered species (marine turtles and marine mammals) were not shown.

The comparison of petroleum hydrocarbon contamination in sea waters in the Gulf of Thailand in recent twenty years indicates a similar range of contamination levels (Table 2).

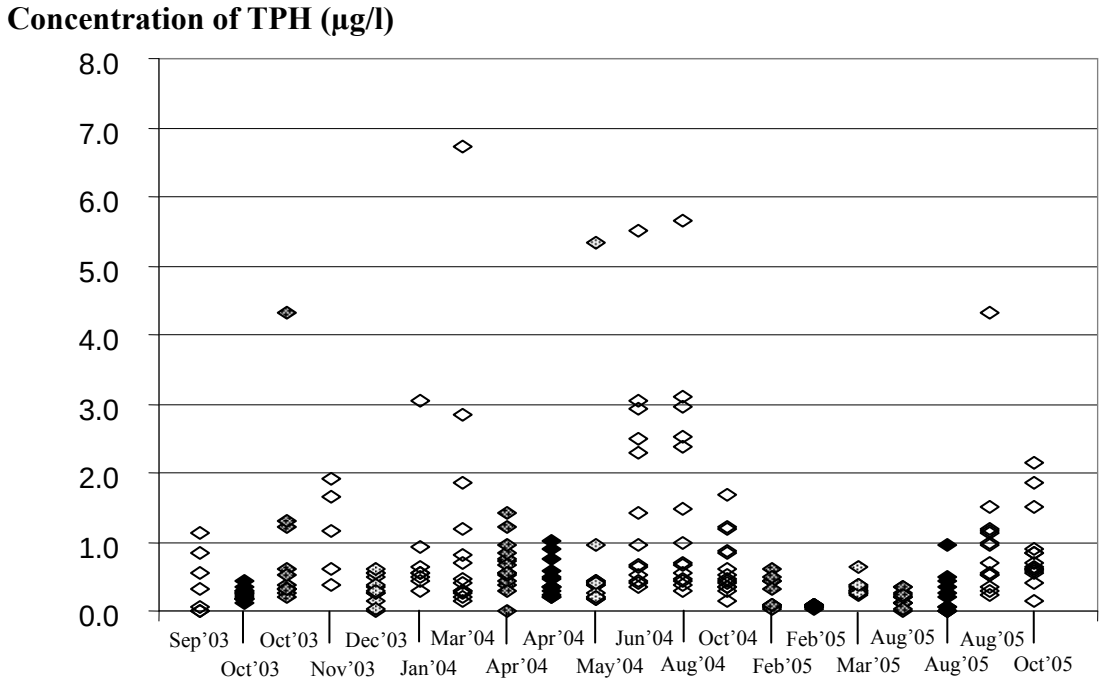

Figure 2. Total petroleum hydrocarbon (TPH) concentrations in water collected from east coast of the Gulf of Thailand between September 2003 and October 2005.
(: Laem Chabang, Chonburi province; : Map Ta Phud, Rayong province; : Chang and Kud Islands, Trat province and : river mouths in Rayong, Chantaburi and

Table 1. Average and standard deviation (SD) of petroleum hydrocarbon in sea water (µg/l) were collected from coast off Laem Chabang, Chonburi province (LC), Map Ta Phud, Rayong province (MP), Chang and Kut Islands, Trat province (KC) and river mouths in Rayong, Chanthaburi and Trat province (RE) between 2003 and 2005.

Period	LC		MP		KC		RE	
	Average	SD	Average	SD	Average	SD	Average	SD
September 2003	-	-	-	-	-	-	0.42	0.45
October 2003	0.24	0.09	0.89	1.19	-	-	-	-
November 2003	-	-	-	-	-	-	1.15	0.65
December 2003	-	-	-	-	0.33	0.20	-	-
January 2004	-	-	-	-	-	-	0.87	0.90
March 2004	-	-	-	-	-	-	1.24	1.82
April 2004	0.47	0.27	0.66	0.37	-	-	-	-
May 2004	-	-	-	-	0.75	1.39	-	-
June 2004	-	-	-	-	-	-	1.67	1.53
August 2004	-	-	-	-	-	-	1.61	1.55
October 2004	-	-	-	-	-	-	0.67	0.43
February 2005	0.07	0.02	0.24	0.21	-	-	-	-
March 2005	-	-	-	-	0.33	0.10	-	-
August 2005	0.25	0.29	0.18	0.11	-	-	1.03	1.02
October 2005	-	-	-	-	-	-	0.88	0.57
Average	0.70							
SD	0.97							
Range	<0.01 – 6.72							

Table 2. Contamination of petroleum hydrocarbon in Thai waters and adjacent areas.

Type	Concentration (µg/l)	Area	Ref.
Total	6.19-14.57	Ship-scrapping, Rayong Province (1986)	7
Dissolved/dispersed	0.65-8.30	Upper Gulf of Thailand	11
	0.07-6.50	Lower Gulf of Thailand	
Total	0.059-6.095	Upper Gulf of Thailand (1986)	12
Dissolved	0.018-5.286	Pattaya, Chonburi province to Trat province	6
	1.96-6.19	Takua Pa mangrove, Pang-nga province	11
	7.67-10.17	Laem Fa-pa mangrove, Chao Phraya River Mouth	13
Total	0.93-4.25	Lower Tha Chin River (1989)	14
	0.05-11.84	Coastal area off Rayong Province	9
	6.01-18.02	Lower Chao Phraya River	15
Total	0.05-4.13	Gulf of Thailand and Eastern Peninsular Malaysia (1996)	16
Total	0.85-6.61	Chang and Kut Islands, Trat province (2001)	17
Total	**<0.01-6.72**	**East coast off the Gulf of Thailand (2003-2005)**	**This study**

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USING BIOLOGICAL METHODS TO MONITOR NEW ZEALAND ESTUARINE ENVIRONMENTAL HEALTH

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Abstract

Estuarine areas are among the most productive ecosystems, and are under increasing pressure from a variety of stressors including urbanisation and municipal and industrial waste effluents. The aquatic and, ultimately, the estuarine environments act as sinks receiving natural and anthropogenic contaminants, and the potential impacts on organisms are not well characterised. Studies characterising the effects of pollution have focused on larger macroinvertebrates, but few have looked at the effects on the meiofauna (micro-invertebrates between 53 and 500 μm in length). In ecotoxicology, both the abundance and distribution of the major taxa, species diversity or community composition are commonly used to characterise the state of various environments. In laboratory tests, individual toxicants are tested on organisms in the aqueous phase. However, in the field many of these contaminants occur as mixtures and are usually associated with the sediment. Most tests are short term and measure biological endpoints such as survival, reproduction and development over a 96-h period. Few studies have investigated the chronic effects of contaminants over the full life cycle of an organism. This report highlights the complexities of determining the effects of contaminants on individual species and communities. Studies of the effects of contamination in estuaries on marine invertebrate species in New Zealand and elsewhere are described. The key area for future research is the development of biological methods to evaluate the impacts of cumulative sources of stressors on the environment. There is a need, both in New Zealand and elsewhere, for a suite of biological indicators that can efficiently and cost-effectively characterise and monitor the health status of marine environments. This report provides information on the development of such methods in New Zealand.

Key words: estuary; meiofauna; ecotoxicology; environmental contamination; toxicant; monitoring; bioindicator; biomarker

1. Introduction

1.1. Estuarine environments

Estuaries are areas where fresh and marine waters meet. They are zones of transition for factors such as salinity and suspended organic matter, and for the distribution of nutrients and oxygen. Fairbridge (1980) defined an estuary as:

> ….an inlet of the sea reaching into a river valley as far as the upper limit of the tidal rise, normally being divisible into three sectors: (a) a marine or lower estuary, in free connection with the open sea; (b) a middle estuary, subject to

strong salt and freshwater mixing; (c) an upper or fluvial estuary, characterised by freshwater but subject to daily tidal action.

The boundaries between these sectors are not fixed as they are subject to tidal movements and seasonal variability.

New Zealand has the fourth largest maritime area, with an exclusive economic zone of some 483 million hectares. Only the United States, French Polynesia and Indonesia have larger areas (Ministry for the Environment 1997). Estuaries and shallow harbours exist in close proximity to all of New Zealand's large coastal cities and these multi-use environments are already showing modification and deterioration from increasing human pressure (Knox 1986). While estuaries are of vital importance to fishes, providing sheltered environments for breeding, feeding, and juvenile development (McDowall 1976), they also function as collection funnels for pollutants from industry, agriculture and urban areas. Fish from urban and industrialised estuaries are exposed to among the highest levels of contaminants of any vertebrate population and therefore serve as relevant models for determining the toxic effects and mechanisms through which environmental toxicants work (Wirgin & Waldman 2004).

Shallow inshore waters, or estuaries, are recognised worldwide for their high productivity – they are generally important for both natural ecosystems and the economies of countries with these resources (Costanza *et al.* 1997). Kennish (1995) stated that estuarine intertidal areas are one of the most productive natural ecosystems on earth, with a gross productivity of up to 10 kcal/m^2 per year. Human life is supported by natural ecosystems and the species that constitute them through conditions and processes known as ecosystem services or nature's services (Daily *et al.* 1997). Ecosystem services are the life-support systems of the planet (Myers 1996; Daily *et al*. 1997), and human life cannot exist without these services and functions. Māori, the indigenous people of New Zealand, have a strong spiritual connection with the land and sea and value these natural ecosystems as an interdependent entity. Some Māori use estuaries and coastal areas as a source of income through activities such as shellfish harvesting and participating in commercial fisheries.

Estuaries are important nursery grounds for bottom-feeding fish such as flounder (*Rhombosolea plebeia*) and mullet (*Aldrichetta forsteri*), and also provide resting areas for pelagic species such as kahawai (*Arripis trutta*) and kingfish (*Seriola grandis*). Apart from providing these important aquatic habitats, estuaries also support a large number of birds that use the intertidal areas as feeding grounds. Estuaries are highly susceptible to a range of water-quality issues, however, as they receive contaminants from multiple sources. There is growing concern about the ways in which pollutants enter and are transported into estuaries where they may possibly accumulate in sediments (Kramer *et al*. 1994).

Estuarine areas are under increasing pressure from a variety of sources, the most

important being the effects of urbanisation, given that approximately 50% of the world's population now live close to the coast (GESAMP 1990). The expansion of urban areas around coastal and estuarine environments is increasing as many of New Zealand's main cities are already situated within estuarine catchments (i.e. Auckland, Whangarei, Tauranga, Napier, Nelson, Christchurch and Invercargill). With urbanisation comes an increase in impervious surfaces (e.g. roads and housing developments) causing increased stormwater runoff containing contaminants from non-point sources, which discharge into coastal and estuarine environments. The primary stormwater contaminants of concern in New Zealand estuaries are zinc (from galvanised steel, tyres), copper (vehicle wiring), lead (vehicle emissions) and polycyclic aromatic hydrocarbons (PAHs) (also originating from vehicle emissions) and these contaminants have accumulated in estuarine sediments (Williamson & Wilcock 1994; ANZECC 2000; ARC 2003). Studies indicate that these contaminants are having an effect on the fauna, particularly sediment-dwelling organisms, by reducing species abundance, increasing contaminant accumulation in shellfish and crustaceans and causing changes in growth and reproductive rates (Ministry for the Environment 1997 and references therein).

There are a variety of sources of contamination, such as stormwater, industrial sites, land clearance and agricultural and harbour activities. Due to increasing soil erosion, runoff from land clearance, dredging and land reclamation, sediment deposition and water turbidity have increased in coastal areas throughout the world (Ellis 1988; Vogt & Schramm 1991; Chou 1996). Sediment accumulation and contamination tend to be most problematic in estuaries near human settlement and where there is poor tidal flushing. Because contaminants typically bind to the finer silty material in estuarine sediments, this creates a reservoir of contaminants, which are a source of pollution to the water column and organisms (Oberholster *et al*. 2005).

It is a challenging task to monitor all the contaminants present in sediment and the marine environment and determine what possible impacts these may have, particularly as information relating to the persistence, bioaccumulation and toxicity of many contaminants is poorly known. The breakdown products of many contaminants add to the effects of the bioaccumulated chemicals already present in the sediment (Coull & Chandler 1992; Kramer *et al*. 1994), which makes identification of which chemicals have a disruptive effect on marine organisms and communities even more difficult (Coull & Chandler 1992; Kramer *et al*. 1994). As these contaminants can have various effects on organisms such as small crustaceans living in the sediment, the long-term effects on an ecosystem may be amplified by bioaccumulation at higher trophic levels, starting with those animals feeding on sediment-dwelling organisms (Kramer *et al.* 1994). Prolonged exposure of organisms to contaminants may cause sublethal effects, including decreased reproductive and growth rates and changes in sex ratio (Coull & Chandler 1992; Cary *et al*. 2004; Chandler *et al*. 2004). These sublethal effects have many consequences for organisms and the ecosystems of which they are a part and may provide useful indicators of environmental health.

1.2. Biomonitoring techniques

Biomarkers are defined as physiological, biochemical, or histopathological alterations that occur as a result of exposure to environmental pollutants, and can be measured at different levels of biological organisation (Hugget *et al*. 1992). Detection of changes at the population/community/ecosystem/human-health levels of biological organisation is the ultimate goal of environmental monitoring, but it is extremely difficult to detect subtle changes at such high levels of organisation. In addition, at these levels it is difficult to characterise cause-and-effect relationships and identify the causative stressor(s). The biochemical/molecular approach involves detection of 'distress signals' (known as biomarkers) at the molecular and cellular levels of organisation, and the linking of these signals to higher-level consequences (Suter 1990). Suites of biomarkers, representing modifications in a number of different physiological and biochemical pathways, are a useful means by which to identify and characterise the impacts of mixtures of contaminants on living organisms (Galloway *et al*. 2004).

Acute (short-term toxicity tests, usually within 24–96 hours of exposure to a substance) and chronic (long-term toxicity tests usually identifying exposure effects on the full life cycle of an organism) sediment toxicity bioassays have been used in several studies to investigate the effects of contaminated estuarine sediment on a variety of benthic estuarine and marine-dwelling organisms (Chandler & Green 1996; Kovatch *et al*. 1999; Stronkhorst *et al*. 2003; Bejarano *et al*. 2004; Oberholster *et al*. 2005; Castro *et al*. 2006). These tests have used organisms such as amphipods (Costa *et al*. 2005; Castro *et al*. 2006; King *et al*. 2006; Manyin & Rowe 2006), midge (Soares *et al*. 2005), water louse (De Lange *et al*. 2005), mayfly nymph (De Lange *et al*. 2005), heart urchin (Stronkhorst *et al*. 1999), copepods (Willis 1999) and oysters (Geffard *et al*. 2001, 2004) to assess the effects of contamination These tests have proven to be successful in identifying sediments with potential toxicological effects to the organisms inhabiting the test sediment.

2. Flounder

Yellowbelly flounder (*Rhombosolea leporina*) are native to New Zealand and a traditional source of food for the indigenous Māori people. Several factors make yellowbelly flounder potentially useful bioindicators of the 'state of health' of the New Zealand estuarine and coastal environment:

1. They are benthic fishes and therefore exposed to contaminated sediments.
2. They are non-threatened and present in sufficient numbers for sampling to have no detectable effect on populations.
3. They are non-migratory.
4. They are not subject to commercial exploitation.
5. They are present in estuaries around New Zealand, enabling comparisons between regions.
6. They have cultural importance as a food source.

The biomarker suite currently being developed for use with the yellowbelly flounder

includes:

1. Liver biotransformation enzymes.

Biotransformation enzymes play a vital role in the detoxification of organic compounds. The cytochrome P450 isoenzymes are of particular importance as they are involved in the phase I metabolism (oxidation, reduction, hydrolysis) of PAHs, polychlorinated biphenyls (PCBs), and steroid hormones. In yellowbelly flounder the induction/inhibition of cytochrome P4501A1 in liver tissue of fishes from different sites has been measured by the EROD (ethoxyresorufin-*O*-deethylase) assay.

2. Biotransformation products.

Exposure of fish to PAHs can be estimated from the concentration of PAH metabolites in the bile. The levels of PAH metabolites in bile samples from yellowbelly flounder have been measured as fluorescent aromatic compounds by fixed-wavelength fluorescence spectroscopy.

3. Stress proteins.

Metallothioneins are small (6–7 kDa), cysteine-rich, non-enzyme proteins involved in metal homeostasis and the detoxification of heavy metals. In yellowbelly flounder, the relative levels of metallothionein mRNA in liver samples from different sites have been measured by real-time, quantitative, competitive, reverse-transcriptase polymerase chain reaction (qc RT-PCR). This technique is one of the most sensitive for detecting differences in the levels of specific mRNA.

4. Reproductive and endocrine function.

Successful reproduction is a fundamental feature of species sustainability and the mechanisms that control reproduction are therefore among the most carefully coordinated and rigidly controlled physiological processes in most animals. One important component of this system is the steroid hormone 17-β-estradiol (E2), which is an important chemical signal in successful female reproduction. One of the proteins produced in response to E2 is the glycophospholipoprotein vitellogenin (Vtg). Vtg is a yolk protein precursor and therefore basal expression in male fish is low to non-existent. Following exposure to E2, however, male fish will initiate the physiological processes usually active only in females required to produce Vtg. Vtg synthesis in male fish is therefore an excellent indicator of exposure to compounds that can directly or indirectly stimulate estrogenic activity. In yellowbelly flounder Vtg mRNA will be measured by real-time qc RT-PCR. Plasma concentrations of the reproductive hormones testosterone (T) and E2 have been measured by radio-immuno assay (RIA) to determine their relationship with Vtg levels.

5. Higher-level physiological parameters.

Molecular and biochemical biomarkers need to be correlated with higher physiological and morphological parameters of growth and reproduction in order to validate their ecological relevance. The higher-order parameters determined for the

yellowbelly flounder include condition factor, gonadosomatic index (GSI), splenosomatic index (SSI), hepatosomatic index (HSI) and age.

3. Marine copepods

Copepods are a group of organisms comprising the diverse ecological group of meiofauna (crustacea, worms etc. between 63 and 500 μm in length) that occur in almost all aquatic habitats (marine, brackish and fresh water), are ubiquitous in the marine environment and occur from tidal pools to the abyssal zone. Copepods live in a wide variety of habitats. Sediments of all types, from mud, sand, gravel and shells, provide habitat for harpacticoid (benthic free-living) copepods and the water column provides yet another habitat for two other free-living Orders, calanoid and cyclopoid copepods. In the benthic zone, harpacticoid copepods have been reported as one of the most abundant groups of organisms, second only to free-living nematodes (Hicks & Coull 1983).

Because harpacticoid copepods have an intimate association and dependence on sediments, high abundance and reproductive rates, short generation times (15–25 days), benthic larvae, and are easily culturable in the laboratory they are good candidates for the assessment of anthropogenic pollution in aquatic systems (Coull & Chandler 1992). Furthermore, as copepods produce multiple generations within a short time frame, the sublethal effects of contaminants on physiological parameters such as, growth, reproduction and fecundity can be easily determined within days to weeks. As copepods can be easily cultured under laboratory conditions, the rapid production of nauplii (juveniles) makes this group of organisms suitable for laboratory bioassays. Research in the USA has shown the enormous potential of using this group of organisms as bioindicators of environmental health (Chandler & Green 1996; Kovatch *et al*. 1999; Bejarano *et al*. 2004).

Throughout New Zealand estuaries, chemical pollution from urban and rural land uses is having impacts on human and wildlife populations, with widespread problems such as soil contamination, aquatic algal blooms and shellfish poisoning. A project is underway to develop and validate methods to assess pollution effects, using a native marine harpacticoid copepod (*Robertsonia propinqua*) as a bioindicator. The developed protocols will provide much-needed tools for environmental consultancies and government authorities to monitor pollution effects in New Zealand estuarine environments. Shellfish species have previously been used to assess the health of estuaries, but their use in environmental assessment programmes has many limitations, including long generation times and reproductive rates, large size, and the difficulty of culturing adults and maintaining juveniles under laboratory conditions. In recent years, the development of biomonitoring protocols using the more appropriate meiofauna has provided great advantages in evaluating the effects of pollution. The development of a copepod bioindicator will enable environmental consultancies and government authorities to offer new services on pollution characterisation at contaminated and estuarine sites.

4. Monitoring estuarine environments in New Zealand

Rapid urban growth has put pressure on the capacities of natural resources and

physical infrastructure, particularly around estuaries (Ministry for the Environment 1997). Urban retrofitting and infilling for agriculture and commercial land development has lead to increased demands on conventional infrastructure (e.g. stormwater piping) and has contributed to the decline of mangrove ecosystems this century (Ministry for the Environment 1997, 2002). The costs of maintaining existing and new stormwater and sewerage systems using conventional design and engineering approaches are escalating. While extensive piped systems remove discharges from a site, urban stormwater discharges (flow peaks and contamination) are unpleasant and are degrading coastal and inland waterways (Curry 1981; Williamson 1991; Wilcock 1994; Snelder & Trueman 1995). After decades of debate over 'cause and effect' relationships, stormwater impacts remain an unresolved priority concern (Parliamentary Commissioner for the Environment 1998; Ministry for the Environment 2002).

Information on the state of contamination in New Zealand water environments is widespread (Williamson 1991; Kingett Mitchell & Associates 1992; Hicks 1993, 1994; Wilcock 1994; Boxall & Maltby 1995, 1997; Snelder & Trueman 1995; Huser & Wilson 1997; Macaskill *et al*. 1996; Mikkelson *et al*. 1996; Ministry for the Environment 1997, 2002; Robien *et al*. 1997; Parliamentary Commissioner for the Environment 1998), but publications providing specific information on levels of sediment contamination in estuarine environments are few (see ARC 2003 and references therein). Monitoring reports have traditionally focused on community composition and the distribution and abundance of macrofauna species (e.g. the common oyster (*Saxostrea glomerata*), estuarine mud crab (*Helice crassa*), estuarine mud snail (*Amphibola crenata*) and some species of fish). There is now a move towards a more detailed monitoring structure that will incorporate parameters such as levels of pollutants present at a particular site, measurements of both point source contaminant levels and the interaction of pollutants on estuarine ecology, effects on the biology of organisms, and implications for environmental health.

Industry in New Zealand is moving towards integrated standard operating procedures (SOPs), but current SOPs have several significant technological gaps. One key gap is their poor ability to effectively characterise any adverse biological effects of exposure to chemicals in the environment. Although land-use practices involving the application of chemicals are heavily regulated, there are still problems associated with soil leaching and runoff from land. The effects of chemical pollutants on terrestrial organisms have been studied, but less information is available on the effects of terrestrial pollution on marine organisms. Appropriate biological methods are needed to properly assess and provide 'early-warning' signals of potential biological effects resulting from chemical pollutants in marine environments. End-users have raised concerns over the limitations of methodologies currently available to monitor pollution in estuarine environments and in particular the power of these methodologies to estimate long-term effects on exposed populations. By using a native copepod as

bioindicator, the developed protocols will be designed for and relevant to New Zealand.

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