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THESIS

USE OF FRESHWATER OLIGOCHAETE (*LIMNODRILUS HOFFMEISTERI*) IN SEDIMENT BIOASSAY

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A Thesis Submitted in Partial Fulfillment of
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The research on using freshwater oligochaete *Limnodrilus hoffmeisteri* in sediment bioassay was carried out to cover four purposes, including investigation on its morphological feature and biological behavior, estimation on its specific growth and biological activity, assessing its tolerance to the toxicity of naphthalene spiked-sediment, and determination on its bioaccumulation and trophic transfer of such chemical contaminated in sediment via a fingerling predator, *Oreochromis niloticus.*

The result showed that *L. hoffmeisteri* collected from Chao Phraya estuary were totally immature. This organism showed important behaviors consisting of active swimming, burrowing, clump living, feeding habit and defecation. The results from laboratorial culturing showed significant increase of number of individual and weight in the treatment added by high level of food indicated the reproduction of *L. hoffmeisteri* occurred after 35 d of the experiment. These increasing values affected the decrease of TOM in the sediment and increase of DO in the overlying water. The results from toxicity test showed acute effect of naphthalene to the worms with the 72 h and 96 h LC_{50} values of 85.11 and 60.26 µg/g wwt, respectively. In addition, sublethal responses found the 96 h EC_{50} values for autotomy and sediment avoidance were 35.48 and 58.88 µg/g wwt, respectively, while the 96 h LOEC reworking activity value was 25 µg/g wwt. The results from bioaccumulation test showed naphthalene was transferred from sediment and accumulated in a brief period in *L. hoffmeisteri* with the peak of residue highest during 24 h of the exposure. Average tissue residue and BAFs were 1038.83±564.56 µg/g dwt and 31.97±17.07, respectively. The study on trophic transfer showed fluctuation of fish residues, distinctly increasing during 6 h after feeding, and then suddenly decreasing if feeding was stopped. This occurrence appeared due to the process of food digestion and metabolism in tilapia. These evidences implied that naphthalene was not biomagnify to *O. niloticus* via eating *L. hoffmeisteri* in this aquatic food chain model.

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Warucha Kanchana-Aksorn

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

Appendix Table **Page**

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

BAFs	=	bioaccumulation factors
BSAFs	=	biota-sediment accumulation factors
d	=	day
DO	=	dissolved oxygen
dwt	=	dry weight
EC_{50}	=	mean effect concentration
h	=	hour
HPLC	=	high performance liquid chromatography
ind	=	individual
LC_{50}	=	median lethal concentration
LOEC	=	lowest observed effect concentration
M	=	molar
MFO	=	mixed function oxigenase
min	=	minute
nd	=	non-detected
NOEC	=	no observed effect concentration
Oc	=	organic carbon
PAHs	=	polycyclic aromatic hydrocarbons
ppb	=	part per billion
ppm	=	part per million
SEM	=	scanning electron microscope
TOM	=	total organic matter
w	=	week
WC	=	water content
wwt	=	wet weight
µg	=	microgram
µm	=	micrometer

USE OF FRESHWATER OLIGOCHAETE (*LIMNODRILUS HOFFMEISTERI*) IN SEDIMENT BIOASSAY

INTRODUCTION

Lower Chao Phraya River, one of the estuaries in Thailand, has shown the rapid increase in population growth and coastal development. The report from Pollution Control Department (2001) revealed that the frequency of low dissolved oxygen and high organic materials tends to be greater, particularly in the urbanized and industrialized areas. The sources of pollution mostly come from urban industrial wastewater, agriculture and domestic sewage disposal. These contribute to the loading of organic pollutants, such as pesticides, heavy metals and petroleum hydrocarbons, distributing to the water, and setting in bottom sediment of the estuary (The Office of Environment Policy and Planning, 2001). The deposition of toxicants in the sediment constitute one of several factors that cause change to the structure of benthic communities since several chemicals are persistent and can bioaccumulate in sediment-ingesting infauna (Suess, 1976).

Polycyclic aromatic hydrocarbons (PAHs) are one of the important organic xenobiotics found in the bottom sediment of Chao Phraya estuary (Wattayakorn, 2003). Previous report by Nokyoo (1995) showed that the concentration of total PAHs in sediment were in the range of 0.58-4.71 µg/g dwt. However, these levels were low in comparison with other major world estuaries (e.g., Mille *et al*., 2007; Oros *et al*., 2007). Sources of PAHs in this area originate from oil pollutions along coastlines including major spills from freighters, tankers and other vessels going aground and during emptying of oil contaminated ballast and bilged water. Apart from these, the normal operation of coastal factories and mills adjacent to estuaries often leads to oil leakage or seepage into water. The runoff due to rainfall also carries oil from the highways, city streets and industrial areas into the estuary.

It is well known that PAHs have a high toxicity for most organisms. The PAHs are lipophilic in nature and gets integrated into biological systems through uptake and accumulated by aquatic organisms with the potential to exert toxic actions (Readman *et al*., 1982). High toxicity of PAHs has been found associated with the water-soluble fractions of aromatic compounds dominated by 2-3 ringed aromatics such as naphthalene (Anderson *et al*., 1974a, 1974b). Naphthalene is a very common semivolatile PAH found in numerous petroleum products. Due to the low molecular weight (128.18), less sensitivity to photo-oxidation and persistent nature in water, naphthalene is considered as extremely toxic to the exposed aquatic animals (Vijayavel and Balasubramanian, 2006a). Several researches on toxic effects of naphthalene have been reported in aquatic biota, such as mysid shrimp *Mysidopsis bahia* (Barron *et al*., 1999), amphipod *Diporeia* spp. (Landrum *et al*., 2003), and mud crab *Scylla serrata* (Vijayavel and Balasubramanian, 2006a, 2006b). Most of all demonstrated on water-borne toxicity, while the data related to toxic effects of naphthalene in sediments are lack of publication.

Aquatic oligochaetes are macrobenthic invertebrate which often associated with aquatic systems rich in organic matter. They also play a major role in processing organic material and are a food source for benthic feeding fish (Kurata, 1994). Most oligochaetes are relatively tolerant to many classes of chemical contaminants (Khangarot, 1991). This tolerance may be a positive attribute for assessing bioaccumulation or the toxicity studies of severely contaminated sites. Many representatives of freshwater oligochaetes were commonly used for their propriety as test organisms for the testing of chemicals, as well as for the ecotoxicological assessment of sediment contamination (Phipps *et al*., 1993). For the laboratory performing to characterize their tolerance to PAHs, apparent documents have been reported in few species, such as the freshwater oligochaete *Lumbriculus variegatus*, and the estuarine oligochaete *Monopylephorus rubroniveus*. These two species efficiently accumulated and highly tolerant to several PAHs exposed in sediment (Leppanen and Kukkonen, 1998b; Sheedy *et al*., 1998; Conrad *et al*., 2002; Weinstein and Sanger, 2003; Hyotylainen and Oikari, 2004).

At this stage, laboratory study on using of aquatic oligochaete in sediment toxicity test is more limited in Thailand and the tropical region. Thus, to effectively assess the ecological risk of sediment–associated PAHs, the study concerning the toxic effects of these chemicals in aquatic oligochaetes is necessary. The main purpose of this research was to use the tubificid oligochaete *Limnodrilus hoffmeisteri* as a proposed test organism in the sediment toxicity testing. This oligochaete species was chosen due to the highest density (greater than 10,000 ind/m^2) established in the lower Chao Phraya river (Kanchana-Aksorn and Petpiroon, 2006). The experiments were performed involving naphthalene, one of the predominant PAHs found in sediments of the Chao Phraya estuary. The study was also to provide some information on the possibility of *L. hoffmeisteri* to bioaccumulate such chemicals in contaminated sediment, and the efficiency on trophic transfer to tilapia fingerling, *Oreochromis niloticus*, a test predator. In addition, some considerable detail of *L. hoffmeisteri* related to the main aims of this work, such as taxonomic characteristics, and biological aspects (e.g. sediment inhabit, behavior, growth, reproduction), is scarce in Thai waters. Despite the current data in distribution studies (Kanchana-Aksorn and Petpiroon, 2006), there are no other published data available on the biology so far. The other targets of this study were to afford background on the morphological appearance of *L. hoffmeisteri* collected from Chao Phraya estuary, accompanied with estimating on their specific growth and biological activity in laboratory culture.

OBJECTIVES

1. To investigate the morphological features and biological behaviors of *Limnodrilus hoffmeisteri* collected from Chao Phraya estuary.

2. To estimate the specific growth and biological activities of *L. hoffmeisteri* in laboratory culture.

3. To assess the acute and sublethal toxicity of naphthalene contaminated-sediment to *L. hoffmeisteri*.

4. To study the bioaccumulation of sediment-associated naphthalene in *L. hoffmeisteri*, and the potential to biomagnify in the food chain model exposure, corporating with *L. hoffmeisteri* and tilapia fingerling *Oreochromis niloticus*, as a prey and predator, respectively.

LITERATURE REVIEW

Methodology of Toxicity Testing

Toxicity tests are used to evaluate the concentrations of the chemical and the duration of exposure required to produce the criterion effect on a living organism with that of a standard preparation. In the laboratory, the test usually follows a stepwise tier approach based on the results of the previous tests. The general test design is required careful control of conditions. Test organisms are exposed in test chambers to various concentrations of the test material. The criteria for effects established before testing are then evaluated by comparing the chemically exposed organisms with the untreated organisms which are exposed.

Aquatic toxicity test methods may be categorized according to length of exposure, test situation, criteria of effects to be evaluated, and organism to be tested. The effects evaluated are biological end points selected. These effect criteria differ depending on the type of toxicity test being conducted and the species used. The statistical approach also changes with the type of toxicity test conducted. Some of the commonly used tests and the end point that they measure are briefly described in the topics below.

1. Acute Toxicity Tests

These are tests designed to evaluate the relative toxicity of a chemical to selected aquatic organisms on short-term exposure to various concentrations of the test material. In fish and other aquatic organisms, acute effects are those that rapidly occur within a few hours, days, or weeks as a result of test exposure. The most common effect criteria used with aquatic organisms is lethality or mortality. The criteria for death are usually lack of movement and lack of reaction to gentle prodding. Experimentally, a 50% response is the most reproducible measure of the toxicity of a test material, and 96 hours or less is the standard exposure time. The measure of acute toxicity most frequently used is the median lethal concentration

(LC_{50}). However, death is not easily determined for some invertebrates, the median effective concentration (EC_{50}) is estimated rather than the LC_{50}. The effect used for estimating the EC_{50} is immobilization defined as lack of movement, loss of equilibrium defined as inability to maintain normal posture (Parrish, 1985).

2. Sublethal Effects Tests

These tests are designed to determine the toxic effects of a chemical to selected aquatic organisms exposed to low concentrations of the test material. In general, organisms are not usually exposed to high toxic concentration of chemicals in the aquatic environment unless they are restricted to the vicinity of a chemical release site. Thereby, a greater biomass is exposed to sublethal concentrations of chemicals than to acutely toxic lethal concentrations (Rand and Petrocelli, 1985). Sublethal effects may be studied in the laboratory by a variety of procedures. The most common effect criteria are behavioral changes, physiological changes, biochemical changes, and histological changes. Some sublethal effects may indirectly result in lethality, but they may have a profound effect on the future survival of the organisms. Behavioral effects that have received considerable attention in aquatic organisms are locomotion and swimming, attraction-avoidance, prey-predator relationship, aggression and territoriality, and learning (Rand, 1985). Physiological and biochemical tests include studies of enzyme inhibition, clinical chemistry, hematology, and respiration (Mehrle and Mayer, 1985). Finally, histological studies are also useful to determine changes in histological structure which may often significantly modify the function of tissues and organs (Meyers and Hendricks, 1985).

3. Chronic Toxicity Tests

Sometimes a chemical does not have adverse effects on aquatic organisms in acute toxicity tests. Chronic toxicity tests permit evaluation of the possible adverse effects of the chemical under conditions of long-term exposure at sublethal concentrations. In a chronic test, the test organism is exposed for an entire reproductive life cycle. The exposure is generally initiated with an egg and continues

through development, such as hatching of the embryo, growth and development of the young organism, attainment of sexual maturity, and reproduction to produce a second generation organism. The criteria for these effects include mortality, growth, reproduction, development of sex products, maturation, spawning success, hatching success, survival, and also behavior. The duration of a chronic toxicity test varies with the life cycle of the species tested (Petrocelli, 1985). The estimated threshold concentration of a chemical within a range was defined by the highest concentration tested at which no significant deleterious effect (NOEC) was observed and the lowest concentration tested at which some significant deleterious effect (LOEC) was observed (Mount and Stephan, 1967).

4. Bioaccumulation Tests

The study on bioaccumulation of chemicals using aquatic organisms is an integral step for estimating the potential of particular species in a bioassay. In addition, the result can be used for evaluating the possibility of food chain accumulation to elsewhere in the aquatic ecosystem (Hyotylainen and Oikari, 2004). Bioaccumulation of chemicals by an aquatic organism might also pose a health hazard to predators, including humans (Hellou *et al*., 1995). Bioaccumulation tests should be undertaken only for chemicals showing a high degree of bioconcentration or for especially hazardous and persistent environmental contaminants. In addition, these tests may be expanded to a simple step food chain representing the transfer of a chemical with lowest trophic level connected to the highest trophic level (Spacie and Hamelink, 1985).

In aquatic test, bioaccumulation is a term describing a process by which chemicals are taken up by organisms. Direct uptake of chemicals from water has been shown for many aquatic organisms including algae, annelid, arthropods, mollusks, and fish. Most xenobiotics are taken into the body by passive diffusion through semipermeable membrane such as gill, lining of the mouth, or gastrointestinal tract (Fromm and Hunter, 1969; Wildish and Zitko, 1971; Wilkes and Weiss, 1971; Bryan, 1976; Jennings and Rainbow, 1979; Carpene and George, 1981). Another

process that xenobiotics are taken into the body of organisms is through consumption of food containing the chemicals. The molecules can apparently be absorbed in the intestine and lipophilic organics in food are expected to transfer (Guthrie, 1980).

The bioaccumulation potential of a chemical compound is greatly affected by the rate of elimination from the organism. If an unaltered chemical can be eliminated rapidly, residues will not accumulate and tissue damage is less likely.
In invertebrates, elimination can proceed by several routes, including transport across the integument or resperatory surfaces, secretion in gallbladder bile, and excretion from the kidney in urine (Spacie and Hamelink, 1985). Furthermore, passive elimination of lipid-soluble chemicals can also occur across the skin and gills (Hunn and Allen, 1974; Allen and Hunn, 1977).

The choice of the model to calculate the uptake of contaminants by an organism depends on the pollution sources organisms exposed to and has to be adapted to the case encountered. If the pollution is dissolved in the water, bioconcentration factors (BCF), calculating the uptake of contaminants from the water, must be used. If the pollution is located in the sediment, biota accumulation factors (BAFs), determining the uptake of contaminants from the sediment can be used. The BCF is the ratio of the concentration of test chemical accumulated in the tissues of the test organism to the measured concentration in the water to which the organisms are exposed, while BAFs are the concentration of a chemical accumulated by an organism divided by the concentration of the same chemical in sediment (Spacie and Hamelink, 1985). In sediment toxicity studies, the design was altered to determine the biota-sediment accumulation factors (BSAFs) calculated by dividing the lipid-normalized tissue concentrations by the organic-carbon normalized sediment concentration (Lee, 1992; Kemble *et al*., 1998). BSAFs were compared with the theoretical values of 1.7 predicted for equilibrium partitioning of hydrophobic organic contaminants in sediment-benthos systems (McFarland and Clarke, 1986; DiToro *et al*., 1991). A BSAF of less than 1.7 indicates less partitioning onto lipids than predicted and a value greater than 1.7 indicates more uptake than can be explained by partitioning theory alone (Lee, 1992). For non-metabolizing chemicals, a BSAF in the

range of 0.15-1.5 is thus expected providing that there is sufficient time for equilibration (Hellou *et al*., 1999).

Aquatic Oligochaetes

1. General Characteristics

1.1 Morphology

Aquatic oligochaete has the same structure as the common terrestrial earthworms classified in Phylum Annelida (Figure 1). The true aquatic oligochaetes are much more delicately constructed and small. The usual length ranges from 1 to 30 mm. The body wall is soft, muscular and covered with a thin cuticle. The prostomium is a presegmental lobe at the extreme anterior end of the body. It projects in a rooflike fashion above the mouth and is sometimes elongated into a proboscis. The body of an oligochaete is divided by a number of segments which is quite variable even in mature individuals of each family. For instance, the threadlike Haplotaxidae have the largest number of segments, sometimes up to 500. At the opposite extreme the Naididae usually have between 7 and 40 segments. The other families, Tubificidae, for example, have an intermediate number of segments, usually consist of 40 to 200 segments. The chitinoid setae are arranged in four bundles on each segment except on segment I. A bundle may consist of from 1 to as many as 20 seta. The morphology of setae and their arrangement which is important as taxonomic characters are very diverse (Figure 2).

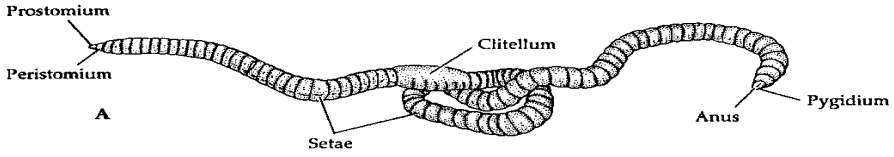


Figure 1 Aquatic oligochaete.

Source: Brusca and Brusca (1990)

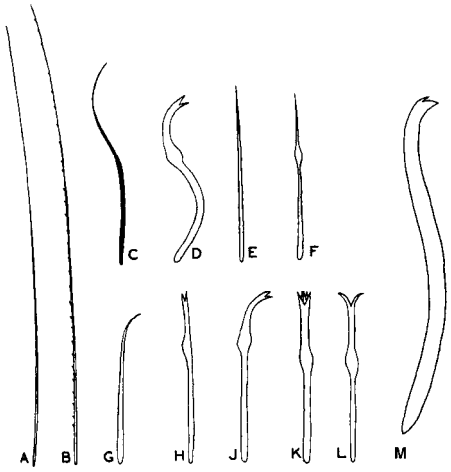


Figure 2 Typical setae of aquatic oligochaete; A and B: hair; C-L: aciculate, A: simple; B: serrate; C and D: sigmoid; D, H and J: bifurcate; G: simple; J: hooked; K: pectinate; L: biuncinate; M: unequal bifurcate.

Source: Smith (2001)

1.2 Locomotion

Usual locomotion of aquatic oligochaete is a crawling movement on or in the superficial layers of the substrate. It involves contractions of the muscular body wall and obtains a purchase with the setae. A few species are effective swimmers. *Pristina* and *Stylaria* can move along just above the substrate in a serpentine manner. *Lumbriculus* undergoes a combination of rapid directional reversals and helical swimming movement presumably to avoid predation (Smith, 2001).

1.3 Feeding

The great majority of aquatic oligochaete obtains food by ingesting quantities of the substrate and the organic component was digested as it passes through the alimentary canal (Smith, 2001). Under some circumstances the food may consist largely of filamentous algae, diatoms or simply miscellaneous plant and animal detritus. Carnivorous forms are poorly known, such as *Chaetogaster* feeding

on microcustaceans, insect larvae and other oligochaete (Smith, 2001). Many Naididae may be herbivorous, for example *Styralia* feeding on algae, diatoms, plant fragment and fresh water sponges (Yoshizawa, 1928).

1.4 Respiration

Most of carbon dioxide and oxygen exchange occurs through the general body surface of oligochaete which is well supplied with capillaries. In the case of most naidids and some tubificids, water is taken into the anus and passed forward by antiperistalsis and ciliary action. This process is thought to constitute an accessory respiratory mechanism (Smith, 2001). Some species of oligochaete have gill on the posterior part of the body which are well supplied with blood vessels. *Dero* has ciliated gills surrounding the anal region whereas *Branchiura* has a finger-like dorsal and ventral gill on each of the posterior segments (Figure 3). Gilled species remain quietly in position with the anterior portion of the body hidden in the substrate and in a tube constructed of fine debris, and projecting up from the substrate for a variable distance. The posterior end projects vertically out of the tube and into the water, then waves about vigorously to circulate the water and make more oxygen available to the body surface (Brinkhurst and Jamieson, 1971).

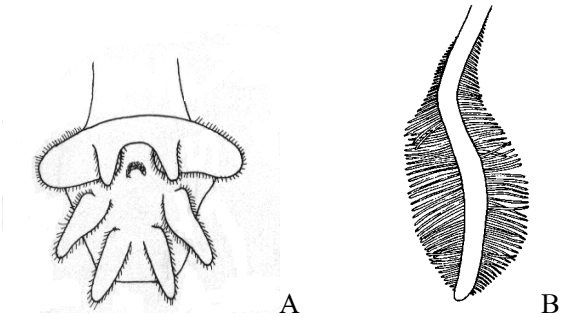


Figure 3 Posterior end of *Dero digitata* (A) and *Branchiura sowerbyi* (B).

Source: Pennak (1989)

1.5 Reproduction

Asexual reproduction is widespread among freshwater oligochaetes. Asexual methods vary including fission by architomy or fragmentation, and more commonly, paratomy or formation of buds followed by fragmentation (Smith, 2001).

In paratomy method, the budding segment grows and undergoes repeated transverse divisions that result in four or more anterior segments of a new posterior worm and several new posterior segments of the anterior parent worm. These regions rapidly become differentiated. The two animals remain attached for a variable length of time, and the more posterior individual may in turn produce a third individual posterior to its budding zone (Figure 4). This process may be repeated, so that in some species there may be chains of as many as four to eight zooids in various stages of development. After an interval, two adjacent and mature zooids separate and become completely independent individuals. This regeneration has been found in most Naididae, which is often reproduced a new individual every 2 or 3 days (Smith, 2001).

A similar ability to reproduce by fission or architomy appears to be best developed in the Tubificidae and Naididae. It has been reported in *Tubifex newaensis* and *Branchiura sowerbyi* (Brinkhurst and Jamieson, 1971). The occurrence of this asexual reproduction in the Lumbriculidae is also well established, especially in *Lumbriculus variegatus* (Cook, 1968). The strategy of this fragmentation is constituted fission of worm into 2 parts, followed by anterior regeneration at the caudal end, then posterior regeneration at cephalic end. The specific location of the fission zone varies greatly form one individual to another within a species. In *Nais elinguis*, the budding segment varies from 12 to 20, and in *Stylaria fossularis*, it varies form 8 to 23 but is usually 18 (Smith, 2001).

Sexual reproduction certainly occurs in aquatic oligochaetes. They are all hermaphroditic, but do not self fertilize. The epidermis in the reproductive zone of the adult worm is modified to form a clitellum forming several kinds of secretory

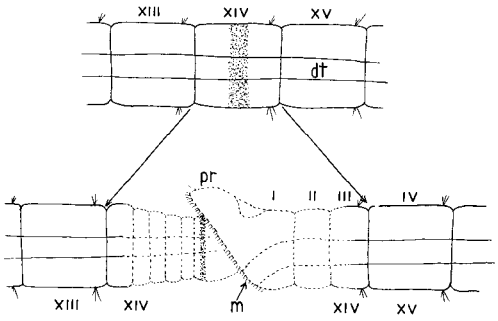


Figure 4 Budding in oligochaete showing segmental derivatives in daughter zooids. dt: digestive tract; m: mouth; pr: prostomium.

Source: Smith (2001)

cells involved with different aspects of copulation. For copulation, worms line up with ventral portions of body touching heads going in opposite directions. Large quantities of mucous are secreted to keep worms together. The seminal groove is used to transfer sperms to the spermathecae of the opposing worm. Some species have penes to help in sperm transfer. The copulation takes a period of time, then worms separate and the clitellum of each worm secretes a cocoon containing fertilized eggs. The cocoon is laid on rocks, vegetation or debris (Figure 5) after secretion and the embryos will develop to juveniles inside it. Once development is completed, young worms hatch and then exit the cocoon (Smith, 2001).

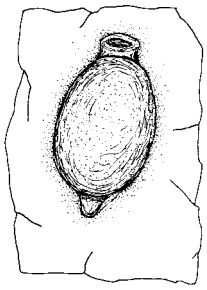


Figure 5 Cocoon of *Stylaria* deposited on a bit of debris.

Source: Pennak (1989)

The study on sexual reproduction of aquatic oligochaetes is not well established. There are limited to notes on the presence of mature specimens in field populations. In tubificids and some lumbriculids, breeding may be restricted to brief periods (Cook, 1968). Fully mature individuals of *Peloscolex ferox* and *Aulodrilus pluriseta* are only present just before breeding commences (Brinkhurst, 1964). Others, such as *Limnodrilus hoffmeisteri* and *Tubifex tubifex* may be found in mature condition and even breeding at all time in some locality, but even these species often have periods of intensive breeding (Brinkhurst, 1980). However, these periods may not be the same from place to another. For example, *Limnodrilus hoffmeisteri* exhibited life cycle which varied from site to site and even from year to year at one site (Kennedy, 1966). Most of aquatic worms became mature and reproduced within 12 months. Aston (1968) found that *Branchiura sowerbyi* take 1 year breeding cycle both in field and in the laboratory. In some instance, worms might take as long as 24 months to mature depending on season and localities. Brinkhurst (1964) found the available evidence that *Tubifex costatus*, *Aulodrilus pluriseta* and *Peloscolex ferox* take 2 years to mature and breed. Most of these studies suggested that the majority of breeding worms die after sexual reproduction. However, in some species, particular on Tubificidae, are known to be capable of surviving as immature worms and breeding a second time by having developed a new set of reproductive organs (Brinkhurst and Kennedy, 1965).

2. Habitat and Distribution

Aquatic oligochaetes are widely distributed. Most genera of the Tubificidae and Naididae are cosmopolitan and several species have been reported from every continent. In contrast, the Lumbriculidae are more limited in distribution, with over 35 species known only from Asia, nearly 40 in Europe and about 10 in North America (Brinkhurst and Jamieson, 1971).

Habitat preferences for aquatic oligochaetes can be established with reference to the physico-chemical parameters of the environment or any other recognizable ecological criteria. Several species of *Peloscolex*, *Tubifex*, *Limnodriloides*,

Thalassodrilus, Clitellio, Adelodrilus, Phallodrilus and *Spiridion* may be classified as marine oligochaetes (Hrabe, 1960). Among the brackish water forms may be considered several species of *Monopylephorus* and other such as *Tubifex costatus, Peloscolex benedeni*, and several *Isochaeta* species (Brinkhurst and Simmons, 1968). At the superficial level, there is an obvious tendency for some naidids and lumbriculids to occur in stony streams whereas tubuficids, which is frequently limited to *Tubifex tubifex, Peloscolex.ferox* and *Limnodrilus hoffmeisteri*, are more often found in the softer sediments of river and lake (Brinkhurst and Jamieson, 1971). Brinkhurst (1964) established that *Tubifex tubifex* is often found in productive or polluted lakes, replacing *Peloscolex ferox*, which is common in oligotrophic or mesotrophic lakes. All of these three species is always found in mud and debris substrates (Figure 6). *Aulodrilus pluriseta* also seems to occur most often in mud rich in plant fragments, whilst *Rhyacodrilus coccineus* and the lumbriculid *Stylodrilus heringianus* being mostly restricted to sandy sediment (Brinkhurst, 1967).

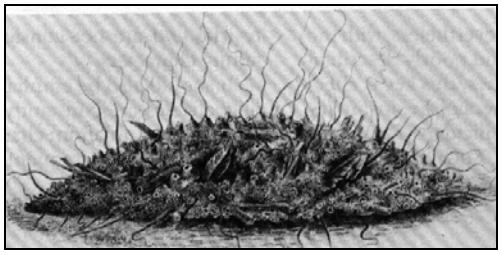


Figure 6 Colony of Tubificidae in mud and debris substrates.

Source: Pennak (1989)

3. Pollution Biology

The diversity of benthic fauna was severely reduced by the degree of pollution suffered. Lacking of oxygen and food supply, accumulation of poisonous products of anaerobic breakdown of organic matter and metabolic wastes are the most limiting factor. Certain species of aquatic oligochaete have the ability to tolerate under these conditions (Lerberg *et al*., 2000). In fact, the distributional patterns of certain

assemblages of oligochaetes are a recognized biological indicator of environmental quality in marine and estuarine systems (Chapman *et al*., 1982; Chapman and Brinkhurst, 1984). The tubificids are able to thrive in low concentrations of dissolved oxygen and can also withstand the complete absence of oxygen for extended period (Brinkhurst and Gelder, 1991; Pennak, 1989). They have a high level of resistance to unfavorable conditions where they form densed populations up to 10,000 ind/m^2 (Robbins *et al*., 1989). For example, the presence of *Limnodrilus hoffmeisteri* in organically enriched localities in tropical regions, suggested that it may be a good indicator of organic pollution, as well as *Potamothrix hammoniensis*, which may be present in significant numbers even in waters mildly polluted with organic matter (Brinkhurst and Kennedy, 1965).

It is known that certain species of oligochaetes assume an important role in polluted areas (Brinkhurst and Jamieson, 1971). They play a major role in the mixing the surface layers of the sediment, forming uniform surface layer and also cycling of metals and organic compounds in the sediment (Burton, 1991). Certain behavioral adaptation of oligochaete could increase their susceptibility. Tubificid oligochaetes can ingest sediment and derive the bulk of their nutrition from bacteria (Brinkhurst and Churan, 1969) and perhaps from algae (Moore, 1978). They also have been reported to protrude their tail out of the sediment in response to varying DO concentration. Tail protrusion and swaying in the water column (Figure 7) for two freshwater tubificids, *Tubifex tubifex* and *Limnodrilus hoffmeisteri*, were greatest at intermediate levels of DO (Alsterberg, 1992). The ability of tubificids to withstand periods of oxygen deprivation including complete anaerobic condition for up to 4 w has been demonstrated under laboratory condition. For instance, *Branchyura sowerbyi* cease when the oxygen saturation is below 22% at 20°C, and worms seem to survive and even to breed in eutrophic lakes in which the bottom sediment became devoid of oxygen for several weeks (Aston, 1966). However, the periods of severe organic pollution may drastically reduce the number of individuals of even the hardiest tubificid species, but the population usually recovers swiftly (Brinkhurst and Jamieson, 1971).

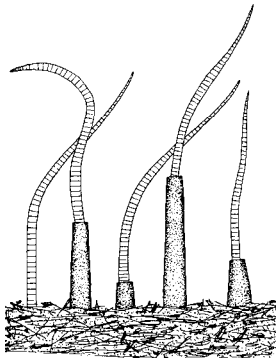


Figure 7 Tail protrusion and swaying in the water column of *Tubifex*.

Source: Pennak (1989)

Polycyclic Aromatic Hydrocarbons

1. Definition and Specific Characteristics

Polycyclic aromatic hydrocarbons (PAHs) are components of crude and refined petroleum and of coal. Their structure compose of carbon and hydrogen arranged in the form of two or more fused benzene rings. Ring pattern and relative stabilities are given in Table 1. Naphthalene ($C_{10}H_8$) which consists of two fused aromatic rings is the PAH with the lowest molecular weight. Substitution of carbon in benzene ring with nitrogen, sulfur, oxygen or other elements gives heterocyclic compounds which are also classified as PAHs. Chemical structures of sixteen PAHs identified by The Environmental Protection Agency (EPA) as priority pollutants are shown in Figure 8.

Crystalline solid of PAHs has high melting point and low vapor pressure. PAHs of higher molecular weight are relatively immobile because of their large molecular volumes and extremely low volatility and solubility. Some of PAHs are considered to be possible or probable human carcinogenic. The connection between PAHs and human cancer is strongly suggested by their occurrence in environment and their carcinogenic pathways. For example, lung cancer mortality that is related to

PAHs has been increasing in many countries (Harvey, 1997). The molecular formulas, relative indices of carcinogenicity, physical and chemical properties of PAHs are shown in Table 2.

Table 1 Ring patterns and relative stabilities of PAHs.

Ring arrangement	Description	Stability
Linear	All rings in line	Least
Cluster	At least one ring surrounded on the three side	Intermediate
Angular	Rings in steps	Most

Source: Blumer (1976)

2. Sources of PAHs

2.1 Pyrolysis

Most PAHs are formed duing incomplete combustion of organic matter (Suess, 1976). The resulting PAHs may be released to the environment in airborne particulates or in solid or liquid by-products of the pyrolytic process. Many domestic and industrial activities involve pyrosynthesis of PAHs. Domestic activities that produce significant quantities of PAHs include cigarette smoking, home heating with wood or fossil fuels, waste incineration, boiling and smoking of foods, and use of internal combustion engines. Industrial activities that produce large quantities include coal coking, such as production of carbon blacks, creosote, coal tar and related material from fossil fuels, petroleum refining such as synthetic fuel production from coal.

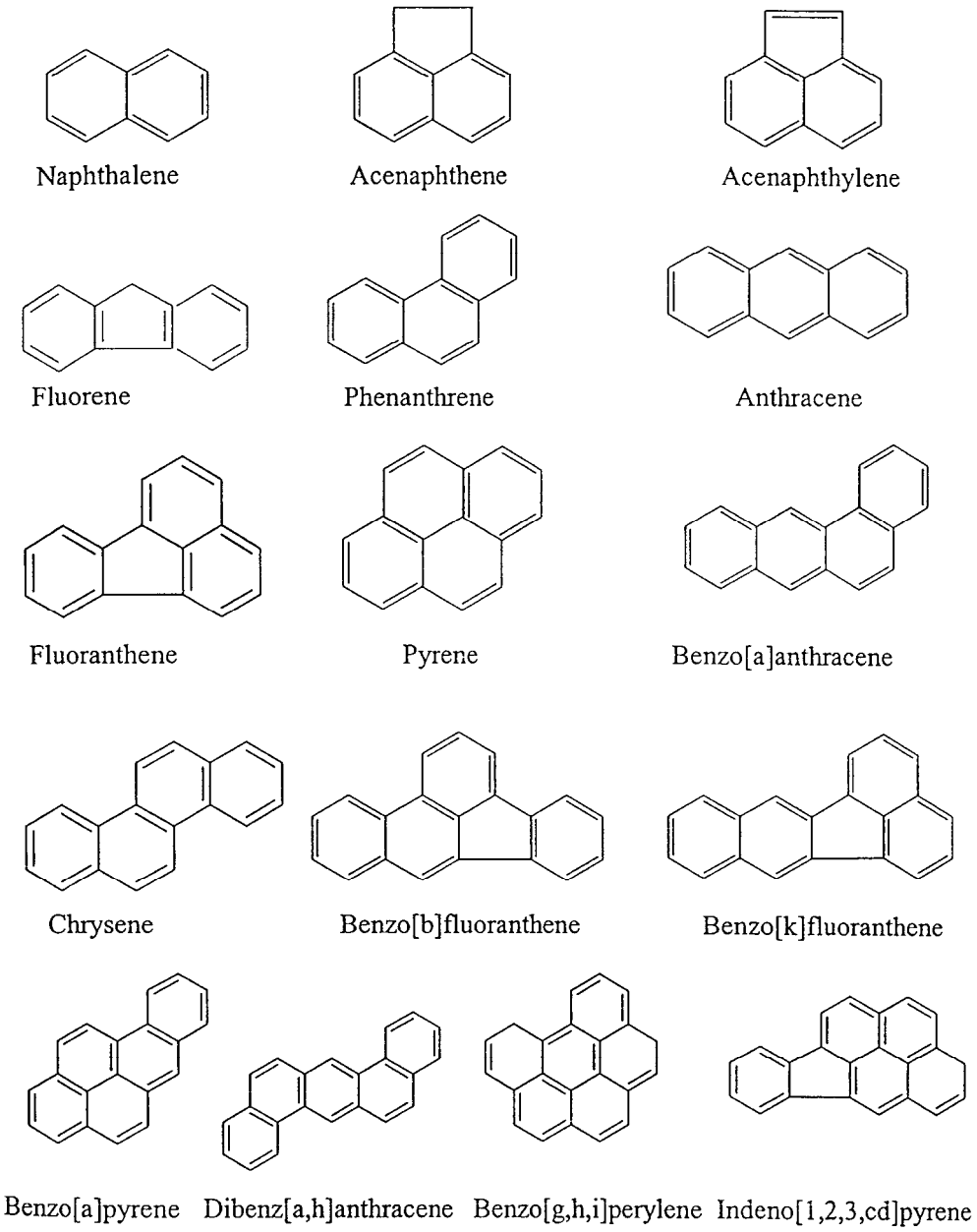


Figure 8 Chemical structures of sixteen PAHs identified by The Environmental Protection Agency (EPA) as priority pollutants.

Source: Harvey (1997)

Table 2 Molecular formulae, relative indices of carcinogenicity, physical and chemical properties of PAHs.

Name	Formula	Weight	Relative index of carcinogenicity	Melting point (°C)	Boiling point (°C)	Water solubility (mg/l)
Naphthalene	$C_{10}H_8$	128	zero	80	218	30
Acenaphthylene	$C_{12}H_8$	152	zero	92	265	3.93
Acenapthene	$C_{12}H_{10}$	154	zero	96	279	3.47
Fluorine	$C_{13}H_{10}$	166	low	116	293	1.98
Phenanthrene	$C_{14}H_{10}$	178	low	101	340	1.29
Anthracene	$C_{14}H_{10}$	178	low	216	340	0.07
Fluoranthene	$C_{16}H_{10}$	202	low	111	-	0.26
Pyrene	$C_{16}H_{10}$	202	low	149	360	0.14
Benzo(a) anthracene	$C_{18}H_{12}$	228	intermediate	158	400	0.014
Chrysene	$C_{18}H_{12}$	228	intermediate	255	-	0.002
Benzo(b) fluoranthene	$C_{20}H_{12}$	252	very high	137	-	1.2×10^3
Benzo(k) fluoranthene	$C_{20}H_{12}$	252	intermediate	217	480	5.5×10^4
Benzo(a) pyrene	$C_{20}H_{12}$	252	extreme high	179	496	3.8×10^3
Dibenzo(a,h) Anthracene	$C_{22}H_{14}$	278	extreme high	262	-	5.0×10^4
Benzo(g,h,i) perylene	$C_{22}H_{12}$	276	intermediate	222	-	2.6×10^4
Indeno (1,2,3,cd) pyrene	$C_{22}H_{12}$	276	high	163	-	0.062

Source: Sim and Overcash (1983)

2.2 Fossil Fuels

This form is diagenesis of sedimentary organic material to form fossil fuels under low to moderate temperature. Coal is generally considered an aromatic material. As much as 75% of the carbon present in bituminous coal may be in aromatic form (Neff, 1985). Crude and refined oils contain significant quantities of PAHs. Typical crude petroleum may contain from 0.2% to more than 7% PAHs. Refined petroleum such as kerosene, gasoline and diesel oil, have relatively low concentrations of tricyclic and larger PAHs, while heavy oil products such as residual

oil and asphalt may contain several percent PAHs (Guerin *et al*., 1978). These PAHs include naphthalene, phenanthrene, fluorine, fluoranthene, benzo (a) anthracene, benzo (a) pyrene, benzo (e) pyrene, dibenzo (c, d) pyrene and perylene.

2.3 Biosynthesis

PAHs are also directly synthesized by organisms particularly bacteria, fungi and higher plants. Many of these compounds are not true PAHs since they contain oxygen, nitrogen, or sulfur substituents. Most of these synthesize are wide variety of polycyclic quinine pigment. Naphthoquinones are common in flowering plants. The largest group of natural occurring polycyclic quinines is the anthraquinones (Gerarde and Gerarde, 1962).

3. Routes of Entry into the Aquatic Environment

PAHs may reach the aquatic environment by a variety of routes including biosynthesis and human activities (Andelman and Snodgrass, 1972). PAHs emitted to the atmosphere during pyrolysis of organic matter are absorbed on microscopic particles and deposited into water (Suess, 1976). Accidental spillage and natural seepage of petroleum represents a quantitatively important input of PAHs to the aquatic environment. Treated and untreated liquid sewage is nearly always discharged to water. Solid residues from activated sludge treatment of wastes may be disposed of in the ocean or in landfills. The estimated total annual input of PAHs to the aquatic environment from the sources discusses above is approximately 230,000 metric tons (Neff, 1979). Liquid domestic sewage usually contains less than 1μg/l total PAHs, industrial sewage 5-15 μg/l (Borneff and Kunte, 1965) and sewage sludge 1-30 mg/kg (Nicholls *et al*., 1979). Surface runoff from land and fallout from the air appear to be the main sources of high molecular weight PAHs in the aquatic environment while petroleum spillage is the main source of total PAHs (Neff, 1985).

4. Distribution in the Aquatic Environment

PAHs entering water from various sources quickly become adsorbed on organic and inorganic particulate matter in water, and large amounts are deposited in bottom sediment. Herbes (1977) said that organic particles tend to adsorb PAHs more readily than clay particle. Once deposited on the bottom, the PAHs are much less subject to photochemical, chemical or biological degradation than they were in the water column (Neff, 1985). However, leaching or biological activities in the sediments may return a small fraction of these PAHs to the water column. The composition and relative concentrations of the PAH assemblage in the sediment may be differed from those in the PAHs source because of differential partitioning of PAHs between sediment and aqueous phases (Hase and Hites, 1977). The distribution of PAHs in aquatic environment is described in Figure 9.

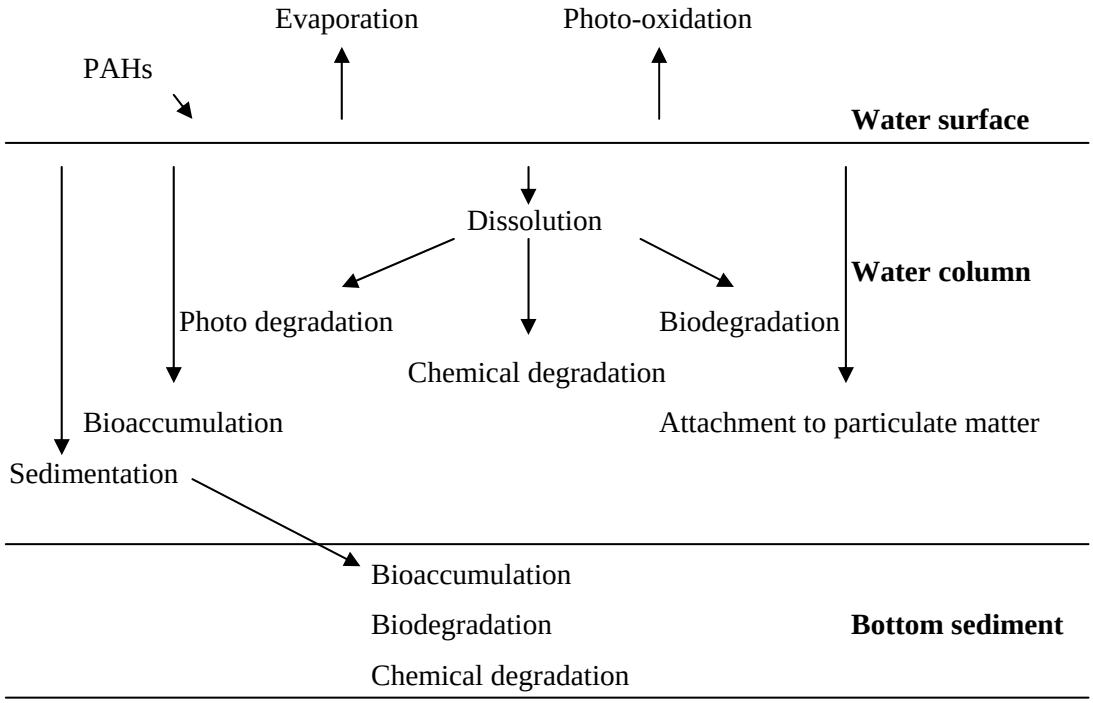


Figure 9 Distribution of PAHs in aquatic environment.

Most of the PAHs entering the aquatic environment are localized in rivers, estuaries and coastal marine waters. Table 3 and Table 4 show the concentration of

several PAHs in water and sediment, respectively, from many aquatic areas. PAHs burden remains relatively near the point sources of PAH and concentrations decrease with distance from the source (Neff, 1985). Sediments receiving drainage from industrial areas may have total PAHs concentrations of 100 mg/kg or more (Bjorseth *et al*., 1979), while those in areas remote from human activity usually has PAHs concentrations in the low ppb range (Shaw *et al*., 1979). Rivers flowing through heavily industrialized areas may contain 1-5 μg/l total PAHs. Unpolluted rivers, ground water, drinking water, seawater usually contain less than 0.1 μg/l total PAHs (Neff, 1979). From these documented data, it may be suggested that relative concentrations of PAH in aquatic ecosystems are generally high in the sediments and low in the water column.

Table 3 Concentration of PAHs in water from many aquatic areas around the world.

Aquatic area / Country	PAHs	Concentration (ng/l)	Reference
River / Norway	Acenaphthene	14 – 1,232	Berglind (1982)
	Benzo(a)pyrene	19 – 311	
	Benzo(b)fluorene	17	
	Fuoranthene	285 – 3,269	
	Indeno(1,2,3-cd)pyrene	17 – 299	
	Naphthalene	50 – 2,090	
	Pyrene	485 – 3,099	
Emscher River / Germany	Anthracene	270	ROWWD (1986)
Elbe River / Germany	Anthanthrene	0.2 - 0.5	Grimmer *et al*. (1981)
	Chrysene	11 – 15	
Rhine River / Germany	Benzo(e)phenanthrene	1.5 - 9.1	Grimmer *et al*. (1981)
	Benzo(ghi)fluoranthene	1.0 – 11	
Ruhr River / Germany	Benzo(k)fluoranthene	20	ROWWD (1986)
Seawater / Germany	Acenaphthylene	0.02 - 1.7	GFOSNH (1993)
	Benzo(e)pyrene	0.02 - 8.8	
	Coronene	0.01 - 1.4	
	Perylene	0.01 - 5.7	
Lake Superior / United States	Benzo(a)anthracene	0.16	Baker and Eisenreich (1990)
	Benzo(b)fluoranthene	0.07	
	Benzo(ghi)perylene	< 0.05	
	Dibenzo(ah)pyrene	< 0.03	
	Fluorene	0.6	
	Phenanthrene	3.5	

Table 4 Concentration of PAHs in sediment from many aquatic areas around the world.

Aquatic area/ Country	PAHs	Concentration (µg/kg dwt)	Reference
Black River / United States	Acenaphthene	1,100	Broman *et al*. (1987)
Rainy River / Canada	Pyrene	nd – 160	Merriman (1988)
Aquatic area in Stockholm / Sweden	Indeno(1,2,3-cd) pyrene	486	Broman *et al*. (1987)
Mediterranean Sea / France	Acenapthylene Benzo(a)anthracene Benzo(ghi)pyrylene Perylene	nd – 2,000 1 – 900 nd – 400 1 – 2,200	Milano *et al*. (1985)
North Sea / United Kingdom	Benzo(a)pyrene	0.2 – 460	Massie *et al*. (1985)
Adriatic Sea / Italy	Dibenzo(a,h) anthracene Naphthalene	0.5 - 4.2 0.7 - 8.6	Marcomini *et al*. (1986)
Boston Bay / United States	Anthracene Pyrene	nd – 507 196 – 66,831	Shiaris and Jambard–Sweet (1986)
Chesapeake Bay / United States	Benzo(a) fluoranthene	2 – 150	Huggett *et al*. (1988)
Vancouver Bay / Canada	Fluorene	100	Environment Canada (1994)
A small Lake in Oslo / Norway	Benzo(e)pyrene	80	Gjessing *et al*. (1984)
Leman Lake / Switzerland	Benzo(ghi) fluoranthene Coronene Triphenylene	75 1 25	Dreier *et al*. (1985)
Gironde estuary / France	Benzo(k) fluoranthene	< 0.1 – 24	Garrigues *et al*. (1987)
Great Barrier Reef / Australia	Chrysene Fluoranthene	< 0.04 - 0.8 < 0.1 - 7.2	Smith *et al*. (1987)
Mabtaphut / Thailand	Total PAHs	0.06 – 1.87 *	Suthanaruk (1991)
The lower Tha Chin River / Thailand	2-6 rings PAHs	2.7 *	Sunwanich (1991)
Gulf of Thailand / Thailand	Total PAHs	13.63 – 123.52	Sarin (1994)
Seashore, Rayong / Thailand	Total PAHs	0.28 *	Tappatat (1995)
Tha Chin River / Thailand	Total PAHs	0.22 – 1.52 *	Chaipuriwong (2001)

Note: * µg/g dwt

5. Transformation by Microorganisms and Aquatic Organisms

PAHs in the aquatic environment are subjected to transformation and degradation by a variety of organism. Bacteria show tremendous adaptability in utilization of organic molecules, such as aromatic hydrocarbons, as a carbon source. Jerina *et al*. (1971) found that PAHs were oxidized by bacteria to dihydrodiols oxidized further in a series of reactions to catechols and eventually to carbon dioxide and water. Many studies have been performed on the ability of marine water column and sediment bacteria to degrade PAHs, which is much more rapidly occurred under aerobic than under anaerobic conditions (Lee *et al*., 1978; Delaune *et al*., 1980; Wade *et al*., 1980). Rate of PAH degradation tends to decrease with increasing PAHs molecular weight (Herbes and Schwall, 1978). Bacteria populations from oil-contaminated areas metabolize PAH more readily than populations from clean areas.

Among the invertebrates, the enzyme system variously known as the cytochrome P-450-dependent mixed function oxigenase (MFO) is responsible for initiating the metabolism of various lipophilic organic compounds including PAHs. This enzyme system is similar to that found in mammalian liver microsomal system (Neff, 1985). Although this system effectively detoxifies certain PAHs, they may be transformed to intermediates that are highly toxic, mutagenic, or carcinogenic to the host (Jerina and Daly, 1974; Huberman *et al*., 1976). However, MFO activity seems to be restricted primarily to some aquatic biota. Marine amphipods, *Rhepoxynius abronius* can metabolize naphthalene and benzo (a) pyrene in sediment exposure (Reichert *et al*., 1985; Meador *et al*., 1995), while another species, *Diporeia* spp. has limited ability to biotransform PAHs (Landrum, 1988). Several species of bivalve mollusks, such as *Mytilus edulis*, *Mya arenaria*, *Ostrea edulis* and *Anodonta* sp., were characterized by low biotransformation capacities (Lee *et al*., 1972a; Vandermeulen and Penrose, 1978; Payne and May, 1979). In polychaete worms, *Nereis diversicolor* can transform benzo (a) pyrene efficiently, but *Leitoscoloplos fragilis* appeared not to transform at all or transformed very slowly (Driscoll and McElroy, 1996). *Nereis diversicolor* also showed high potential of pyrene metabolizing capacity (Christensen *et al*., 2002), as same as *Nereis virens* with benzo (a) anthracene (McElroy, 1985),

Capitella capitata sp.1 with fluoranthene (Forbes *et al*., 1996), *Arenicola marina* with pyrene (Christensen *et al*., 2002), *Armandia brevis* with naphthalene (Meador *et al*., 1995) and *Abarenicola pacifica* with benzo (a) pyrene (Weston, 1990). In contrast with some aquatic oligochaetes species, slow biotransformation rate to metabolize PAHs is observed including *Lumbriculus variegatus* with pyrene and benzo (a) pyrene (Harkey *et al*., 1994a; Leppanen and Kukkonen, 2000b) and *Monopylephorus rubroniveus* with fluoranthene (Weinstein *et al*., 2003).

In fish, several studies reported that many species have a MFO enzyme system, such as mosquito fish *Gambusia affinis* (Chamber, 1979), killi fish *Fundulus heteroclitus* (Stegeman, 1979) and rainbow trout *Salmo gairdneri* (Stegeman and Chevion, 1980), etc. Most MFO activity is localized in the liver, gills and kidney (Lindstorm-Seppa *et al*., 1981). When the parent compounds are broken down, the polar metabolites are then ultimately excreted via the gallbladder or the urine (Howard, 1989; Varanasi *et al*., 1989; Hellou *et al*., 1999; Klumpp *et al*., 2002).

6. Accumulation and Release by Aquatic Organisms

PAHs can be accumulated by aquatic organism (Vijayavel and Balasubramanian, 2006b). The concentrations detected in tissues of both freshwater and marine animals are generally found in the low ppb range, except near point sources of PAH pollution (Neff, 1985). For instance, the surveys of PAHs in marine bivalve mollusks along the Pacific coast of Canada and the United States have confirmed a high degree of correlation with industrial, urban and recreational uses of the coastal water (Dunn and Stich, 1976; Dunn and Young, 1976; Mix and Schaffer, 1979). Residues of total PAHs in many aquatic species investigated from field survey were recorded in Table 5. Additionally, lower molecular weight compound, such as naphthalene, was also observed. For example, naphthalene constituted 75-80% of total PAHs found in the muscle, liver and gonads of American plaice and yellow tail flounder (Hellou and Warren, 1996), and 57% of total PAHs found in edible clams *Tapes philippinarum* and *Venus gallina* (Binelli and Provini, 2003). Average naphthalene residue in fish was determined at a concentration of 8.1 µg/g (Anyakora

et al., 2005), while in shore crabs collected from the San Francisco bay area mean concentration of this chemical were 7.4 ng/g (Miles and Roster, 1999). Concentration of naphthalene was also detected from the animals in the United States waters, ranging from 5 to 176, 4 to 10 and less than 1 to 10 ng/g in oysters, mussels and clams, respectively (Bender and Huggett, 1989).

Release of PAHs from tissues of contaminated organisms may be passive, reflecting an equilibrium distribution between the aqueous phase and lipophilic compartments in contact with it. By another way, it may be active, involving metabolic transformation of PAHs to polar water-soluble metabolites which are more readily excreted (Neff, 1985). Several reports revealed accumulation and release of PAHs from solution by aquatic organisms in the laboratory. For example, freshwater oligochaete *Lumbriculus variegatus* uptake four PAHs in its tissue and the depuration occurred most rapidly for fluorine, followed by anthracene and fluoranthene, but no apparent depuration of pyrene was observed during the 96 h depuration period (Sheedy *et al*., 1998). Marine polychaete worms *Neanthes arenaceodentata* and *Arenicola marina* rapidly accumulated naphthalene from the water, and both species rapidly released naphthalene from their tissues when returned to clean water (Rossi, 1977; Lyes, 1979). Similary, the water flea *Daphnia pulex* accumulated and released PAHs rapidly due to reaching equilibrium concentration within 24 h (Herbes and Risi, 1978; Southworth *et al*., 1978). Fish is an aquatic organism that can metabolize and excrete PAHs even more rapidly because the equilibrium concentrations were often reached in their tissues with 24 h or less (Lee *et al*., 1972; Anderson *et al*., 1974). Most of the PAHs were excreted via the gallbladder or the urine (Lee *et al*., 1972b; Statham *et al*., 1976).

The accumulation and release of PAHs from sediment by benthic organisms has been focused on some species. Most studies have shown that an increase in the sediment-chemical contact time decreased the bioavailability of these compounds. Ferguson and Chandler (1998) found that estuarine polychaete, *Strebospio benedicti*, increased PAHs body burdens at the beginning, followed by a decline from 12 to 28 d of the exposure period. Fluorene, phenanthrene and pyrene, were more available to

Table 5 Concentration of PAHs in aquatic organisms.

Organism / Location	Concentration (µg/kg dwt)	Reference
Oyster / Barataria Bay	1,900	Wade *et al*. (1989)
Spider crab / Barataria Bay	56	
Mollusc (*Mytilus galloprovincialis*)/ Arcachon Bay	900 – 41,000	Baumard *et al*. (1998)
Mollusc / Black Sea	20 – 750	Shchekaturina *et al*. (1995)
Bivalve / Seashore, Middle East Region	570 – 2,600 *	Fowler *et al*. (1993)
Fish / Seashore, Middle East Region	9.6 – 31 *	
Fish / Arabian Gulf	2.51 – 563.6	Al-Yakoob *et al*. (1993)
Oyster / Rowley Shelf	< 0.010 – 0.150 *	Pendoley (1992)
Fish / Naples Bay	94 – 1,930 **	Cocchieri *et al*. (1990)
Crustacean / Naples Bay	185 – 295 **	
Mussel (*Perna viridis*) / The lower Tha Chin river, Thailand	12.5 – 81.0	Sunwanich (1991)
Mussel (*Perna viridis*) / The lower Chao Phraya river, Thailand	15.64-76.8	Nokyoo (1995)
Fish (*Pampus argenteus*) / Gulf of Thailand	53 *	EVS Environment Consultants (1999)

Note: * µg/g dwt; ** µg/kg wwt

two species of amphipod, *Pontoporeia hoyi* (Landrum, 1989), and *Diporeia* spp. (Landrum *et al*., 1992; Harkey *et al*., 1994b) in dosed sediments aged less than 1 w. Naphthalene tissue residues of freshwater oligochaete *Lumbriculus variegatus* generally peaked by the starting and tended to decline to the end of the exposure (Brunson *et al*., 1998). In the same way, accumulation of naphthalene from sediment by marine worm *Arenicola marina* was rapid and almost complete depuration of the accumulated naphthalene was observed after 24 h (Lyes, 1979). And also, *Lumbriculus variegates* accumulated pyrene and benzo (a) pyrene in dosed-sediment and these compounds were found to be depurated within 72 h in clean sediment (Leppanen and Kukkonen, 2000b).

7. Toxic Effects in Aquatic Organisms

PAHs readily have biological effects in aquatic organisms. They may interact physically with hydrophobic sites in the cell, causing molecular deformation and perturbation. Alternatively, PAHs metabolites may undergo a variety of spontaneous or enzyme-mediated chemical reaction leading to cell damage, mutagenesis, teratogenesis and cancer (Neff, 1985). Furthermore, these chemicals appear to bind selective to the surface of plasma membranes (Roubal and Collier, 1975). This binding causes perturbations in surface organization, increasing membrane permeability (Goldacre, 1968). By disrupting, PAHs might also affect the activity of the many enzymes bound to plasma membranes and essential for cell function.

The toxicity of PAHs will be proportional to the concentration associated with the membrane surface. Less soluble aromatics tend to have s greater effect at a given concentration than more soluble ones (Neff, 1985). Although acute toxicity of PAHs to aquatic organisms increases as molecular weight increases, the relationship is not absolute. Only low molecular weight including naphthalene, fluoranthene and pyrene are acutely toxic to aquatic organisms, while the higher molecular weight PAHs are not acutely toxic. Variability in response to water-borne PAHs and sediment-associated PAHs in several species of aquatic animals were shown in Table 6 and Table 7, respectively.

It should be recognized that the acute toxicity data do not adequately reflect the potential impact of chronic low-level PAH contamination produced sublethal responses in aquatic organisms. These sublethal effects may be detrimental to the long-term survival. In addition, chronic exposure to certain PAH, such as 4-6 rings and highly angular configurations, may induce mutation and cancer in sensitive species (Zedeck, 1980). Several experimental conducted on sublethal effect of PAH in aquatic biota have reported particularly on naphthalene. The behavioral response had been studied in blue crabs *Callinectes sapidus* which detected extremely low concentrations of naphthalene in solution and responded by increased antennular flicking rate (Pearson and Olla, 1980). Donahue *et al*. (1977) found that several

Table 6 Acute toxicity of water-borne PAHs to aquatic animals.

PAHs	Species	LC_{50} (ppm)	Duration (h)	References
Acenaphthene	Water flea *Daphnia magna*	41	48	LeBlanc (1980)
Acenaphthylene	Red killifish *Oryzias latipes*	6.1	96	Yoshioka and Ose (1993)
Anthracene	Bluegill *Lepomis macrochirus*	0.0045	96	Oris and Giesy (1986)
Naphthalene	Polychaete *Neanthes arenaceodentata*	3.8	96	Rossi and Neff (1978)
	Dungeness crab (zoeae) *Cancer magister*	2.0	96	Caldwell *et al*. (1977)
	Amphipod *Elasmopus pectenicrus*	2.7	96	Lee and Nicol (1978)
	Copepod *Eurytemora affinis*	3.8	24	Ott *et al*. (1978)
	Grass shrimp *Palaemonetes pugio*	2.4	96	Neff *et al*. (1976)
	Sheephead minnow *Cyprinodon variegatus*	2.4	24	Anderson *et al*. (1974a)
	Coho salmon fry *Oncorhynchus kisutch*	3.2	96	Moles (1980)
	Mud crab *Scylla serrata*	18	96	Vijayavel and Balasubramanian (2006b)
Fluorene	Polychaete *Neanthes arenaceodentata*	1.0	96	Rossi and Neff (1978)
	Grass shrimp *Palaemonetes pugio*	0.32	96	Kennish (1992)
	Amphipod *Gammarus pseudoliminaeus*	0.6	96	
Phenanthrene	Polychaete *Neanthes arenaceodentata*	0.6	96	Kennish (1992)
	Zooplankton *Artemia nauplii*	0.677	24	Abernethy *et al*. (1986)
Phenanthrene	Water flea *Daphnia magna*	0.843	96	Eastmond *et al*. (1984)
Fluoranthene	Polychaete *Neanthes arenaceodentata*	0.5	96	Rossi and Neff (1978)
	Bluegill *Lepomis macrochirus*	4.0	96	Buccafusco *et al*. (1981)

Table 6 (Continued)

PAHs	Species	LC_{50} (ppm)	Duration (h)	References
Chrysene	Polychaete *Neanthes arenaceodentata*	1.0	-	Rossi and Neff (1978)
Benzo(a)pyrene	Polychaete *Neanthes arenaceodentata*	1.0	-	Rossi and Neff (1978)
Dibenz(ah) anthracene	Polychaete *Neanthes arenaceodentata*	1.0	-	Rossi and Neff (1978)

Table 7 Acute toxicity of sediment-associated PAHs to aquatic animals.

PAH	Species	LC_{50} (ppm dwt)	Duration (d)	References
Naphthalene	Amphipod	1,757	1028	Landrum *et al*. (2003)
	Diporeia spp.	1,266		
Fluoranthene	Oligochaete *Monopylephorus rubroniveus*	>191,765 *	10	Weinstein *et al*. (2003)
	Polychaete *Streblospio benedicti*	65.6	10	Weinstein and Sanger (2003)
	Copepod *Schizopera knabeni*	14,200 *	10	Lotufo (1998)
	Copepod *Coullana* sp.	8,800 *		
	Amphipod *Rhepoxynium abronius*	2,229 *	10	Swartz *et al*. (1990)
	Amphipod *Corophium spinicorne*	2,833 *		
	Midge *Chironomus tentans*	1,740 *	10	Seudel *et al*. (1993)
	Amphipod *Hyallella azteca*	1,480 *		
Pyrene	Oligochaete *Lumbriculus variegatus*	>226 **	7	Kukkonen and Landrum (1994)

Note: * ppm oc; ** ppm wwt

PAHs abolished positive phototaxis in nauplii of barnacle *Balanus amphitritniveus* at concentrations ranging from about 0.14 ppm dimethylnapthalene. The studies on physiological changes including growth, reproduction and larval development also have been observed. Laughlin and Neff (1979) reported that duration of larval development to the megalops stage of mud crab *Rhithropanopeus harrisii* was decreased by exposure to sublethal concentrations of naphthalene. Ott *et al*. (1978) reported that marine copepod *Eurytemora affinis* was statistically significant reduction in life span, brood size, and total number of nauplii produced when exposed to 29 d 10 µg/l naphthalene. Woodward *et al*. (1983) found that cutthroat trout *Oncorhynchus clarki* exhibited significant reductions in survival and growth at the concentration between 1 and 17 µg/l total naphthalene. The study on mud crab *Scylla serrata* showed that naphthalene causes disturbance in the normal physiology of the crab at a concentration of 10 mg/l, including increase of the consumption of oxygen, decrease of respiratory enzymes in hepatopancreas, ovary and gills (Vijayavel and Balasubramanian, 2006a). Moreover, the biochemical constituents and all marker enzymes in this crab species were decreases in hepatopancreas and ovary, while in hemolymph, were increased (Vijayavel and Balasubramanian, 2006b).

Use of Aquatic Oligochaete in Sediment Toxicity Tests

1. Criteria for Selection

Test species used for evaluation of sediment toxicity and bioaccumulation of anthropogenic contaminants should meet certain criteria to fit satisfactorily for sediment bioassay. These criteria include ecological relevance, wide geographical distribution, taxonomic relation to indigenous animals, good availability and ability to assess chronic endpoint, incorporation of all relevant routes of exposure, tolerance of sediment characteristics and suitability for bioaccumulation assays (Giesy and Hoke, 1989). Oligochaetes fulfill most of the criteria and have been successfully employed in bioassays (Kukkonen and Landrum, 1994). Due to their true sediment-ingesting behavior, they are excellent test organisms for studying bioaccumulation of hydrophobic sediment-bound contaminants. Ingestion of contaminated particles and

their exposure to digestive fluids may be the principle route for contaminant accumulation (Weston, 1990; Mayer *et al*., 1996). Feeding on subsurface sediments and egesting onto the sediment surface of oligochaetes are important roles for recycling deposited material. Their reworking can considerably modify the structure of sediments (McCall and Fisher, 1980) and can even lead to release of sediment-bound contaminants to overlying water (Reible *et al*., 1996).

2. Background Information on Toxicity and Bioaccumulation Testing of Sediment-Associated PAHs

Several studies documented on the application of using aquatic oligochaetes in sediment toxicity and bioaccumulation assays are briefly revealed as described below.

Kukkonen and Landrum (1994) studied toxicokinetics and toxicity of sediment-associated pyrene to *Lumbriculus variegates*. The worms were exposed to pyrene-dosed Lake Michigan sediment. The results were found that they accumulated pyrene rapidly and achieved apparently steady state within 48 to 168 h. The uptake clearances decreased with increasing pyrene concentration. The worms avoided the sediment at high concentrations (269 μg/g), which reduced accumulation and likely minimized the mortality response. Bioavailability apparently declined for exposures in sediment stored 1.5 months attributed to both a decline in lipid content during the experiment and changes in pyrene bioavailability.

Brunson *et al*. (1998) assessed the bioaccumulation of sediment-associated PAHs of the upper Mississippi River using 28 d laboratory exposure with the freshwater oligochaete *Lumbriculus variegatus*. The experiment was conducted under flow-through reconstituted water system. Residues measured in the worms after exposure were compared to contaminant concentration in field-collected oligochaetes. The results were found that two low molecular weight PAHs, naphthalene and phenanthrene, and two high molecular weight PAHs, pyrene and chrysene, were the PAHs of highest concentration in field-collected oligochaetes. Mean BSAFs for PAHs were within a range of about 1.0-2.6.

Leppanen and Kukkonen (1998b) investigated the relative importance of ingested sediment and pore water as sources of bioaccumulation routes for pyrene in *Lumbriculus variegatus*. The worms were exposed to radiolabeled pyrene spiked lake sediment for 28 d and the egestion rate was followed. The results showed that pyrene accumulated mainly (61% of the body burden) through ingested material and bioavailability was decreased when exposure time increase. The data signified the importance of ingested material in bioaccumulation of hydrophobic chemicals in *Lumbriculus variegatus*

Leppanen and Kukkonen (2000a) studied the importance of sediment-chemical contact time in bioaccumulation of pyrene and benzo(a)pyrene to *Lumbriculus variegatus*. The results showed that increase in sediment-chemical contact time decreased pyrene and benzo(a)pyrene uptake clearance for both feeding and nonfeeding animals. Ingest of sediment considerably increased accumulation of both compounds indicating the importance of feeding behavior in bioaccumulation of sediment-associated chemicals.

Leppanen and Kukkonen (2000b) studied bioaccumulation, depuration and biotransformation of radiolabelled pyrene and benzo(a)pyrene were studied in *Lumbriculus variegatus* in spiked Lake Mekrijarvi (Eastern Finland) sediment. The results showed that bioaccumulation of both chemicals was fast and an apparent steady level was reached with a week. Biotransformation results showed the relative proportion of parent compound in tissues decreased continuously during the exposure. Approximately 60% of pyrene and 90% of benzo(a)pyrene was still in the parent compound.

Conrad *et al*. (2002) studied the effect of sediment-chemical contact time on routes of uptake in *Lumbriculus variegatus* using sediment spiked with ^{14}C-labelled pyrene. The results showed that there was a decline in bioavailability with time separated in 3 stages process, including a rapid initial decline (40% decreases) during the first period, an intermediate stage with stable levels, and an ultimate decline (70% decreases) at the end of the exposure. The dietary route of uptake for pyrene varied

during the sediment aging process, reflecting the changes in the physico-chemical interactions between the pyrene, sediment and pore water

Weinstein *et al*. (2003) studied bioaccumulation and toxicity of sediment-associated fluoranthene in the estuarine oligochaete *Monopylephorus rubroniveus* using both in the presence and absence of ultraviolet radiation. It was found that little mortality was observed following 10 d exposure with the LC_{50} higher than 3912 μg/g sediment dwt. Bioaccumulation of this chemical was high with mean BAFs varied between 1.6-23.0. This result demonstrated that *Monopylephorus rubroniveus* was highly tolerant of fluoranthene in the presence of sediment and could bioaccumulate fluoranthene to high levels.

Hyotylainen and Oikari (2004) studied bioaccumulation of PAHs in the crosote-contaminated sediment of Lake Jamsanvesi in a 28 d laboratory-exposure using *Lumbriculus variegatus* as a bioassay. Chemical analyses showed that benzo (k) fluoranthene, anthracene and fluorine were the main PAH compounds present in the tissue of oligochaete, just as in the sediment. BSAFs of the individual PAHs varied from 1.2 to 5.7. It could be concluded that this worm species had a marked ability to accumulate and retain PAHs from creosote-contaminated sediment.

Filipowicz *et al*. (2007) studied the potential for dietary transfer of sediment-associated fluoranthene from *Monopylephorus rubroniveus* to grass shrimp *Palaemonetes pugio* using both in the presence and absence of sublethal water-borne concentrations of the metabolic inhibitor, piperonyl butoxide (PBO). It was found that all grass shrimp bioaccumulated fluoranthene with the higher in the presence of PBO. These results demonstrated that sediment-associated fluoranthene was transferred through the diet from oligochaetes to grass shrimp, but the biomagnification was low due to low trophic transfer coefficients (TTCs) calculated of 0.02 and 0.01 in the presence and absence of PBO, respectively. Finally, the presence of PBO enhanced fluoranthene bioaccumulation.

Environmental Conditions in the Lower Chao Phraya River

1. General Information

The lower Chao Phraya river is an important estuary located in Bangkok and Samutprakarn Province. This estuary occupied the area approximately 58 km extending from the mouth of the river to Rama VII Bridge. The mouth of the river is opened to the upper Gulf of Thailand. Water change in this area was controlled by diurnal tides. The volume and salinity of water are mainly affected by influence of precipitation, freshwater and seawater. The bottom of the estuary was constituted of fine sediment mainly composed of mud.

2. Water Qualities and Sediment Properties

The Office of Environment Policy and Planning (2001) reported that water qualities in the lower Chao Phraya river were in rather bad condition. Most of all parameters were within the Thailand National Water Quality Classification for industrial purpose, except for DO, biochemical oxygen demand (BOD) and ammonia-nitrogen (NH_3-N) which declined below the criteria. The measurement of water quality recorded during 1994 and 1999 for DO, BOD and NH_3-N were 1.6, 3.2 and 1 mg/l, respectively.

It is obvious that the rapid increase in population and coastal development lead to water quality change. Ngamprayad (1999) found that the frequency of low DO tends to be greater in urbanized and industrialized zones. Pollution sources, such as urban runoff, sewage disposal, and industrial effluents, contribute to the loading of organic pollutants to the water and bottom sediment of the estuary (Wattayakorn, 2003). According to Pollution Control Department (2000), the average BOD loading from domestic waste in Chao Phraya river was approximately 19-1,377 mg/l while the industrial loading estimated 230-29,000 mg/l. It is evidence that the industrial activity was the major source of organic matter in this area followed by the domestic waste.

Changes in bottom sediment in the lower Chao Phraya river were fluctuated accordingly due to the industrial and domestic activities along the river bank. High sedimentation occurred along the shoreline since the bulks of organic matter were released to the water column. During the occurrence, there was significant shift in sediment characteristics. High organic content appeared in urbanized and industrial zones between 4.34 and 10.84%, while silt and clay fractions were recorded high value in the coastal area from 41.44 to 99.22% (Kanchana-Aksorn and Petpiroon, 2006).

3. Contamination of PAHs

Anthropogenic sources of PAHs in the lower Chao Phraya river were from petroleum, sewage and industrial wastewaters, boating activities and incomplete combustion of fossil fuels. Previous studies established that the contamination of PAHs have been found in water, sediment and aquatic biota. Wattayakorn (2003) reported that the concentration of total identified PAHs were found in the water samples ranging from < 5-1,500 ng/l, and several PAHs, such as naphthalene, acenaphthylene, acenaphthene, fluorine, phenanthrene and anthracene, were identified. Nokyoo (1995) reported that the sediment samples contained 2-6 rings PAHs and the average concentration of total PAHs ranged between 1.93–2.14 µg/g dwt. Mussel *Perna viridis* samples collected from the mouth of Chao Phraya river in this study contained PAHs including naphthalene, biphenyl, fluorine, anthracene, fluoranthene, pyrene and chrysene with individual PAHs concentrations ranging from 15.64-76.8 ng/g dwt.

4. Benthic Communities

The settling and the accumulation of PAHs in the bottom sediments of the lower Chao Phraya river cause change to the structure of benthic communities (Suess, 1976). It can promote the abundance of some tolerant species recognized a pollution indicator (Chapman and Brinkhurst, 1984; Heip, 1992; Peterson *et al*., 1996). Previous study on the macrobenthic communities reported that oligochaete was

especially abundant in this area (Ngamprayad, 1999). The predominant species in this system was the tubificid oligochaete *Limnodrilus hoffmeisteri*, and the abundance could reach densities greater than 10,000 ind/m^2 (Kanchana-Aksorn and Petpiroon, 2006). This finding suggests that *L. hoffmeisteri* is tolerant to multiple stresses including high organic sediment, low DO water and PAHs contamination.

MATERIALS AND METHODS

Materials

1. Instruments

1.1 Stereo microscope (Nicon, SMZ-2B, Japan)
1.2 Compound microscope (Nicon, E400, Japan)
1.3 SEM (Jeol, JSM 5600 LV, Japan)
1.4 Critical point dryer (Hitachi, HCP-2, Japan)
1.5 Ion coater (Eiko, IB-2, Japan)
1.6 Digital camera (Fujifilm, FinePix S9600, Japan)
1.7 Hot air oven (Memmert, ULE 500, Germany)
1.8 Muffle furnace (Vulcan, 3-1750, USA)
1.9 Microwave extraction system (CEM Corporation, MARS-X, USA)
1.10 Rotary evaporator (Buchi, R-205, Switzerland)
1.11 Homogenizer (Kinematica, PT-2100, UK)
1.12 Centrifuge (Jouan, MR 23i, France)
1.13 Magnetic stirrer (HL, MS-115, India)
1.14 HPLC (Shimadzu, LC 2010, Japan)
1.15 C_{18} column (3.9 mm×15 cm, alphaBond, Alltech, USA)
1.16 Photodiode array detector (Shimadzu, LC-10ADVP, Japan)
1.17 Separatory funnel
1.18 Mortar and pestle
1.19 Stainless sieve (0.5, 0.25 and 0.063 mm mesh, Endecotts, England)
1.20 Analytical balancer (4 digits, Mettler-Toledo, AB 204-S, Switzerland)
1.21 Micrometer (range: 0-75 mm, INSIZE, IS13101, China)
1.22 DO meter (Eutech, Cyberscan DO 6000, Singapore)

2. Chemicals and Reagents

2.1 Naphthalene ($C_{10}H_8$, 99% purity, AR grade, Ajax Finechem, Australia)
2.2 Acetone (C_3H_6O, AR grade, J. T. Baker, USA)
2.3 Cyclohexane (C_6H_{12}, AR grade, Lab-Scan, Thailand)
2.4 Hexane (C_6H_{14}, AR grade, Fisher Chemicals, USA)
2.5 Acetonitrile (CH_3CN, HPLC grade, Lab-Scan, Thailand)
2.6 Ethanol (CH_3CH_2OH, AR grade, Merck, Germany)
2.7 Sodium sulfate (Na_2SO_4, AR grade, Ajax Finechem, Australia)
2.8 Magnesium chloride ($MgCl_2$, AR grade, Ajax Finechem, Australia)
2.9 Glutaraldehyde ($C_5H_8O_2$, EM grade, EMS, UK)
2.10 Osmium tetroxide (OsO_4, EM grade, EMS, UK)
2.11 Phosphate buffer (pH 7.2)
2.12 Olive oil
2.13 Deionized water

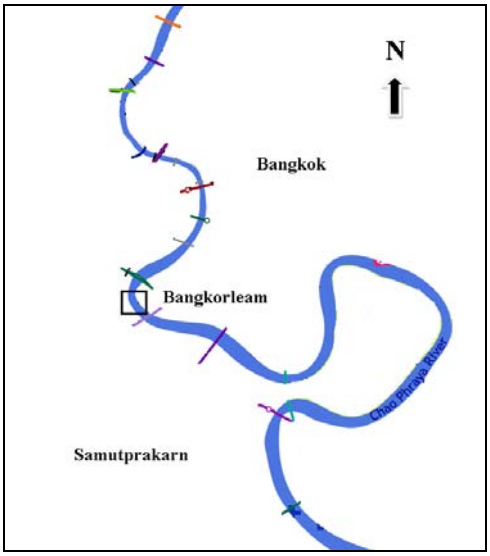
Methods

1. Test Organism and Sediment

1.1 Worms

Limnodrilus hoffmeisteri was manually collected in April 2006 from Chao Phraya river bank located at Tok road waterside, Bangkorlaerm district, Bangkok, Thailand (Figure 10). High densities of oligochaetes were found among debris within 5 m of the river edge. A large quantity of sediment containing worms was scraped with small shovel several times. The sediments were washed through 0.5 mm sieve with regional spring water. Worms were transported to the laboratory and carefully sorted under stereo microscope. Before setting up the experiments, worms were classified according to Brinkhurst and Jamieson (1971), and the identification was also confirmed by Christer Erseus (pers. comm.), the expert on zoological systematics of Class Clitellata (Phylum Annelida), from University of Gothenburg, Sweden, using

the technique of mitochondrial 16s ribosomal DNA markers (Beauchamp *et al*., 2001) which was developed to examined phylogenetic relationships among these cryptic species. The DNA sequences of two specimens from Chao Phraya estuary were compared with each other, and also compared with the sequences of *L. hoffmeisteri* samples from middle Europe and Estonia (northern Europe). Additional sequences for *L. udekemianus* obtained from a laboratorial culture which originally collected from Kirgizstan (Central Asia), was also included in these analyses.



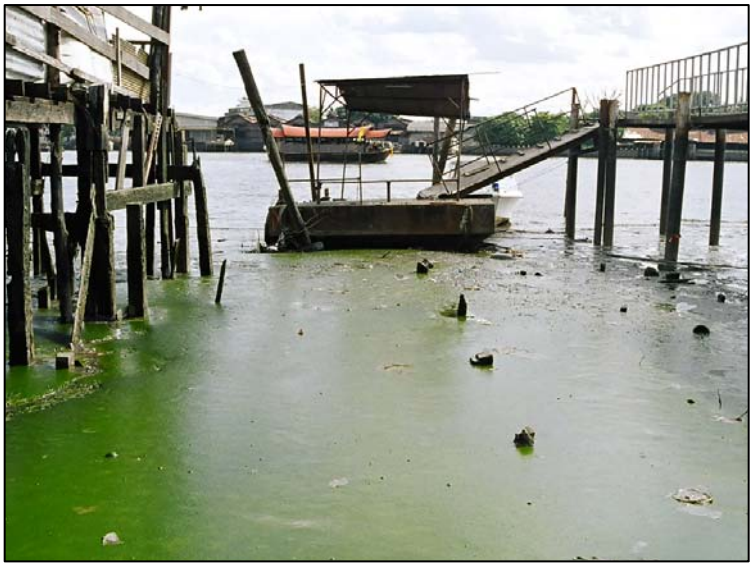


Figure 10 Sampling site at Tok road waterside, Bangkorlaerm district, Bangkok, Thailand.

In the laboratory, a first group of 500 worms was separated in a petridish containing tap water for studying morphological feature and biological behaviors. A second group of about 20,000 worms was acclimated in 20×30×15 cm^3 plastic box containing 2-3 cm depth of wet sieved sediment with >500 μm particles removed. This depth should allow for natural burrowing behavior of the worm (Dermott and Munawar, 1992). Water brought from the sampling site was used for rearing at a depth of approximately 8 cm. The worms were fed on weekly diet with decomposing ground fish food and maintained at room temperature under a daily photoperiod of 12 h. The water body was gently aerated via a pipette positioned 2 cm above the sediment surface. The overlying water was changed manually once a week to avoid accumulation of organic material. The last group of 300 worms was divided into 3 replicates and analyzed for their background naphthalene concentration by using the technique of HPLC (Filipowicz *et al*., 2007).

1.2 Sediment

Test sediment was collected from the same location described above using a small shovel. Extraneous materials and any organisms seen by naked eyes were removed using a forceps. A small fraction of sediments was divided into 3 replicates and characterized by determination of granular composition using wet sieving method (Zhang *et al*., 2004), TOM using loss on ignition method (Parsons *et al*., 1984), WC using oven-drying method (Gardner, 1986) and background naphthalene concentration using the technique of HPLC (Filipowicz *et al*., 2007). The remaining sediments were washed through 0.5 mm mesh sieve and a large plastic container was used to allow for the retention of sediment fines. After the suspended fines settled, the overlying water was decanted carefully. The particles were mixed, turned them back into the sediment, and stored in a refrigerator at 4 °C prior to use in the experiment.

2. Investigation on the Morphological Features and Biological Behaviors

A group of 15 worms was brought from the first separation. They were concisely observed for general characteristics using a stereo microscope. The first group of 10 worms were cleaned with tap water and then leaved in a petri dish containing distilled water. As oligochaetes tend to continually loose weight when weighed in air, the weighing technique of Lundkvist (1978) was modified. Individual wet weight of the worms was measured by carefully dipping each worm on the edge of a glass beaker, soaking up on absorbent paper sheets to remove some of the surface water, placing them in a small pre-weighed container of olive oil, and weighting with an analytical balancer. The animals were individually rinsed with distilled water to clean the remaining olive oil and then narcotized in 5% magnesium chloride. The specimens were size-estimated by measuring length from the anterior end to the posterior end using micrometer. Thereafter, they were fixed and then transferred to preserve in 70% ethanol. The specimens were temporary mounted on microscope slides and taxonomic description was done under a compound microscope. The last group of 5 worms was prefixed with 2.5% glutaraldehyde overnight at 4 °C, washed 3 times with 0.1 M phosphate buffer (pH 7.2), postfixed with 1% osmium tetroxide 2 h, washed 3 times with distilled water, and dehydrated in an ethanol series (10, 30, 50, 70, 90 and 100%). They were then subjected to dry using a critical point dryer 30 min, coated with gold by an ion coater 5 min, and examined by a SEM.

For behaviors observations, the first microcosm was established by placing a part of sediment into 600 ml glass beaker to produce a depth of 2-3 cm, and covering with water from the collected site. At the start of the experiment, a group of 10 worms was sieved and transferred to the beaker. Swimming pattern, burrowing behavior, and trace leaving of active worms were observed. Once the worms bury into the substrate, the air was pressed into the beaker by an additional air bubble that was applied to produce water circulation. The beaker was kept under this condition for 1 h and the results of worm settlement were observed. After finishing this step, the beaker was then placed on a table without aeration for duration of time until the water current was absent. The beaker was covered with a sheet of paraffin which

prevents the diffusion of atmospheric air into the overlying water. Living pattern of the worms was observed half an hour later. The second microcosm was set by placing a group of 50 worms into a petri dish filled with spring water and small decaying materials such as plant debris or lump of soft sediment. Clumping behavior of these worms was observed using stereo microscope. A part of the worms was separated to another dish and then a portion of same sediment was added to make food supply. Feeding and excretory behaviors were observed under stereo microscope.

3. Estimation on the Specific Growth and Biological Activities

3.1 Preparation of Sediment and Test Organism

The day before the experiment starts, sieved sediment was taken off the refrigerator and then manually mixed in cleaned plastic tub. Fresh oligochaetes acclimated for 1 d in the plastic container were used in this test. The worms were isolated by sieving sediment through a 0.5 mm mesh sieve and the content on the sieve was then placed in an enamel tray with tap water. Individual worm was gently picked and transferred with fine forceps. They were first clean with tap water and then placed in a petri dish containing distilled water to excrete their fecal pellets. Non-sexually mature individuals of approximately similar size (length ≈ 2.0-2.5 cm) were used in the majority of experiment. Weight of each 100 individuals from a total of 5,400 test organisms were estimated at the start of the experiment to determine population sizes by the method of Lundkvist (1978). Each group of these animals was placed in another dish containing distilled water prior to being return to the experimental vessels.

3.2 Experimental Design

There were two experimental treatments and two control sediments prepared for this study (Table 8) consisting of worms placed to the sediment added low level of food (E-WL), worms added to the sediment enriched with high level of food (E-WH), worms added to the sediment without the supplement of food (E-WN)

and no worms placed to the sediment added high level of food (E-NH). The source of food added to the sediment in this experiment was ground fish dry pellets. In the treatment with high level of food added to the sediment, the total amount of food during the period of the test was twice (6 g) as much that of the treatment with low level of food (3 g).

Table 8 Conditions for the estimation on specific growth and biological activities of *L. hoffmeisteri*.

Treatment	Duration (d)	Initial size of worm (ind/beaker)	Amount of food addition (g dwt)
E-WL	0	100	0.5
	7	100	0.5
	14	100	0.5
	21	100	0.5
	28	100	0.5
	35	100	0.5
	42	100	-
E-WH	0	100	1.0
	7	100	1.0
	14	100	1.0
	21	100	1.0
	28	100	1.0
	35	100	1.0
	42	100	-
E-WN	0	100	-
	7	100	-
	14	100	-
	21	100	-
	28	100	-
	35	100	-
	42	100	-
E-NH	0	-	1.0
	7	-	1.0
	14	-	1.0
	21	-	1.0
	28	-	1.0
	35	-	1.0
	42	-	-

The static renewal test system was chosen for this experiment. Each experimental unit consisted of 600 ml glass beakers filled with homogenized sieved sediment in the depth of 2-3 cm. Before the addition of 400 ml deionized water into

each experimental unit, a little portion of sediment from the E-WH and E-NH was kept and divided into three subsamples for measuring initial TOM at the start of the experiment (o h). When sediment settles, 100 worms were placed to each test beaker except the E-NH. After the worms had burrowed into the substrates, the beakers were kept on a table without aeration. Each test vessel was covered with a sheet of polyvinylchloride cling film, which prevented the dissolve of oxygen pass into the test medium. The experiment was carried out for 6 w with 6 test durations: 7, 14, 21, 28, 35 and 42 d. Three replications were set up for each treatment and each of test duration. The organic matter was added to the sediment every week after water renewal. The produced fecal pellets were removed from the sediment surface with a pipette every day. The test was conducted at room temperature in a daily photoperiod of 12 h. Light was provided from cool-white fluorescent lamps. Mean values of pH in the overlying water measured during the experiment were 6.09±0.77.

3.3 Biological and Environmental Analyses

At the end of each 7 d interval, the overlying water in the E-WH and E-NH was measured for DO concentration by penetrating the oxygen probe through a sheet of polyvinylchloride cling film to locate the tip of the probe about 2 cm over the sediment surface. Change in oxygen concentrations was measured by using oxygen meter connected to the probe. After measurement, the oxygen probe was taken off, the volume of the water was gently poured, and the sediment was spread in an aluminum tray. A small part of sediment in the E-WH and E-NH was removed from each of the experimental unit to estimate TOM using loss on ignition method (Parsons *et al*., 1984). The remaining sediments of the E-WH and the whole sediments of the E-WL and E-WN were wet sieved (0.5 and 0.25 mm mesh) and the worms were sorted under stereo microscope. The number of individual worm in each test vessel was counted and then measured total wet weight. Each animal was narcotized in 5% magnesium chloride and size-estimated by measuring length (Raburu *et al*., 2002). Thereafter, they were fixed and then preserved in 70% ethanol for the observation on morphological characteristics under stereo microscope.

3.4 Data Analysis

All values were reported as mean values ± standard deviation. Two-factor ANOVA was applied to analyze the differences of biological factors (number of individual and weight of the worms) and environmental factors (TOM in the sediments and DO in the overlying waters). Multiple comparisons were adopted to examine when significant different from two-factor ANOVA was detected using Student-Newman-Keul (S-N-K) test. The Pearson correlation coefficient (r) was calculated to determine the relationship between the biological and environmental factors. A significance level of 0.05 was employed to detect all statistics in this study.

4. Acute and Sublethal Toxicity Testing of Sediment-Associated Naphthalene

4.1 Preparation of Test Animals

L. hoffmeisteri was taken from the acclimation in the plastic container. They were separated by a 0.5 mm mesh sieve and the individual was picked with fine forceps. The worms were placed in a petri dish filled with distilled water to evacuate their gut contents for 6 h (Lyes, 1979; Widdows *et al*., 1983). A similar size (length ≈ 2.0-2.5 cm) of active worms were chosen and transferred to another dish containing distilled water before placing in the test chambers.

4.2 Sediments Spiking

Sieved sediment was taken off the refrigerator and then manually mixed in cleaned plastic tub. Naphthalene-spiked sediment was obtained by introducing 1.0 g naphthalene dissolved in 20 ml ethanol to 500 g sediment. The spiked sediment was gently stirred for 1 h at room temperature using magnetic stirrer prior to being serially diluted with unspiked sediment to produce the required concentrations. Two controls were prepared for the experiments: an unaltered sediment control and a solvent control. Sediment for the solvent control was prepared by adding 10 ml

ethanol to 250 g unspiked sediment and stirring the mixture for 1 h. The sediment was kept at 4 °C until the start of the experiment.

4.3 Test Conditions

The test was evaluated using a 96 h static short-term sediment toxicity test. There were five replicate test chambers per treatment. Each test chamber consisted of 600 ml glass beaker with 2-3 cm depth sediment and 400 ml of overlying water. A hundred of worms per test chamber were used as test organisms. They were not fed during the exposure period. No aeration was provided for each test chamber. The water overlying was prepared using deionized water. Test chambers were covered with a sheet of polyvinylchloride cling film to prevent the evaporation of naphthalene out off the test medium. All test chambers were conducted at room temperature with a daily photoperiod of 12 h and kept under the following conditions: pH, 6.91±0.25; dissolved oxygen, 2.51±0.41 mg/l.

4.4 Exposure Study

1) Range-finding Test

Since there was no reliable data on the toxic level of naphthalene to *L. hoffmeisteri*, preliminary test was conducted to find the critical range at first. This range was defined as an interval between the highest concentration that killed all worms and the lowest concentration at which all worms survived during the exposure time (96 h). To determine the critical range, concentration of naphthalene 0.1, 1, 10, 100 and 1,000 µg/g sediment wwt were prepared. The observed result was performed to determine the concentrations for the definitive test.

2) Definitive Test

Suitable range for the definitive test was determined with five nominal concentrations (6.25, 12.5, 25, 50 & 100 µg/g sediment wwt as shown in

Table 9). Spiked sediment was subsampled to each test chamber and the overlying water was gently poured over. The sediment and water mixtures were allowed to settle overnight in the dark (ASTM, 1995). Test worms were selected from the petri dish and placed in the overlying water of the test chamber by using a soft forceps. They could swim, crawl, and then burrow onto the test medium.

Table 9 Conditions of the study on the acute and sublethal toxicity of sediment-associated naphthalene to *L. hoffmeisteri*.

Treatment	Components	Nominal concentration (μg/g sediment wwt)
1	Sediment + Naphthalene + Ethanol	100
2	Sediment + Naphthalene + Ethanol	50
3	Sediment + Naphthalene + Ethanol	25
4	Sediment + Naphthalene + Ethanol	12.5
5	Sediment + Naphthalene + Ethanol	6.25
Negative control	Sediment	0
Solvent control	Sediment + Ethanol	0

To assess the endpoints of the experiment, the mortality was checked daily and dead worms were removed from test chambers as soon as they were observed. Test worms were considered dead when there was complete immobilization and no response to pressing with blunt grass rod. Worms were also checked visually every 24 h to monitor any sublethal effects causing morphological and behavioral changes including autotomy, sediment avoidance and reworking activity. Guidance and method for evaluating the effects of naphthalene on *L. hoffmeisteri* in this study followed the work of Meller *et al*. (1998) as shown in Table 10. Morphological changes of the worms were determined using stereo microscope.

Table 10 Description of endpoints for short-term sediment toxicity test using *L. hoffmeisteri*.

Endpoint	Description
Mortality	Animals were recorded as dead when they did not respond to a gentle mechanical stimulus to the front end
Autotomy	Autotomy starts with a local constriction of the circular muscles, which can be seen macroscopically. The segments behind the constriction are completely autotomized (Kaster, 1979). Autotomy was defined as all animals showing either constriction and/ or loss of segments.
Sediment avoidance	According to Keilty *et al*. (1988) a worm was considered unburrowed if more than an estimated 75% of its body was visible on the sediment surface.
Reworking activity	The animals leave traces while digging through the sediment (so-called galleries). To estimate the reworking activity, a qualitative comparison on replicate level was performed. The reworking activity of all animals in one test chamber was defined reduced when the visible number of galleries was distinctly lower than in the control chamber (Figure 12)

Source: Meller *et al*. (1998)

4.5 Data Analysis

The effect rates in percentages of mortality, autotomy and sediment avoidance for each concentration (i) and each test duration (j) were calculated according to the following equations;

$$\text{Mortality in ij} = \frac{(\Sigma \text{ dead animals in ij})}{(\Sigma \text{ exposed animals in ij})} \times 100 \quad (1)$$

$$\text{Autotomy in ij} = \frac{(\Sigma \text{ dead animals in ij} + \Sigma \text{ animals showing autotomy in ij})}{(\Sigma \text{ exposed animals in ij})} \times 100 \quad (2)$$

$$\text{Sediment avoidance in ij} = \frac{(\Sigma \text{ animals showing sediment avoidance in ij})}{(\Sigma \text{ survival animals in ij})} \times 100 \quad (3)$$

The LC_{50} and EC_{50} for each endpoint, and their corresponding 95% confidence at each test concentration were estimated using log-probit analysis (Finney, 1971) after 24, 48, 72 and 96 h. Since the endpoints mortality, autotomy and sediment avoidance was small, a NOEC for each endpoint was defined as the highest concentration exhibiting an effect<10%, and a LOEC, as the lowest concentration with an effect ≥10%. The effect of the endpoint reworking activity was met when all replicates of a specific concentration level indicated reduced reworking activity (Figure 11). NOEC reworking activity was defined as the highest concentration with no replicate demonstrating reduced reworking activity, and LOEC reworking activity, as the next higher concentration.

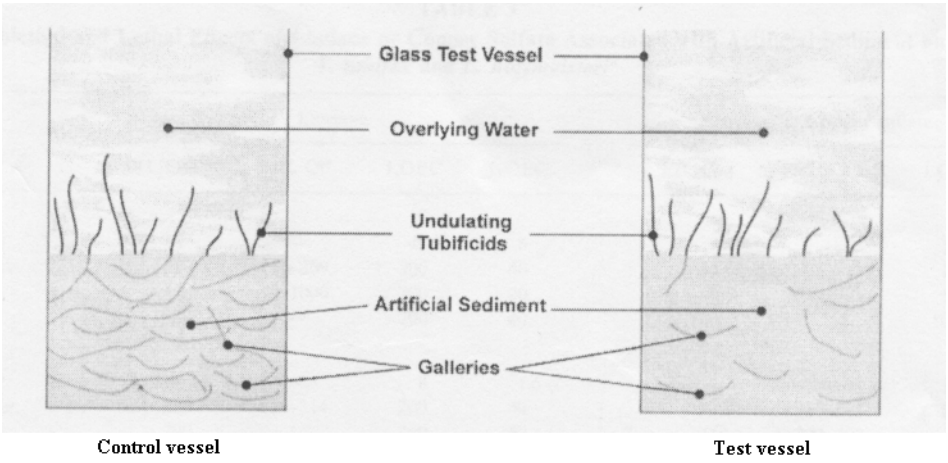


Figure 11 Schematic figure of a test vessel showing reduce reworking activity and a control vessel.

Source: Meller *et al*., 1998

5. Study on the Bioaccumulation and Trophic Transfer of Naphthalene in an Aquatic Food Chain

5.1 Bioaccumulation Test

1) Preparation of Test Organisms

L. hoffmeisteri was brought from the culture in the plastic container. The worms were sieved from sediment through a 0.5 mm mesh sieve. Each worm was picked with fine forceps and placed in a petri dish containing distilled water to excrete their fecal pellets. Sufficient time for emptying their gut was 6 h. (Lyes, 1979; Widdows *et al*., 1983). Active worms of similar size (length ≈ 2.0-2.5 cm) were randomly selected and then placed in another dish containing distilled water prior to being return to the test chambers.

2) Sediments Spiking

Sieved sediment kept in the refrigerator was taken off and then manually mixed in cleaned plastic tub. Naphthalene-spiked sediment was obtained by introducing 1.0 g naphthalene dissolved in 20 ml ethanol to 500 g sediment. The spiked sediment was gently stirred for 1 h at room temperature using magnetic stirrer prior to being serially diluted with unspiked sediment to produce the required concentrations. Based on the results of the acute toxicity test, the optimal concentration for naphthalene spiked in sediment was 25 μg/g sediment wwt. This dose was chosen due to the highest contaminant concentration at which the oligochaetes could survive, thereby ensuring sufficient prey biomass to the predator. However, this level would not be considered toxic by generating severe toxic effects in the prey. The negative control was prepared for the experiment by using unspiked sediment. The sediment was stored at 4 °C before the experiment start.

3) Test Conditions

The bioaccumulation of sediment-associated naphthalene in *L. hoffmeisteri* was run with the treatment plus a control in the component of oligochaetes maintained in spiked and unspiked sediments, respectively (Table 11). The exposure was conducted using static system. The uptake period of the exposure was estimated comparing between 0, 24, 48, 72 and 96 h. Three replicate test chambers were performed for each uptake period in the treatments and a control. Each test chamber consisted of 600 ml glass beaker with 2-3 cm depth sediment and 400 ml of overlying water. A hundred of worms per test chamber were used as test organisms. They were not fed during the exposure period. No aeration was provided for each test chamber. The water overlying was prepared using deionized water. Test chambers were covered with a sheet of polyvinylchloride cling film to prevent the evaporation of naphthalene out off the test medium. The experiment was conducted at room temperature with a daily photoperiod of 12 h and kept under the following conditions: pH, 6.94±0.18; dissolved oxygen, 2.30±0.42 mg/l.

Table 11 Experimental design used in the bioaccumulation test.

Experimental units	Conditions	Exposure period (h)	Number of worm (ind/beaker)
Treatment	Spiked sediment	24	100
		48	100
		72	100
		96	100
Control	Negative sediment	24	100
		48	100
		72	100
		96	100

4) Exposure Study

Both spiked and unspiked sediments were subsampled to each test chamber. Prior to experiment, a small portion of sediments in all replicates from the treatment and control sediments was kept to air dry in the dark for 48 h to determine initial concentration at the start of the experiment using the technique of HPLC

(Filipowicz *et al*., 2007). A large portion remaining was weighted and then gently poured over with the overlying water. The sediment and water mixtures were allowed to settle overnight in the dark before the test organisms were added (ASTM, 1995). Each of 100 organisms was picked using a soft forceps and rinsed with deionized water. Thereafter, they were placed to each test chamber to bioaccumulate naphthalene in the sediments.

Bioaccumulation of naphthalene in *L. hoffmeisteri* was conducted to measure transfer of naphthalene from sediments to the worms at the end of each exposure time. The overlying waters from each test chamber were gently poured, filtered through a Millipore HA filter (0.045 mm), kept in glass beakers sealed with a sheet of polyvinylchloride cling film, and then store in the dark. The sediments were spread on trays and a little portion was kept for naphthalene residues analysis using the technique of HPLC (Filipowicz *et al*., 2007). The worms were wet sieved with tap water and removed by using soft forceps. They were rinsed with deionized water prior to being samples for naphthalene tissue residues.

5.2 Examination on Trophic Transfer

1) Experimental Design

A naphthalene trophic transfer study was conducted using *L. hoffmeisteri* as the prey item and the fingerling of tilapia, *Oreochromis niloticus*, as the predator. In order to achieve the aim of this study, the experiment was performed consisting of two treatment units. The first treatment (T-A) composed of fish fed naphthalene-contaminated oligochaetes only one time at the start of the experiment (0 h). The second treatment (T-B) composed of fish fed naphthalene-contaminated oligochaetes two times at 0 and 6 hours of the experiment, respectively. Feeding time was chosen based on the preliminary data of the process of food digestion observed in this fish species (Jauncey and Ross, 1982). Two control units (C-A and C-B) were set in a manner generally similar to that described above for the treatments except fish fed control oligochaetes (Table 12). A 12 h exposure period was used to determine

uptake of naphthalene into fish, and six sampling times (2, 4, 6, 8, 10 and 12 h) were provided to evaluate the amount of naphthalene transferred to fish through the worm diet. All experimental units were carried out with three replicates.

Table 12 Experimental design used in the trophic transfer experiment.

Experimental Units	Worm diet	Feeding time (h)	Exposure period (h)	Number of fish (ind/beaker)
Treatments				
T-A	Naphthalene-contaminated	0	2	1
			4	1
			6	1
			8	1
			10	1
			12	1
T-B	Naphthalene-contaminated	0, 6	2	1
			4	1
			6	1
			8	1
			10	1
			12	1
Controls				
C-A	Control worm	0	2	1
			4	1
			6	1
			8	1
			10	1
			12	1
C-B	Control worm	0, 6	2	1
			4	1
			6	1
			8	1
			10	1
			12	1

2) Test Organisms

Fish used in the trophic transfer experiment were of same age and size conditions in order to reduce any bias during the experiment. The fingerling, 17 w old and 7 cm in length, were purchased from Pathumthani Inland Fisheries Research and Development Center, Pathumthani Province. Three individual fish were sampled for measuring weight and then kept for background analysis of naphthalene

contaminant level prior to experiment using the technique of HPLC (Filipowicz *et al*., 2007). The remaining juveniles were acclimated in 5 l glass aquaria filled with reconstituted freshwater for 1 w at room temperature. They were fed with dry food pellets and held in aerated water. One day before the start of the experiment the feeding was terminated.

L. hoffmeisteri used for the trophic transfer experiment were obtained from the worm culture. They were sieved and transferred to petri dishes containing distilled water to excrete their fecal pellets before the start of the experiment.

3) Worm Culture

Naphthalene contaminated and control oligochaetes were produced by establishing two oligochaete cultures. The first culture consisted of naphthalene spiked sediment and the second culture contained control sediment that was not spiked with naphthalene. For the naphthalene-spiked culture, 500 g wwt sediment was spiked with 1.0 g naphthalene that was dissolved in 20 ml of ethanol. The spiked sediment was gently stirred for 1 h at room temperature using magnetic stirrer prior to being serially diluted with unspiked sediment to produce the required concentrations (25 µg/g sediment wwt). For the control culture, unspiked sediment was prepared. The sediments were kept at 4 °C until the start of the experiment.

The naphthalene-spiked and control sediments were placed in separate glass beaker (600 ml) and 400 ml of overlying water was added. The sediment and water mixtures were allowed to settle overnight prior to adding the oligochaetes. The worms were added to the beakers before the start of the trophic transfer experiment which allowed them in spiked sediment to bioaccumulate naphthalene. Previous study in the bioaccumulation test had demonstrated that *L. hoffmeisteri* had high tissue residues of naphthalene after 24 h exposure to the spiked sediment during the 96 h test period. Therefore, 24 h was the appropriate bioaccumulation period which was chosen for uptaking naphthalene in the worms.

4) Exposure Study

Trophic transfer of naphthalene from oligochaetes to fish was assessed using static system under control condition. At the start of the experiment, a total of 72 fish was individually measured for initial weight and then placed in its own aquaria. Test aquaria consisted of 1,000 ml glass beakers with 800 ml of deionized water. Either naphthalene contaminated or control worms were rinsed with deionized water, blotted dry, and then fed to each fish during a period of 15 min. Prey items that were not consumed were removed from each container by using a small rubber tube. The experiment was conducted at room temperature under a daily photoperiod of 12 h. Air bubbles were used to maintain dissolved oxygen level kept at 5.83±0.06 mg/l. The values of pH in the water were 7.19±0.05.

At each sampling time, the fingerlings were removed from the experiment and control aquaria. General observations on fish health and condition were noted. They were gently rinsed with deionized water prior to being samples for naphthalene tissue residues.

5.3 Chemical Analysis

1) Extraction

Naphthalene in water was extracted by agitation using liquid-liquid extraction (Weinstein *et al*., 2003). Fifty ml of water samples were transferred into a separatory funnel and 5 ml of normal hexane was added. Samples were mechanically shaken for a minimum of 2 min and the solvent phase was removed from the water by drawing into volumetric flasks. An emulsion was broken up by mixing it with sodium sulfate. The extracts were concentrated by rotary evaporator and the residues were then dissolved in acetonitrile to a final volume of 1 ml prior to HPLC analysis.

Sediments were extracted by following the method of Bangkedphol (2004). About 1 g wwt sediment was removed, placed on aluminum foils and allowed

to air dry at room temperature in the dark for 48 h. Dry sediments were weighted and homogenized using a mortar and pestle. About of 0.25 g of sediment was subsampled and placed into the lined extraction vessel for sample preparation. Each sample was covered by 20 ml of cyclohexane: acetone (3:2) mixture and placed in the microwave extraction system. The time of irradiation was 15 min and temperature of irradiation was 140% of boiling of acetone (79 °C). After the extraction was finished, the vessels were allowed to cool to room temperature and pressure before opening. The extracts were cleaned up by centrifuging at 5,500 rpm for 15 min and the supernatants were pipetted into glass tubes. The solvent was evaporated at 20 °C by rotary evaporator and transferred into 5 ml volumetric flask using acetonitrile as solvent. Each of prepared samples was analyzed for naphthalene contaminant level. Sediment concentrations were reported on a dry weight basis.

Tissues of *L. hoffmeisteri* were extracted following the method of Weinstein *et al*. (2003) and Filipowicz *et al*. (2007) with some modification. The worms were placed on aluminum foils and rinsed with deionized water to remove naphthalene loosely bound to the external surface. For determination of dry weight, the animals were gently touched against the edge of the holding dish, blotted dry to remove excess water, allowed to air dry at room temperature in the dark for 8 h and weighed (Weinstein et al., 2003). Naphthalene was immediately extracted by grinding dried samples in a homogenizer with 5 ml extraction fluid using cyclohexane: acetone (3:2) mixture. The extracts were centrifuged at 5,500 rpm for 15 min and the supernatants were transferred to glass tubes. The solvent was evaporated by using rotary evaporator and the extracted substances were finally dissolved in acetonitrile to a final volume of 1 ml before quantification. Oligochaete tissue residues were reported on a dry weight basis.

The amounts of muscle tissue from each fish were removed after rinsing with deionized water. All samples were dissected, cut into small pieces and placed on aluminum foils. The muscles were allowed to air dry at room temperature in the dark for 24 h and weighed. Fish tissue residues were determined in a manner generally similar to that described above for oligochaete except the extraction was

performed with 20 ml extraction fluid, and the last volume was adjusted to 5 ml. Fish tissue residues were reported on a dry weight basis.

2) Determination

All extracted samples were filtered through a Millipore HA filters (0.045 mm) and then analyzed for naphthalene residues using the technique of HPLC (Filipowicz *et al*., 2007). Five µl of sample were injected directly onto a C_{18} column. An isocratic elution was performed with acetonitrile: water (75:25) at a flow rate of 1.5 ml/min. The photodiode array detector was used at an excitation wavelength of 275 nm. Corresponding peaks were recorded and quantified using LC solution software. Analytical data were quantified using the external standard method of quantification. Naphthalene in the samples was identified by a combination of a retention time match and chromatogram match against the calibration standards. The concentrations were determined using linear regression with a six point standard calibration curve (0.02, 0.2, 2, 20, 200 and 2000 µg/ml). Level of naphthalene detected from this quantification was reversed to the real concentration existing on the extracted samples by the method of calculation using the integration of a volume of injected samples, a final volume of extracted samples in acetonitrile, and a quantity of samples brought to extract (Appendix A).

5.4 Data Analysis

All concentration values determined in the bioaccumulation test were reported as mean ± standard deviation. For the comparison of changes, naphthalene concentrations in each compartment were converted to percentages of the total measured naphthalene to estimate the relationship between concentration and time in sediment-water and sediment-worm tissues. One-way ANOVA was used for comparing means of worm residues between each of the exposure time. Multiple comparison was adopted to examined when significant difference from one-way ANOVA was detected using S-N-K test. The Pearson correlation coefficient (*r*) was calculated to determine the relationship of the residues between sediments and

oligochaetes. Significance level of 0.05 was employed to detect statistical difference. BAFs were calculated as described by Lee (1992) and was obtained by the following equation:

$$BAF = \frac{\text{Naphthalene concentration in worm tissue}}{\text{Naphthalene concentration in sediment}} \quad (4)$$

Bioaccumulation of naphthalene in the fingerling at each of exposure durations was expressed as mean ± standard deviation. Percentages of the muscle concentration were determined at each of the exposure time. Significant differences of naphthalene concentrations in muscular tissues comparing between two treatments at each exposure time were evaluated by two-factor ANOVA and S-N-K test was used to analyzed when the results from two-factor ANOVA was significant different. All statistically significant differences were expressed as $p<0.05$.

RESULTS AND DISCUSSION

Results

1. Background Information of Test Organism and Sediment Characteristics

L. hoffmeisteri collected from Tok road waterside had the average value of naphthalene concentration of 95.42 ± 7.78 μg/g dwt (n = 3). The characteristics of sediment from field sampling were summarized in Table 13. The main portion of the sediments had a grain size of < 0.063 mm. Concentration of naphthalene in the sediments was calculated on a μg/g dwt basis with the mean value of 0.236 ± 0.01 μg/g dwt (n = 3). The TOM and WC levels were also reported.

Table 13 Characteristics of sediment samples collected from Tok road waterside.

Measurements	Mean ± SD (n=3)
Granular composition (%)	
Sand particle	23.69 ± 3.88
Fine particles*	76.29 ± 3.86
TOM (%)	8.23 ± 0.37
WC (%)	26.47 ± 1.30
Naphthalene concentration (μg/g dwt)	0.236 ± 0.01

Note: * particles less than 0.063 mm (silt-clay fraction)

2. Morphological Features and Biological Behaviors

2.1 Morphological Features

The morphological features of *L. hoffmeisteri* collected from Chao Phraya estuary were investigated in laboratory. Figure 12 showed the characteristics of

whole live worms under stereomicroscope. The worms had slender, cylindrical body, which the body wall was so thin. Both two ends of their bodies were pointed. These worms had reddish bodies which were split into segments. Each segment composed of short bristles setae. They could move by stretching and pulling their bodies along in a worm-like fashion. They coiled tightly when disturbed.

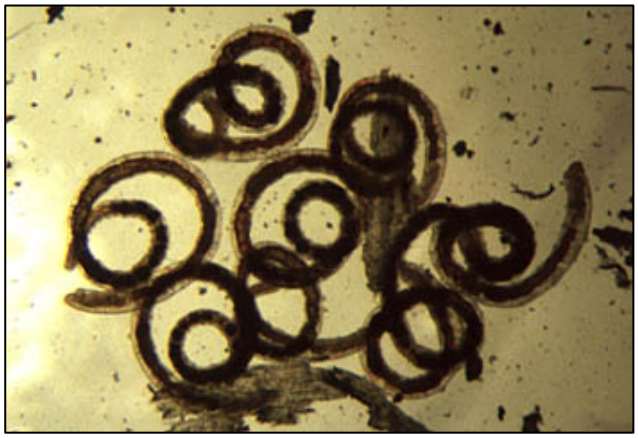


Figure 12 Features of the whole live worm under stereo microscope, 65x.

Figure 13 depicted some morphological features of *L. hoffmeisteri* observed from this study. The active worms were characterized by a total length between 20-30 (25.52±3.29) mm. Size of these worms measured on wet weight basis was varied from about 3.154 to 4.557 (3.813±0.46) mg wwt. The worms had clearly segmented body of which the total count ranging from 50-90 (64.8±13.34) segments. The head was composed of distinguish triangular prostomium, whereas the tail was round. Setae could be found on most segments. All bifid crochets were found in both dorsal and ventral bundles, which the upper tooth was less shorter and thinner than the lower. Hair setae and pectinate setae were absent from *L. hoffmeisteri*. Anterior dorsal bundles were composed of 5-6 setae while ventral bundles were composed of 6-7 setae. Posterior setae had become reduced in number falling to 2-3 setae which were shorter than the anterior. Although a few of specimens appeared to be sexually maturing which the genital structures began to develop in segment XI, no sign of partly or fully developed penis sheaths was present through the body wall.

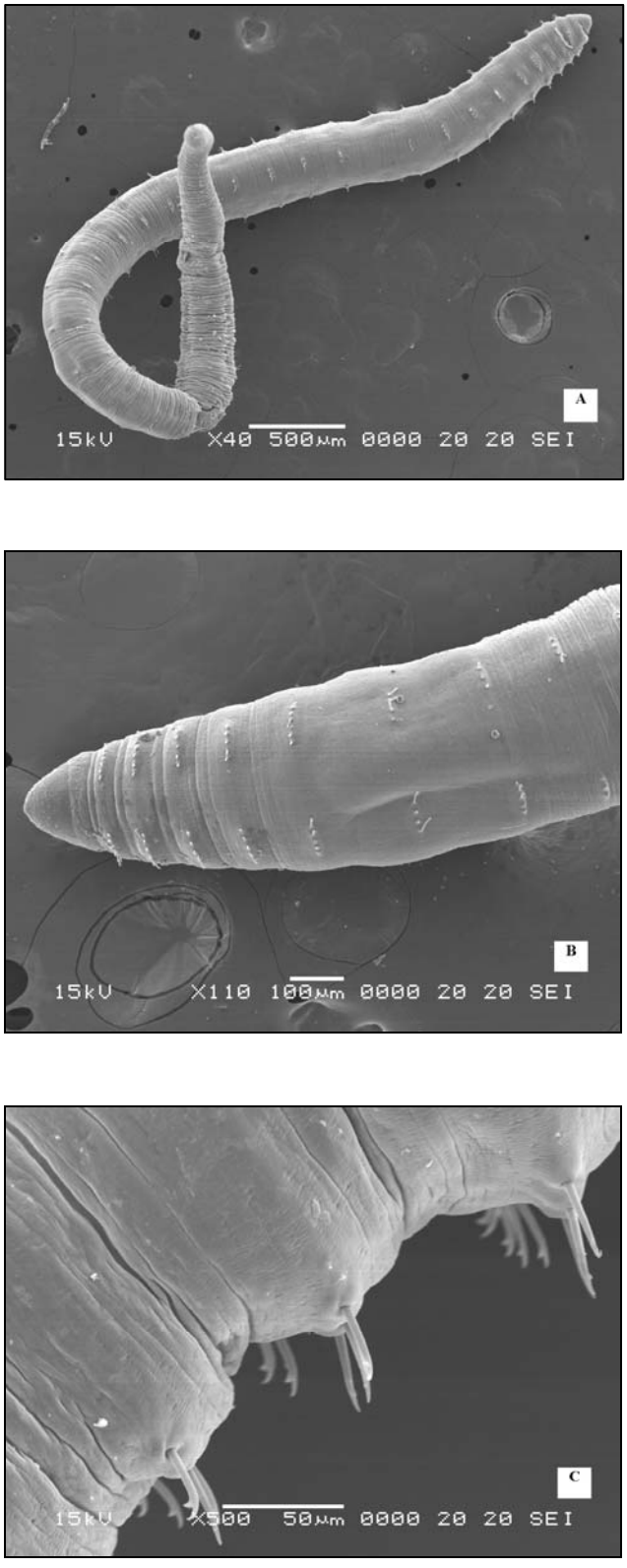


Figure 13 Morphological features of the whole specimen (A), the anterior segment (B), and the setae (C) of *L. hoffmeisteri* under SEM.

2.2 Biological Behaviors

In the water column, active worms could swim by making corkscrew movements with their body. During such swimming, worms rapidly twisted their body into a helical coil that moved in wave-like fashion along the body. When falling to the bottom, they crawled around and then entirely burrowed into the sediment within a half an hour after the start of the investigation. The anterior portion of the worm was hidden in the substrate and the posterior end was projected vertically out of thin mucous tubes secreted by them into the sediment-water interface. The worms were vertically distributed throughout the whole sediment. They generally penetrated as deep as 4-5 cm in the sediment but mostly concentrated in the first centimeter. They inhabited temporally burrows and then left traces while digging through the sediment (Figure 14). Dense population of *L. hoffmeisteri* often accompanies with an accumulation of fecal pellets produced by the worms. From the observation, the worms began to defecate within 2 h after the start of the investigation. Long and cylindrical feces were passed through the posterior end and deposited on the top of the sediment surface. They had been shown to defecate several times since large volumes of processed sediment were brought. However, feeding habit of the worms was not seen in this microcosm.

Figure 14 Burrowing behavior and dense population of *L. hoffmeisteri* in the test chamber.

Living style of *L. hoffmeisteri* was observed after its settlement in the sediment. During the first period of the investigation, the oxygen level in the current water was essentially saturated at > 5 mg/l. At these high DO levels, the posterior end of the worm body slightly moved and they tended to remain within the sediment without noticeable fecal pellets mounding at the sediment surface. After 2 h of the investigation, aeration of the feed water was stopped and the oxygen level was kept at < 2 mg/l. In this condition, it was showed the result in movement of *L. hoffmeisteri* to the sediment surface. Irrigation movement by these organisms was occurred via the tail of the worm wafting to the water overlying the burrow (Figure 15).

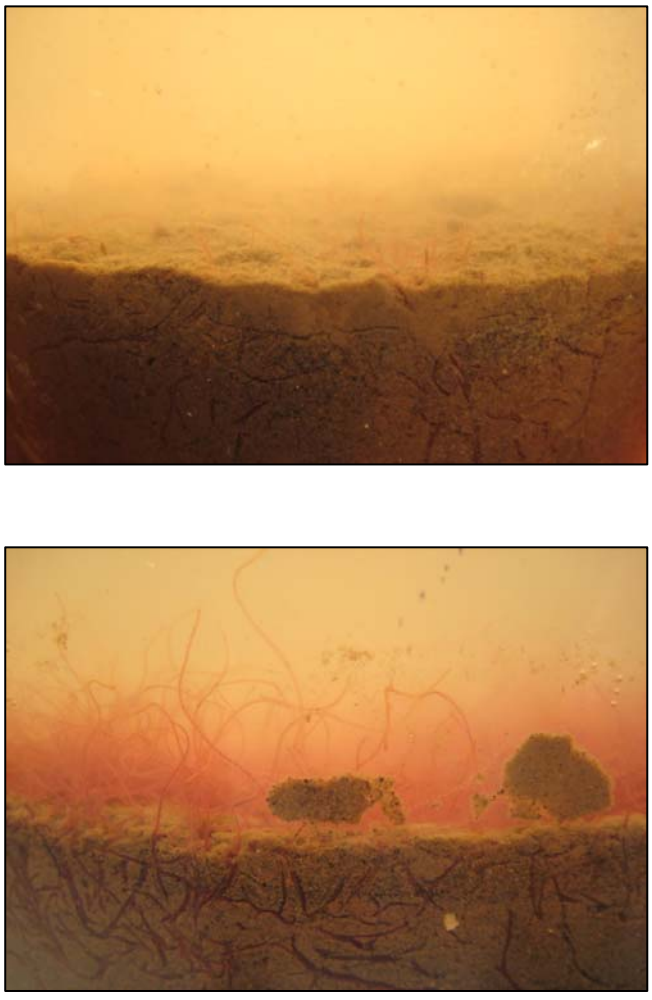


Figure 15 Living of *L. hoffmeisteri* in the sediment under high DO (A) and low DO (B) level.

Clumping behavior of *L. hoffmeisteri* was observed by placing worms in a petri dish of water. Figure 16 showed cluster of this organism under stereo microscope. They were found to be crawled and sought out for cramped microhabitats that tend to maximize contact of the worm's body with the substrate. Some of them used plant debris or lump of soft sediment placed in the petri dish for gripping. Thereafter, they stretched and then wiggled their tails into the water surrounding. These worms showed rapid response by coiling up their tails when a sudden vibration occurred or they were stimulated by soft touch using glass rod.

Figure 16 Cluster of *L. hoffmeisteri* under stereo microscope, 65x.

Feeding habit of *L. hoffmeisteri* was appeared as shown in Figure 17. The worms began to feed almost immediately. They seem to ingest the particle discriminately such as small bits of plant debris mixed with a quantity of mud contained in the petri dish. Such sediment particles and decaying materials were gathered by the mouth at the anterior end of the worm and were passed forward through the gut. These particles were discharged into the water as fecal pellets.

Figure 17 Feeding behavior of *L. hoffmeisteri* under stereo microscope, 65x.

3. Specific Growth and Biological Activities

3.1 Growth and Reproduction of the worms

The number of individual and weight of the worms indicated fluctuation patterns throughout the period of the experiments. Number of individual gradually decreased in the early stage (14 d from the start of the experiment) to the mean values of 82.00±1.00 and 88.00±8.89 ind/beaker, and weight remained with the mean values of 0.323±0.012 and 0.355±0.038 g wwt/beaker in E-WL and E-WH, respectively. These values slightly increased from 21 d to the end of the experiment in E-WL while significantly increased between 35 and 42 d from the start of the experiments in E-WH. Number of individual and weight of the worms in E-WN showed continuously decreasing from the first day through the end of the experiment. According to two-factor ANOVA, there was significant difference in number of individual and weight determined at each duration in E-WL, E-WH and E-WN ($p<0.05$). At the end of the test, mean number of individual of the worms were 91.33±2.08 (E-WL), 111.33±7.64 (E-WH) and 76 ± 2.65 (E-WN) ind/beaker, respectively (Figure 18). Mean weight of the worms were 0.359±0.006 (E-WL), 0.415±0.038 (E-WH) and 0.275±0.018 (E-WN) g wwt/beaker, respectively (Figure 19).

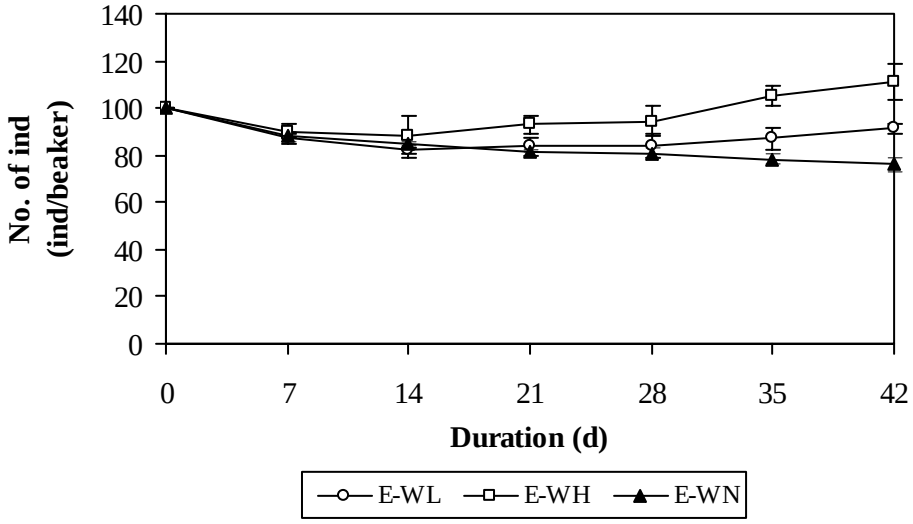


Figure 18 Number of individual of *L. hoffmeisteri* in E-WL, E-WH and E-WN.

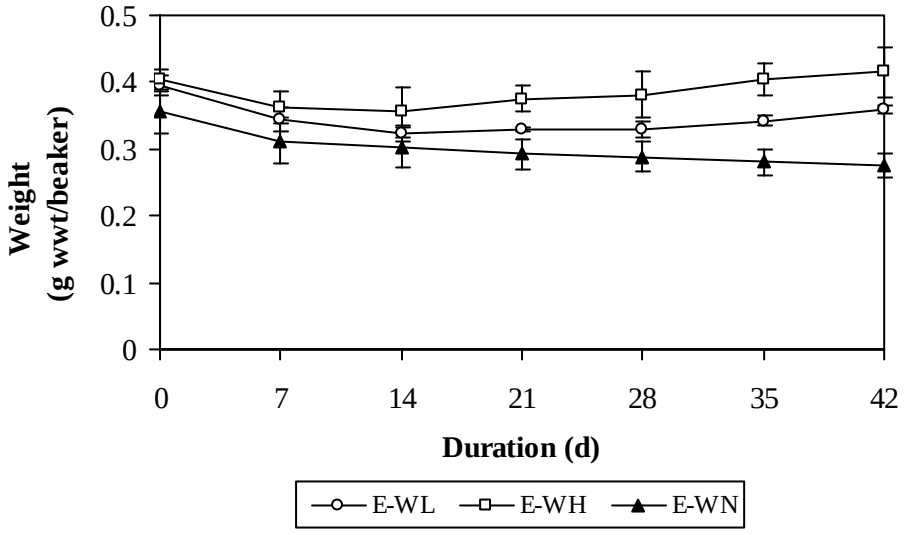


Figure 19 Weight of *L. hoffmeisteri* in E-WL, E-WH and E-WN.

Length frequency of *L. hoffmeisteri* of E-WL, E-WH and E-WN was recorded as shown in Figure 20, 21 and 22, respectively. Size of the worms was categorized into 4 length classes varying from 0.3-0.5, 1.0-1.5, 2.0-2.5 and 3.0-3.5 cm. Immature worms established at the start of the experiment were categorized in the length class of 2.0-2.5 cm. In E-WH, the result showed a small number of individual

worms in this old class whereas a large number of big and long in size of adult group (3.0-3.5 cm) was rather present when growth and reproduction were determined around 35-42 days of the experiment (Figure 23). In addition, sign of reproduction was obviously noticed due to the occurrence of a few numbers of tiny and newly hatched worms (0.3-0.5 cm) in E-WH (Figure 24), while such condition was not found in E-WL and E-WN. However, either indication of sexual maturity or evidence of cocoon production was not observed.

3.2 Total Organic Matter of the Sediments

Mean TOM of the sediment in the test vessels were shown in Figure 25. In E-NH, the levels of TOM were slowly increased from the start (0 d) through the end (42 d) of the experiment. The levels of organic matter were between 1.84±0.06 and 2.64±0.03%. On the contrary of E-WH with *L. hoffmeisteri* colonies, the levels of organic matter of the sediment slightly increased and kept constant from 1.83±0.13 to 2.27±0.02% until 28 d, and these levels were obviously decreased from 35 d to 42 d after the start of the experiment to the level of 1.83±0.04%. Mean percentage of organic matter thoroughly measured the experiment in E-NH (2.37±0.27%) was significantly higher than that of E-WH (2.10±0.19%). Two-factor ANOVA estimated the difference of TOM of the sediments in each of the test duration (7, 14, 21, 28, 35 and 42 d) was also shown significantly ($p<0.05$).

3.3 Dissolved Oxygen of the Overlying Waters

Figure 26 showed the fluctuations in DO concentration of the overlying water from the test vessels of the experiment. Mean concentration of DO was less than 3 mg/l in both treatment and the control sediments. In E-WH, the levels of DO were gradually decreased during 21 d from the start of the experiments. Mean values were ranged from 2.04±0.22 to 1.37±0.22 mg/l. The DO levels were then distinctly increased after 28 d from the start of the experiment to the mean values of 2.07±0.12 mg/l at the end of the experiment. In contrast with E-NH, the fluctuation of DO was still stable during the first 7 d from the start of the experiment and then gradually

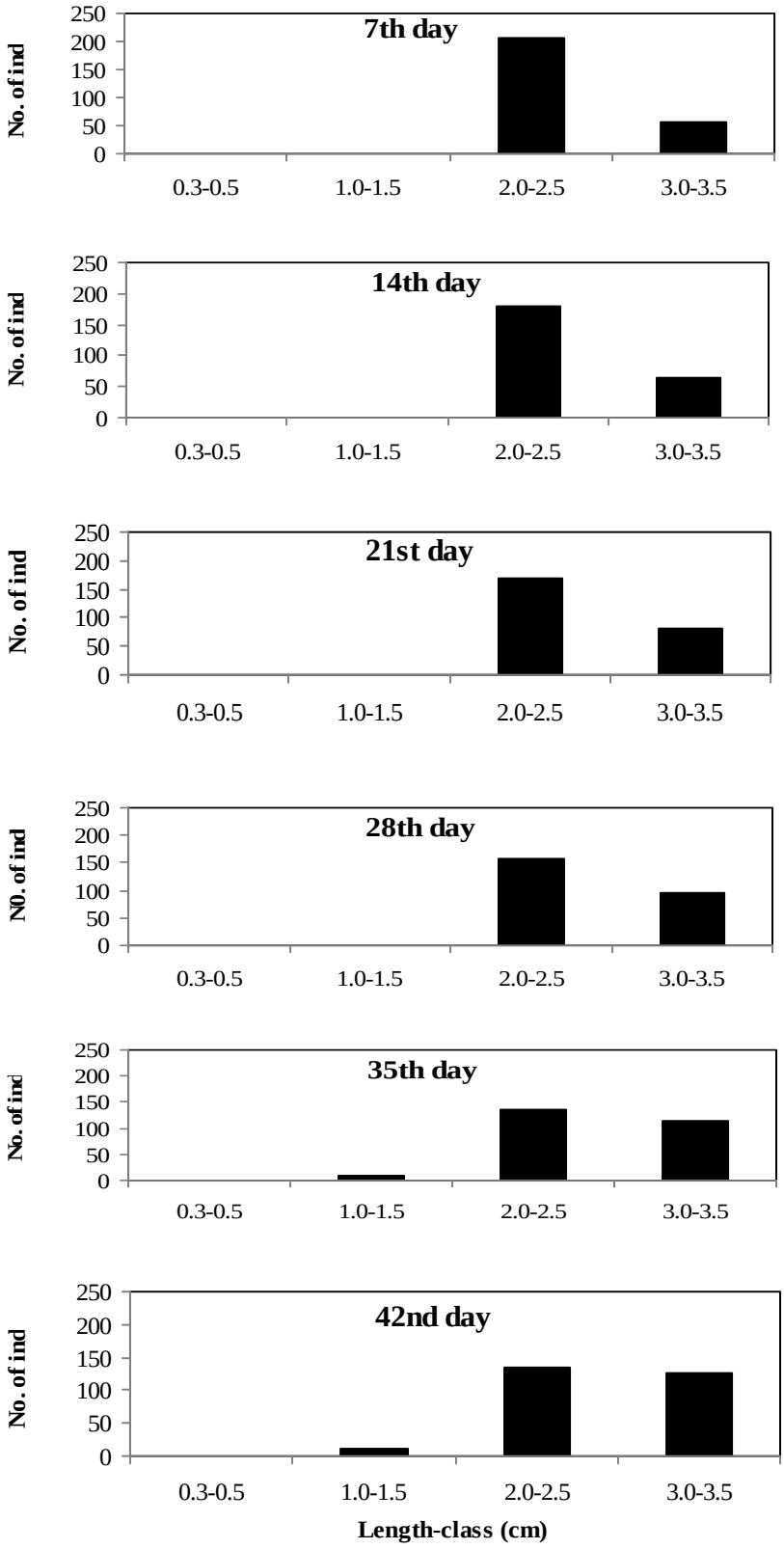


Figure 20 Length frequency of *L. hoffmeisteri* in E-WL.

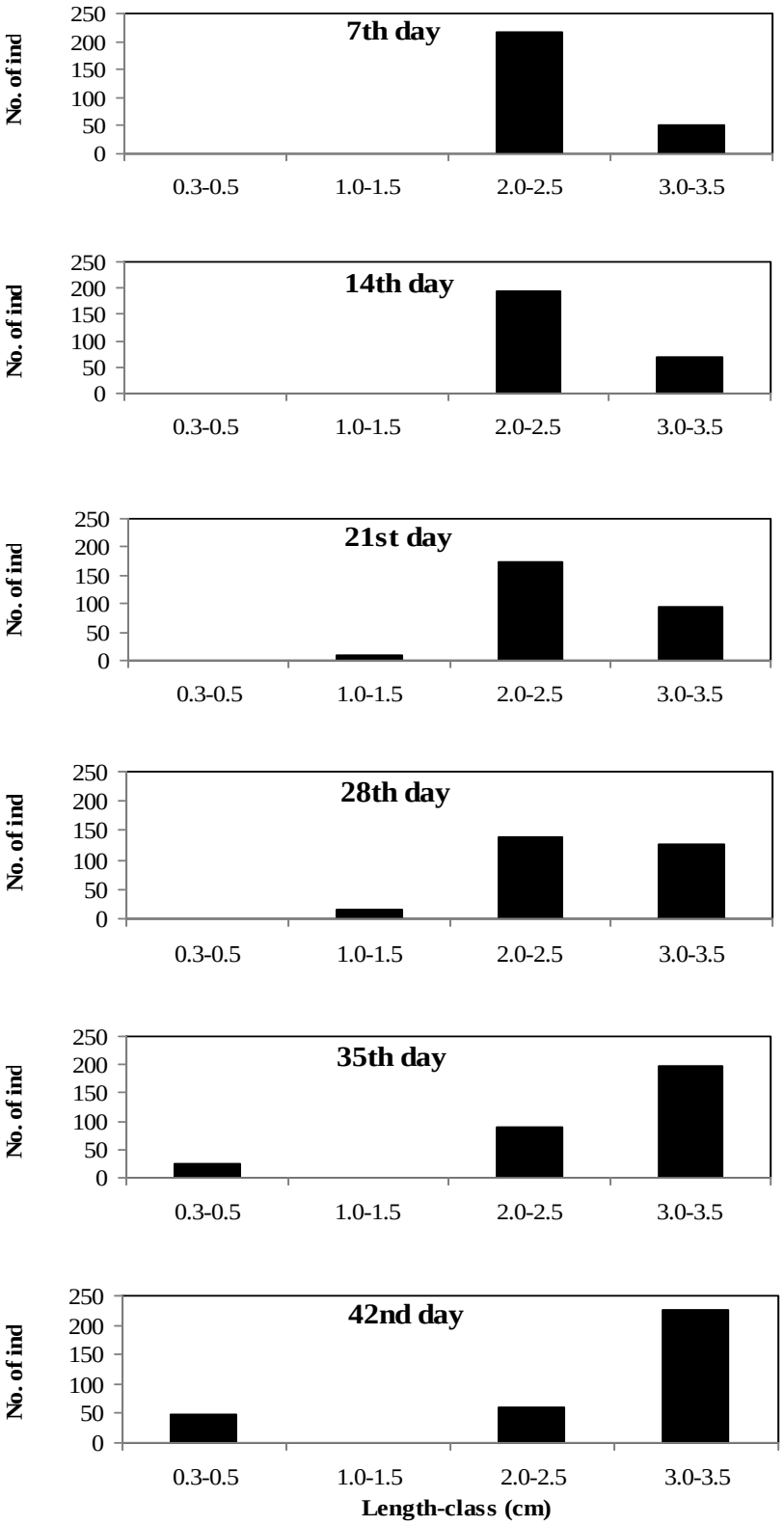


Figure 21 Length frequency of *L. hoffmeisteri* in E-WH.

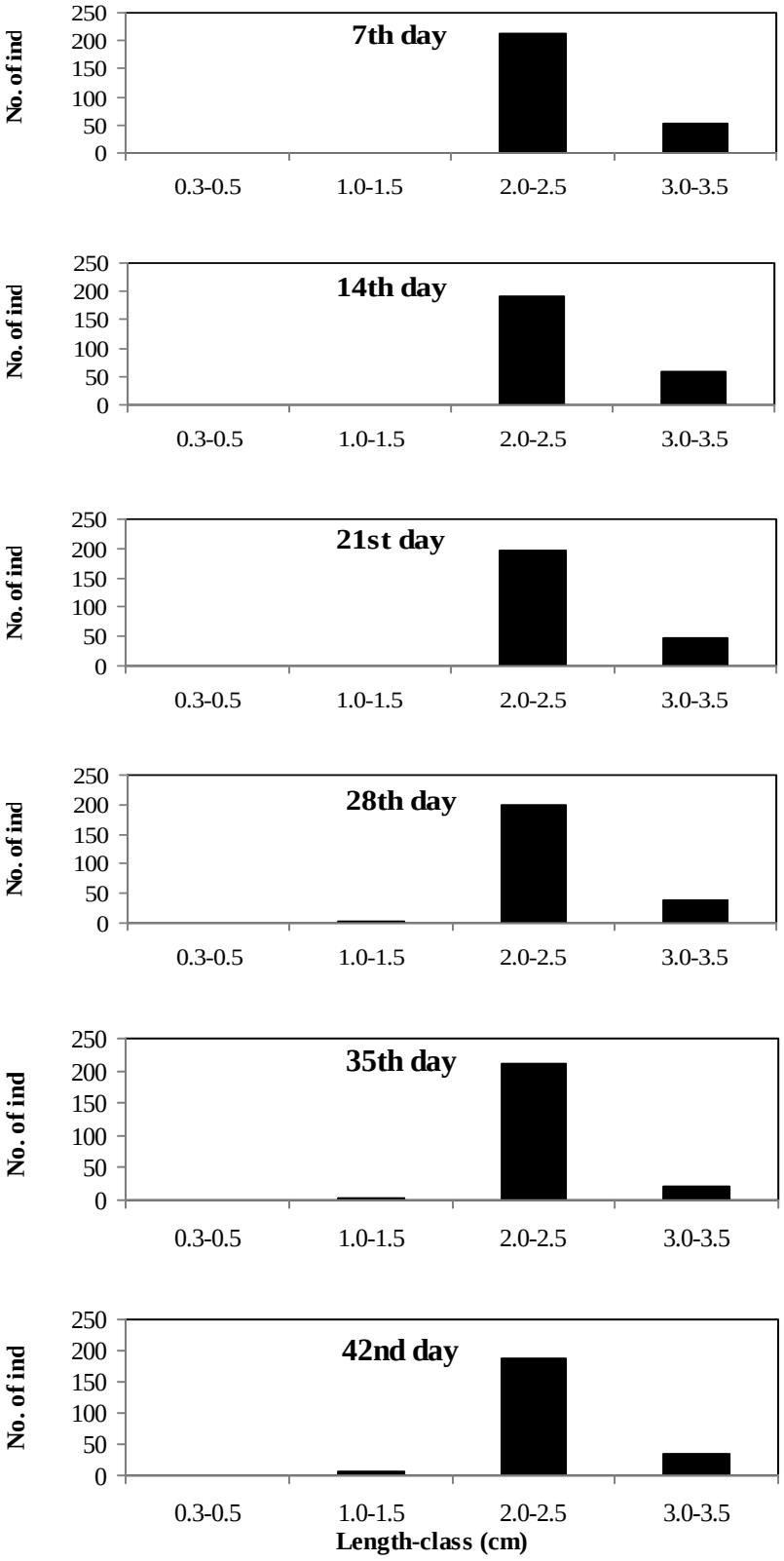


Figure 22 Length frequency of *L. hoffmeisteri* in E-WN.

Figure 23 Adult worm (3.0-3.5 cm) in E-WH during 42 d of the experiment under stereo microscope, 65x.

Figure 24 A cluster of tiny newly hatched worms (0.3-0.5 cm) in E-WH during 42 d of the experiment under stereo microscope, 65x.

decreased thereafter to the mean value of 1.007±0.03 mg/l at the end of the experiment. Total mean value of DO in E-WH (1.81±0.26 mg/l) was significantly higher than that of E-NH (1.37±0.35 mg/l). The analysis of two-factor ANOVA estimated the difference of DO in each of the test duration (7, 14, 21, 28, 35 and 42 d)

was also shown significantly. Comparing to the decrease of TOM in sediment, consequent increases of DO concentration during the late period in E-WH were clearly observed.

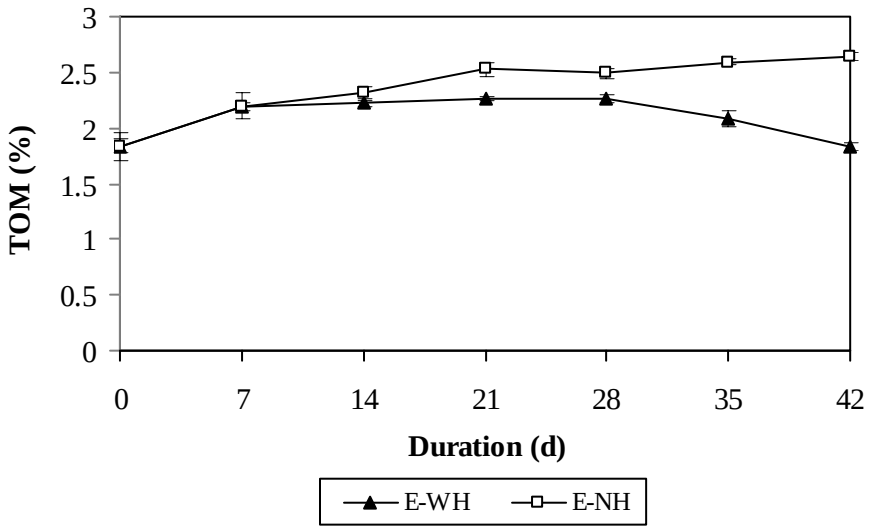


Figure 25 TOM of the sediment in E-WH and E-NH.

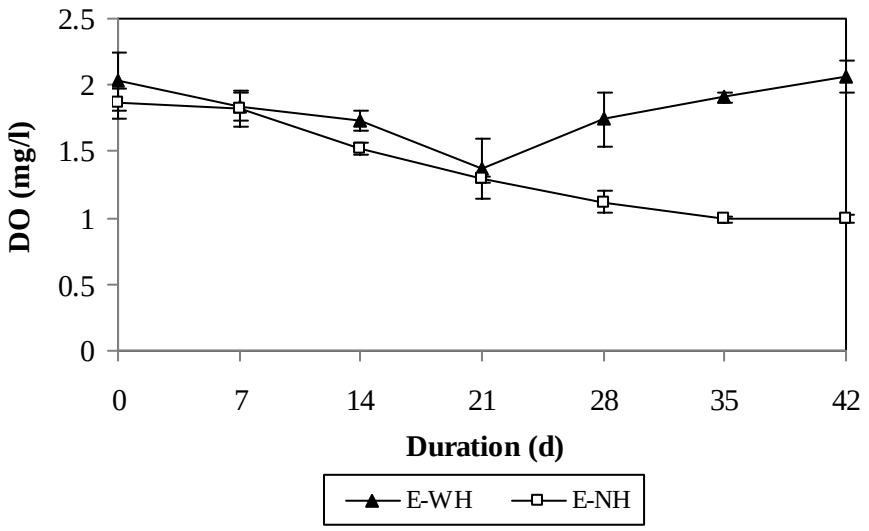


Figure 26 DO of the overlying water in E-WH and E-NH.

3.4 Relationship between Oligochaete Population and Environmental Characteristics

From the determination of correlation coefficient using Pearson correlation (*r*), it was found that number of individual and weight of *L. hoffmeisteri* in E-WH significantly showed negative correlation with TOM (r = -0.74 and -0.58, respectively). On the contrary, significantly positive correlations between number of individual and DO (r = 0.62), accompanied with weight and DO (r = 0.47) were present in this analysis.

4. Acute and Sublethal Toxicity of Sediment-Associated Naphthalene

4.1 Acute Toxicity

The first experiment was aimed at determining the relationship between naphthalene exposure level and naphthalene toxicity. Mortality was 0% at the three lowest exposure levels (0.1-10 µg/g wwt) and 100% at the 1,000 µg/g wwt exposure level. The animals in the highest exposure level did not burrow into the sediment, but remained on the surface. Signs of toxicity in these animals were consistent with affected organisms exhibiting violent twitching, then spiraling, and finally relaxation. The body of reddish worms turned pale before absolutely died (Figure 27). During the regular exposure phase of the second experiment, no effect of *L. hoffmeisteri* was observed when used concentration up to 6.25 µg/g wwt, as well as both negative control and solvent control. The worms were apparently stressed at the four highest exposure concentrations between 12.5-100 µg/g wwt.

An overview of all endpoint values were given in Table 14. The LC_{50} values decreased with the increasing of exposured time from 48 to 96 h. It clearly reveals that the toxic effect of naphthalene was in relation to the exposure concentrations (Figure 28). Due to the lack of toxicity in the experiments with exposure time of 24 and 48 h, LC_{50} values are reported as >100 µg/g wwt. The sign of mortality happened at 48 h of exposure with the LOEC value of 50 µg/g wwt. The

Figure 27 Dead worms under stereo microscope, 65x.

behaviors of *L. hoffmeisteri* when recorded as dead were different from the first experiment. In control sediments, posterior end projected vertically into the water, and then waved vigorously. In contradiction, worms were observed protruding their tails above the surface of the sediment-contaminated naphthalene but did not respond to a gentle mechanical stimulus to the posterior end (Figure 29).

4.2 Sublethal Toxicity

Observation of the worms exposed to sediment-associated naphthalene revealed that naphthalene caused autotomy. The sign of symptom occurred at 96 h of exposure at concentration of 25 µg/g wwt. The posterior region of the worm became first constricted, and then isolated from the body. The isolated part then degenerated and the tail was lost (Figure 30). In contrast, the control worms appeared to have no morphological alterations with the posterior part had normal caudal regions. The percentages of worms undergoing this process of autotomy increased proportionally with the duration of the experiment and the naphthalene concentration. The 48, 72, and 96 h EC_{50} were found to be 83.17, 46.77, and 35.48 µg/g wwt, respectively.

Table 14 Lethal and sublethal effects of naphthalene associated with sediment on *L. hoffmeisteri*.

Endpoint	Data analysis	Exposure time (h)			
		24	48	72	96
Mortality	LC_{50}	>100	>100	85.11	60.26
	95% CI			83.92-86.30	58.83-61.69
	NOEC	50	25	25	25
	LOEC	100	50	50	50
Autotomy	EC_{50}	>100	83.17	46.77	35.48
	95% CI		82.00-84.34	45.14-48.40	33.51-37.45
	NOEC	25	25	25	12.5
	LOEC	50	50	50	25
Sediment	EC_{50}	>100	>100	97.72	58.88
avoidance	95% CI			96.74-98.70	57.37-60.39
	NOEC	50	25	25	25
	LOEC	100	50	50	50
Reworking	EC_{50}				
activity	95% CI				
	NOEC	50	25	25	12.5
	LOEC	100	50	50	25

Sediment avoidance of the worms was observed within 48 h from a value of 50 µg/g wwt onward. The EC_{50} values were 97.72 and 58.88 µg/g wwt for 72 and 96 h, respectively. To describe this behavioral change, in negative control and solvent control, the worms were vertically distributed throughout the whole sediment. By contrast, the animals in the treatments with naphthalene did not burrow immediately throughout the whole contaminated sediment. These worms remained in a group on the surface of the contaminated sediment (Figure 31), and then burrowed just into the surface. The observation also demonstrated that most animals initially burrowed into the spiked sediment and then some of them returned to the sediment surface during the following 96 h. The body part of these worms became white, with disintegration. The color of the worms was paler than of worms in clean sediment.

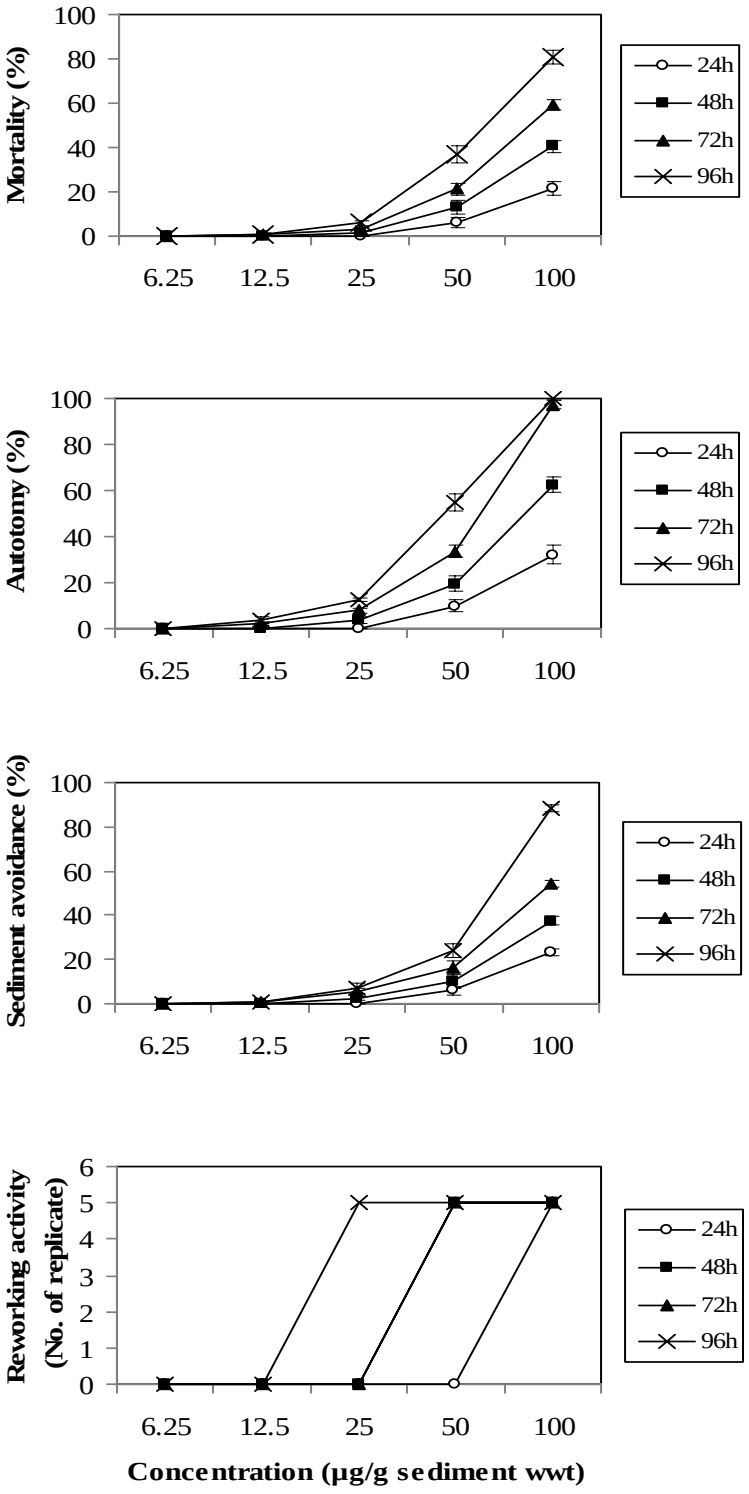


Figure 28 Mortality, sediment avoidance, autotomy and reworking activity of *L. hoffmeisteri* after 24, 48, 72 and 96 h of test exposure in sediments.

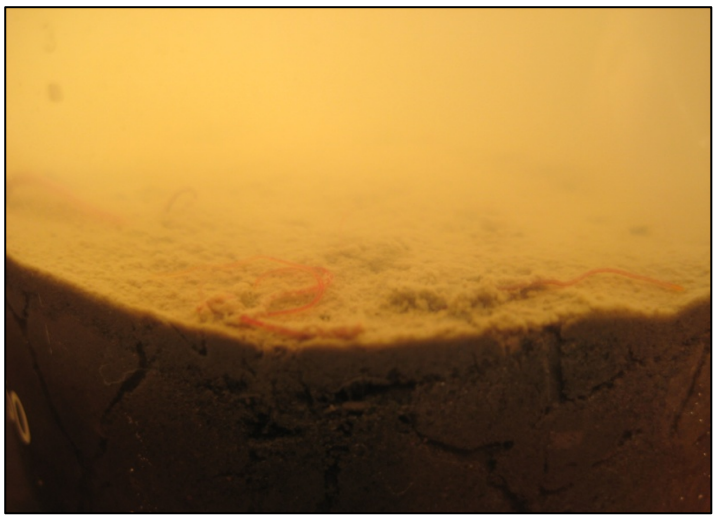


Figure 29 Mortality of *L. hoffmeisteri* exposed to sediment-associated naphthalene in the test chamber.

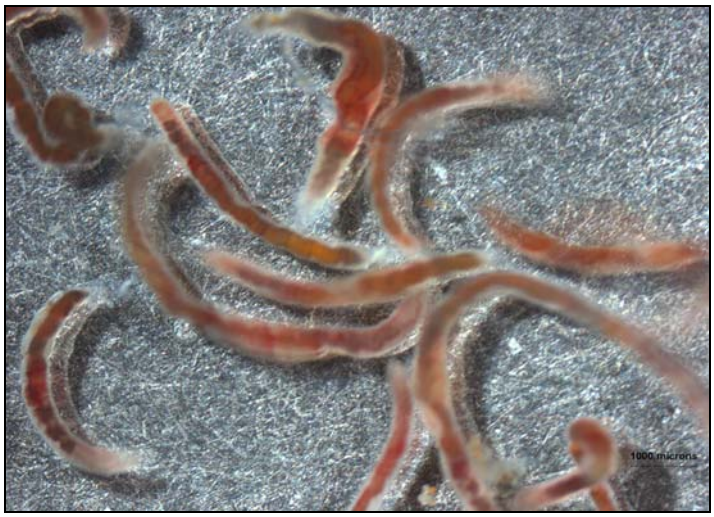


Figure 30 Autotomy of *L. hoffmeisteri* exposed to sediment-associated naphthalene under stereo microscope, 65x.

For the explanation on the reworking activity, the worms were found to entirely burrow onto sediment within few minutes with anterior portion hidden in the substrate. They inhabited temporally burrows which may or may not be vertical, and then left traces while digging through the sediment. Complicated and densed burrows

Figure 31 Sediment avoidance *L. hoffmeisteri* exposed to sediment-associated naphthalene.

of *L. hoffmeisteri* can be observed in the sediment with a depth more than 3 cm. The reworking activity of all animals was defined as reduced when the visible number of traces was distinctly lower than in the control sediments. From the assumption above, the results of this study showing that naphthalene influenced the reworking activity at sediment concentrations of 50 µg/g wwt within 48 and 72 h, and at sediment concentrations of 25 µg/g wwt within 96 h.

5. Bioaccumulation and Trophic Transfer of Naphthalene in an Aquatic Food Chain

5.1 Bioaccumulation Test

Initial concentrations of naphthalene in the treatment were achieved. Spiked sediments analyzed at the beginning of the experiment (o h) contained naphthalene ranging between 31.89±1.10 and 33.06±0.82 µg/g dwt, while most of these values in the control sediments ranged from 0.01±0.002 to 0.02±0.005 µg/g dwt. These concentrations in the treatment sediments decreased steadily over the course of the ranging from 21.40±0.51 to 1.88±0.37 µg/g dwt in 24 to 96 h of the

bioaccumulation test, respectively (Figure 32) with the mean value of 12.26±7.59 µg/g dwt.

The results of the study clearly showed that the overlying water above the sediment in the treatment contained naphthalene after the experiment start. The concentrations gradually increased over the course of the test period, ranging from 3.39±0.24 to 10.55±0.09 µg/ml in 24 to 96 h of the exposure, respectively (Figure 33) with the average value of 6.55±2.84 µg/ml.

Figure 34 depicted accumulation of naphthalene from laboratory-exposed oligochaetes. Following the 96 h exposure, worms significantly bioaccumulated naphthalene in direct proportion to the sediment concentrations to which they were exposed (r = 0.98). Tissue residues of naphthalene in worms varied among the exposure periods, ranging from 1749.72±66.98 to 264.11±3.02 µg/g dwt in the 24 to 96 h exposure, respectively. It was found that the concentration reached a maximum after 24 h of the exposure and decreased thereafter through the end of the experiment. The bioaccumulation was found to be significantly decreased with increasing exposure time ($p<0.05$). Mean value of tissue residue in exposed worms was 1038.83±564.56 µg/g dwt. In the control units, the tissues of non-exposed worms were contained detectable concentration with the mean values of 0.42±0.53 µg/g dwt. BAF calculated from this study was found to be decreased when exposure time increased, with the highest value of 52.96±2.95 at the shortest exposure period, and the lowest value of 8.29±0.37at the longest exposure period (Figure 35). Mean BAF was 31.97±17.07 after 96 h of exposure to 25 µg/g sediment wwt.

The comparison of the amount of naphthalene expressed as percentages of the whole measured naphthalene was analyzed in all compartments. To calculate this relationship, the amount of naphthalene detected in spiked-sediment at the initial stage of the experiment (0 h) was assumed equal to 100% of the total measured naphthalene (Table 15). From this idea, it was apparently found that the concentration detected in the treatment sediment decreased from 64.73% to 5.89% over the 24 to 96 h exposure. Oppositely, the percentage of total naphthalene in the

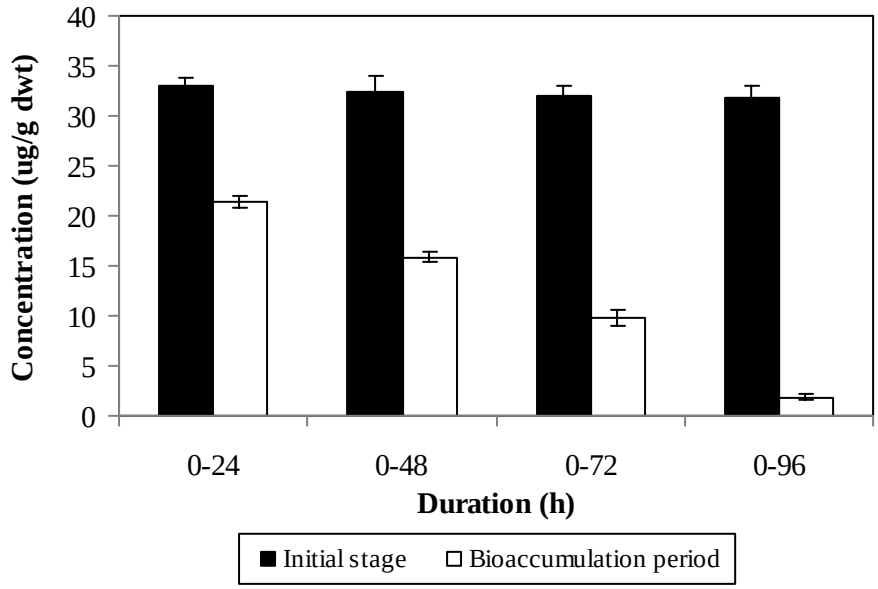


Figure 32 Mean concentration of naphthalene in the treatment sediments at the initial stage (0 h) and during the bioaccumulation period (24, 48, 72 and 96 h).

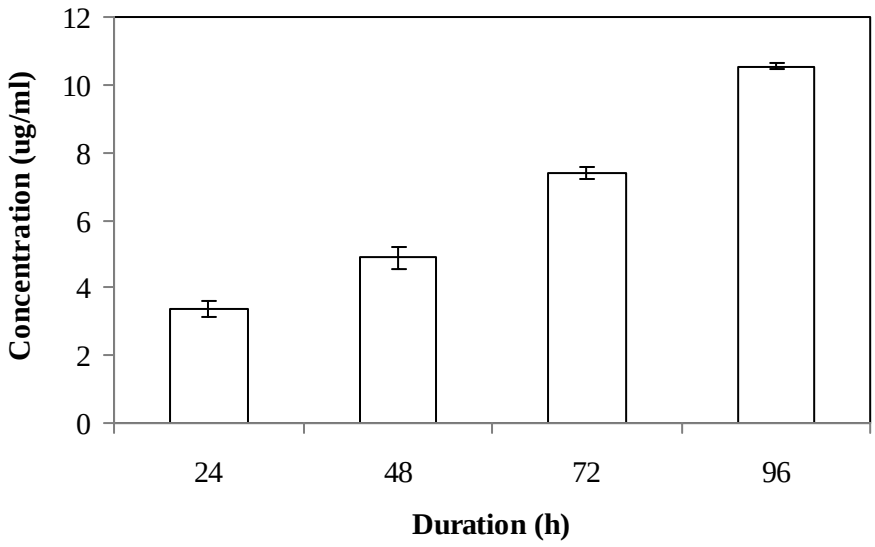


Figure 33 Mean concentration of naphthalene in the overlying water during the bioaccumulation period.

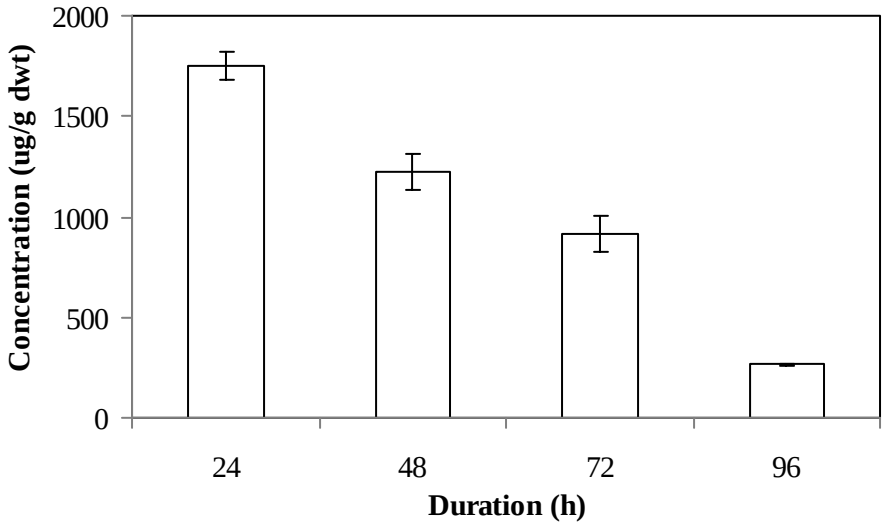


Figure 34 Mean concentration of naphthalene in tissues of oligochaete during the bioaccumulation period.

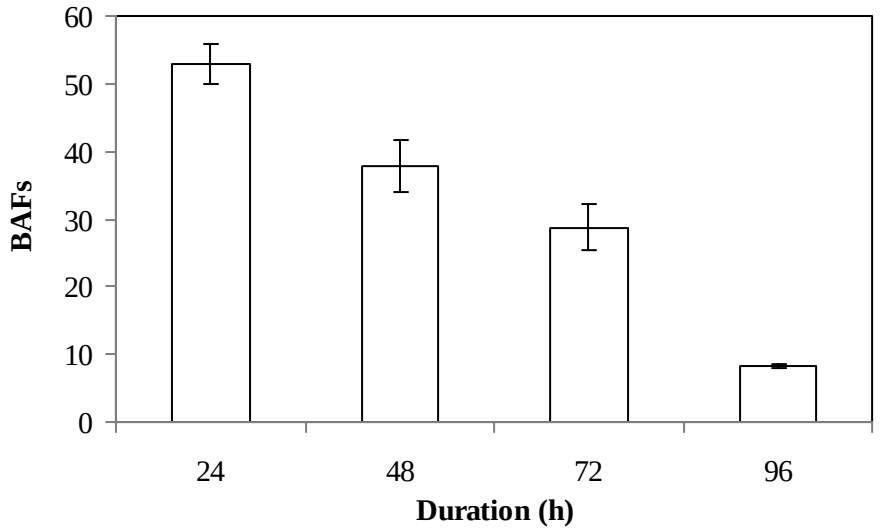


Figure 35 Mean BAFs values during the bioaccumulation period.

overlying water (400 ml) of the treatment was increased from 27.89% to 88.61% at the same test period. For the whole body residue existed in *L. hoffmeisteri*, it was obviously found that naphthalene accumulated in the worm tissue had dropped after 24 h through the end of the experiment. This accumulated fraction was found to be

small with the range decreasing from 2.86% to 0.43%. The decline of naphthalene in the sediment from this study corresponded to the release and transfer of naphthalene from sediment to water and worm, respectively.

Table 15 Change in naphthalene concentration in the different compartments during the bioaccumulation period.

Duration (h)	Change in naphthalene concentrations (%)		
	Sediment	Water	Worm
0	100.00	-	-
24	64.73	27.89	2.86
48	48.99	40.41	1.85
72	30.89	61.80	1.46
96	5.89	88.61	0.43

5.2 Trophic Transfer of Naphthalene in an Aquatic Food Chain

Throughout the trophic transfer experiment, mortality and other toxic effects were not observed in the fingerlings of the treatment and the control units. Background naphthalene concentration in fish determined prior to experiment was not detected from this study.

During the 12 h exposed period, the result showed that fish in T-A and T-B bioaccumulated naphthalene in their tissues (Figure 36). In T-A, the residue gradually increased after 2 h of the exposure with the highest peak at 6 h, and dramatically decreased after 8 h until the end of exposure. The average residue in T-A was ranged between 1.40±0.07 and 12.28±0.98 µg/g dwt. On the contrary with T-B, fish residues changed with the same trend as found in T-A except the second increase of tissue concentration when the time reached 12 exposed hours. The highest concentration was 11.66±1.10 µg/g dwt at 6 h exposure, followed by 10.52±0.57 and 9.98±0.46 µg/g dwt after 8 and 12 h of the experiment, respectively. Statistical

analysis from two-factor ANOVA showed significant difference of fish tissue concentrations compared between T-A and T-B, accompanied with the comparison at each of the exposure time ($p<0.05$). The result of fish fed control oligochaetes did not show a prevalence of this chemical in their muscles.

Percentages of the muscle concentration in the fingerling were determined. To estimate the accumulation, the highest concentration found in muscle tissue of T-A and T-B at 6 h exposure period was supposed to be 100%. Consequently, the fluctuation of their residues was revealed with the difference of percentage of total naphthalene found at each of the exposure time. As shown in Table 16, it was notable that the fingerling in T-A were able to assimilate naphthalene accumulated in worm during the 6 h experimental period. Thereafter the depuration was occurred due to the decrease of percentage of total residues. The uptake trend observed in T-B showed additional percentage of muscle residues prominently recovered at the end of the exposure (12 h).

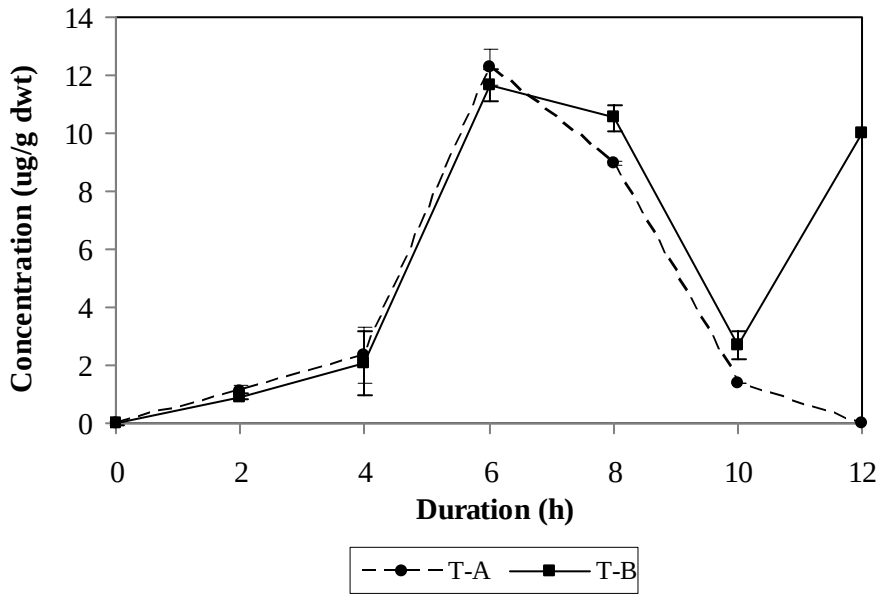


Figure 36 Mean concentration of naphthalene in tissues of fish during the 12 h trophic transfer exposure period.

Table 16 Change in naphthalene concentration in the fingerling muscles between the first treatment (T-A) and the second treatment (T-B) during the trophic transfer exposure period.

Duration (h)	Change in naphthalene concentration in the fingerling muscles			
	T-A		T-B	
	µg/g dwt	%	µg/g dwt	%
2	1.094	8.91	0.916	7.86
4	2.348	19.12	2.066	17.72
6	12.280	100.00	11.660	100.00
8	8.951	72.89	10.524	90.26
10	1.402	11.42	2.682	23.01
12	nd	-	9.975	85.55

Discussion

1. Background Information of Test Organism and Sediment Characteristics

Sediment particle determination in this study showed the majority of silt-clay fraction. The TOM levels were considerably medium (≤10%) while the values of WC were quite low (20-40%) (Tyson, 1995). These results indicate slightly organic contamination in this area.

The average values of naphthalene in *L. hoffmeisteri* collected from Chao Phraya estuary was 95.42 ± 7.78 µg/g dwt. This level is higher than previous study by Nokyoo (1995) reporting that the concentration of naphthalene in mussel samples, *Perna viridis*, ranged from 28.57-56.21 ng/g dwt. Furthermore, naphthalene in this oligochaete tissue was found to be higher than in the sediments (0.236 ± 0.01 µg/g dwt). However, mean concentration of naphthalene in the sediments from this study was in the range found in the year 1995 with the values between 0.051-0.365 µg/g dwt (Nokyoo, 1995). These results suggest that naphthalene was adsorbed by

sediment particles and accumulated in *L. hoffmeisteri*, but it was expected not to be toxic to this oligochaete worm.

2. Morphological Features and Biological Behaviors

2.1 Morphological Features

In this study, only immature worms were obtained and examined on the morphological features. These collected specimens were similar to the work of Raburu *et al*. (2002) with immature worms dominated the population of *L. hoffmeisteri* throughout twelve months in Lake Naivasha, Kenya. Although most of the tubificids are sexually reproducing hermaphrodites, it is hardly to get complete mature worms from field collections. According to the systematic by Brinkhurst and Jamieson (1971), the identification of species of the Tubificidae was made on the basis of the shape of the penis sheaths which is applicable only in mature specimens. This problem has led to scarcity of data on determining age of individuals and certainly justified the scientific name of the worm because immature *L. hoffmeisteri* does not possess the penis tube on the ninth segment (Brinkhurst, 1966).

As the result of none mature specimens presented in this worm population, both morphological characteristics coupled with genetic analysis were adopted to identify the species name before the start of this investigation. The result from using the technique of mitochondrial 16s ribosomal DNA markers (Beauchamp *et al*., 2001) to examine phylogenetic relationships confirmed by C. Erseus (personal communication) was clearly demonstrated that the worm corresponded to the named species *L. hoffmeisteri* Claparede, 1862, the most commonly collected freshwater tubificid worldwide (Wetzel and Taylor, 2001). The responsive relationship between two specimens of *L. hoffmeisteri* from Bangkok was 99.6% similar to each other. Moreover, both specimens were 99.8% similar to *L. hoffmeisteri* individual which was collected from middle Europe. However, all these 3 worms were about 8.5% different from a specimen of *L. hoffmeisteri*, which was from Estonia. In addition, interspecific variation observed between the populations of *L. hoffmeisteri* and a single worm of

L. udekemianus also showed that *L. udekemianus* was about 10% different from the Estonian *L. hoffmeisteri,* and about 12.5% different from the Bangkok coupled with the middle Europe *L. hoffmeisteri*. Based on this preliminary data, it is indicated that Bangkok *L. hoffmeisteri* is the same species as the middle European worm, but different from the one from Estonia. Owing to the comparison with material from other sites, intraspecific variation was observed in populations of *L. hoffmeisteri* between Bangkok and Estonia, suggesting that morphologically cryptic species may exist within the different geographic region (Brinkhurst and Jamieson, 1971; Kathman and Brinkhurst, 1998).

The population of *L. hoffmeister* established in Chao Phraya estuary indicates that environmental conditions in the estuary may reflect organic enrichment associated with chemical contamination. Since this worm species is known to be able to tolerate unfavorable conditions such as low levels of DO and high organic pollution (Chapman and Brinkhurst, 1984; Brinkhurst and Kennedy, 1965). Therefore, they can be used as a biological indicator for a polluted river ecosystem. This finding is agreed with the suggestions by Yap *et al*. (2003) and Azrina *et al*. (2006) that *Limnodrilus* sp. found at the down stream of the Semenyih River and the Langat River, respectively was due to anthropogenic impacts. Harrel and Smith (2002) also found evidence that some organic enrichment at the upper stations of the Neches River estuary was indicated by the dominance of *L. hoffmeisteri* and depressed oxygen concentration. Similarly, Khan *et al*. (2007) reported that organic pollution indicator *L. hoffmeisteri* fairly dominated at stations connecting to the drainage opening to the Mouri river.

2.2 Biological Behaviors

The results from the observation of biological behavior were found that *L. hoffmeisteri* showed active swimming in the water and distinctly crawling into the sediment. These appearances were supported by the works of Drewes and Fourtner (1989), Drewes (1997) and Drewes and Cain (1999) that worm could swim for short distances in water by making corkscrew movements with their body, calling

undulatory swimming. Also, the peristaltic crawling was present when worm had a movement by getting extra traction from bristles that can be projected from the side of its body. The explanation of such phenomena in most oligochaetes is there are muscles arranged into two distinct layers in the body wall - the circular and longitudinal muscle layers. A nerve cord below the intestine along the entire length of the body worm has a major role of controlling these muscles that cause it to move. Because of no hard skeletal elements to which these muscles attach, the forces were produced since worms contract simply act upon the inner fluid-filled body compartments (Jamieson, 1981). This design is referred to as a hydrostatic skeleton, a typical manner of many burrowing invertebrates which lack appendages (Barnes, 1974). When the worm crawls, chaeta are protruded in those segments that are undergoing shortening and thickening due to longitudinal muscle contraction. This helps to anchor these segments to the substrate. In contrast, chaetae are retracted in segments that are undergoing elongation and thinning due to circular muscle contraction (Jamieson, 1981).

Clump living of *L. hoffmeisteri* population found in this observation indicates an orientation behavior which is referred to as thigmotaxis (Drewes and Fourtner, 1989). In nature, suitably cramped microhabitats may be established including tight spaces between submerged, decaying leaves or crevices in submerged (Stephenson, 1930). These preferred habitats offer worm protection from predators as well as access them to food (Brinkhurst and Gelder, 1991). Additionally, these organisms seemed to be vigilant in the environmental surroundings. Worms showed rapidly pulled back their tail if they were touched by using glass rod. This reflex response in *L. hoffmeisteri* is as possible as other oligochaetes since there are mechanosensory neurons present in these animals to detect touch, vibration and pressure. Drewes and Cain (1999) described this mechanism in which when a tail segments of oligochaete worm was touched, it responded reflexively by waving of muscle contraction that move in a direction opposite to the actual direction of body propulsion in the term retrograde. Furthermore, those tails holding up to the water surface were quickly withdrawn when they felt threatened. Since these worms have tiny photoreceptor cells scattered along their tail segments (Drewes and Fourtner,

1989), such an approaching of shadow or an occurring of vibration may signal a life-threatening attack by a predator. As a defense, the worms sense these stimuli and initiate a rapid escape response in which reflexively withdraws their tail by sudden contraction.

L. hoffmeisteri adopt a vertical position in the sediment with their anterior part in the mud and their posterior end free in the water. This burrowing behavior is normally found in most oligochaete due to their feeding habit. It is well known that oligochaete worms have a complete digestive tract with a mouth and an anus (Brinkhurst and Jamieson, 1971). Fragment of dead organisms and small decaying materials settled down on the sediments become a valid food source for the worms (Brinkhurst and Churan, 1969). From this observation, undigested materials ingested by worms at the front end of the body were passed through the gut, and the exceed particles were finally laid down onto the Petri dish via the hind end of the body. Kaster *et al*. (1984), Ewald *et al*. (1997) and Othman *et al*. (2002) reported that oligochaetes feed by ingesting sediment-anterior end buried in the sediment, and posterior end protruding out of thin tubes into the sediment-water interface where the defeacted materials are deposited, in a manner similar to this observation. Due to bringing sediment from depth to the sediment surface in the form of fecal pellets via feeding habit by such animals, it is suggests that conveyor belt species like oligochaetes seem to be an organism playing an important role in both modifying and recycling sediment materials.

Cluster of *L. hoffmeisteri* on the sediment surface was obviously seen in this observation. According to the worm penetration, they comprehensively pierced as deep as 4-5 cm but mostly condensed near the first centimeter of the sediment. This result was similar to Fisher *et al*. (1980), demonstrated that tubificid worms preferred to feed on the level of sediment from 5-9 cm. Based on these results, it is implied that accumulation of organic matter near the top of the sediment layer may be a factor affecting aggregation of worm on foraging food particles.

The existence of *L. hoffmeisteri* in the sediments under the different level of DO in the overlying water was clearly observed. If the overlying water was well aerated, the presence of dense colony of *L. hoffmeisteri* was found beneath the sediment surface without movement of the posterior end accompanied with no accumulation of fecal pellets. As this result, it is assumed that worms might be survived and excreted feces within the sediment layer. These appearances were agreed with Reible *et al*. (1996), observed that freshwater oligochaete *T. tubifex* penetrated to about 3.0-3.5 cm below the sediment surface and subsequently defecated. In contradiction, the consequence in waving movement on the tail of *L. hoffmeisteri* due to low level of DO was recorded after aeration of the feed water was stopped. This result was consistent with the works of Leynen *et al*. (1999) and Fisher and Beeton (1975). It is well known that aquatic oligochaetes usually stick their tails up to the water surface and vibrate in common shallow water. This adaptation mechanism is arisen from breath of the worms through their skin by using dorsal surface of their tails (Pennack, 1989). There are sensory structures in the body wall calling chemosensory cell which is might be useful in detecting the level of dissolved oxygen in the water column (Jamieson, 1981). Therefore, the undulatory movement of the tail end of the worm was then occurred to maximize the oxygen gradient in the water. Consequently, tubificid oligochaetes can still survive for long periods of time even when there is little oxygen in the water.

3. Specific Growth and Biological Activities

3.1 Specific Growth

The result of this study indicated that the reproduction of *L. hoffmeisteri* occurred around 35 d after the start of the experiment. It is feasible that worms grew and then reach the period of reproduction at that time which the worms probably spawn. This appearance may be resulted from the variability of reproduction of *L. hoffmeisteri* depended on the aging cycle of individuals (Raburu *et al*., 2002; Yun-jun and Yan-ling, 2002). Without the information on its life cycle in Chao Phraya estuary, it is impossible to determine the age of the population used in this

study. However, it might be assumed that new offspring of *L. hoffmeisteri* may be produced due to the completion of their life cycle reaching. Ongoing studies investigating the life cycle and production of *L. hoffmeisteri* in Chao Phraya estuary is warranted.

Number of individual and weight of the worms were more increased in E-WH than in E-WL since more level of food was input to the sediment. These results suggest that the quantity of food addition strongly influenced growth and reproduction of *L. hoffmeisteri*. Because this oligochaete species can thrive on soft sediments rich in organic matter, dense colony of *L. hoffmeisteri* could feed on available organic material assimilated in the sediment (Collado and Schmelz, 2001). Martin *et al*. (1999) stated that the quantity of food is a factor affecting density and distribution of aquatic oligochaetes. It was also supported by the work of Leppanen and Kukkonen (1998a) who found that the oligochaete *Lumbriculus variegatus* decreased reproduction in sandy sediment with low organic carbon content. Similar response in reproduction was observed also by Hickey and Martin (1995) between sandy and muddy New Zealand sediments, and Phipps *et al*. (1993) between sediments with low (1-2%) and high (8-10%) organic carbon content.

However, abundant organic material was not immediately available for the growth and reproduction of *L. hoffmeisteri* in the first stage of this experiment although the same amount of food was added in the sediments. This suggestion was arisen from the decrease of number of individual and weight of the worms in both E-WL and E-WH around 14 d, followed by the appearance of specific growth in E-WH during 35 d from the start of the experiment. On the other hand, it could be said that weak animals may be died, while strong worms may lose weight and attend to reach sexual maturity and fecundity in the early stage (d 14). Subsequently, individual worm grew up and reproduced new offspring in the later period (d 35) of the experiment. These results agree with the data of White *et al*. (1987), in which the slowing of feeding of *Stylodrilus heringianus* was connected with reproductive behavior, and Leppanen and Kukkonen (1997), in which the ingestion of

Lumbriculus variegatus ceased at least two days before fragmentation.

The size group of *L. hoffmeisteri* established from the experiment was separated into four categories based on length measurement, consisted of big and long adult worms (3.0-3.5 cm), two sizes of immature worms (2.0-2.5 and 1.0-1.5 cm) and small newly hatched worms (0.3-0.5 cm). The index of size observation in this study is in contrast to previous study concerning the determination of this worm species by using the width of the ninth segment which contains the penis tube (Raburu *et al*., 2002). However, by the same author, length measurement was used to group another freshwater oligochaete, *Branchiura sowerbyi*. The method of classification using length-frequency was adopted since there was no sign of sexual maturity and evidence of any cocoon appearing in individuals. Furthermore, truly distinction of immature, breeding and mature worms was so difficult in this study. Ladle (1971) found that immature and breeding worms are not easy to distinguish either by chaetal characteristics and the presence of spermatophores. Classification of *L. hoffmeisteri* is further complicated by the fact that sexually mature worms resorb the penis tube after reproduction (Kennedy, 1966; Ladle, 1971; Aston, 1973; Poddubnaya, 1980) and revert to an immature condition which cannot be differentiate from a juvenile worm. From this reason, the size group of *L. hoffmeisteri* from this study was then established only by grouping the worms into four categories as stated in the results.

3.2 Biological Activities

In E-WH, the accumulation of TOM of the sediment had been observed around 28 d, followed by the decline of organic matter occurring between d 35 and d 42 of the experiment with an increasing of *L. hoffmeisteri* colonies. These results indicated that such an increase in number of individual and weight of worms had enhanced the decrease of TOM in the sediment. The number of individual and weight of the worms increased due to individual growth and reproduction. In addition, these worms provide an affect to sediment geochemical processes via bioturbation (Rice and Rhoads, 1989). Bioturbation is the process of sediment mixing by benthic animals (Gage and Tyler, 1991). Oligochaetes are assumed to have a significant

impact on this process due to their feeding habit (Davis, 1974; Soster *et al*., 1992) with ingesting subsurface sediments and egesting onto the sediment surface (Martin *et al*., 2005). The same phenomenon may be true in the present study as well, corresponding with the worms in E-WH had removed amount of the sediment onto the surface. Such behavior could promote the vertical distribution of sediment particles which effects total property of sediment (McCall and Fisher, 1980; McCall and Tevesz, 1982). From this observation, it could be suggested that the biological activities of *L. hoffmeisteri* such as feeding behavior encouraging the increase of number of individual and weight, apparently contribute to the recycling deposited material in the sediment.

The result from this study indicates that *L. hoffmeisteri* was able to live in less oxygenated water (less than 3 mg/l) due to non aerated water overlying the sediment under the laboratory control system. This result agrees with the data of Pearson and Rosenberg (1978) who reported that oligochaetes are able to withstand such a period of hypoxia. Similar to the work of Hunter and Arthur (1978) who cited tubificid *Peloscolex benedeni* is commonly found concomitant with a precipitous reduction. An explanation for this tolerance was since most tubificids, including *L. hoffmeisteri*, have erythrocruorin, a red blood pigment, which effectively extracts oxygen dissolved in the water (Pennak, 1989). As they live by projecting their posterior ends vertically into the water, these tails wave continuously to circulate the water which makes more oxygen available to the body surface when low oxygen saturation was detected (Brinkhurst and Jamieson, 1971). Therefore, in this case *L. hoffmeisteri* can survive under such condition of low DO.

The decline of DO in the overlying water in E-WH around 21 d of the experiment was affected by the accumulation of the organic matter added in the sediment. The occurrence of hypoxic condition reflected the increase of the activity of microorganism in the sediment (Maitland, 1978). In the later period *L. hoffmeisteri* individual grew up and started reproduction related to the decrease of the level of the TOM in the sediment. Such an increase in number of individual and weight of the worms seemed to affect the increase of DO in the overlying water. In contradiction,

low level of DO had thoroughly occurred in E-NH with no worm colony. From this result, it could be implied that the decrease in TOM in sediment and consequent increases of DO concentration in E-WH might reflect the bioturbation process improved by the worms. This biological activity was also observed by Levin *et al*. (2003) with the burrowing behavior of freshwater oligochaete *Olavius crassitunicatus* to be caused an effect on the increase of DO. Thus, the biological activities of *L. hoffmeisteri* from this study should therefore consider encouraging the decomposition of organic matter in the sediment which provides an aerobic condition through the connecting water.

The results of this preliminary study suggest that *L. hoffmeisteri* has possibility to reproduce under laboratory conditions supplemented with organic sediment. Additionally, the biological activities such as feeding habit and burrowing behavior have significant effect on the status of the whole sediment and presumably on the overlying water. These observations are available to use *L. hoffmeisteri* as a biological indicator since high level of food has promoted to the specific growth, and also behavioral change occurring in the presence of less oxygenated water. Based on these obtainable data, it could be determined that *L. hoffmeisteri* collected from Chao Phraya estuary might be adopted for further study which extremely focuses on using as an organism in sediment toxicity test, as well as successfully employed as the bioassay in the temperate region (e.g. Keilty *et al*., 1988; Meller *et al*., 1998; Flores-Tena and Martinez-Tabche, 2001).

4. Acute and Sublethal Toxicity of Sediment-Associated Naphthalene

4.1 Acute Toxicity

All statistical values for *L. hoffmeisteri* obtained in this study are expressed as µg naphthalene/g wet sediment because this work was carried out toxicity tests with naphthalene dissolved in ethanol and spiked in sediments. In addition, as *L. hoffmeisteri* is benthic species, the decision was made to evaluate naphthalene toxicity in sediments. In contrast, most reports on toxicity test of

naphthalene have received considerable attention for water-column which the statistical values were reported as mg/l or µg/l, such as the work of Barron *et al*. (1999), Landrum *et al*. (2003) and Vijayavel and Balasubramanian (2006a, 2006b).

The 72 and 96 h LC_{50} values from this study were 85.11 and 60.26 µg/g wwt. Although aquatic oligochaete are commonly used in several sediment quality assessments (e.g., Kukkonen and Landrum, 1994; Weinstein *et al*., 2003), lack of studies have been conducted in toxicity test using naphthalene spiked sediment exposures with these organisms. Due to these limitations, it is difficult to determine how much it was for the toxicity of sediment-associated naphthalene on these animals and other benthic invertebrates. However, a few data revealed toxicokinetics of naphthalene in sediment using some aquatic species has been reported, such as zebra fish *Brachydamio rerio* (Djomo *et al*., 1996) and mollusk *Corbicula fluminea* (Narbonne *et al*., 1999). The results of this experiment give the first information on tolerance of *L. hoffmeisteri* to naphthalene for using as a sediment bioassay. It is also suggested that more laboratories attributed to the toxic effect of naphthalene need to be done to better assess the condition and suitability of the oligochaete worms used in sediment toxicity testing.

The LC_{50} values of naphthalene obtained in this study may be compared with those of other PAHs (Table 17). The results presented here are different from those of previous studies. In the present study, *L. hoffmeisteri* had a 96 h LC_{50} of 60.26 µg/g wwt. This is considerably lower than LC_{50} values for the other aquatic oligochaetes. For example, Weinstein *et al*. (2003) reported no mortality in the estuarine water tubificid oligochaete *Monopylephorus rubroniveus* to fluoranthene sediment concentrations as high as >191,765 $\mu g/g_{oc}$ dwt following 10 d of exposure. Similarly, the freshwater oligochaete *Lumbriculus variegatus* had a 7 d LC_{50} value of >226 µg/g wwt to sediment-associated pyrene (Kukkonen and Landrum, 1994). These values indicate that naphthalene is more toxic than other PAHs. It was presumed that the cause of high acute effects of naphthalene on aquatic invertebrates is based on its low molecular weight and high hydrophilicity (Vijayavel and Balasubramanian, 2006a). Due to its characteristics, naphthalene is commonly native

in water, and thus is available for the uptake by organisms. Anderson *et al*. (1974) supported that middle distillates were extremely toxic to the exposed aquatic animal because of higher concentrations of two- and three-ring aromatics.

Although these results suggest that naphthalene was highly toxic to aquatic organisms in sediment exposure, less toxicity of this chemical correlated with other PAHs was presented. Geyer *et al*. (1981) reported that aromatic hydrocarbons compounds with low solubility were more toxic than compound with high solubility. Karcher *et al*. (1988) indicated that naphthalene was the least toxic due to its lowest coefficient of volatilization and may be rapidly volatilized. Moreover, it was found that the low adsorption rates of naphthalene in sediment, compared with anthracene, phenanthrene, pyrene and benzo (a) pyrene, were a factor affecting the decrease of PAHs bioavailability for zebra fish *Brachydamio rerio* (Djomo *et al*., 1996) and for mollusk *Corbicula fluminea* (Narbonne *et al*., 1999). Our results from this study could not be compared with these authors due to emphasized on specific naphthalene. Certainly, comparative sediment toxicity study among various PAHs including naphthalene on aquatic oligochaetes are areas worthy of further investigation.

Table 17 LC_{50} values of PAHs in sediments determined in some aquatic oligochaetes.

PAHs	Test organism	Duration	LC_{50}	Refernece
Pyrene	*L. variegatus*	7 d	>226 µg/g wwt	Kukkonen and Landrum (1994)
Fluoranthene	*M. rubroniveus*	10 d	>191,765 *	Weinstein *et al*. (2003)
Naphthalene	*L. hoffmeisteri*	96 h	60.26 µg/g wwt	This study

Note: * $\mu g/g_{oc}$ dwt

4.2 Sublethal Toxicity

The results from microscope examination revealed that naphthalene induced autotomy of the caudal region of *L. hoffmeisteri* with the threshold level of 25

µg/g wwt at the end of exposure time. Autotomy is a morphological response to chemical exposure which is also known for oligochaetes by which the animal may protect itself against the increase of internal concentrations of toxicants (Roberts and Dorough, 1984; Lucan-Bouche *et al*., 1999). However, loss of segments may leads to death of the single individual after extended exposure period. This phenomenon was also observed by Lucan-Bouche *et al*. (2000) who found segmentation and disintegration of the rear part of the body of *T. tubifex* exposed to copper induced fragmentation. The data of this study verify that autotomy of *L. hoffmeisteri* is a valid endpoint of sublethal toxicity induced by naphthalene.

From the investigation of this study, it is known that *L. hoffmeisteri* have the ability to avoid stress induced by naphthalene associated with sediment at the concentration of 50 µg/g wwt. Keilty *et al*. (1998) found that burrowing behavior of oligochaetes, an excellent response variable, is an advantage endpoint for assessing pollutant impact on benthic communities. This previous work stated that a worm was considered unburrowed if more than an estimated 75% of its body was visible on the sediment surface. However, a few published works has been present on freshwater oligochaete behavioral responses to toxicant contaminated sediment. For example, McMurtry (1984) who indicated that sublethal doses of copper and zinc increased movement of *T. tubifex* and *L. hoffmeisteri* from the treated sediment to surrounding uncontaminated sediment. Similar to the work of Meller *et al*. (1998) who observed that these two worm initially burrowed into sediment contaminated with lindane and copper sulfate and later returned to the sediment surface during the following 24 h of exposure.

The experiment with naphthalene contaminated sediment described here demonstrates that the reworking activity of the examined *L. hoffmeisteri* was reduced within 96 h at the concentration of 25 µg/g wwt. This phenomenon is harmonious with Meller *et al*. (1998) who noticed a change in the reworking behavior of tubificids when exposed to sediment-associated lindane and copper sulfate. Decline of reworking activity also occurred accompanied with a reduction of worm biomass which reflect decreased feeding rates. These incidents were confirmed by Keilty *et al*.

(1988) who investigated subacute responses of *L. hoffmeisteri* exposed to endrin in sediment. Likewise, Lotufo and Fleeger (1996) demonstrated that sediments-contaminated pyrene and phenanthrene caused reduction of egestion rate and reproduction of *L. hoffmeisteri*. As a result of this study, however, neither worm biomass nor feeding rate has been assessed. For further investigations it should be to attend in these terms as mentioned above to the better explanation of a reduction in reworking activity which affects the population dynamics of *L. hoffmeisteri*.

In the present study, lethal as well as sublethal effects demonstrated a dose-response relationship. It was found that the EC_{50} values of autotomy for *L. hoffmeisteri* are nearly about 2 times lower than the LC_{50} values, while the EC_{50} values of sediment avoidance are similar to the LC_{50} values. This level of response suggests that naphthalene have more potential to change morphological feature than directly killing individuals. In contrast, from that level, it could be said that evaluation of burrowing behavioral alteration is as good as assessing on mortality. The observation also implies that loss of the posterior end of the worms may be more sensitive indicator of environmental impact of chemical pollutants.

There are many other factors that may have some influences on the toxic effects of naphthalene in this study such as experimental procedures, chemical and physical characteristics conditions (Reynoldson *et al*., 1996). Another factor which may affect the toxicity of naphthalene is different constituent corporating in the species of organisms used. For instance, Sager and Pucsko (1991) found that different feeding behavior of animals could be an important factor which may have affected the toxic stress. Reynoldson *et al*. (1996) suggested that genetic variability of organisms may cause differences in the responses to toxicant. Moreover, Klerks and Bartholomew (1991) stated that distinct physiological mechanisms of oligochaetes seem to have a key function with respect to the availability of toxicants within the organisms. From these documented data, it is recommended that more laboratory works are necessary to examine the interaction between these variability and the animal bioassay. The benefit of the anticipated data could be available for

evaluating the toxicity of sediment polluted with naphthalene. In addition, a better comparison of toxicity data among related study can be made.

The range of naphthalene concentrations in the sediment exposures of this study are well above those typically found in Chao Phraya estuary in the year 1995, which the value ranging from 0.106 to 0.216 µg/g dwt (Nokyoo, 1995). Based on the results appeared here, the lowest values of 96 hours NOEC and LOEC were 12.5 and 25 µg/g wwt, respectively. According to the data above, it can be confirmed that *L. hoffmeisteri* are safe for naphthalene contamination in the lower Chao Phraya river.

5. Bioaccumulation and Trophic Transfer of Naphthalene in an Aquatic Food Chain

5.1 Bioaccumulation Test

Decrease of naphthalene in the sediments gradually marked after the first 24 h in the test chambers while concentrations in the water were increased after 24 h through the end of the exposure. This appearance is similar to the work of Ferguson and Chandler (1998) found that concentration of fluoranthene, benzo (a) anthracene and benzo (a) pyrene in sediment decreased throughout the 28 d exposure period. From the apparent result, it is suggest that naphthalene may losses from sediment by dissolution in water which flowed through the experimental system. Both correspond to the release of naphthalene from sediment to water over the whole experiment period. It is well known that naphthalene is one of the PAH compounds classed as a water soluble fraction (WSF). According to its structural chemistry consisted of 2-ring aromatic with low molecular weight, this specific aspect controls its solubility (Elder and Dresler, 1988) related to dissolution capacity in water as shown in this study.

Sediment-bound pollutants may be released by a number of means, including reworking by benthic invertebrates (Jernelov, 1970). Infaunal annelid worm play a significant role in the biogeochemical cycling of sediment-bound organic

pollutants in aquatic ecosystems (McElroy *et al*., 1987). Their feeding habit of digging by head down into the sediment and defecate on the sediment surface have perhaps the greatest impact on sediment conditions (Robbins *et al*., 1979; Robbins, 1982). Moreover, these sediment dwellers can cause significant particle and contaminant movement by disturbing the surface. Their actions alter sediment porosity using advective processes driven from below or by currents in the overlying water (McCall and Fisher, 1980). In addition, Reible *et al*. (1996) found that the movement of the tails of tubificid oligochaetes to the surface to improve oxygen uptake led to an increase of pyrene released to the overlying water to about 50% of that processed. Contaminants in the dissolved state or associated with colloidal organic matter in the pore water will then be moved. Based on this evidences, it is indicated that naphthalene in the sediment may removed by reworking activity of dense population of *L. hoffmeisteri.*

Another factor affecting decrease of naphthalene concentration in sediment may be due to biodegradation. There is some data showing the loss of PAH especially on the water soluble aromatics such as naphthalene with the major degradation path being biological (Douglas *et al*., 1996; Prince *et al*., 2003). According to the process of sediment preparation before the bioaccumulation test start, the microorganisms may be present in the sediment test medium due to 4 °C storage temperature. Leppanen and Kukkonen (2000a) said that sediment samples should store frozen before the extraction to slow down possible degradation of PAHs. However, it could not anticipate that the degradation product would be occurred in this experiment because the method of the study was not covered expected naphthalene derivative compounds. Nevertheless, it was visible from the present results that sediment chromatogram showed a single peak with the same retention time as the naphthalene standard present on the HPLC trace (Appendix Figure A3). Based on this evidence, it might be suggested that biodegradation of naphthalene in the sediment was not occurred or the rate of occurrence was probably slow because of decreasing concentration by the time exposure.

The present study showed the result occurring from the decrease of naphthalene in sediment apparently caused accumulation into worms in the treatment over the 96 h exposure. The BAFs calculated for sediment naphthalene bioaccumulation were 31.97±17.07 that was higher than a range of about 1.2-10 for most benthic fauna (Landrum, 1989). According to this study, BAFs proved that naphthalene were accumulated by *L. hoffmeisteri* with high reported values.

Concentration of naphthalene in the worm was found to be significantly decreased with decreasing sediment concentration (r = 0.98). This finding was corresponded with the work of Vijayavel and Balasubramanian (2006b) reported that bioaccumulation of naphthalene in an estuarine edible crab *Scylla serrata* was found to be decreased with decreasing concentration of naphthalene in water exposure. Compatible with the report on a model predicted the bioaccumulation of hydrophobic organic chemicals in aquatic food web by Gobas (1993), the uptake rate of naphthalene depended on their concentrations in water. Therefore, the result from this study could be suggested that the uptake rate of naphthalene in *L. hoffmeisteri* may depend on their concentrations in sediment.

The bioaccumulation of naphthalene was found to be decreased with increasing exposure time. Uptake and depuration of naphthalene was rapid during 24 h of the exposure. This finding is consistent with previous studies that have measures PAH bioaccumulation in the other annelid species. Ferguson and Chandler (1998) reported that estuarine polychaete *Strebospio benedicti* body burdens increased at the beginning, followed by a decline from 12 to 28 d of the exposure period when conducted in PAH-contaminated sediment. Probably, a function of decreased bioavailability of particular PAH may play a role determining the bioaccumulation (Lyes, 1979; Ferguson and Chandler, 1998). Several studies have shown that an increase in the sediment-chemical contact time decreased the bioavailability of these compounds. For instance, PAHs, such as fluorene, phenanthrene and pyrene, were more available to two species of amphipod, *Pontoporeia hoyi* (Landrum, 1989), and *Diporeia* spp. (Landrum *et al*., 1992; Harkey

et al., 1994b), in dosed sediments aged less than 1 w. Based on these documents, it may be concluded that the reduced uptake of naphthalene from sediment by *L. hoffmeisteri* in this study might be occurred after 24 h of the exposure period.

The result in the present study corroborates with those of previous studies concerning the rapid loss of naphthalene from the tissue of many organisms. Bates *et al*. (1997) observed that when naphthalene contaminated invertebrates were transported to uncontaminated waters, it was found to depurate rapidly from 24 h to few weeks based on the life stages and exposure concentration. This finding was also confirmed in the laboratory studies using annelids. Brunson *et al*. (1998) reported that naphthalene tissue residues of freshwater oligochaete *Lumbriculus variegatus* generally peaked by the starting and tended to decline to the end of the bioaccumulation test. Harmoniously, accumulation of naphthalene from sediment by marine worm *Arenicola marina* was rapid and almost complete depuration of the accumulated naphthalene was observed after 24 h (Lyes, 1979). By comparing with other higher molecular weight PAHs, the loss was shown in the same trend with decreasing of such compounds but the elimination was appeared later. For example, the parent compound of pyrene and benzo (a) pyrene accumulated in *Lumbriculus variegatus* was depurated within 72 h in clean sediment (Leppanen and Kukkonen, 2000b). These supported evidences could be implied that high elimination of naphthalene was possibly caused the rapid lo**ss** of this chemical from the tissue of organisms.

Mean tissue residue of naphthalene reported here for *L. hoffmeisteri* in the 96 h sediment exposure was 1038±564.56 µg/g dwt with mean BAF value of 31.97±17.01. These values are considerably higher than that reported for other annelids species exposed to the higher molecular weight PAHs. Nevertheless, time for exposure in this study is distinctly different from the previous reports due to short test period. For instance, the estuarine oligochaete *Monopylephorus rubroniveus* accumulated fluoranthene-contaminated sediments with tissue residues and BAFs ranged from 5,127-6,431 µg/g dwt and 23.0-1.6, respectively, in the 223-3,912 µg/g treatment by the 10 d exposure (Weinstein *et al*., 2003). The tube-dwelling spionid

polychaete *Streblospio benedicti* had mean fluoranthene tissue residues 356.3 µg/g at a sediment concentration 75.8 µg/g with the BAF 4.7 after 10 d exposure (Weinstein and Sanger, 2003). For the chronic test with the same spionid species, Chandler *et al*. (1997) found that the bioaccumulation of fluoranthene in this worm using 28 d exposure were 2.46 and 32.9 µg/g, corporate with BAF of 9.5 and 13.7 at the sediment concentration of 0.26 and 2.4 µg/g, respectively.

For the comparison of naphthalene accumulation in other infaunal benthic invertebrates, tissue residues found in the present study were still greater than those reported from previous studies. Vittor (2004) found that naphthalene was the only PAH detected in the tissues of *Nereis virens* after the 28 d exposure with total average concentrations between 25.8 and 34.2 µg/kg wwt. Roesijadi *et al*. (1978a, 1978b) found that amount of total naphthalene accumulated by the sipunculid worm *Phascolosoma agassizii*, and two clam species, *Macoma inquinata* and *Protothaca staminea*, was quite low after 60 d exposed to contaminated sediment with the concentration of 0.25, 2.24-2.68 and 0.18 ppm, respectively.

The accumulation of PAH contaminants in aquatic biota may be depended on many factors concerning with the properties of this chemical. As knowing that the uptake of a contaminant in an organism is related to its water solubility represented by the octanol-water partition coefficient (Kow) which has shown a positive correlation with the bioaccumulation potential of organic chemical (Landrum and Robins, 1990; Meador *et al*., 1995). In addition, Kow is adopted to estimate the potential for an organic chemical to move from water into lipid in aquatic organism (ATSDR, 1990). It is suggested that compound with high log Kow (5) have high BAF, and contaminants with lower or higher logKow are either eliminated quickly or not bioavailable due to extensive sorbtion to particulate matter in the sediment, respectively (Landrum, 1989). Similarly, Watanabe *et al*. (2005) also reported that the bioaccumulation potential is high for high molecular weight PAHs comprised of four rings such as benzo (a) anthracene, followed by phenanthrene, comprised of three fused rings aromatic, and is lowest for naphthalene comprised of two fused rings aromatic which is the lowest molecular weight PAH compound. However, Bates *et al*.

(1997) said that naphthalene is moderately hydrophobic with log Kow of 3.34, and this chemical may thus have a tendency to adsorb to particulate matter and accumulate in biota if the period of exposure is no longer. From these documents, it is suggested that naphthalene body burden may depends on the period of time exposure. With an estimation by 96 h test period using in this study, it could be said that naphthalene remained in the test sediment tends to transfer and then bioaccumulate in the tissue of *L. hoffmeisteri*.

The route of uptake of a chemical to an organism will have a major influence on the bioaccumulation of many chemical contaminants. Aquatic animals may be exposed to such contaminants via several routes, such as, the food by ingestion, through the overlying water, by pore water, by the respiratory system, and by dermal absorption. The latter two routes of entry is called skin absorption for some invertebrate species which do not have very specialized respiratory organs (Conrad *et al*., 2002). James and Kleinow (1994) stated that the different uptake route would affect bioaccumulation. Several previous studies have suggested that pore water is the most important route of uptake for infaunal benthic organisms, including oligochaetes (Roesijadi *et al*., 1978a; Oliver, 1984; Knezovich and Harrison, 1988). The chemical was accumulated via pore water or via direct contact of integument with sediment particles and then entered to the body of oligochaetes (Leppanen and Kukkonen, 1998b). Although these animals received their body burden both through interstitial water and sediment ingestion, the low molecular weight PAHs, such as naphthalene (128.18), is more available to such organisms that mainly accumulate from pore water due to only 2-ring PAH with hydrophilic character. This is agreed with the work of Lyes (1979), Landrum (1989), Weston (1990) and Meador *et al*. (1995). In comparison with highly hydrophobic PAHs, strongly associated with particulate matter are accumulated in deposit-feeder as a result of sediment ingestion (Lyes, 1979; Landrum, 1989; Boese *et al*., 1990; Schrap and Opperhuizen, 1990; Weston, 1990; Meador *et al*., 1995). Chemicals with greater hydrophobicity are more tightly associated to solids which in turn increase the importance of ingested material in accumulation (Leppanen and Kukkonen, 2000a). This is in agreement to previous studies conducted with other oligochaete species. Leppanen and Kukkonen (1998b)

reported that ingested sediment contributed up to 61% of the total pyrene body burden in *Lumbriculus variegatus* during 8 d sediment exposure. An increased accumulation of pyrene and benzo (a) pyrene in this worm tissue after 816 h test was caused by this feeding behavior (Leppanen and Kukkonen, 2000a). In similar with Weinstein *et al.* (2003) who observed that ingestion was an important route of uptake fluoranthene for *M. rubroniveus* when exposed to sediment contamination. Based on these evidences, it is assumed that naphthalene uptake across the body surface is the predominant route of uptake of *L. hoffmeisteri* in this study.

Assessment of bioaccumulation and fate of PAHs in oligochaetes needs information on biotransformation capability. The metabolism of PAHs composes of two phases. The first step involves cytochrome P450-dependent MFO system which inserts oxygen atom on PAH ring. The second, various enzymes may catalyze further reactions and result in water-soluble products which can be excreted (Varanasi *et al.*, 1989). Although very little information exists on the metabolism of PAHs in oligochaetes and the main mechanisms are also unknown (Lee, 1998), the ability to metabolize PAHs has been reported supporting with low biotransformation of these animals. Current information in the literature has indicated that *Lumbriculus variegatus* is not able or has slow biotransformation rate to metabolize pyrene and benzo (a) pyrene (Harkey *et al*., 1994a; Leppanen and Kukkonen, 2000b). Similarly, the high BCF reported for *Monopylephorus rubroniveus* may be the result of the inability to metabolize fluoranthene (Weinstein *et al*., 2003). However, from the apparent result it may not be assumed that *L. hoffmeisteri* is probably not able to biotransform naphthalene despite no sign of metabolites compounds present in the HPLC trace (Appendix Figure A3). Certainly, apparently more research in the future is need to investigate whether naphthalene are biotransformed by this oligochaete species.

5.2 Trophic Transfer of Naphthalene in an Aquatic Food Chain

In the present study, the results showed that fish from the treatments were naphthalene contaminated via trophic transfer. This apparent data was resembled to

various studies showed that fish can accumulate hydrophobic chemicals through the diet by feeding (Gobas *et al*., 1993; Connelly, 1991). Previous reports also recommended that 2,3-ring PAHs with logKow less than 5, such as naphthalene, were particularly dominant accounting for about 90% of total PAH found in muscle tissue of fish (Thomann and Komlos, 1999; Liang *et al*., 2007). Additionally, it was demonstrated that the percentage of parent and derivative naphthalene from fish tissue was more than 72% (Wolfe *et al*., 2001). However, the tissue residues investigated from this study was quite low. The highest peak was detected only at 6 and 12 h exposure since absolute success in the process of food digestion and assimilation in Tilapia was observed between 5-6 hours after feeding meal (Jauncey and Ross, 1982). Thereby, low tissue residues may be resulted from the rapid uptake as well as depuration of this chemical characterized in this fish species.

The results of the present study demonstrate that naphthalene in estuarine sediments seem to be not biomagnified from *L. hoffmeisteri* to fish through dietary transfer. The muscle residue was distinctly decreased when exposure time increase without worm-contaminated dietary feeding. These results corroborated with the work of Filipowicz *et al*. (2007) determined the potential for dietary transfer of sediment-associated fluoranthene from tubificid oligochaete *M. rubroniveus* to grass shrimp *Palaemonetes pugio*. This report was found that fluoranthene bioaccumulation was observed in grass shrimp but biomagnifications of fluoranthene in the food chain was unlikely because of low trophic transfer coefficients (TTCs) value. Suedel *et al*. (1994) stated that biomagnification is likely to occur when the TTC values in predator-prey relationships are > 1.0. Harmoniously, benzo (a) pyrene and 7,12-dimethylbenzo (a) anthracene were not bioaccumulated in the seabass fish *Dicentrarcus labrax* with only negligible quantities found after 75 d exposure feeding with mussel *Mytilus galloprovincialis* (D'Adamo *et al*., 1997). In contrast, some of previous works demonstrated that sediment-associated PAHs can be significantly transferred to bottom-feeding organisms through the diet. For example, winter flounder *Pseudopleuronectes americanus* fed polychaetes *Nereis virens* dosed with benzo (a) pyrene accumulated both the parent compound and its metabolites (McElroy and Sisson, 1989). In addition, the studies of water borne PAHs can also be

transferred through the diet. Rotifers *Brachionis plicatilis* bioaccumulated naphthalene and phenanthrene in their tissues after fed algae *Isochrysis galbana* cultured in such contaminants -dosed water (Wolfe *et al*., 1996, 1998, 1999). Similarly, Wolfe *et al*. (2001) reported that naphthalene was uptaken by larva topsmelt *Atherinops affinis* via trophic transfer by eating this rotifer population during the exposure. Likewise, clams *Mercenaria mercenaria* fed diatoms *Thalassiosira pseudomana* cultured in benzo (a) pyrene-spiked water bioaccumulated that PAH in their tissues (Dobroski and Epifano, 1980). Based on these evidences, it was concluded that the difference observed in tissue PAH distributions between the two species could result from different trophic levels, different diet, and also different bioavailability of the compound as well (Baumard et al., 1998).

Biotransformation capacities of fish had shown a greater impact on bioaccumulation and biomagnification of PAHs via a trophic transfer (Baumard *et al*., 1998). As a detoxification mechanism, MFO enzymes, with the cytochrome P450 monooxygenase system, play a major role in the metabolism and activation of PAHs to convert lipophilic contaminants into water soluble forms, which can then be more readily excreted (Lee *et al*., 1972b; Djomo *et al*., 1996). Several studies reported that many species of fish have a MFO enzyme, such as mosquito fish *Gambusia affinis* (Chamber, 1979), killi fish *Fundulus heteroclitus* (Stegeman, 1979) and rainbow trout *Salmo gairdneri* (Stegeman and Chevion, 1980), which parent compounds are broken down to metabolite that are ultimately excreted in the bile (Howard, 1989; Varanasi *et al*., 1989; Hellou *et al*., 1999; Klumpp *et al*., 2002). Opposite with invertebrates, biotransformation of PAHs naturally occurs at slower rate due to lower concentration of cytochrome P450 (Jame, 1989; D'Adamo *et al*., 1997; James and Boyle, 1998; Lee, 1998). According to these evidences, it could be exhibited from this study that the gradual decrease of naphthalene in the muscle tissue during the exposure may be the result of induced biotransformation enzymes, and subsequent elimination via the bile of the Tilapia. This is also agreed with the work of Anderson (1979), James (1989), Burkhard *et al*. (1994) and D'Adamo *et al*. (1997). In addition, it may be indicated higher residue of naphthalene bioaccumulated by those invertebrates stated before

(Dobroski and Epifano, 1980; Wolfe *et al*., 1996, 1998, 1999) due to the less metabolism compared to the fingerling in the present study.

It could be summarized from this study that although naphthalene bioconcentrated to a moderated degree for brief periods in *L. hoffmeisteri*, it will not efficiently transferred from one trophic level to the next via consuming by *Oreochromis niloticus* fingerling. Hence, bioaccumulation and biomagnification of naphthalene in this aquatic model food chain is not expected to occur.

CONCLUSION AND RECOMMENDATIONS

Conclusion

From the experimental results and discussion of this study, the conclusion can be drawn as follows:

The investigation on the morphological features of *Limnodrilus hoffmeisteri* collected from Chao Phraya estuary revealed that the specimens were totally immature. By using 10 individuals, the worms had a total length between 20-30 (25.52±3.29) mm with a total segment ranging from 50-90 (64.8±13.34) segments. The range of weight was varying from 3.154 to 4.557 (3.813±0.46) mg wwt. All bifid crochets were found, which the upper tooth was shorter and thinner than the lower. Anterior dorsal bundles were composed of 5-6 setae while ventral bundles were composed of 6-7 setae. Posterior setae had become reduced in number falling to 2-3 setae which were shorter than the anterior. The results from biological behavior observation were found that *L. hoffmeisteri* showed active swimming in the water. They burrowed and left traces while digging through the sediment. Cluster of *L. hoffmeisteri* on the sediment surface was found that they comprehensively pierced as deep as 4-5 cm. The anterior portion of the worms was hidden in the substrate due to their feeding habit, and the posterior end was vertically projected and excreted fecal pellets. Dense colony of *L. hoffmeisteri* was found beneath the sediment surface without movement of the posterior end when the overlying water was well aerated. On the contrary, movement of the tail end was appeared when low level of DO was recorded. Clump living of *L. hoffmeisteri* was also found by furnishing cramped microhabitats.

The estimation on the specific growth and biological activities of *L. hoffmeisteri* was done under laboratory condition corporating with the different level of food addition in the sediments. It could be concluded that the number individual and weight of the worms increased in the treatment with high level of food (E-WH) than in the treatment with low level of food (E-WL) after 35 d of the

experiment, indicated the reproduction of *L. hoffmeisteri*. Average number of individual and weight of the worms at the end of the experiment were highest in E-WH (111.33±7.64 ind/beaker and 0.415±0.038 g wwt/beaker), followed by E-WL (91.33±2.08 ind/beaker and 0.359±0.006 g wwt/beaker) and the control sediment with no food supplement; E-WN (76 ± 2.65 ind/beaker and 0.275±0.018 g wwt/beaker), respectively. The size group of *L. hoffmeisteri* was separated into four categories based on length measurement varying from 0.3-0.5, 1.0-1.5, 2.0-2.5 and 3.0-3.5 cm. A large number of big and long in size of adult worms and a few numbers of small and short newly hatched juveniles were the major group observed when the reproduction occurred in E-WH. The observation on biological activities showed the decline of TOM in the sediment and the increase of DO concentration in the overlying water from E-WH after 35 d of the experiment, while the control sediment added high level of food but no *L. hoffmeisteri* colony (E-NH) showed opposite trend. Mean percentage of TOM was 2.10±0.19 and 2.37±0.27% in E-WH and E-NH, respectively. Average value of DO was 1.81±0.26 and 1.37±0.35 mg/l in E-WH and E-NH, respectively. These data could be summarized that the increase of number of individual and weight of *L. hoffmeisteri* enhanced the decrease of TOM in the sediment and subsequently increase of DO in the overlying water.

The study on acute and sublethal toxicity of naphthalene-spiked sediment in *L. hoffmeisteri* was measured using a 96 h static short-term sediment toxicity test. The result from lethal effect showed that the 72 h and 96 h LC_{50} values were 85.11 and 60.26 µg/g wwt. Mortality occurred during 48 h of exposure with the LOEC value of 50 µg/g wwt. Sublethal effects of autotomy was found that the 48, 72, and 96 h EC_{50} values were 83.17, 46.77, and 35.48 µg/g wwt, respectively. These values are nearly about 2 times lower than the LC_{50}. The sign of symptom occurred when the posterior region became constricted and isolated from the worm body. The 72 and 96 h EC_{50} of sediment avoidance were 97.72 and 58.88 µg/g wwt, respectively. Abnormal behavior showed that worms remained on the surface of the contaminated sediment and then burrowed just into the surface. The decline of reworking activity was observed with the visible number of traces leaving in the treatments was distinctly lower than in the control sediments. The 48 and 72 h LOEC value was 50

µg/g wwt, and the 96 h LOEC value was 25 µg/g wwt, respectively. These data could be concluded that sediment-associated naphthalene posed risks to *L. hoffmeisteri.* Change in morphology and behavior on *L. hoffmeisteri* due to concentration of naphthalene in sediment could become a useful monitor in the detection of the presence of this pollutant in the laboratory.

The examination on the bioaccumulation of sediment-associated naphthalene in *L. hoffmeisteri* was studied using a 96 h static test system. The result showed that the concentrations in sediment decreased from 21.40±0.51 to 1.88±0.37 µg/g dwt, while the residue in the overlying water increased from 3.39±0.24 to 10.55±0.09 µg/ml in 24 to 96 h of the exposure. The worm bioaccumulated naphthalene in direct proportion to the sediment concentrations to which they were exposed. Tissue residue was highest at 24 h and then decreased through the end of the experiment. Mean value of tissue residue in exposed worms was 1038.83±564.56 µg/g dwt. BAFs showed similar trend corresponded with the tissue residue and the average value was 31.97±17.07. It could be summarized that naphthalene was released from sediment, transferred to water, and accumulated in a short period in *L. hoffmeisteri*. The experiment on the food chain transfer of naphthalene accumulated in this organism via a fingerling predator *Oreochromis niloticus* was examined using a 12 h test period. The result showed that the residues in T-A gradually increased with the highest peak at 6 h, and dramatically decreased during 8 h until the end of exposure time. Average value was ranged between 1.09±0.06 and 12.28±0.98 µg/g dwt. On the contrary with T-B, fish residues changed with the same trend as found in T-A except the second increase of tissue concentration when the time reached 12 exposed hours. The highest concentration was 11.66±1.10 µg/g dwt at 6 h exposure, followed by 10.52±0.57 and 9.98±0.46 µg/g dwt after 8 and 12 h of the experiment, respectively. It could be concluded that naphthalene accumulated in *L. hoffmeisteri* would not efficiently transferred to the fingerling predator. Thereby, the bioaccumulation and biomagnification of naphthalene in this food chain model was not expected to occur.

Recommendations

1. *Limnodrilus hoffmeisteri* collected from the lower Chao Phraya estuary in this study were all immature. In the future, more sampling at the same site is needed to possibly afford at least a group of mature *L. hoffmeisteri*.

2. The study on biological aspects of *L. hoffmeisteri* such as life history, population dynamics and other biological factors (e.g. worm size, feeding and metabolic activities) are needed to investigate for further works, as well as concentrating on environmental characteristics (e.g. laboratorial conditions, temperature variability and the quantity and quality of food adding in sediments), representing important factors which may influence growth or reproduction of *L. hoffmeisteri*.

3. The data of this study provide information about short-term sediment toxicity tests with a freshwater oligochaete *L. hoffmeisteri* using naphthalene as a chemical pollutant. Further experiments over longer periods are necessary to evaluate chronic effects and modify some guidelines for better studying.

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APPENDICES

Appendix A

Methods of Preparation and Analysis

Analysis of Sediment Prior to Experiment

Granular Composition

Sediment grain size was characterized by using wet sieving method (Zhang *et al*., 2004). A 0.063 mm mesh sieve was used to obtain a grain size distribution for the sediments. Samples were air dried for 48-72 hours and ground with a mortar and pestle. Each sample was agitated on the sieve by water. Sediment retained on the sieve was dried again and weighed. Grain sized greater than 0.063 mm and less than 0.063 mm comprised the sand and silt/clay fractions, respectively.

Total Organic Matter

TOM of sediment samples were determined using loss on ignition method (Parsons *et al*., 1984). The sediments were placed in ceramic crucibles of known weight and dried in the 105 °C hot air oven for 24 h. The crucible was weighed and then immediately placed in the muffle furnace for 3 h at 550 °C. The crucibles were removed from the muffle furnace, cooled in a desiccators, and weighed. The weight of ash lost divided by the dried sediment weight was used to quantify TOM.

Water Content

WC in sediment samples were determined using oven-drying method (Gardner, 1986). A known amount of wet sediment was placed on an aluminum foil of known weight. The foil was placed in a tray and kept in the 105 °C hot air oven for 24 h. The foil was weighed again and the mass of water lost was calculated for each sample. The mass of water lost divided by the initial sediment weight was used to quantify sediment WC.

Dilution of Spiked Sediment

The stock spiked-sediment of 2000 µg naphthalene /g sediment wwt was prepared by introducing 1.0 g naphthalene dissolved in 20 ml ethanol to 500 g sediment. From the definitive test determined that range concentration of naphthalene used in toxicity tests were 6.25, 12.5, 25, 50 and 100 µg/g. In order to find a volume of prepared substrate equal to 200 g/beaker, the spiked sediment was diluted with unspiked sediment to produce the required concentrations by the following step,

-Naphthalene concentration was 6.25 µg/g used sediment 199.375 g with stock spiked-sediment 0.625 g.

-Naphthalene concentration was 12.5 µg/g used sediment 198.75 g with stock spiked-sediment 1.25 g.

-Naphthalene concentration was 25 µg/g used sediment 197.50 g with stock spiked-sediment 2.5 g.

-Naphthalene concentration was 50 µg/g used sediment 195.00 g with stock spiked-sediment 5 g.

-Naphthalene concentration was 100 µg/g used sediment 190.00 g with stock spiked-sediment 10 g.

Preparation of Naphthalene Standard Solution

The standard solution of naphthalene was prepared by weighting 0.2 g of naphthalene dissolved in 100 ml acetronitrile in a volumetric flask. This was the concentration of 2000 µg/ml of naphthalene solution. This stock solution was diluted into the desired concentrations with acetonitrile using the formula,

$$N_1V_1 = N_2V_2,$$

N_1 = concentration in stock solution
V_1 = volume in stock solution
N_2 = concentration in diluted solution
V_2 = volume in diluted solution

By using the formula, the standard solution series of naphthalene concentrations were derived as below,

-Naphthalene concentration was 200 µg/ml used acetonitrile 90 ml with the 2000 µg/ml stock solution 10 ml.

-Naphthalene concentration was 20 µg/ml used acetonitrile 90 ml with the 200 µg/ml stock solution 10 ml.

-Naphthalene concentration was 2.0 µg/ml used acetonitrile 90 ml with the 20 µg/ml stock solution 10 ml.

-Naphthalene concentration was 0.2 µg/ml used acetonitrile 90 ml with the 2.0 µg/ml stock solution 10 ml.

-Naphthalene concentration was 0.02 µg/ml used acetonitrile 90 ml with the 0.2 µg/ml stock solution 10 ml.

Naphthalene Standard Calibration Curve Report

Quantitative method : External standard

Function : f(x) = 8350.32x + 0

Rr = 0.99; Rr = 0.99

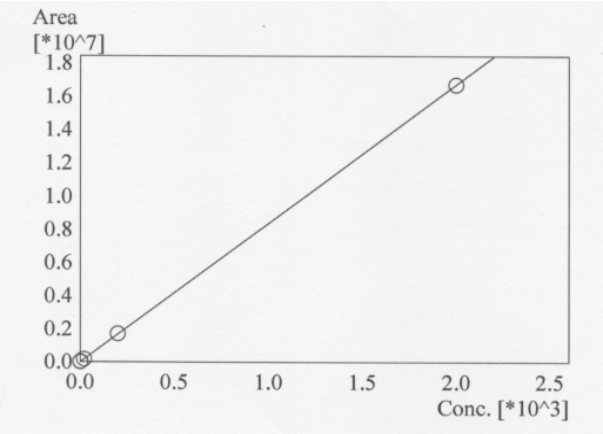
Mean RF = 10451.2; RFSD = 4348.71; RFRSD = 41.6095

Fit typy : Linear

Zero through : Through

Weighted regression : None

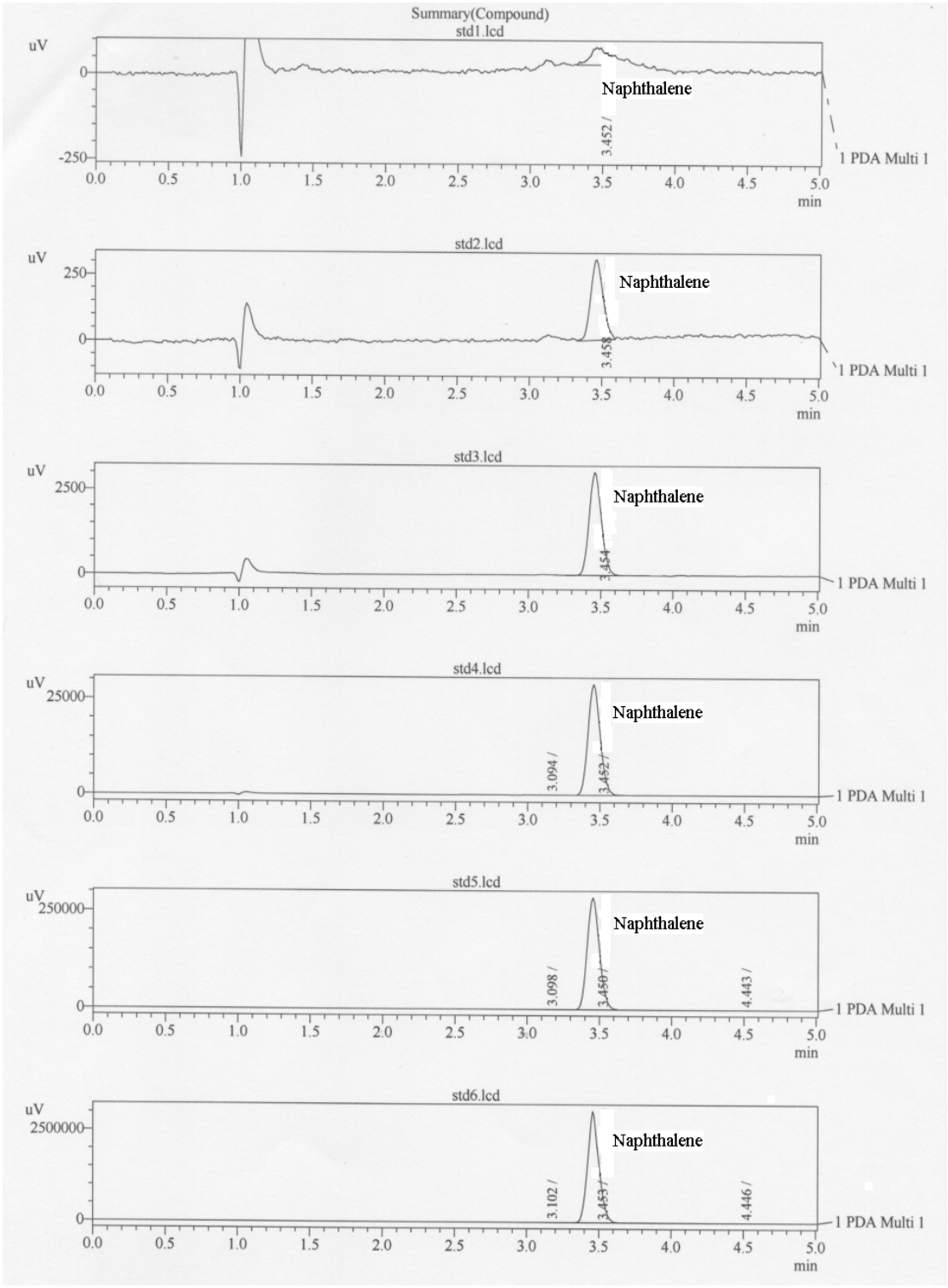
Detector name : PDA



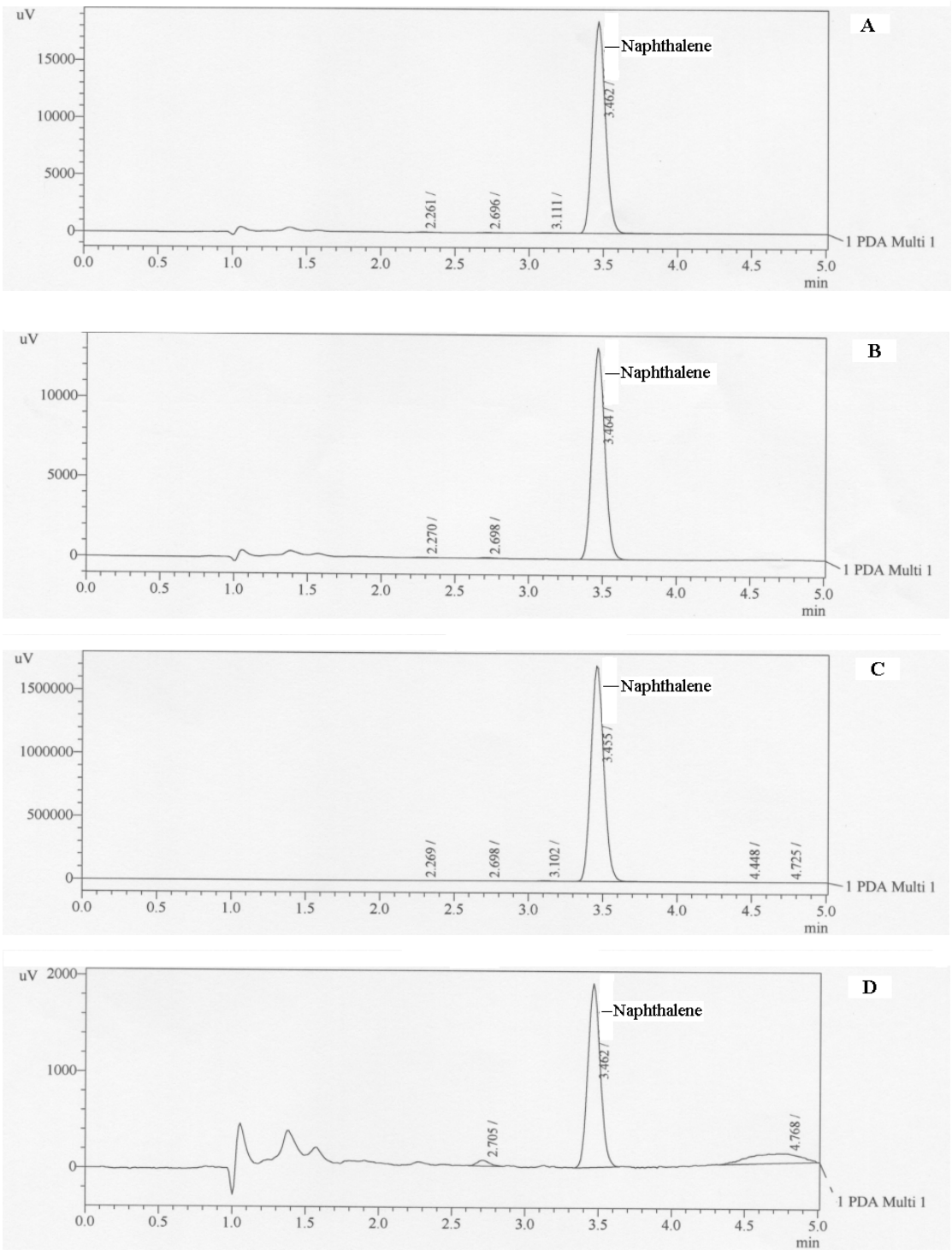
Appendix Figure A1 Naphthalene standard calibration curve.

Appendix Table A1 Summary data of naphthalene standard calibration curve.

Data file	Retention time	Area	Height	Concentration (ppm)	Tailing factor	Resolution
Std 1. lcd	3.452	386	52	0.020	0.000	0.000
Std 2. lcd	3.458	1790	303	0.198	1.239	0.000
Std 3. lcd	3.454	17982	3033	2.000	1.222	0.000
Std 4. lcd	3.452	171714	29001	19.991	1.227	2.028
Std 5. lcd	3.450	1703756	287441	199.983	1.228	2.086
Std 6. lcd	3.453	16697218	3060949	1999.591	1.252	2.098
Mean	3.453	3098808	563463	370.297	1.234	2.071
%RSD	0.082	216.05	218.054	216.584	0.945	1.812
SD	0.003	6694984	1228653	802.003	0.012	0.038



Appendix Figure A2 HPLC chromatogram of naphthalene standard solution.



Appendix Figure A3 HPLC chromatogram of naphthalene in the samples.
A: sediment; B: water; C: oligochaete tissue;
D: fingerling muscle

Calculation of Naphthalene Concentration in Extracted Samples

The calculation of naphthalene concentration in extracted samples was determined by using the formula:

$$C = cV/Mv$$

C = concentration of naphthalene in extracted sample (μg/ml or μg/g dwt)

c = concentration of naphthalene appeared on HPLC trace (μg)

M = mass∗ or volume∗∗ of sample brought to extract

v = volume injected to HPLC (μl)

V = final volume in acetonitrile (μl)

Note: ∗ = mass of sediment, or worm tissue, or fish muscle (g)

∗∗ = volume of water (ml)

Appendix B

Experimental Results

Appendix Table B1 Characteristics of sediment and background naphthalene concentration in sediment and oligochaete worm from Chao Phraya estuary.

Parameters	Replicatation		
	1	2	3
Granular composition (%)			
Sand	27.92	22.86	20.33
Silt-clay	72.08	77.14	79.67
TOM (%)	8.23	7.86	8.59
WC (%)	26.11	25.38	27.91
Naphthalene concentration (µg/g dwt)			
Sediment	0.234	0.249	0.225
L. hoffmeisteri	98.63	101.08	86.54

Appendix Table B2 Length, number of segment and weight of *L. hoffmeisteri* measured from the investigation on the morphological features.

Individuals	Length (mm)	Segment (number)	Weight (g wwt)
1	20	50	3.154
2	22	55	3.296
3	22.5	56	3.441
4	24	60	3.582
5	25.7	67	3.724
6	26.2	68	3.867
7	27.8	75	4.023
8	28.4	80	4.167
9	28.6	83	4.319
10	30	90	4.557

Appendix Table B3 Number of individual (ind/beaker) of *L. hoffmeisteri* observed from the estimation on the specific growth in laboratory condition.

Duration	E-WL			E-WH			E-WN		
(d)	1	2	3	1	2	3	1	2	3
0	100	100	100	100	100	100	100	100	100
7	85	88	89	87	93	90	86	88	90
14	81	83	82	98	85	81	84	85	86
21	84	87	80	89	94	96	82	82	81
28	88	86	78	87	99	97	80	83	79
35	90	89	82	102	104	110	79	80	76
42	92	93	89	103	118	113	75	79	74

Appendix Table B4 Weight (g wwt/beaker) of *L. hoffmeisteri* observed from the estimation on the specific growth in laboratory condition.

Duration	E-WL			E-WH			E-WN		
(d)	1	2	3	1	2	3	1	2	3
0	0.3901	0.3812	0.4106	0.4011	0.4204	0.3908	0.3287	0.3452	0.3908
7	0.3315	0.3344	0.3649	0.3480	0.3906	0.3510	0.2827	0.3037	0.3517
14	0.3159	0.3154	0.3364	0.3921	0.3570	0.3159	0.2762	0.2934	0.3361
21	0.3276	0.3306	0.3280	0.3564	0.3948	0.3744	0.2695	0.2904	0.3164
28	0.3432	0.3268	0.3198	0.3483	0.4158	0.3783	0.2634	0.2906	0.3101
35	0.3510	0.3382	0.3362	0.3785	0.4263	0.4030	0.2586	0.2903	0.2936
42	0.3588	0.3534	0.3648	0.3803	0.4557	0.4087	0.2541	0.2867	0.2852

Appendix Table B5 Length-class of *L. hoffmeisteri* determined in E-WL from the estimation on the specific growth in laboratory condition.

Duration (d)	Replicates	Length-class (cm)			
		0.3-0.5	1.0-1.5	2.0-2.5	3.0-3.5
7	1			65	20
	2			73	15
	3			68	21
14	1			57	24
	2			61	22
	3			63	19
21	1			57	27
	2			58	29
	3			55	25
28	1			55	33
	2			52	34
	3			49	29
35	1		2	50	38
	2		5	43	41
	3		3	43	36
42	1		3	49	40
	2		4	44	45
	3		4	43	42

Appendix Table B6 Length-class of *L. hoffmeisteri* determined in E-WH from the estimation on the specific growth in laboratory condition.

Duration (d)	Replicates	Length-class (cm)			
		0.3-0.5	1.0-1.5	2.0-2.5	3.0-3.5
7	1			73	14
	2			76	17
	3			69	21
14	1			75	23
	2			64	21
	3			55	26
21	1		3	51	35
	2		4	61	29
	3		2	62	32
28	1		5	36	46
	2		7	54	38
	3		4	49	44
35	1	8		29	65
	2	11		32	61
	3	7		30	73
42	1	13		15	75
	2	19		32	67
	3	15		14	84

Appendix Table B7 Length-class of *L. hoffmeisteri* determined in E-WN from the estimation on the specific growth in laboratory condition.

Duration (d)	Replicates	Length-class (cm)			
		0.3-0.5	1.0-1.5	2.0-2.5	3.0-3.5
7	1			71	15
	2			68	20
	3			74	16
14	1			69	15
	2			63	22
	3			60	22
21	1			70	12
	2			63	19
	3			64	17
28	1		3	68	9
	2		1	68	14
	3			63	16
35	1		1	70	8
	2		2	69	9
	3			71	5
42	1			60	11
	2		4	64	13
	3		2	64	10

Appendix Table B8 TOM (%) of the sediments measured from the study on the biological activity of *L. hoffmeisteri* in laboratory condition.

Duration (d)	E-WH			E-NH		
	1	2	3	1	2	3
0	1.98	1.78	1.73	1.77	1.86	1.88
7	2.19	2.17	2.23	2.21	2.09	2.31
14	2.21	2.20	2.25	2.26	2.32	2.37
21	2.27	2.25	2.48	2.53	2.59	2.10
28	2.26	2.23	2.30	2.45	2.47	2.54
35	2.08	2.04	2.17	2.56	2.58	2.62
42	1.83	1.79	1.86	2.61	2.63	2.67

Appendix Table B9 DO (mg/l) of the overlying water measured from the study on the biological activity of *L. hoffmeisteri* in laboratory condition.

Duration (d)	E-WH			E-NH		
	1	2	3	1	2	3
0	1.85	2.28	1.98	1.74	1.85	1.98
7	1.71	1.93	1.87	1.68	1.82	1.96
14	1.74	1.65	1.79	1.47	1.56	1.52
21	1.12	1.45	1.53	1.29	1.31	1.27
28	1.52	1.76	1.94	1.03	1.18	1.15
35	1.95	1.88	1.91	0.98	1.02	0.99
42	2.03	2.21	1.98	1.03	1.01	0.98

Appendix Table B10 Acute toxicity of naphthalene contaminated-sediment to *L. hoffmeisteri* expressed as mortality (ind/beaker).

Concentration (µg/g wwt)	Replication	Duration (h)			
		24	48	72	96
6.25	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
12.5	1	0	0	0	1
	2	0	0	1	2
	3	0	0	0	0
	4	0	0	1	1
	5	0	0	0	0
25	1	0	2	4	7
	2	0	3	4	6
	3	0	1	3	5
	4	0	0	2	5
	5	0	1	3	6
50	1	5	11	19	33
	2	3	9	18	32
	3	7	14	24	39
	4	9	17	23	41
	5	7	15	22	39
100	1	21	39	56	77
	2	19	40	58	78
	3	18	37	60	83
	4	24	44	62	81
	5	26	42	61	85

Appendix Table B11 Sublethal toxicity of naphthalene contaminated-sediment to *L. hoffmeisteri* expressed as autotomy (ind/beaker).

Concentration (µg/g wwt)	Replication	Duration (h)			
		24	48	72	96
6.25	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
12.5	1	0	0	2	5
	2	0	0	2	5
	3	0	0	1	2
	4	0	0	3	4
	5	0	0	2	3
25	1	0	4	7	12
	2	0	6	8	13
	3	0	4	8	12
	4	0	2	7	13
	5	0	4	9	14
50	1	9	18	31	51
	2	6	15	31	52
	3	10	19	35	56
	4	13	23	37	60
	5	12	22	33	55
100	1	31	60	95	99
	2	31	64	98	100
	3	27	58	98	100
	4	35	67	99	100
	5	37	63	97	100

Appendix Table B12 Sublethal toxicity of naphthalene contaminated-sediment to *L. hoffmeisteri* expressed as sediment avoidance (ind/beaker).

Concentration (µg/g wwt)	Replication	Duration (h)			
		24	48	72	96
6.25	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
12.5	1	0	0	0	0
	2	0	0	0	1
	3	0	0	1	1
	4	0	0	0	0
	5	0	0	1	1
25	1	0	2	5	9
	2	0	3	7	8
	3	0	3	6	5
	4	0	1	2	4
	5	0	2	5	7
50	1	5	6	10	12
	2	3	5	9	13
	3	6	10	13	15
	4	6	11	15	18
	5	8	13	16	18
100	1	20	25	23	19
	2	17	21	22	17
	3	16	20	21	15
	4	19	23	24	18
	5	18	22	20	16

Appendix Table B13 Naphthalene in the sediment from the study on the bioaccumulation of sediment-associated naphthalene in *L. hoffmeisteri*.

	Duration (h)	Replication	Weight [a] (g dwt)	Concentration [b] (µg/g dwt)	Amount [c] (µg)	Concentration [d] (µg/g dwt)	Amount [e] (µg)
Treatment	24	1	148.45	32.16	4774.15	21.50	3191.68
		2	146.72	33.79	4957.67	21.86	3207.14
		3	146.87	33.24	4881.95	20.85	3062.19
	48	1	150.05	30.83	4626.04	15.35	2302.61
		2	149.65	32.59	4877.09	16.37	2450.38
		3	147.35	33.88	4992.22	15.96	2351.71
	72	1	149.89	31.75	4759.01	9.03	1353.51
		2	150.12	33.09	4967.47	9.87	1481.68
		3	148.94	30.90	4602.25	10.68	1590.68
	96	1	147.79	31.82	4702.68	2.17	320.70
		2	148.64	33.05	4912.55	1.46	217.01
		3	149.58	30.82	4610.05	2.01	300.66

Note: a = Total weight of sediment in each test vessel.

b = Concentration of naphthalene in the sediment determined using the technique of HPLC at the start of experiment.

c = Total amount of naphthalene in the sediment calculated by multiplying a total weight of sediment (a) with a concentration of naphthalene determined in each test vessel (b) at the start of experiment.

d = Concentration of naphthalene in the sediment determined using the technique of HPLC at the end of each exposure time.

e = Total amount of naphthalene in the sediment calculated by multiplying a total weight of sediment (a) with a concentration of naphthalene determined in each test vessel (d) at the end of each exposure time.

Appendix Table B13 (Continued)

	Duration (h)	Replication	Weight [a] (g dwt)	Concentration [b] (µg/g dwt)	Amount [c] (µg)	Concentration [d] (µg/g dwt)	Amount [e] (µg)
Control	24	1	149.48	0.025	3.82	nd	-
		2	145.28	0.029	4.17	nd	-
		3	147.57	0.017	2.54	nd	-
	48	1	146.19	0.015	2.23	nd	-
		2	148.46	0.020	3.01	nd	-
		3	149.18	0.021	3.18	nd	-
	72	1	149.81	0.015	2.34	nd	-
		2	148.77	0.021	3.18	nd	-
		3	150.02	0.019	2.96	nd	-
	96	1	150.13	0.019	2.96	nd	-
		2	149.26	0.028	4.15	nd	-
		3	148.36	0.023	3.43	nd	-

Note: a = Total weight of sediment in each test vessel.

b = Concentration of naphthalene in the sediment determined using the technique of HPLC at the start of experiment.

c = Total amount of naphthalene in the sediment calculated by multiplying a total weight of sediment (a) with a concentration of naphthalene determined in each test vessel (b) at the start of experiment.

d = Concentration of naphthalene in the sediment determined using the technique of HPLC at the end of each exposure time.

e = Total amount of naphthalene in the sediment calculated by multiplying a total weight of sediment (a) with a concentration of naphthalene determined in each test vessel (d) at the end of each exposure time

Appendix Table B14 Naphthalene in the overlying water from the study on the bioaccumulation of sediment-associated naphthalene in *L. hoffmeisteri*.

	Duration (h)	Replication	Volume [a] (ml)	Concentration [b] (µg/ml)	Amount [c] (µg)
Treatment	24	1	400	3.58	1432.45
		2	400	3.48	1392.87
		3	400	3.13	1250.76
	48	1	400	4.89	1953.84
		2	400	5.22	2086.21
		3	400	4.54	1817.52
	72	1	400	7.19	2874.31
		2	400	7.56	3023.88
		3	400	7.39	2957.35
	96	1	400	10.52	4208.78
		2	400	10.65	4261.21
		3	400	10.48	4190.15
Control	24	1	400	0.0027	1.099
		2	400	0.0025	1.037
		3	400	0.0026	1.058
	48	1	400	0.0028	1.114
		2	400	0.0027	1.099
		3	400	0.0027	1.099
	72	1	400	0.0028	1.114
		2	400	0.0027	1.099
		3	400	0.0029	1.147
	96	1	400	0.0036	1.431
		2	400	0.0038	1.525
		3	400	0.0040	1.613

Note: a = Total volume of overlying water in each test vessel.

b = Concentration of naphthalene in the overlying water determined using the technique of HPLC.

c = Total amount of naphthalene in the overlying water calculated by multiplying a total volume of overlying water (a) with a concentration of naphthalene determined in each test vessel (b).

Appendix Table B15 Naphthalene in the oligochaete tissue from the study on the bioaccumulation of sediment-associated naphthalene in *L. hoffmeisteri*.

	Duration (h)	Replication	Weight [a] (g dwt)	Concentration [b] (µg/g dwt)	Amount [c] (µg)
Treatment	24	1	0.0784	1769.39	138.72
		2	0.0815	1675.09	136.52
		3	0.0793	1804.67	143.11
	48	1	0.0723	1232.09	89.08
		2	0.0658	1312.16	86.34
		3	0.0817	1126.19	92.01
	72	1	0.0825	819.88	67.64
		2	0.0772	932.25	71.97
		3	0.0697	1001.87	69.83
	96	1	0.0803	262.39	21.07
		2	0.0781	262.35	20.49
		3	0.0753	267.59	20.15
Control	24	1	0.0675	1.4682	0.0991
		2	0.0791	1.3299	0.1052
		3	0.0817	1.0318	0.0843
	48	1	0.0749	0.3818	0.0286
		2	0.0753	0.1899	0.0151
		3	0.0646	0.2461	0.0159
	72	1	0.0792	0.1099	0.0087
		2	0.0751	0.1318	0.0099
		3	0.0822	0.1241	0.0102
	96	1	0.0669	0.0269	0.0018
		2	0.0803	0.0286	0.0023
		3	0.0768	0.0156	0.0012

Note: a = Total weight of oligochaete tissue in each test vessel.

b = Concentration of naphthalene in the oligochaete tissue determined using the technique of HPLC.

c = Total amount of naphthalene in the oligochaete tissue calculated by multiplying a total weight of oligochaete tissue (a) with a concentration of naphthalene determined in each test vessel (b).

Appendix Table B16 BAF values determined from the study on the bioaccumulation of sediment-associated naphthalene in *L. hoffmeisteri*.

Duration (h)	Replication	BAFs
24	1	55.00
	2	49.57
	3	54.29
48	1	39.96
	2	40.26
	3	33.24
72	1	25.82
	2	28.17
	3	32.42
96	1	8.24
	2	7.94
	3	8.68

Appendix Table B17 Naphthalene in the fingerling muscle from the study on trophic transfer of naphthalene via the oligochaete diet (T-A).

	Duration (h)	Replication	Weight [a] (g dwt)	Concentration [b] (µg/g dwt)	Amount [c] (µg)
Treatment A	2	1	0.843	1.164	0.981
		2	0.781	1.052	0.822
		3	0.819	1.067	0.874
	4	1	0.789	2.511	1.981
		2	0.828	2.471	2.046
		3	0.835	2.063	1.723
	6	1	0.827	12.996	10.748
		2	0.774	12.677	9.812
		3	0.851	11.166	9.503
	8	1	0.792	9.622	7.621
		2	0.815	8.872	7.231
		3	0.824	8.360	6.889
	10	1	0.849	1.479	1.256
		2	0.769	1.381	1.062
		3	0.836	1.348	1.127
	12	1	0.758	nd	-
		2	0.804	nd	-
		3	0.812	nd	-

Note: a = Total weight of fingerling muscle in each test vessel.

b = Concentration of naphthalene in the fingerling muscle determined using the technique of HPLC.

c = Total amount of naphthalene in the fingerling muscle calculated by multiplying a total weight of fingerling muscle (a) with a concentration of naphthalene determined in each test vessel (b).

Appendix Table B17 (Continued)

	Duration (h)	Replication	Weight [a] (g dwt)	Concentration [b] (µg/g dwt)	Amount [c] (µg)
Control A	2	1	0.821	nd	-
		2	0.756	nd	-
		3	0.698	nd	-
	4	1	0.802	nd	-
		2	0.763	nd	-
		3	0.787	nd	-
	6	1	0.751	nd	-
		2	0.821	nd	-
		3	0.819	nd	-
	8	1	0.772	nd	-
		2	0.793	nd	-
		3	0.804	nd	-
	10	1	0.827	nd	-
		2	0.814	nd	-
		3	0.795	nd	-
	12	1	0.809	nd	-
		2	0.781	nd	-
		3	0.792	nd	-

Note: a = Total weight of fingerling muscle in each test vessel.

b = Concentration of naphthalene in the fingerling muscle determined using the technique of HPLC.

c = Total amount of naphthalene in the fingerling muscle calculated by multiplying a total weight of fingerling muscle (a) with a concentration of naphthalene determined in each test vessel (b).

Appendix Table B18 Naphthalene in the fingerling muscle from the study on trophic transfer of naphthalene via the oligochaete diet (T-B).

	Duration (h)	Replication	Weight [a] (g dwt)	Concentration [b] (µg/g dwt)	Amount [c] (µg)
Treatment B	2	1	0.821	0.952	0.782
		2	0.774	0.875	0.677
		3	0.793	0.922	0.731
	4	1	0.832	2.183	1.817
		2	0.845	2.002	1.692
		3	0.769	2.013	1.548
	6	1	0.773	12.913	9.982
		2	0.805	10.836	8.723
		3	0.818	11.231	9.187
	8	1	0.834	10.937	9.822
		2	0.816	9.870	9.754
		3	0.788	10.767	9.484
	10	1	0.765	3.177	2.431
		2	0.809	2.525	2.043
		3	0.837	2.344	1.962
	12	1	0.758	9.978	7.564
		2	0.772	10.435	8.056
		3	0.814	9.512	7.743

Note: a = Total weight of fingerling muscle in each test vessel.

b = Concentration of naphthalene in the fingerling muscle determined using the technique of HPLC.

c = Total amount of naphthalene in the fingerling muscle calculated by multiplying a total weight of fingerling muscle (a) with a concentration of naphthalene determined in each test vessel (b).

Appendix Table B18 (Continued)

	Duration (h)	Replication	Weight [a] (g dwt)	Concentration [b] (µg/g dwt)	Amount [c] (µg)
Control B	2	1	0.789	nd	-
		2	0.831	nd	-
		3	0.807	nd	-
	4	1	0.792	nd	-
		2	0.776	nd	-
		3	0.842	nd	-
	6	1	0.833	nd	-
		2	0.796	nd	-
		3	0.808	nd	-
	8	1	0.785	nd	-
		2	0.769	nd	-
		3	0.833	nd	-
	10	1	0.777	nd	-
		2	0.791	nd	-
		3	0.852	nd	-
	12	1	0.843	nd	-
		2	0.767	nd	-
		3	0.782	nd	-

Note: a = Total weight of fingerling muscle in each test vessel.

b = Concentration of naphthalene in the fingerling muscle determined using the technique of HPLC.

c = Total amount of naphthalene in the fingerling muscle calculated by multiplying a total weight of fingerling muscle (a) with a concentration of naphthalene determined in each test vessel (b).

Appendix C

Data Analysis

Appendix Table C1 Two-factor ANOVA determined in mean number of individual of oligochaete between level of food addition (E-WL, E-WH, E-WN) and test duration (7, 14, 21, 28, 35, 42 d).

Source of variation	Sum of squares	df	Mean sum of squares	F
Organic food level	2271.62	2	1135.81	66.38*
Test duration	398.09	5	79.62	4.65*
Interaction	1341.79	10	134.18	7.84*
Error	616.00	36	17.11	
Total	4627.50	53		

Appendix Table C2 S-N-K test of mean number of individual of oligochaete compared at each level of food addition (E-WL, E-WH, E-WN).

Duration (d)	Pair	Different value	*p*	*q*	W*p*	
7	x2, x3	2	2	2.86	6.84	
	x2, x1	2.67	3	3.44	8.22	
	x3, x1	0.67	2	2.86	6.84	
14	x2, x3	3	2	2.86	6.84	
	x2, x1	6	3	3.44	8.22	
	x3, x1	3	2	2.86	6.84	
21	x2, x1	9.33	2	2.86	6.84	*
	x2, x3	11.33	3	3.44	8.22	*
	x1, x3	2	2	2.86	6.84	
28	x2, x1	10.33	2	2.86	6.84	*
	x2, x3	13.66	3	3.44	8.22	*
	x1, x3	3.33	2	2.86	6.84	
35	x2, x1	18.33	2	2.86	6.84	*
	x2, x3	27	3	3.44	8.22	*
	x1, x3	8.67	2	2.86	6.84	*
42	x2, x1	20	2	2.86	6.84	*
	x2, x3	35.33	3	3.44	8.22	*
	x1, x3	15.33	2	2.86	6.84	*

Note: * Significant difference at $p<0.05$

x1: E-WL; x2: E-WH; x3: E-WN

Appendix Table C3 Two-factor ANOVA determined in average weight of oligochaete between level of food addition (E-WL, E-WH, E-WN) and test duration (7, 14, 21, 28, 35, 42 d).

Source of variation	Sum of squares	df	Mean sum of squares	F
Organic food level	0.0731	2	0.0366	52.28*
Test duration	0.0031	5	0.0006	0.86
Interaction	0.0042	10	0.0004	0.57
Error	0.0266	36	0.0007	
Total	0.1070	53		

Appendix Table C4 S-N-K test of average weight of oligochaete compared at each level of food addition (E-WL, E-WH, E-WN).

Duration (d)	Pair	Different value	*p*	*q*	W*p*	
7	x2, x1	0.0196	2	2.86	0.0429	
	x2, x3	0.0505	3	3.44	0.0516	
	x1, x3	0.0309	2	2.86	0.0429	
14	x2, x1	0.0325	2	2.86	0.0429	
	x2, x3	0.0531	3	3.44	0.0516	*
	x1, x3	0.0206	2	2.86	0.0429	
21	x2, x1	0.0465	2	2.86	0.0429	*
	x2, x3	0.0831	3	3.44	0.0516	*
	x1, x3	0.0366	2	2.86	0.0429	
28	x2, x1	0.0509	2	2.86	0.0429	*
	x2, x3	0.0924	3	3.44	0.0516	*
	x1, x3	0.0415	2	2.86	0.0429	
35	x2, x1	0.0608	2	2.86	0.0429	*
	x2, x3	0.1223	3	3.44	0.0516	*
	x1, x3	0.0615	2	2.86	0.0429	*
42	x2, x1	0.0559	2	2.86	0.0429	*
	x2, x3	0.1396	3	3.44	0.0516	*
	x1, x3	0.0837	2	2.86	0.0429	*

Note: * Significant difference at $p<0.05$

x1: E-WL; x2: E-WH; x3: E-WN

Appendix Table C5 Two-factor ANOVA determined in average TOM of the sediment between present of worm (E-WH, E-NH) and test duration (7, 14, 21, 28, 35, 42 d).

Source of variation	Sum of squares	df	Mean sum of squares	F
Present of worm	0.866	1	0.866	288.67*
Duration	0.203	5	0.041	13.67*
Interaction	0.667	5	0.133	44.33*
Error	0.063	24	0.003	
Total	1.799	35		

Appendix Table C6 S-N-K test of average TOM of the sediment compared between present of worm (E-WH) and absent of worm (E-NH).

Duration (d)	Pair	Different value	*p*	*q*	W*p*	
7	x2, x1	0.01	2	2.92	0.09	
14	x2, x1	0.1	2	2.92	0.09	*
21	x2, x1	0.13	2	2.92	0.09	*
28	x2, x1	0.23	2	2.92	0.09	*
35	x2, x1	0.5	2	2.92	0.09	*
42	x2, x1	0.81	2	2.92	0.09	*

Note: * Significant difference at $p<0.05$

x1: E-WH; x2: E-NH

Appendix Table C7 Two-factor ANOVA determined in average DO of the overlying water between present of worm (E-WH, E-NH) and test duration (7, 14, 21, 28, 35, 42 d).

Source of variation	Sum of squares	df	Mean sum of squares	F
Present of worm	2.16	1	2.16	216*
Duration	0.92	5	0.18	18*
Interaction	1.46	5	0.29	29*
Error	0.31	24	0.01	
Total	4.85	35		

Appendix Table C8 S-N-K test of average DO of the overlying water compared between present of worm (E-WH) and absent of worm (E-NH).

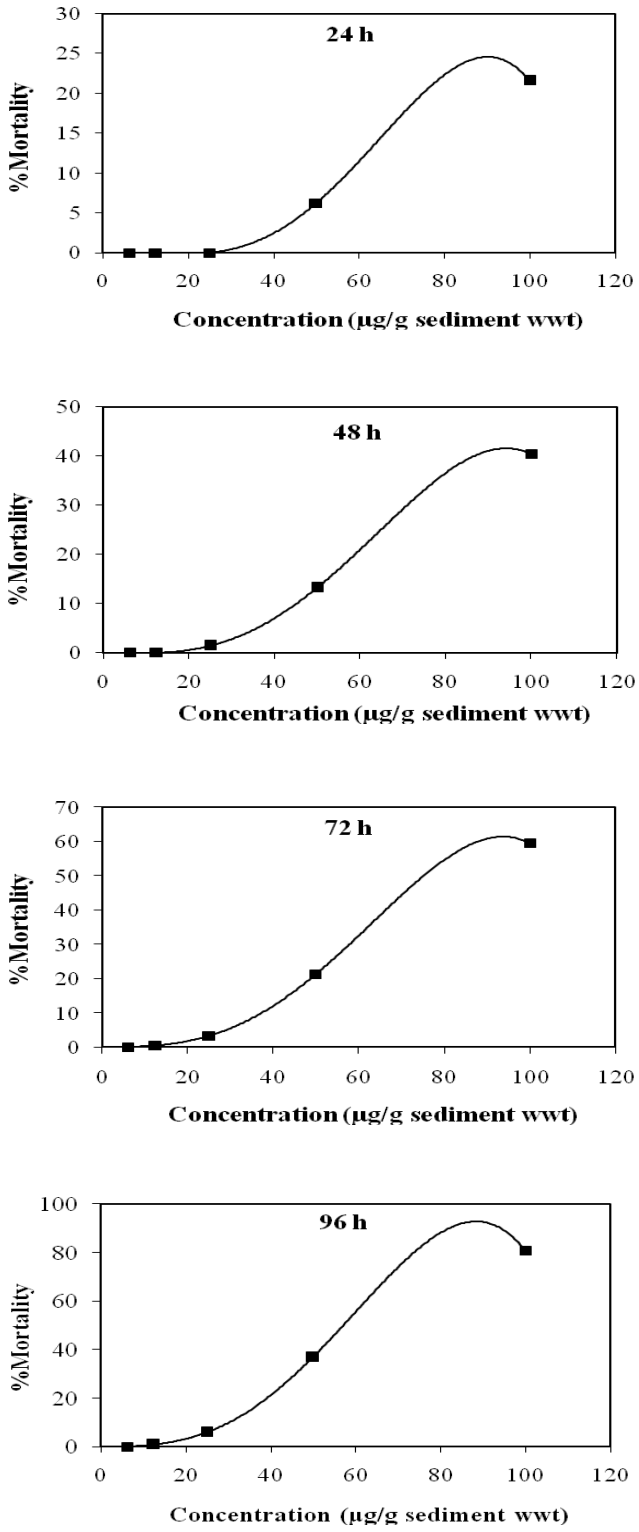
Duration (d)	Pair	Different value	*p*	*q*	W*p*	
7	x1, x2	0.02	2	2.92	0.17	
14	x1, x2	0.21	2	2.92	0.17	*
21	x1, x2	0.08	2	2.92	0.17	
28	x1, x2	0.62	2	2.92	0.17	*
35	x1, x2	0.92	2	2.92	0.17	*
42	x1, x2	1.07	2	2.92	0.17	*

Appendix Table C9 Pearson correlation coefficient (*r*) and significant level ($p<0.05$) between biological factor (X) and environmental factor (Y).

Factors		*r*	|*u*|	t	
X	Y				
Number of individual	TOM	-0.74	4.44	2.12	*
Weight	TOM	-0.58	2.85	2.12	*
Number of individual	DO	0.62	3.15	2.12	*
Weight	DO	0.47	2.15	2.12	*

Note: * Significant difference at $p<0.05$

x1: E-WH; x2: E-NH



Appendix Figure C1 Dose-response relationship between concentration of naphthalene in sediments and % mortality of *L. hoffmeisteri*.

Probit Analysis of Mortality

Equation : $Y = aX + b$

while; Y : Probit value at 50% mortality = 5

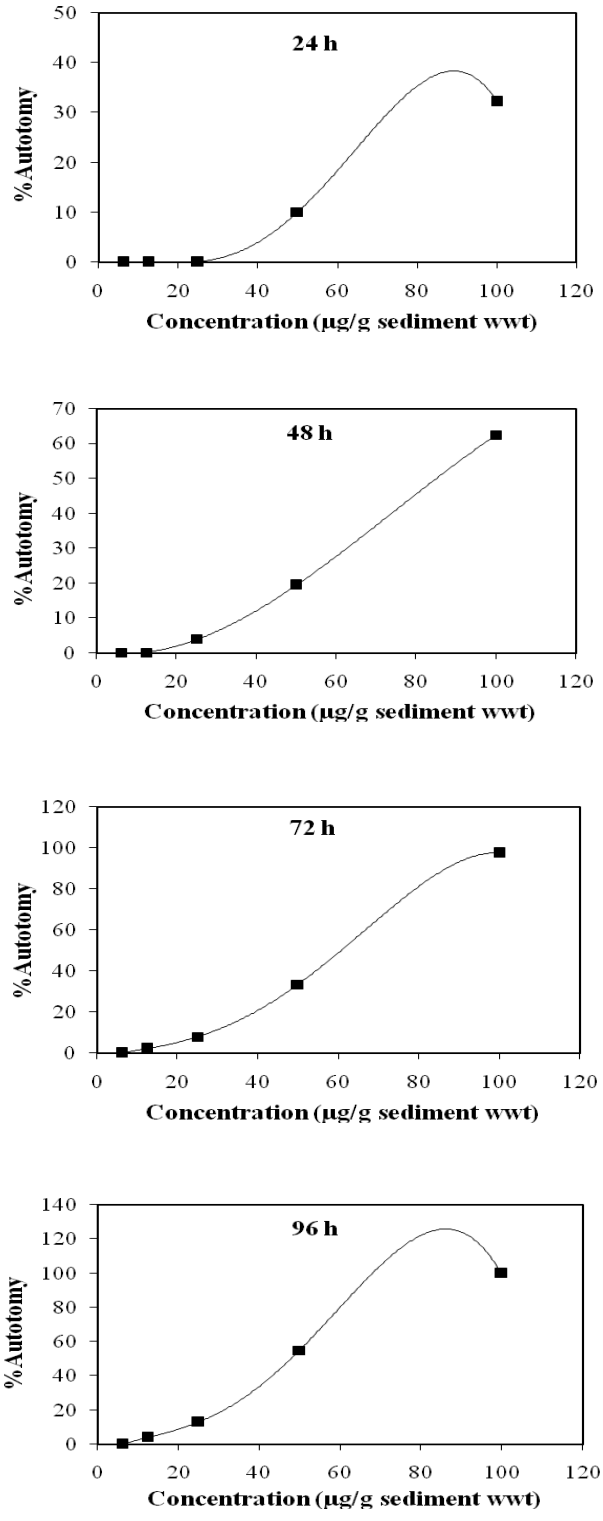
X : Log concentration

a : slope

b : intercept

Appendix Table C10 Probit analysis of mortality of *L. hoffmeisteri* exposed to naphthalene contaminated-sediment.

Concentration (µg/g wwt)	Log concentration	Test duration (h)							
		24		48		72		96	
		%	probit	%	probit	%	probit	%	probit
6.25	0.796	0		0		0		0	
12.5	1.097	0		0		0.4		0.8	
25	1.398	0		1.4	2.67	3.2	3.12	5.8	3.36
50	1.699	6.2	3.45	13.2	3.87	21.2	4.19	36.8	4.67
100	2.000	21.6	4.19	40.4	4.75	59.4	5.23	80.8	5.88
	intercept	-0.58		-2.01		-1.68		-2.36	
	slope	2.39		3.41		3.46		4.13	
Calculation	log LC_{50}	2.33		2.05		1.93		1.78	
	LC_{50}	213.79		112.20		85.11		60.26	
	SD	0.523		1.039		1.055		1.259	
	CI	0.721		1.176		1.188		1.426	



Appendix Figure C2 Dose-response relationship between concentration of naphthalene in sediments and % autotomy of *L. hoffmeisteri*.

Probit Analysis of Autotomy

Equation : $Y = aX + b$

while; Y : Probit value at 50% autotomy = 5

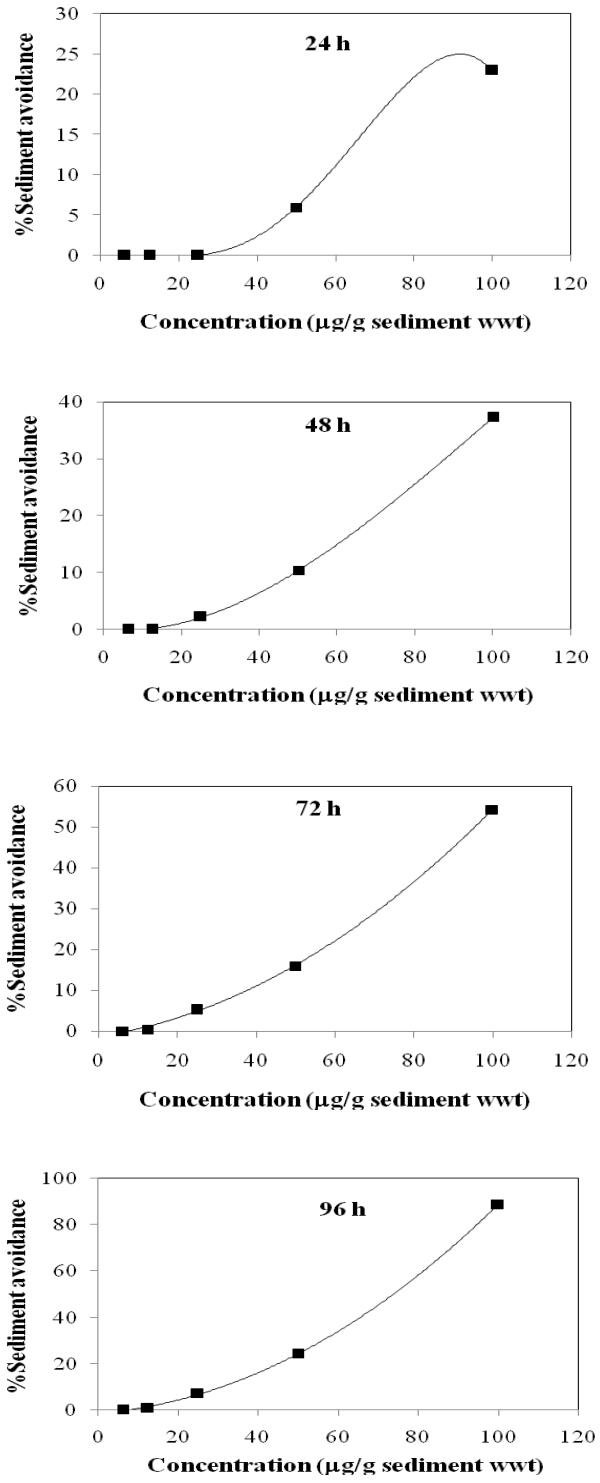
X : Log concentration

a : slope

b : intercept

Appendix Table C11 Probit analysis of autotomy of *L. hoffmeisteri* exposed to naphthalene contaminated-sediment.

Concentration (µg/g wwt)	Log concentration	Duration (h)							
		24		48		72		96	
		%	probit	%	probit	%	probit	%	probit
6.25	0.796	0		0		0		0	
12.5	1.097	0		0		2	2.95	3.8	3.12
25	1.398	0		4	3.25	7.8	3.52	12.8	3.82
50	1.699	10	3.72	19.4	4.12	33.4	4.56	54.8	5.1
100	2.000	32.2	4.53	62.4	5.31	97.4	6.88	99.8	7.88
	intercept	-0.69		-1.49		-2.07		-2.96	
	slope	2.61		3.38		4.24		5.15	
Calculation	Log EC_{50}	2.18		1.92		1.67		1.55	
	EC_{50}	151.35		83.17		46.77		35.48	
	SD	0.573		1.031		1.660		2.013	
	CI	0.789		1.166		1.627		1.969	



Appendix Figure C3 Dose-response relationship between concentration of naphthalene in sediments and % sediment avoidance of *L. hoffmeisteri*.

Probit Analysis of Sediment Avoidance

Equation : $Y = aX + b$

while; Y : Probit value at 50% sediment avoidance = 5

X : Log concentration

a : slope

b : intercept

Appendix Table C12 Probit analysis of sediment avoidance of *L. hoffmeisteri* exposed to naphthalene contaminated-sediment.

Concentration (µg/g wwt)	Log concentration	Duration (h)							
		24		48		72		96	
		%	probit	%	probit	%	probit	%	probit
6.25	0.796	0		0		0		0	
12.5	1.097	0		0		0.4		0.6	
25	1.398	0		2.23	2.95	5.17	3.36	7.01	3.52
50	1.699	5.97	3.36	10.4	3.72	15.9	4.01	24.1	4.29
100	2.000	22.9	4.23	37.3	4.67	54.2	5.10	88.5	6.18
	intercept	-1.38		-1		-0.68		-2.74	
	slope	2.81		2.82		2.86		4.37	
Calculation	Log EC_{50}	2.27		2.12		1.99		1.77	
	EC_{50}	186.21		131.83		97.72		58.88	
	SD	0.615		0.861		0.871		1.333	
	CI	0.86		0.973		0.984		1.505	

Appendix Table C13 One-way ANOVA determined in average concentration of naphthalene in oligochaete tissue compared between each exposure time (24, 48, 72, 96 h).

Source of variation	Sum of squares	df	Mean sum of squares	F
Exposure time	3462760.79	3	1154253.59	216.19*
Error	42711.37	8	5338.92	
Total	3505472.16	11		

Appendix Table C14 S-N-K test of average concentration of naphthalene in oligochaete tissue compared between each exposure time (24, 48, 72, 96 h).

Pair	Different value	*p*	*q*	W*p*	
x1, x2	526.24	2	3.26	137.54	*
x1, x3	831.73	3	4.04	170.45	*
x1, x4	1485.61	4	4.53	191.12	*
x2, x3	305.49	2	3.26	137.54	*
x2, x4	959.37	3	4.04	170.45	*
x3, x4	653.88	2	3.26	137.54	*

Appendix Table C15 Pearson correlation coefficient (*r*) and significant level ($p<0.05$) between naphthalene in sediment (X) and naphthalene in oligochaete tissue (Y).

Factors		*r*	
X	Y		
Sediment	Oligochaete	0.98	*

Note: * Significant difference at $p<0.05$

x1: 24 h; x2: 48 h; x3: 72 h; x4: 96 h

Appendix Table C16 Two-factor ANOVA determined in average concentration of naphthalene in fingerling muscle compared between oligochaete meal (T-A, T-B) and exposure time (2, 4, 6, 8, 10, 12 h).

Source of variation	Sum of squares	df	Mean sum of squares	F
Oligochaete meal	35.74	1	35.74	127.64*
Exposure time	618.73	5	123.75	441.96*
Interaction	120.84	5	24.17	86.32*
Error	6.77	24	0.28	
Total	782.08	35		

Appendix Table C17 S-N-K test of average concentration of naphthalene in fingerling muscle compared between oligochaete meal (T-A, T-B).

Duration (h)	Pair	Different value	*p*	*q*	W*p*	
2	x1, x2	0.18	2	2.92	0.89	
4	x1, x2	0.28	2	2.92	0.89	
6	x1, x2	0.62	2	2.92	0.89	
8	x2, x1	1.57	2	2.92	0.89	*
10	x2, x1	1.28	2	2.92	0.89	*
12	x2, x1	9.98	2	2.92	0.89	*

Note: * Significant difference at $p<0.05$

x1: T-A; x2: T-B

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