이학석사 학위논문

Effects of Benzo(a)pyrene on the Spawners and Next Generation of Pacific Oysters, *Crassostrea gigas*

지도교수 강 주 찬

이 논문을 이학석사 학위논문으로 제출함

2003년 2월

부 경 대 학 교 대 학 원

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주 심 이학박사 김 기 홍 

위 원 이학박사 정 현 도 

위 원 이학박사 강 주 찬 

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Benzo(a)pyrene 이 참굴 모패와 다음 세대에 미치는 영향

최 은 정

부경대학교 어병학과

광범위한 오염물질로 알려진 다환방향족탄화수소 (PAHs, Polycyclic aromatic hydrocarbons)는 도시화 산업화등 인간활동의 증가로 인해 최근들어 연안에 축적이 점차 증가하는 추세에 있다. 그 중 benzo(a)pyrene (BaP)는 강한 발암성과 변이원성을 가지며 인체와 포유류에 대해 내분비계 장애를 일으키는 물질로 알려져 있다. 이 화합물은 분자구조적인 특성으로 인해 수중에서 높은 소수성 (hydrophobicity)을 보여, 퇴적물이나 부유생물을 포함한 부유물 (DOMs, dissolved organic matters)에 강하게 분획된다. 굴양식장에서의 BaP 문제는 굴이 여과섭식을 하는 관계로 먹이생물 또는 DOM 에 부착 (absorption/adsorption)된 BaP 가 보다 쉽게 굴에 노출된다는 데 있다고 볼 수 있다. 따라서 본 연구에서는 먹이생물을 매개로하여 양식굴에 BaP 를 노출시켜, 본 화합물에 대한 굴의 축적(bioaccumulation)과 배설(depuration)을 GC/MS (gas chromatography/mass spectrometry)로 정량하고, BaP 축적에 따른 참굴모패와 다음 세대인 유생의 부화률, 유생성장, 생존 및 부착 등

의 영향을 통해 BaP 가 양식굴에 미치는 직접 및 간접적인 영향을 이해하고자 하였다.

BaP 에 노출된 먹이생물 (*Tetraselmis suecica, Isochrysis galbana, Phaeodactylum tricornutum*)은 굴에 대한 뛰어난 BaP 매개자로의 역할을 하였다. BaP 에 노출된 먹이생물의 농도별 축적상관관계는 대조구에서 Y = - 0.0085x + 3.2299 (r^2 = 0.0105), 50 ppb 에서 Y= 387.11x - 573.08 (r^2 = 0.9870), 500 ppb 에서 Y= 1035.2x + 3798.6 (r^2 = 0.8813), 그리고 5000 ppb 에서는 Y= 831.95x + 12561 (r^2= 0.7084)로 나타났다. 또한 배설상관관계는 50 ppb 에서 Y= -324.91x + 19597 (r^2 = 0.9748), 500 ppb 에서 Y= -152.8x + 34590 (r^2 = 0.3294), 그리고 5000 ppb 에서는 Y= -84.982x + 33632 (r^2 = 0.6487)로 나타났다. 이 결과는 참굴에서 BaP 축적은 먹이생물의 BaP 농도에 따라 지배되고 있음을 나타내며, 반면에 배설의 경우 일정농도 이상의 수준 (500 ppb)에서는 유의한 차이가 없음을 시사하고 있어, 참굴이 BaP 를 biodegradation/biotransformation 할 수 있는 농도의 범위를 짐작하게 해 주고 있다.

참굴 모패의 혈청학적 특성, 즉 hemocyte membrane stability 와 hydrolytic enzyme activity 를 측정하여 BaP 가 참굴모패에 미치는 영향을 구명하였다. 그 결과 hemocyte 의 neutral red retention (NRR) time 은 50 ppb 이상의 농도에서 대조구에 비해 유의한 수준으로 감소하는 경향을 나타내었다. 반면, acid phosphatase 와 alkaline phosphatase 는 노출된 BaP 농도에 유의적인 변화는 관찰되지 않았다. BaP 노출에 따른 참굴의 모패로부터 수정시킨 유생의 부화

율은 5000 ppb 에서 유의한 감소를 나타냈고, 유생의 생존율은 500 ppb 이상에서 유의한 감소를 보였으며, 유생의 성장 및 부화율은 50 ppb 이상의 농도에서 유의수준으로 감소하는 경향을 나타내었다. 이러한 결과는 종합적으로볼 때, 양식 참굴에서 BaP 는 먹이생물을 매개로했을 때, 최소 500 ppb 이상에서는 참굴의 biodegradation/biotransformation 의 범위에 노출된 모패에 대한 영향 뿐만 아니라 그 다음 세대인 유생에 까지 영향을 미침을 시사하고 있다.

I. INTRODUCTION

1.1 General

It is well known that a wide variety of toxic chemicals are present in the global oceans (Clark, 1986; Giam and Ray, 1987). Contamination of the coastal marine environment has received increasing attention over a last couple of decades. Included among these are natural products as well as compounds of anthropogenic origin: marine toxins, inorganic and organic metals, petroleum and combustion derived hydrocarbons, chlorinated pesticides, halogenated aromatic hydrocarbons, and many others. These contaminants can be found bound to sediments, dissolved in water (including pore water), in the sea-surface microlayer, and within various marine organisms, including marine animals used for foods for human consumption. The highest concentrations of these chemicals are often found in urban harbors and other coastal areas (Farrington et al., 1983; Weaver, 1984; Dethlefson, 1988). However, there are more generalized contaminations in the global sense. For example, persistent organic and inorganic pollutants have been documented even in remote locations such as in polar oceanic regions and in the deep sea (Stegeman et al., 1986; Muir et al., 1988; Mason and Fitzgerald, 1990; Ballschmitert et al., 1997; Stegeman et al., 2001). Therefore, the possible environmental impacts of the pollutants have been taken into considerable concern among environmental physiologists.

Techniques based on measurements of the physiological and biochemical processes can provide the environmental physiologists with a good tool for the monitoring the healthy of organisms living in coastal and even in the open oceanic waters. Changes and fluctuations in measurement

might function as an early warning of the pollutants and provide information on the nature of the pollutants and the toxicity stress caused by the pollutants.

Of the chemicals, polycyclic aromatic hydrocarbons (PAHs) are a group of the most widespread chemical contaminants occurring in the marine environments, particularly in bottom sediments, overlying waters, and biota, especially those in estuarine and coastal waters. PAHs have been the focus of numerous studies because they are potentially carcinogenic, mutagenic, and teratogenic to aquatic organisms (Livingstone et al., 1990; Cavalieri et al., 1993a,b; Bigger et al., 1994; Maccubbin, 1994; Venier and Canova, 1996; Akcha et al., 2000; Law et al., 2002; White, 2002). These harmful effects of the chemicals can be particularly alarming when the marine organisms are of cultured species, including oysters, mussels, and kinds of fish. PAHs have been dectected in a wide variety of environmental samples, including air (Freeman and Cattell, 1990; Sexton et al., 1985; Greengerg et al., 1985), soil (Jones et al., 1898a, 1989b, 1989c; Wilson and Jones, 1993), sediments (Youngblood and Blumer, 1975; Laflamme and Hites, 1978; Shiaris and Jambard-Sweet, 1986), water (Cerniglia and Heikamp, 1989), oils, tars (Nishioka et al., 1986) and foodstuffs (Lijinsky, 1991; Dipple and Bigger, 1991).

1.2 Polycyclic aromatic hydrocarbons

The term PAH generally refers to hydrocarbons containing two or more fused benzene rings in linear, angular or clustered arrangements (Sims and Overcash, 1983; Albert and Ravendra, 2000). The chemical sources in the marine environments are diversified. For example, PAHs are introduced into the environment via natural and antropogenic combustion processes.

Volcanic eruptions and forest and praire fires are among the major sources of naturally produced PAHs. However, anthropogenic activities have dramatically increased the quantity of PAHs in the environment, with the majority emitted from fossil fuel combustion sources such as automobiles, coking plants, asphalt production, and manufacturing facilities that use fossil fuels (Basu and Saxena, 1987). In aquatic environment, they are accumulated throughout atmospheric deposition directly on water surfaces or indirectly via land runoff and sewage wastewater. Many of them exhibit a potential for bioaccumulation and have a negative effect on aquatic organisms (Kirso et al., 1988; Warshawsky et al., 1995; Livingstone et al., 2000; White, 2002), including the genotoxic carcinogens to several biological systems of organisms when the chemicals are persistent in the marine environment.

PAHs, like many other lipophilic organic environmental pollutants, have low vapor pressure and high octanol-water partition coefficients (Cerniglia, 1992; Krasnoschekova et al., 1992). Generally, solubility and hydrophobicity of PAHs decreases with an increase in number of fused benzene rings. In addition, volatility decreases with an increasing number of fused rings (Wilson and Jones, 1993). Therefore, PAHs are rapidly absorbed by particulated matters and by living organisms. In water, PAHs may be present in the vapor phase, dissolved phase, micelle form, sorbed to "dissolved" or colloidal organic matter, sorbed to particles, or incorporated into biota (McCarthy et al., 1989; Broman et al., 1990).

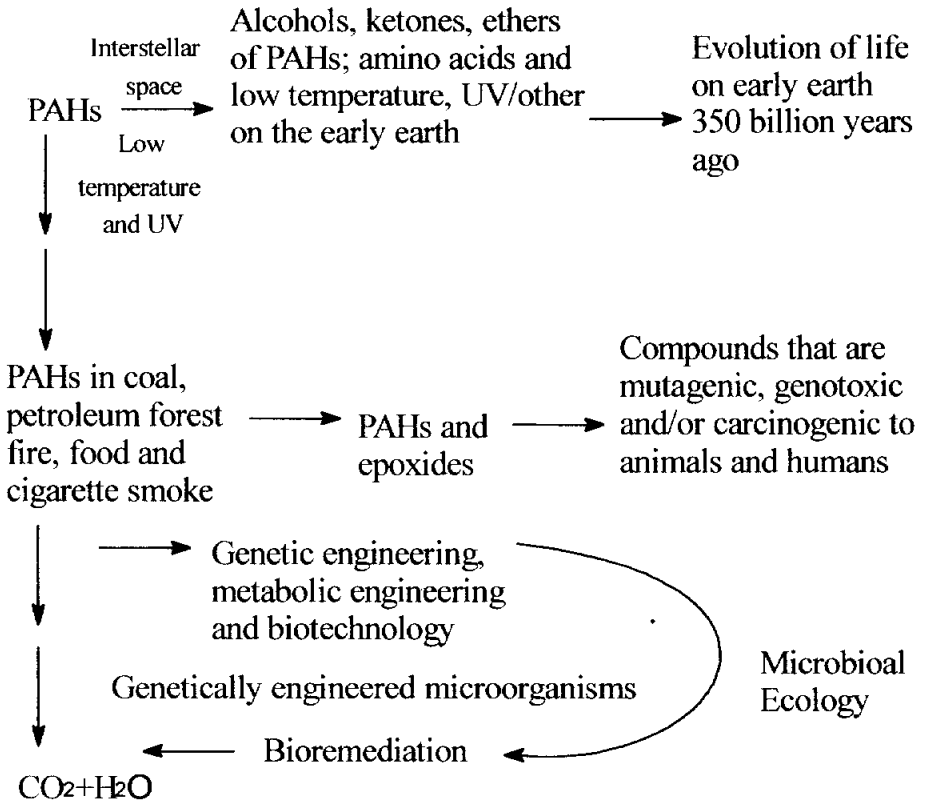

Fig.1. Fate, toxicity, and remediation of polycyclic aromatic hydrocarbons (PAHs) from the environment. A wide variety of PAHs are abundant in nature owing to incomplete combustion of organic matters. The PAHs from extraterrestrial matter are also oxidized and reduced owing to prevalent astrophysical conditions and resulting in the formation of various organic molecules, which are the basis of early life on primitive earth. The microorganisms (naturally occurring or genetically engineered) can mineralize toxic PAHs into CO_2 and H_2O (from Samanta et al., 2002)

PAHs consist of hydrogen and carbon arranged in the form of two or more fused benzene rings in linear, angular, or cluster arrangements with unsubstituted groups possibly attached to one or more rings. Two general types of PAHs have been well characterized; fused and linked ring compounds. One of such high molecular weight PAHs in the fused ring compounds is benzo(a)pyrene (BaP), a five-ring compound. BaP has been classified by the US Environmental Protection Agency (USEPA) as a priority pollutant, a compound selected on the basis of its known or suspected carcinogenicity, teratogenicity or acute toxicity (Albert and Ravendra, 2000). BaP is a five-ring PAH where the fused rings are arranged in a clustered manner. BaP has a low aqueous solubility (0.0038 mg l^{-1}) and vapour pressure (5.0×10^{-7} torr), and a high octanol: water partitioning coefficient (6.04), which suggest its preference to non-aqueous phases. The chemical peroperties of PAHs, including BaP, have a tendency rendering the chemical to portion into the nonaqueous phases. For example, The total concentration of 16 PAHs vary from 4228 to 29,325 ng/Kg in water of a local aquaculture farming area, while vary from 115 to 1134 ng/g dry weight in the sediment of the area (Zhou and Maskaoui, 2003). The summeries of the physical and chemical properties and metabolic pathway of some species of PAHs, including BaP are shown in Table 1 and Fig. 1.

Table 1. Structure and physico-chemical properties of some three-, four-, five- and six-ring polycyclic aromatic hydrocarbons (adapted from Sims and Overcash, 1983)

PAH	No.of rings	Mp[a] (℃)	Bp[b] (℃)	Sol[c] (mgl^{-1})	$LogK_p$[d]	Vapour pressure
Phenanthrene	3	101	340	1.29	4.46	6.8×10^{-4}
Anthracene	3	216	340	0.07	4.45	2.0×10^{-4}
Fluoranthene	4	111	250	0.26	5.33	6.0×10^{-6}
Benz(*a*)anthr-acene	4	158	400	0.014	5.61	5.0×10^{-9}
Pyrene	4	149	360	0.14	5.32	6.8×10^{-7}
Chrysene	4	255	488	0.002	5.61	6.3×10^{-7}
Benzo(*a*)-pyrene	5	179	496	0.0038	6.04	5.0×10^{-7}
Dibenz(*a,h*)-anthracene	5	262	524	0.0005	5.97	1.0×10^{-10}
Benzo(*g,h,i*)-perylene	6	222	-	0.0003	7.23	1.0×10^{-10}
Indeno(1,2,3-*c,d*)pyrene	6	163	536	0.062	7.66	1.0×10^{-10}

[a]mp: melting point

[b]bp: boiling point

[C]Sol: aqueous solubility

[d]log Kp: logarithm of the octanol: water partitioning coefficient

- : not reported.

Table 2. PAH concentrations (ng/g dry weight) in sediments from different locations (modified from Moon et al., 2001)

Location	Range	Reference
Korean coasts	6.4-1,214	Moon et al. (2001)
Kyeonggi Bay, Korea	9.1-1,400	Kim et al. (1999)
Casco Bay, USA	16-21,000	Kennicutti et al. (1994)
Washington coast, USA	29-460	Prahl and Carpenter (1983)
Chesapeake Bay, USA	0.56-180	Foster and Wright (1988)
San Fransicso Bay, USA	2,902-29,590	Pereira et al. (1996)
Manukau Habour, New Zealand	16-5,300	Holland et al. (1993)
Humber Plume, North Sea	700-2,700	Klamer and Fomsgaard (1993)
Adriatic Sea	18-580	Caricchia et al. (1993)
White Sea	13-208	Savinov et al. (2000)
Baltic Sea	9.5-1,900	Witt (1995)
Western Baltic Sea	3-30,000	Baumard et al. (1999a)
Arcachon Bay, France	32-4,120	Baumard et al. (1999b)

Table 2. Continuted

Location	Range	Reference
Daya Bay, China	115-1,134	Zhou and Maskaoui (2003)
Gironde Estuary, France	3.5-853	Soclo et al. (2000)
Lazaret Bay, France	100-50,000	Benlahcen et al. (1998)
Pearl River and Estuary, China	11-707	Bixian et al. (2001)
Malburger Haven	2,353,000	Huis in't Veld et al. (1995)
Rijks Binnenheven, The Netherlands	3,335,000	Huis in't Veld et al. (1995)
Ajaccio habour, Corsica	20,000	Baumard et al. (1998c)
Rhine-Muese Delta	60,100	Hendrix et al. (1998)
Kitimat Arm, BC, Canada	1,310-9,890,000	Paine et al. (1996)
Geore's river, Australia	1-1,500	Brown and Maher (1992)
Weatern Mediteranean, Australia	9-114	Lipiatov and saliot (1991)
Bedford harbor, Massachusetts	640-8,400	Pruell et al. (1990)

1.3 Benzo(a)pyrene in marine organisms

In spite of higher concentrations from different sources in terrestrial environments, the concentrations in water are extremely low. This is because they are characterized by low water solubility and high octanol-water partitioning coefficient (Albert and Ravendra, 2000). Bioaccumulation of PAHs by marine organisms is strongly dependent on bioavailability of the chemicals (i.e. partitioning of the chemicals between sediment, water and food), as well as physiology of and trophic transfer of them to the organisms (Meador et al., 1995). Accumulation kinetics of BaP in the bivalves was not only related water solubility of the chemical but also depended on the structure and physicochemical properties of the chemicals. Therefore, it is quite a reasonable to expect that the accumulation kinetics of the chemical varies with the PAH species.

Regarding PAH accumulation in the sediment of marine environment, due to their hydrophobic nature, PAHs accumulate in fine grain sediments, partitioning to organic carboncoated particles (Meador et al., 1995). As such, sediments may be considered as a reservoir for PAH accumulation in aquatic environment (Albert and Ravendra, 2000). The concentration of PAHs in sediments may range over several orders of magnitude (from a few $\mu g\ kg^{-1}$ up to g kg^{-1}), depending on the proximity of the waterway to antropogenic activity, water currents, and water usage (Table 2).

For sediment ingesters, the amount of the xenobiotic BaP taken up depends on the amount of sediment ingested, so that high tissue concentrations are found when sediment ingestion high. Uptake of xenobiotics from ingestion of sediment particles depends on the feeding rate of the animal, assimilation efficiency, feeding selectivity, and concentration of xenobiotics in ingested particles (Benoit et al, 1999; Kukkonen and Landrum, 1995).

Uptake of PAHs by mollusks is very fast in the first 24h for low molecular species. Depurations are also dependent on the molecular properties of the PAH species. For example, bivalve concentration of PAHs decreases significantly after the first day for anthracene and phenanthrene, after the second day for pyrene, and after second week for BaP (Narbonne et al., 1999).

Baumard et al. (1999) found a seasonality of the chemical kinetics in marine organisms, observing higher concentrations of PAHs in mussel in March (870 ng/g) over those in October (250 ng/g). The observed higher concentrations of PAHs in March are probably due to higher feeding rates of the filter-feeders during spring (Deslou-Paoli et al., 1987). In other words, the higher filtering rates of the mussels in the spring induced a greater exposure to the PAHs in the water column. Environmental factors, such as temperature, oxygen content, pH, and salinity can also influence the uptake of PAHs by marine organisms due to their effects on the bioavailability of the compounds. In addition, changes in the organisms behavior, seasonal rhythms, nutritional quality, and stress can also influence PAH accumulations in the marine organisms. Lemke and Kennedy (1997) investigated the effects of the smoltification process (the transformation of the freshwater parr to the seaward migrating smolt) in coho salmon on the uptake, distribution and metabolism of BaP. Smoltification consists of various morphological, physiological, and behavioral changes that occur to adapt the fish for the marine environment. They observed that the rate of BaP uptake increased three- to four-fold (0.01-0.04 μg/g/h) from February to April, proposing that the increased oxygen consumption during the smoltification process led to the enhanced absorption of BaP.

The size, ingestion rate, growth rate, membrane permeability,

ventilatory rate, gut residence time, and osmoregulation of the organisms are biological factors that influence the bioaccumulation or uptake of PAHs by the organisms. In reality, the measurements of the chemical toxicity are complicated. The chemical toxicity can vary from one organism to another as well as within the age, sex, diet, physiological status, or health status of the organism (Hodgson, 1997). The complex response of the marine organisms against PAHs makes overall understanding of the chemical toxicity difficult.

1.4 Bivalve metabolism of benzo(a)pyrene and its implications

PAH concentrations of the bivalve animals are strongly correlated with those of bottom sediment. Law et al. (2002) determined concentrations of PAHs in sediment and mussels in the vicinity of a former gaswork site. Very high concentrations of the chemicals were found in the substrate. Higher PAH concentrations were observed from mussels from sites showing higher bottom pollutions, marking up to extreme concentration, 458,000 μg/Kg dry weight mussel, depending on the pollution.

However, there is still an incomplete understanding of the metabolism and mechanisms of reactive intermediate formation from these xenobiotic chemicals in marine invertebrates compared to knowledge of such processes in mammals. The ability of aquatic organisms to uptake BaP from contaminated ambient environments is well documented. Besides the animal bioaccumulation of BaP, trophic transfer of the chemical to the animals is of importance when bivalves are concerned. This is because of their filter feeding behaviors. Therefore, coupled to this uptake is the potential for trophic transfer of BaP from their preys, the phytoplankton.

Hence, when BaP fate of bivalves is considered, it is necessary to assess the availability of the chemical to the animals, the mobilization in various compartments of the contaminated systems, and the capability of the exposed animals to metabolize and excrete the chemical (McElroy, 1990). Because of their relative insolubility in water and strong adsorption to particulate matters, BaP tends to concentrate in bottom sediment (Baumard et al., 1999b). These biochemical properties of the BaP render benthic invertebrates inhabiting in the polluted environments to be exposed to the chemical continuously. However, less information is available on the chemical fate in the aquatic animals.

Albert and Ravendra (2000) fully reviewed biodegradation of PAHs where they explained that numerous genera of micro-organisms had been observed to oxidize kinds of PAH. They also summarized that while there was a great diversity of organisms capable of degrading the low molecular weight PAHs, such as naphthalene, acenaphthene, and phenanthrene, relatively few genera had been observed to degrade the high molecular weight PAHs, such as BaP. Regarding the biodegradation of BaP by marine bivalves, owing to its water insolubility and strong adsorption to particulate matters, the animals inhabiting polluted environments are, often continuously exposed to BaP. However, less information is available on ability of the animals to metabolize and excrete BaP than is available on higher marine organisms. Bivalves have lower enzyme activities metabolizing BaP and typically bioaccumulate the chemical with little alteration (Wade et al., 1989). Therefore, some populations of mussels (*Mytilus* sp.) and oysters (*Crassostrea* sp. and *Ostrea* sp.) have been successfully employed as sentinel organisms to monitor PAH pollution in the coastal waters (Akcha et al., 2000).

However, accumulated informations on the biotransformation of BaP

by marine mussels are available. BaP, as in most PAHs, is known to undergo a wide variety of metabolic transformations in bivalves. Most of the transformations are to be catalyzed by the cytochrome P450 monooxygenases found predominantly in the endoplasmic reticulum and in nuclear membranes. Aromatic molecules are oxidized to phenols and dihydrodiols sometimes via epoxide intermediates. Evidence suggests that the formation of 7,8-and 9,10-epoxides is crucial step in the carcinogenic activity of BaP. Further metabolism of 7,8-and 9,10-epoxides is necessary for the production of the ultimate carcinogen believed to form a nucleic acid adduct.

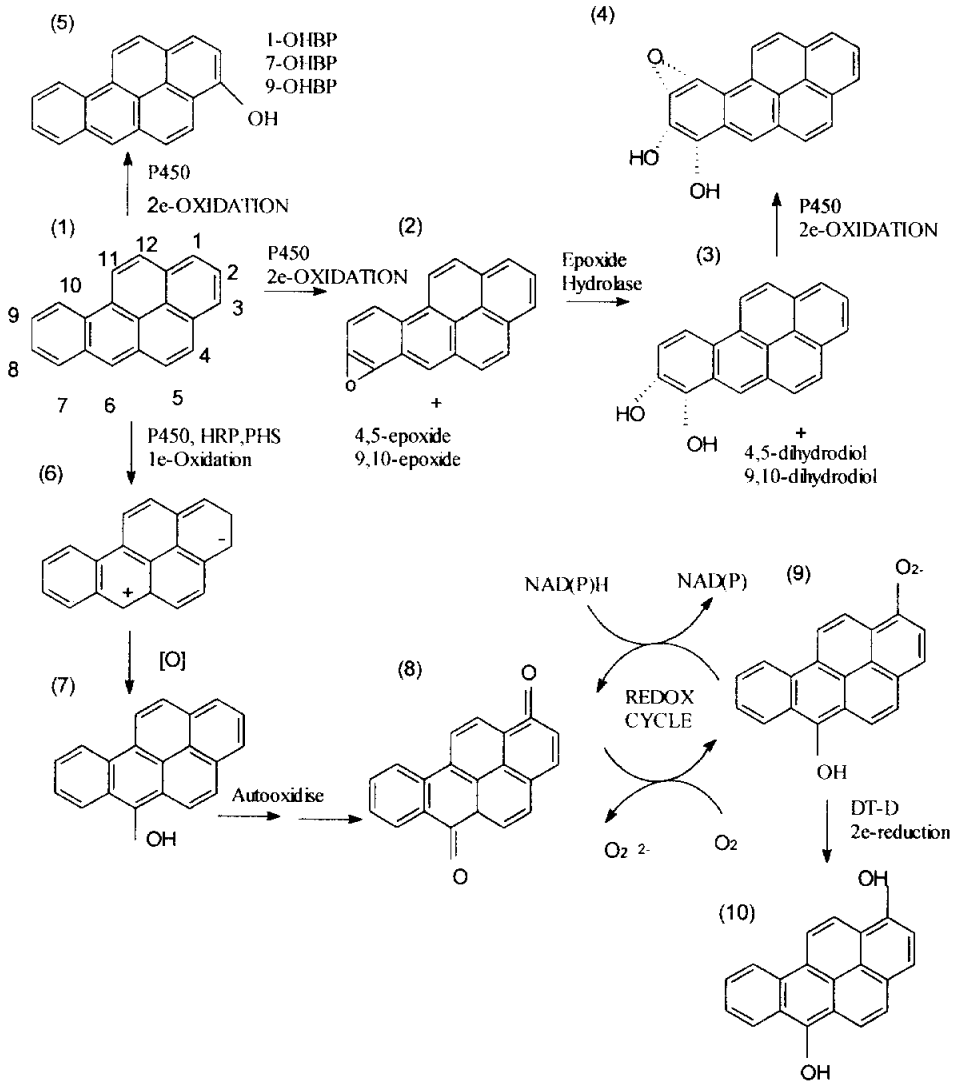

Fig. 2. The pathway involved in the metabolism of benzo(a)pyrene. (1) BaP. (2) BaP 7,8-epoxide. (3) BaP 7,8-dihydrodiol. (4) BaP 7,8-dihydrodiol 9,10 epoxide (BaPDE). (5) various phenols (e.g. 3-hydroxy-BaP). (6) BaP cation radical. (7) 6-hydroxy-BaP. (8) various quinones (e.g. BaP 1,6-quinone). (9) BaP semiquinone. (10) BaP hydroquinone. Abbr.: DT-D, DT-diaphorase; P-450, cytochrome P-450; HRP, horseradish peroxide; PHS, prostaglandin H synthase (from Mitchemore et al., 1998).

Metabolism of PAHs occurs via the cytochrome P450-mediated mixed function oxidase system with oxidation or hydroxylation as the first step (Stegeman et al., 2001). The resultant epoxides or phenols might get detoxified in a reaction to produce glucoronides, sulfates or glutathione conjugates. Some of the epoxides might metabolize into dihydrodiols, which in turn, could undergo conjugation to form soluble detoxification products or be oxidized to diol-epoxides (Samanta et al., 2002). PAHs, including BaP, may be metabolized to reactive intermediates via two major mechanisms (see Fig. 2). The first involves a 2-electron oxidation catalysed by NAD(P)H cytochrome P450 (CYP1A in mammals) and epoxide hydrolase ultimately to form reactive epoxides, in particular trans-7,8-diol-9,10-epoxides which can produce bulky DNA adducts and DNA strand breaks (SB) (Bigger et al., 1994; Maccubbin, 1994). A second mechanism involves an 1-electron oxidation step which can be catalysed by hydroperoxides and cytochrome P450 to form cation radicals, which themselves may bind to DNA and other macromolecules (Livingstone et al., 1990; Cavalieri et al., 1993a,b; Venier and Canova, 1996). Quinones and semiquinone radicals may also be formed by this oxidation, giving rise to further potential DNA adducts and the ultimate production of ROS via redox cycling (Venier and Canova, 1996). Although the rate of BaP metabolism in bivalves is relatively low compared to vertebrates, studies on digestive gland microsomes have indicated these two possible routes of cytochrome P450-catalysed NAD(P)H-dependent BaP metabolism, leading to the formation of phenols and diols and quinones (Livingstone et al., 1997).

Bivalves and other aquatic organisms can take up such contaminants from bottom sediments, suspended particulate material, the water-column

and food-sources (Livingstone, 1991, 1992, 1993; Varanasi et al., 1992). Because of their marked bioaccumulation of contaminants, the analysis of bivalve tissue has for many years been used in pollution monitoring as a measure of environmental contamination. Mussel species in particular have been used extensively in so-called 'Mussel Watch' monitoring programmes because of their filter-feeding and sessile habit, geographical distribution, available numbers, and resistance to general stress (Widdows and Donkin, 1992). In addition they are capable of withstanding baseline levels of pollution and are abundant in estuaries where much human contact with the aquatic environment occurs (Sheehan, 1995). As a matter of fact, the body burden of the chemical contaminants and their consequent biological effects may not simply relate to the external concentration of such compounds, depending upon physiological processes (i.e. metabolism, feeding activity, gametogenesis, etc.) and behavioral reactions (i.e. valve closure) of the mussels, as well as seasonal factors (i.e. temperature), and the combined action of several pollutants both in the environment and in the organisms. For the above reasons, and also for the procedural differences within the published studies, literature data on bioaccumulation and metabolism of common pollutants in mussels are variable or even questionable (Lee et al., 1972; Kurelec, 1987; Nasci et al., 1989; Valerio and D'Ambrosio, 1989; Livingstone, 1991; Michel et al., 1994).

Monitoring the health of the cultured organisms has gained recent interest from researchers engaging in the aquaculture business of bivalves, including the Pacific oyster, *Crassostrea gigas*, one of the most commercially important bivalves cultured in Korea. A sheer number of tools have been offered for the bivalve health monitoring. Typically, the biometry and biochemical composition of bivalves have been served as a

tool for monitoring the animal growth or physiological status because bivalves undergo marked seasonal changes associated with both environmental factors and the annual reproductive cycles (Holland 1978; Pieters et al., 1979; Holm and Shapiro, 1984; Gallager and Mann 1986; Ruiz et al. 1992; Kang et al., 2000). More detailed and specified methods have been subsequently suggested, such as digestive tubule condition (Winstead, 1995), vesicular connective tissue condition, RNA/DNA ratio (Kenchington 1994, Paon and Kenchington 1995), and larval lipid composition (NFRDI Report, 1997). The methods suggested, however, are long term-based. Therefore, development of sensitive and easy-to-touch methods is strongly required. One approach currently being applied is the use of "biomarker", defined as biological responses to a chemical or chemicals that give a measure of exposure or toxic effect. Even if the term has been basically employed in environmental toxicology, it can also afford to provide an early warning of animal health caused by the chemicals (Camus et al., 2000).

Recent understanding of the metabolic physiology of bivalves against xenobiotics brought more sensitive measurements for biomarkers. Akcha et al. (2000) measured enzymatic activity and DNA adduct formation in the BaP-contaminated mussels; CYP1A-like immunopositive protein titration and BaP hydroxylase activity for Phase I biotransformation, GST for conjugation activities, CAT and DTD for potential biomarker of oxidative stress, and AchE for neurotoxicity of BaP exposure. In reality, the extent available for biomarker is increasing.

Generally, blood serves as a good tool for health monitoring of vertebrates. The information from the invertebrates, however, is still lacking. In bivalve, the morphological heterogeneity has been well documented, probably suggesting different functions from different

morphological subpopulations (Renwrantz et al., 1979; Cheng, 1990; Tripp, 1992; Carballal et al., 1997; Lopez, 1997). However, most studies have focused on the hemolymph role in the defence mechanism against exogenous pathogen, summarizing that the hydrolytic lysosomal enzymes of the hemolymph function as one of the immune capacity indicators in many molluscan bivalves (Moore and Gelder, 1985; Cheng, 1988; Beckmann et al., 1992; Bachere, 1995; Dyrynda et al., 1995; Anderson, 1996; Torreilles et al., 1997; Xue and Renault, 2000).

PAHs are one of the key environmental pollutants ubiquitous throughout the global environs, threatening physiological integrity of all kinds of life. They are released into the environment in large part due to human activities such as the combustion of wood and fossil fuels. BaP, a species of the PAH family, is known as a potent carcinogen (Sutherland et al., 1995). It can affect the lysosomal membrane stability of bivalve (Lowe et al., 1995a; Grundy et al., 1996). The neutral red retention (NRR) assay is a chemosensitivity procedure in which cell survival/viability is based on the ability of viable cells to incorporate and bind NR. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates in lysosomes. Alteration of a cell surface or sensitive lysosomal membrane leads to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by an action of xenobiotics result in a decreased uptake and binding of NR, thus, being used for the generation of cellular biomarker to xenobiotics in the ambient environments (Ringwood et al., 1998; Nicholson, 2001; Petrovic et al., 2001).

1.5 BaP in phytoplankton

Understanding of BaP accumulation in phytoplankton under diverse ecological condition is critical in the prediction of the biological fate of the chemical in the aquatic environment (Wang and Dei, 2001). This is because the algae are playing a crucial role in transfer and detoxification of BaP in the aquatic system. Serving as a valuable food sources for the higher trophic level, phytoplankton plays a very important role in supporting the growth of many aquatic biota. The bioaccumulation mechanisms of BaP and other carcinogenic PAHs in marine organisms is of interest in aquaculture, and in the utilization of these marine resources for human food, thus making it essential to understand the fate of such organic contaminants within these organisms (Moore et al., 1986). Unicellular photosynthetic algae have been recently implicated in the metabolism and degradation of foreign organic compounds (Kumar et al., 1988; Peterson et al., 1994). Thus, phytoplankton, like other aquatic microorganisms, may be important in reducing the exposure of higher organisms to the contaminants via processes of adsorption, bioaccumulation and/or metabolism to non-toxic and/or water-soluble forms (Quensen et al., 1998). Conversely, as a food source, phytoplankton may facilitate uptake of water-insoluble contaminants into organisms, so increasing the possibility of toxicity. Therefore, it is not surprising that most of the contaminants in aquatic biota are accumulated by dietary consumption through the food web rather than by direct uptake from water (Evans et al., 1982; Thomann and Connolly, 1984; Van der Oost et al., 1988).

Based on the findings that bivalves accumulate considerable parts of BaP from water and algal food (Barry et al., 1995; Wang and Simpson, 1996), possible modes of BaP uptake in mussels (i.e, soluble and

particulate-bound) were proposed by manipulating the *Isochrysis galbana* concentrations of BaP. Because of their filtering behaviors for feeding, bivalves can accumulate xenobiotics from algal foods (Okay et al., 2000). Interestingly, algae can be easily exposed to the xenobiotics through both aborption and adsorption pathways. These behaviors can be manifested in hours or within a day in some useful marine algae.

BaP metabolisms of terrestrial plants and algae take different ways: The major pathway in terrestrial plants is thought to be mediated by peroxidases and active oxygen species and to a lesser extent by cytochrome P450 (Van der Trenck and Sandermann, 1980; Sandermann, 1988), while in algae such as *Selenastrum capricornutum* metabolism is via a dioxygenase pathway (Schoeny et al., 1988). Degradation of BaP results in the formation of *cis*-4,5-, 7,8-, 9,10-, and 11,12-BaP-dihydrodiols (Table 3). The fate of the metabolites produced by algae and terrestrial plants is different. Algae frequently release the conjugates into the surrounding water, while terrestrial plants store them in the plant body which could result in the consumption by animals and man (Cockerham and Shane, 1994). In summary, metabolism of xenobiotics, including BaP, proceeds in plants in three phases: Phase I (transformation) mostly done by cytochrome P-450 monooxygenases; Phase II (conjugation) mostly done by glutathione S-transferases (GSTs) and glucosyltransferases (GTs); and Phase III (compartmentation) mostly in cell wall fractions or in vacuole (Pflugmacher et al., 1999). However, little information is available on the activities of these enzymes in lower algae.

Table 3. Benzo(a)pyrene degradation by algal isolates (modified from Albert and Ravendra, 2000)

Organism	Growth Substrate	Initial BaP Conc.	Incubation Time	Metabolites Produced	Reference
Selenastrum Capricornu-utum	Glucose Yeast Extract	1.2 mg L^{-1}	4 days	*Cis*-4,5-, 7,8-, 9,10-, and 11,12-BaP-dihydrodiols	Lindquist and Warshawsky (1985)
Selenastrum Capricornu-utum	Glucose Yeast Extract	0.4 mg L^{-1}	24 h	*Cis*-4,5-, 7,8-, 9,10-, and 11,12-BaP-dihydrodiols	Warshawsky et al. (1988)

1.6 Research objectives

To understand BaP effects on the spawners and the next generation of Pacific oyster, *Crassostrea gigas,* serial experiments were carried out; 1) bioaccumulation kinetics of BaP in the adult oyster fed BaP-carrying algal food, 2) depuration kinetics of BaP after 28-day exposure, 3) lysosomal membrane stability of hemocyte in the adult oyster fed BaP-carrying algal food, 4) hatching rate of the fertilized egg, larval growth, larval survival, and attachment of eye-spotted larvae.

II. MATERIALS AND METHODS

2.1 Organisms and Experimental Scheme

A. Oysters

A total of 800 healthy spawners of Pacific oyster, *Crassostrea gigas*, weighing 95.9 ± 21.46 g (mean ± SD) in total weight, were collected from a local oyster farm, Tongyoung, Korea (Table 4). Upon arrival at the laboratory, the oysters were roughly cleaned, removing epifauna on the shells and then acclimated to aquaria under flowing water for 1 week prior to start of the experiment. A total of 390 oysters, 90 oysters each, thereafter, were distributed into four 200 L flow-through aquaria (water volume, 120 L) for the BaP exposure via algal food and depuration.

Table 4. Biometry of *Crassostrea gigas* used

Item measured	Mean	Standard deviation (±)
Shell length (mm)	122.9	12.8
Shell width (mm)	62.9	6.5
Shell height (mm)	30.7	4.6
Shell weight (gr.)	95.9	21.5
Soft body weight (gr.)	22.3	4.4

B. Preparation of benzo(a)pyrene-carrying algae

Three species of algal food organism, *Isochrysis galbana*, *Tetraselmis*

suecica, and *Phaeodactylum tricornutum* originated from the Food Organism Laboratory of Namhae Marine Hatchery, National Fisheries Research and Development Institute (NFRDI), Namhae, Korea were acclimated to the Food Organism Room of NFRDI, Pusan, Korea. The species were cultured in 3 L round flasks in f/2 medium (Guillard and Ryther, 1962) with white light (PAR) at about 140 μmol m^{-2} s^{-1} and a 24 L: 0 D cycle as inocula. Algae at mid-logarithmic growth phase were added to 30L round chambers (culture volume, 20 L) to make final cell densities, 400 to 500 × 10^5 cells mL^{-1} for *I. galbana* and 200 to 300 × 10^5 cells mL^{-1} for *T. suecica* and *P. tricornutum.* The cultures were maintained in the f/2 medium at 20 ± 0.5°C for 24 hours after addition of acetone-vectored BaP concentrations; 0 ppb (acetone vehicle only), 50 ppb, 500 ppb, and 5000 ppb.

C. Oyster exposure to benzo(a)pyrene

Oysters were exposed to 4 different concentrations of xenobiotic benzo(a)pyrene (Sigma) for 28 days and then depurated for another 28 days. For the exposure, each of four oyster cultures (control, 50 ppb, 500 ppb, and 5000 ppb oysters) was fed with 20 L of 400 to 500 × 10^5 cells mL^{-1} of *I. galbana,* 200 to 300 × 10^5 cells mL^{-1} of *T. suecica,* or *P. tricornutum* grown in four different concentrations of BaP, 0, 50, 500, 5000 ppb, for 24 hours, respectively. Oysters were basically fed with *I. galbana* and *T. suecica* alternatively with occasional *P. tricornutum* feeding.

Feeding scheme was 4 times a day with an interval of 8 hours. Just before feedings were practiced, the flowing waters were stopped, keeping the water volumes 80, and then 20 L BaP-carrying algae were added. Each

feeding was continued within 30 minutes by the time all the algae added to make total volume 100 L Each feeding was continued within 30 minutes by the time all the algae added were consumed by the cultured oysters. After the feeding, the tanks were maintained in flow-through manner to keep culture volume 120 L until next feeding.

The sand and charcoal-filtered seawater was used throughout the culture. Water qualities were in the range of 11-14 (± 0.5)°C for temperature and 1.0268-1.0274 for specific gravity in this study (Table 5).

D. Depuration of benzo(a)pyrene

Once water temperature was established at 25 (± 0.5)°C after the water temperature transition, the temperature remained throughout the depuration of the oysters. During the depuration, oysters were also conditioned to gain sexual maturation by feeding on pure *I. galbana* and *T. suecica* supplied 3 to 5 times according to the feeding behavior of the oysters. As in the water temperature transition period, *P. tricornutum* and *Chaetoceros gracilis* were also supplied to the culture tanks as an occasional food for the nutritional valance of the oysters.

2.2 Determination of benzo(a)pyrene in digestive gland

According to Moon et al. (2002) pooled digestive glands of five oysters were used per sample for BaP analysis. A bottom side of the oyster shells was removed by cutting the base of the adductor mussel with a special care not to hurt any part else of muscle. The oyster tissues were wraped with an alumium foil and frozen at -70℃. The oysters were homogenized with an Ultra-disperser. Homogenized samples were freeze-dried and

internal standard (ES 2044, Cambridge Isotope Laboratories, Inc.) was spiked. They were decomposed in 200 mL of 1 N KOH ethanolic solution of 2 hours by mechanical shaking. The digest was liquid-liquid extracted with twice using 100 mL of *n*-hexane (Ultra resisue analysis, J.T. Baker) after addition of water and 50 g of anhydrous Na_2SO_4. The extracts were reduced to small volume in a rotary evaporator and then adjusted to a volume of 10 mL. The extracts of samples were purified using an activated silica gel (Art No. 7734, 70-230 mesh, Merck) column chromatography with successive elutants of *n*-hexane and 15% methylene dichloride (Pesticide residue analysis, Cica-Merck) in *n*-hexane. The second fraction was concentrated to less than 1 mL, and left at a room temperature for one or two days to evaporate to 100-200 $\mu\ell$. The residues were dissolved with 100 $\mu\ell$ of *n*-nonane (Pesticide residue analysis, Fluka) and determined for PAHs.

The aromatic fraction was analyzed by gas chromatography coupled to mass spectrometry. An Agilent 6890 series GC (Agilent, USA) equipped with a split/splitless injector was used (splitless time: 2 min; flow: 70 mL/min). The injector temperature was maintained at 250℃. The GC temperature program was from 80℃ (1 min) to 300℃ (10 min) at 5℃/min. The carrier gas was helium at a constant flow rate of 1.2 mL/min. The capillary column used was DB-5MS (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness, J & W Scientific). The GC was coupled to an 5973N mass selective detector (MSD). The mass spectrometer was operated under the selected ion monitoring (SIM) mode using molecular ions of the investigated PAHs (electron impact at 70 eV, 2000 V, 1.5 scan/s, dwell time/ion: 40 ms). The interface temperture was 250℃.

The spiked internal standard was dected with no interfering peak. In order to assess the accuracy of the determination by experimental

procedure and instrument, the certified mussel homogenate (1974, NIST) was analyzed as Standard Reference Materials (SRMs) in this study. The recovery result for BaP was 93.5 ± 3.4%. Procedural blanks were processed in the same manner as real samples, and they were below 10 % of analytes abundance. Blank was run before and after the injection of standard solutions to checks for any carryover. The calculated detection limit (S/N ratio = 5) for BaP in oyster sample was 5 pg/g dry weight.

2.3 Cytotoxicity of benzo(a)pyrene

A. Oysters

A total of 500 healthy adults of *C. gigas*, weighing 85.58 ± 18.47 g (mean ± SD) in total weight, were collected from a local oyster farm, Tongyoung, Korea. Upon arrival at the laboratory, the oysters were acclimated to aquaria under flowing seawater for 1 week prior to the start of the experiment. A total of 350 oysters, 35 oysters each, thereafter, were distributed into 50 L flow-through aquaria (water volume, 40 L) for the BaP exposure via algal food and depuration. Another 390 animals were evenly distributed to 10 rectangular bottles (water volume, 30 L) for the immersing exposure to BaP.

B. NR retention

The retention of the neutral red within the lysosomes of the oyster hemocytes was determined using an adaptation of the established protocol (Lowe et al., 1995a). A stock solution of neutral red (Sigma) was prepared by dissolving 10 ㎎ in 1 mL of dimethyl sulfoxide (DMSO). After

filtering through a 0.5 μm millipore filter (Whatman), the solution was stored in a dark container at 4℃. A working solution of NR was prepared immediately before use, by diluting CMFS (calcium magnesium free saline, final dye concentration 40 $\mu g\ mL^{-1}$). Hemolymph extracted from each oyster was diluted 1:1 with CMFS. A 50 μL aliquote of the cell suspension was dispensed onto a microscope slide for 15 min in a light-proof humidity chamber to allow cells attach. A 40 μL of the neutral red working solution was added and coverslip was applied. The slides were returned to the humidity chamber for further 15 min. Then, the slides were examined under light microscope. The endpoint of the assay was defined as the time at which 50 % of the cells had started to lose dye from their lysosomes.

C. Determination of hydrolytic enzyme activity in hemolymph

The hemolymph withdrawn directly from the pericardial cavity of oysters with 1 mL syringe equipped with a needle was pooled in an ample and diluted 1:3 in cold modified Alsever's solution (Bachere et al., 1988). A volume of suspension with 2.5×10^6 cells was centrifuged at 750 g for 10 min and the pellet was resuspended in 1.4 mL of distilled water. A 65 μL of the suspension was deposited in each well of a plate containing the substrates for each enzyme reaction. After incubation at 37℃ for 4 hr, the enzyme activities were disclosed with reagents of the API ZYM System (bioMerieux). The enzyme concentration was estimated according to a color scale given by the manufacturer.

2.4 Benzo(a)pyrene effects on reproduction of oyster

A. Water temperature transition for conditioning maturation

After 28-day exposure to BaP, the oysters were conditioned to make reproductive system developed by temperature manipulation. For this work, water temperature was raised to 25 (± 0.5)°C from previous temperature, 14 (± 0.5)°C for 5 days. Temperature raising was achieved with the aid of a heating tank with 2-ton capacity connected to the culture tanks. During the water temperature transition, of pure *I. galbana* and *T. suecica* were supplied 3 to 5 times according feeding behavior of the oysters. *P. tricornutum* and *Chaetoceros gracilis* were also fed occasionally for the nutritional valance of the oysters.

B. Production of gametes and fertilization

Spawners of the oyster were conditioned to be reproductively mature by manipulating the water temperature to 25 (± 0.5)°C from previous 14°C for 5 days and keeping them in the favoring condition for another 28 days. During the conditioning period, spawners were carefully managed not to stimulate premature spawning. After 28-day of reproductive conditioning, eggs and sperms were stripped from ripe oysters with quality-good gametes under the microscopy. The fertilized eggs were collected using a net (mesh size, 20 μm), rinsed in the sterilized seawater, and distributed evenly in a hatching tank.

C. Larval oyster culture and larval measurements

The newly hatched larvae collected were distributed evenly into 12 larval culture chambers (carrying capacity, 20 L) at a density of 40,000 larvae per chamber. The larval cultures were divided into 4 groups for control, 50 ppb, 500 ppb, and 5000 ppb culture, with each group of 3 cultures. D-shaped larvae were fed with *I. galbana* for the first week and thereafter with *I. galbana*, *T. suecica*, *P. tricornutum*, and *C. gracilis* twice a day. All the culture water were replaced by new ones on the every two-day basis. The cultures were continued until first appearance of the eye-spotted larvae in the culture chamber.

Each culture was sampled randomly for the measurement of larval survivals. Larval survival was measured daily, using three 10 mL subsamples on each occasion. Counts and measurements were made under a profile projector (Nikon V-12, Japan).

D. Spat attachment, culture, and measurements

As soon as any of the cultures showed an eye-spotted larva, the cultures were served with the previously cleaned oyster shells as a collector on the basis of 1000 oyster larvae per shell. Collectors were removed from the culture chambers 2 days after the attachment, and set in the flow-through system. Two to three times of feeding per day were practiced with the addition of *I. galbana*, *T. suecica*, *P. tricornutum*, and *C. gracilis*. The feeding times continued for 1 hour.

The spat survivals were measured 10 days after the installation of the collectors in the culture chamber with the aid of exaggerating apparatus.

2.5 Statistics

Statistical analysis of the data was carried out, using Student's *T*-test in the Sigma Plot Software.

III. RESULTS

3.1 Manipulation of water quality

Table 5. Water quality in the animal culture tanks during benzo(a)pyrene accumulation, temperature transition, and depuration/sexual maturation

Water quality	BaP exposure for four weeks	Water temperature transition for 5 days	Depuration/ maturation/ conditioning for 4 weeks
Water temperature (℃)	11-14 (± 0.5℃)	2℃ increase per day	25 (± 0.5)℃
Specific gravity	1.0268-1.0274	1.0268-1.0274	1.0268-1.0274
Flowing rate (L/hr)	500	400	400

Water quality, particularly in water temperature, in the culture tanks was manipulated for the oyster control for the specific physiological status. Table 5 shows the water qualities manipulated for the BaP exposure, water temperature transition, and depuration/sexual maturation induction. The water temperature during the chemical exposure ranged from 11~14 (± 0.5)℃, depending on the environmental water temperature. After the chemical exposure, the water temperature was manipulated to increase up to 25 (± 0.5)℃ on the basis of 2℃ increase per day. The temperature increased was maintained throughout the depuration or sexual maturation of the oyster. Salinity expressed as a specific gravity remained in

1.0268~1.0274 throughout the experiment. The culture system was basically operated by flowing-through fashion. The flowing rates were 500 L/hr for BaP exposure, 400 L/hr for water temperature transition, and 300 L/hr for depuration or sexual maturation of the oyster.

3.2 Determination and identification of benzo(a)pyrene by GC and GC/MS

The BaP in the digestive gland of the oyster was determined, using gas chromatography coupled with mass spectrometry.

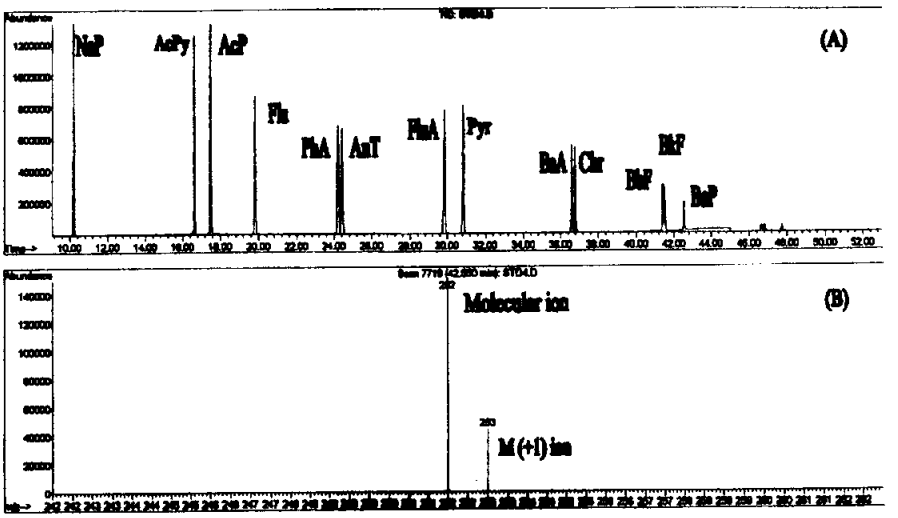

Fig. 3. (A) expresses GC chromatogram of standard PAHs, including naphthalene, acenapthylene, acenaphthene, fluorene, phenanthrene, anthrancene, fluoranthene, pyrene, benz(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, indeno(*1,2,3-c,d*)pyrene, dibenzo(*a,h*)anthracene, and benzo(*g,h,i*)perylene. (B) represents GC/Mass identification of benzo(*a*)pyrene. The fragment at *m/z* 252 is molecular ion with a ratio of M+1 to M, 21.4.

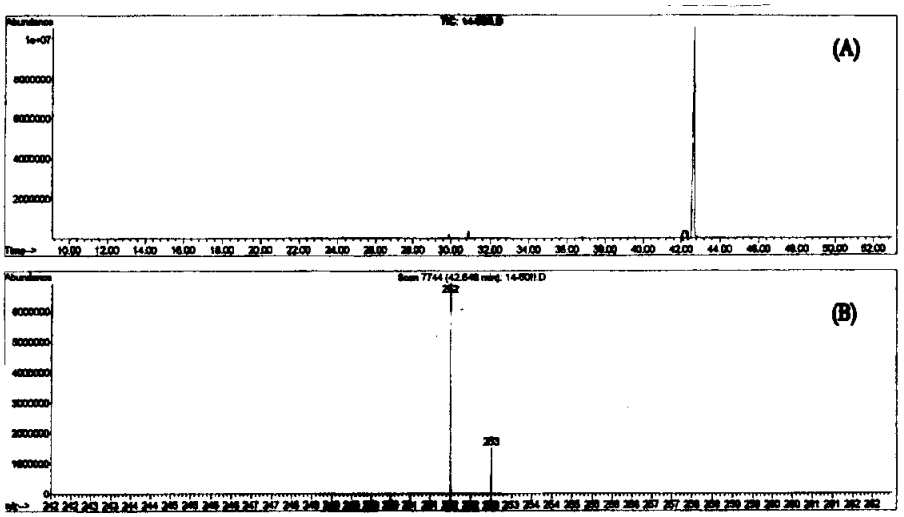

Fig. 4. shows GC chromatogram of benzo(*a*)pyrene (A) and GC/Mass identification of molecular and M+1 ions (B) in the sample from benzo(a)pyrene-exposed oysters at 50 ppb.

3.3 Bioaccumulation and depuration of benzo(a)pyrene by oyster

BaP-treated *C. gigas* with the chemical-carrying algae for 28 showed elevated concentrations of BaP in the oyster digestive gland (Table 6). The concentrations of BaP of control remained low, lower than 5.0 ng/g dry weight. In the 4-time feeding experiment, the level increased sharply 172, 1071, and 5994 ng/g dry weight digestive gland for 50 ppb, 500 ppb, and 5000 ppb oysters, respectively. The increase by day 1 was clearly concentration-dependent. The increases continued by day 14 over the three cultures in a similar manner as the previous values, marking 5245, 21700, and 29971 for 50 ppb, 500 ppb, and 5000 ppb, respectively. The increase after day 21 differed among the cultures. For example, 50 ppb oysters continued to bioaccumulate the chemical by the end of the accumulation experiment, by showing 8026 ng/g digestive gland BaP by day 21 and 9866 ng/g digestive gland BaP ng/g digestive gland BaP by the end of the experiment. The concentration increase of 500 ppb culture was by 21 day, while that of 5000 ppb culture by 14 day. The oysters fed BaP carrying algal foods bioaccumulated the chemical in a concentration-dependent manner for accumulation; $Y = - 0.0085x + 3.2299$ ($r^2 = 0.0105$) for control, $Y= 387.11x - 573.08$ ($r^2 = 0.9870$) for 50 ppb, $Y= 1035.2x + 3798.6$ ($r^2 = 0.8813$) for 500 ppb, and $Y= 831.95x + 12561$ ($r^2= 0.7084$). The depuration, however, was not clearly expressed for 500 and 5000 ppb oysters; $Y= -324.91x + 19597$ ($r^2 = 0.9748$) for 50 ppb, $Y= -152.8x + 34590$ ($r^2 = 0.3294$), and $Y= -84.982x + 33632$ ($r^2 = 0.6487$).

Depurations were notable in the three experimental cultures. This was particularly evident in the culture of 50 ppb oysters. The maximum BaP concentration of 50 ppb culture was by day 28. This figure, thereafter,

started to decrease with the commencement of the depuration to 9069 ng/g by depuration day 7 and to 1191 by the end of the depuration. The levels in the depuration of 500 ppb and 5000 ppb cultures were 25123 and 29126 ng/g digestive gland BaP, respectively.

The BaP accumulation and depuration in the digestive gland were expressed for the 4 concentrations (Fig. 5). In the modes of bioaccumulations of BaP, the oysters exposed to higher concentrations of BaP accumulated the chemical more significantly but depurated in a slower manner. In the low exposure, both accumulation and depuration were significant, but never reached the bottom level within 28 days of the depuration.

Table 6. Benzo(a)pyrene accumulation in digestive gland of *Crassostrea gigas* treated with different concentrations of the chemical

BaP* Conc.	*Day exposed*								
	0	1	3	7	14	21	28	D7**	D28**
0	1.5	-	-	4.5	5.0	3.0	4.5	_	1.5
50	1.5	172.0	296.1	1601.5	5245.7	8025.9	9866.4	9069.4	1191.0
500	1.5	1070.9	5138.6	14413.5	21700.3	29433.7	27638.8	32736.5	25122.5
5000	1.5	5994.0	13690.7	25907.0	29971.4	29356.5	32010.6	29646.5	29125.6

* Benzo(a)pyrene concentration of vectoring algal culture. Twenty-liter of algal vectoring culture was supplied to 100 L of oyster culture to make 120 L of total culture volume during the algal vectoring chemical exposure, using *Tetraselmis suecica, Isochrysis galbana*, and *Phaeodactylum tricornutum* pre-exposed to the chemical concentrations for 24 hr.

** D represents day after depuration after 5-day water temperature transition.

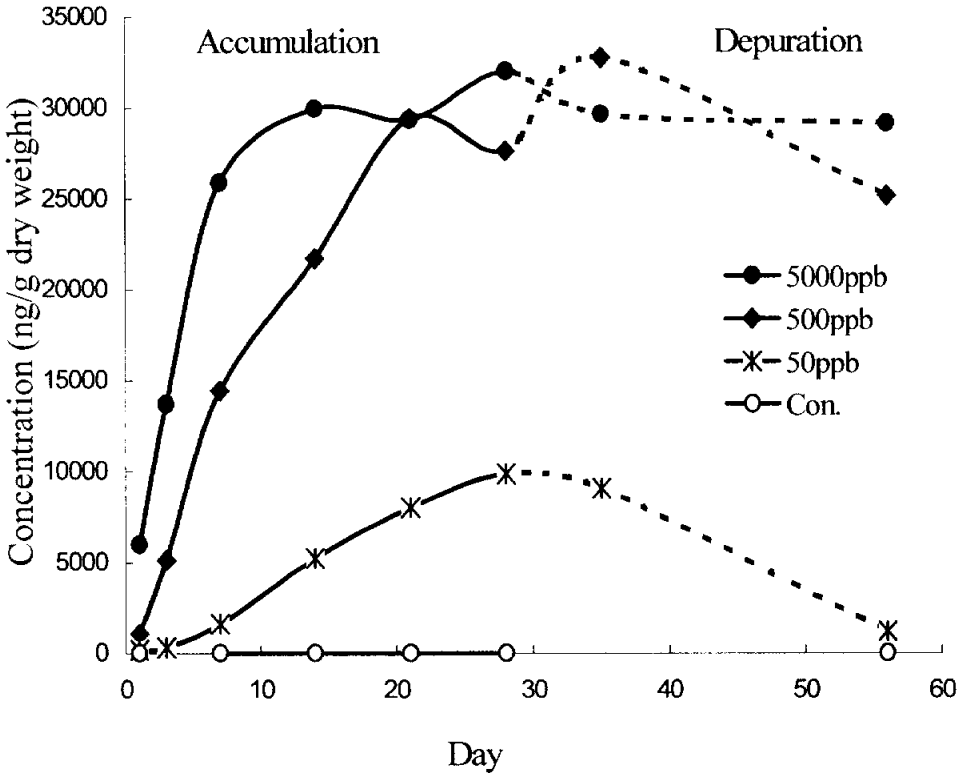

Fig. 5. Benzo(a)pyrene concentration in pooled digestive gland of Pacific oyster. Twenty-liter of algal vectoring culture was supplied to 80 L of oyster culture to make 100 L of total culture volume during the algal vectoring chemical exposure, using *Tetraselmis suecica, Isochrysis galbana,* and *Phaeodactylum tricornutum* pre-exposed to the chemical concentrations for 24 hr. Solid and break lines stand for accumulation and depuration curves, respectively.

Table 7. Linear regression equations for benzo(a)pyrene levels in the digestive gland of *Crassostrea gigas* for 28-day accumulation and 28-day depuration, where Y = BaP levels (ng/g, dry weight); x = days

BaP* conc.	*Linear Regression Equations*	
	Accumulation (day 0 – 28)	Deputation (day 28 – 56)
Con.	Y = - 0.0085x + 3.2299 (r^2 = 0.0105)	
50	Y= 387.11x - 573.08 (r^2 = 0.9870)	Y= -324.91x +19597 (r^2 = 0.9748)
500	Y= 1035.2x + 3798.6 (r^2 = 0.8813)	Y= -152.8x + 34590 (r^2 = 0.3294)
5000	Y= 831.95x + 12561 (r^2= 0.7084)	Y=-84.982x + 33632 (r^2 = 0.6487)

* Benzo(a)pyrene concentration in pooled digestive gland of Pacific oyster. Twenty-liter of algal vectoring culture was supplied to 80 L of oyster culture to make 100 L of total culture volume during the algal vectoring chemical exposure, using *Tetraselmis suecica, Isochrysis galbana*, and *Phaeodactylum tricornutum* pre-exposed to the chemical concentrations for 24 hr.

3.4 Benzo(a)pyrene effects on lysosomal membrane stability

Hemocytes were withdrawn from *Crassostrea gigas* exposed to different concentrations of BaP, and determined the lysosomal membrane properties of the oyster for a development of cellular biomarker to the xenobiotic chemical. Table 8 shows cellular viabilities of the oyster hemocytes expressed as a Neutral Red Retention (NRR) in minute. The NRR of the xenobiotic-free control lysosomes was 133 ± 6.06 (mean ± SD) minutes just after 10-day acclimation in captivity. It showed a trend decreasing along the progress of the experiment even if a little deviation from the trend was noticed; 123 ± 10.41 by week one, 103 ± 7.63 by week four, and 93 ± 5.77 minute by week eight. This explains the oysters have intrinsic stress from the captive life in the laboratory although the first significant decrease ($P<0.05$) was noticed by day 21. In overall sense, the NRR was adversely proportional to concentration and duration of the chemical exposed. The decreasing trend was much evident in the oysters exposed to higher concentrations of the chemical. For example, the first significant decrease from control ($P<0.05$) was from day 21 for control, day 7 for 50, day 3 for 500, and day 1 for 5000 group. A further significant decrease of $P<0.01$ was found from day 3 for 5000 group, while from day 7 for 500 and day 21 for 50 group. No significant decrease of $P<0.01$ was noticed in the control during the exposure test. Interestingly, a recovering trend was observed during the depuration, and the trend was more prominent in the oysters exposed higher BaP concentrations. The NRR recovery in the oyster exposed to highest concentration from 35 to 45. This was significant, particularly considering that the intrinsic stress coming from captive culture in the laboratory was from 103 to 93 during the recovery in control.

Table 8. Lysosomal membrane stability of *Crassostrea gigas* affected by benzo(a)pyrene concentrations expressed as NRR in minute (mean ± SD)

Bap Conc. (ppb)*	*Day after exposure*							
	0	1	3	7	14	21	28	D28**
Con. (0)	133 (6.0)	132 (7.6)	135 (5.0)	123 (10.4)	121 (7.6)	110^{+} (13.2)	103 (7.6)	93 (5.8)
50	-	128 (2.9)	132 (10.4)	107^{+} (20.2)	110 (8.7)	92^{++} (7.7)	83 (7.6)	82 (12.6)
500	-	127 (7.6)	85^{+} (18.0)	67^{++} (7.6)	70 (5.0)	68 (7.7)	57 (5.0)	55 (5.0)
5000	-	113^{+} (12.6)	63^{++} (15.3)	57 (2.9)	57 (5.8)	52 (10.4)	35 (13.2)	45 (5.0)

*Benzo(a)pyrene concentration of vectoring algal culture. Twenty liters of which was supplied to 80 L of oyster culture to make 100 L of total culture volume during the alga-vectored chemical exposure, using *Tetraselmis suecica, Isochrysis galbana*, and *Phaeodactylum tricornutum* pre-exposed to the chemical concentrations for 24 hr.

**D represents day after depuration.

$^{+}$ First appearance of statistically significant decrease from control ($P<0.05$) in the Sigma Plot Software.

$^{++}$ First appearance of statistically significant decrease from control ($P<0.01$).

3.5 Benzo(a)pyrene effects on hydrolytic enzyme activity

Nineteen hydrolytic enzyme activities of the hemocyte of *C. gigas* fed BaP-treated algae were studied, using API ZYM System. The control hemocytes of *C. gigas* showed enzyme activities of alkaline phosphatase, esterase (C4), acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, β -glucuronidase, and N-acetyl-β -glucosaminidase. The hydrolytic enzyme activities varied, but remained significant in all experimental groups throughout the experiment (data not shown). All the enzyme activities, however, failed to show some trends accordingly to the concentrations and durations of the chemical exposed except for activation of acid phosphatase and alkaline phosphatase which showed some potential available for the generation of enzymatic biomarker.

Table 9 exhibit the hydrolytic activities of acid phosphatase of oysters exposed to BaP concentrations. Acid phosphatase activity was stimulated by the increased chemical concentrations. In the lower concentration (50 ppb), the activity increased with duration, while in the higher concentrations, the level by day 1 was already over 30 nM which pertained throughout the experiment.

Table 10 show the hydrolytic activities of alkaline phosphatase exposed to BaP. Alkaline phosphatase activity appeared to be stimulated by the chemical concentrations. Higher concentration of BaP stimulated the enzyme activity from the beginning of the experiment. The increased enzyme activity showed a decreasing tendency, thereafter particularly in the oysters exposed to higher concentrations of the chemical. For example, the 500 ppb and 5000 ppb groups reached maximum level by day 7, and thereafter, both of the groups showed decreased enzyme activity. The decrease in the activity was more significant in the 5000 group.

Table 9. Benzo(a)pyrene effects on the expression of acid phosphatase activity in the hemolymph of *C. gigas*

Bap Conc. (ppb)*	*Day after exposure*							
	0	1	3	7	14	21	28	D28**
Con.	2.0***	2.5	3.5	2.5	2.5	3.5	2.5	2.0
50	-	2.5	2.0	2.5	3.0	4.0	4.5	4.5
500	-	4.0	4.0	4.5	4.0	3.0	4.5	5.0
5000	-	4.5	5.0	4.0	4.5	5.0	4.5	5.0

*Benzo(a)pyrene concentration of vectoring algal culture. Twenty liters of which was supplied to 80 L of oyster culture to make 100 L of total culture volume during the alga-vectored chemical exposure, using *Tetraselmis suecica, Isochrysis galbana*, and *Phaeodactylum tricornutum* pre-exposed to the chemical concentrations for 24 hr.

** D represents day after depuration.

***Acid phosphatase activity determined by API ZYM System was represented as a level of nM of substrate hydrolyzed in the enzyme reaction, where, 1=5nmol, 2=10nmol, 3=20nmol, 4=30nmol, 5≤40nmol.

Table 10. Benzo(a)pyrene effects on the expression of alkaline phosphatase activity in the hemolymph of *C. gigas*

Bap Conc. (ppb)*	*Day after exposure*							
	0	1	3	7	14	21	28	D28**
Con.	3.0***	3.0	2.5	3.0	2.5	2.5	2.0	2.0
50	-	3.0	3.5	4.0	4.0	5.0	4.5	4.0
500	-	4.5	4.5	5.0	3.5	1.0	1.0	2.5
5000	-	5.0	4.5	5.0	2.0	1.5	1.0	1.0

*, ** As in Table 9.

***Alkaline phosphatase activity is as in Table 9.

3.6 Survival of larvae from benzo(a)pyrene-treated spawners

Oyster larvae were obtained from four spawners which were previously exposed to different concentrations of BaP and then cultured under water temperature of 25± 0.5℃ until any of the cultures showed first occurrence of eye-spotted larva (Fig. 6). Eye-spotted larvae first appeared in the culture of intact control by day 14. In the 14-day culture, larval survival of the control was quite a normal. A few of the larvae showed mortality in the first 8 days, still showing 83% of survival rate by the time. Most significant mortalities were found from day 10 ($P<0.01$) with the particularly significant mortality just before the first appearance of the eye-spotted larva, finally showing 21% of survival from previous measurement, 43%.

The larval survival of 50 ppb was still similar to that of control. Interestingly, it was a little bit higher over control without statistical difference. However, the mortality by day 10 was much more significant, representing 47% by day 10 from previous measurement, 86% by day 8. The final survival rate of 50 ppb-larvae was 14%, which was significantly low compared with control one ($P<0.05$), 21%. The difference of potential survival rate between the two larvae at the time of eye spot stage might be more significant, considering that none of the 50 ppb-larvae reached eye spot stage by the time of day 14.

The survival of 500 ppb-larvae was slightly lower than those of control and 50 ppb-larvae for the first 6 days. However, this was not statistically significant. The first significant difference was found from day 8 where the survival rate of 500 ppb-larvae was 62%, quite low from control and 20 ppb-larvae, 80 and 83%, respectively. Thereafter the survivals declined sharply to make 27% by day 10, 14% by day 12, and 4% by day 14. These figures were significantly lower than those from control and 50 ppb-larvae.

The larval survival of 5000 ppb group started to decline from day 4, finally reaching 0.2% at the end of the experiment via 82, 66, 29, 11, and 4% by days 4, 6, 8, 10, and 12, respectively. The mass mortality in the 5000 ppb-larvae resulted in total crash before none of the larvae reached eye-spotted stage.

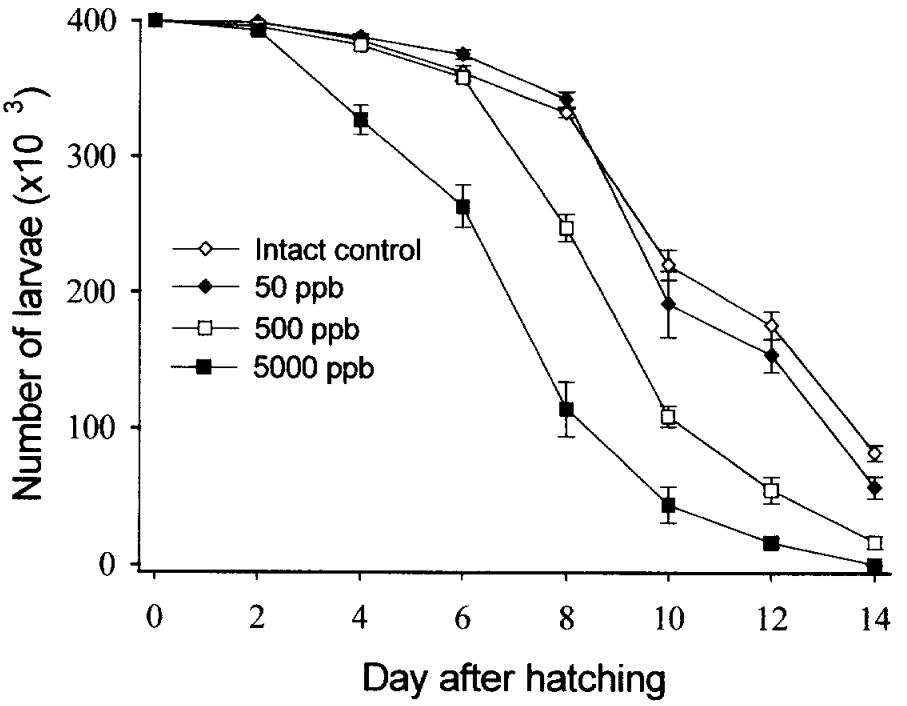

Fig. 6. Larval survival of *Crassostrea gigas* from different spawners under influence of different concentrations of benzo(a)pyrene for 28 days and then under maturation-inducing condition without the chemical for another 28 days. Benzo(a)pyrene exposure to the spawners was achieved by "algal vectoring method" in which the spawners were fed 3 species of algal foods grown under 4 concentrations of benzo(a)pyrene, 0, 50, 500, and 5000 ppb. Maturation of the spawners was gained by raising temperature to 25 (± 0.5)℃ from the previous temperature regime (see Table 5). The culture of the oyster larvae continued until first appearance of an eye-spotted larva from any cultures. Vertical bar represents mean± standard error.

3.7 Growth of larvae from benzo(a)pyrene-treated spawners

Oyster larvae were obtained from four spawners which were previously exposed to different concentrations of BaP and then cultured under water tempaerature of 25 ± 0.5℃ until any of the cultures showed first occurrence of eye-spotted larva (Fig. 7). In the 14-day culture, where eye-spotted larvae first appeared in the culture of intact control, larval growths were distinctly separated by two groups, control and 50 ppb-larvae for faster group and 500 ppb- and 5000 ppb-larvae for slower group. Besides the growth difference between the two groups, the latter group was characterized by higher variation in size distribution over the former one.

The sizes of the just hatched larvae ranged 0.0736, 0.0747, 0.0731, and 0.0735 mm in shell length for control, 50 ppb, 500 ppb, and 5000 ppb-larvae, respectively. The larval growth was steady to reach 0.2361 mm in shell length by the end of the experiment in control when first appearance of eye-spotted larvae was noticed. The maximum size of the eye-spotted larvae was 0.3310 mm in shell length for control, while minimum remained still small, 0.1240 mm in shell.

The size difference between control and 50 ppb larvae started to be noticed from day 6, although it was not significant in statistics. The first significant difference between the two subgroups was marked from day 8 ($P<0.05$). The sizes of the 50 ppb larvae at the end of the experiment were 0.2189 mm with extreme sizes, 0.3260 for upper extreme and 0.0780 mm in shell length for lower extreme. Although upper extreme reached 0.3260 mm in shell length, the abundance of the size was very low and none of the larvae reached the stage with eyespot.

Larval growths of 500 and 5000 ppb group were almost stagnant by day 6, the growth for the first 6 days were 0.0767 mm in shell length for

500 ppb larvae and 0.0789 mm for 5000 ppb larvae. Considering the initial sizes, the net size growths remained 0.0036 mm for 500 ppb larvae and 0.032 mm for 5000 ppb larvae. These figures were considerably lower than those from control and 50 ppb larvae, where the net growths for the control and 500 were 0.0205 and 0.0146 mm in shell length, respectively.

The size difference between 500 and 5000 ppb larvae first noticed from day 10 without statistically significant meaning. The larval growths in the last four days were 0.1053, 0.1254, and 0.1392 mm in shell length for 500 ppb larvae and 0.0937, 0.1065, and 0.1172 mm for 5000 ppb-larvae. The final net size gains of the four cultures were 0.1625, 0.1442, 0.0661, and 0.0437 mm in shell length for control, 50 ppb, 500 ppb, and 5000 ppb-larvae, respectively. Although a few larvae from 500 ppb and 5000 ppb cultures reached larger than 0.2300 mm (maximum 0.2620 mm for 500 ppb and 0.2310 for 5000 ppb-larvae), considerable parts of the larvae remained un-grown. This was particularly true in 5000 ppb-larvae, where notable parts of the larvae remained in the size of initial control.

C. gigas were stressed with concentrations of BaP for 28 days and then conditioned to reach maturation-inducible status by manipulating water temperature and by supplying them with suitable algal foods in the flow-through maturation tanks for another 28 days. Table 7 summarizes the culture condition, larval growth, and spat attachment on the collector. The oysters treated with concentrations of BaP by means of algal vectoring method successfully achieved maturation status, even though considerable percent of 5000 ppb-spawners failed to reach a favorable status.

The gametes obtained by stripping method showed hatching rates of 88.5, 86.0, 82,0, and 19.0% for control, 50 ppb, 500 ppb, and 5000 ppb gametes, respectively. The survival rates of the hatched larvae were 20.5, 11.5, 0.2, and 0% of initial 400,000 individuals contained in the 30 L

culture chamber for control, 50 ppb, 500 ppb, and 5000 ppb larvae, respectively. Days to first appearance of eye-spotted larvae at water temperature 25 (± 0.5)℃ were 14 for control, 15 for 50 ppb larvae, and 19 for 500 ppb larvae. None of the larvae from 5000 ppb spawners failed to reach eye-spotted stage.

The shucked oyster shell were installed as a collector in the larval culture on the basis of 1,000 larvae at the time the first eye-spotted larval appearance to find the BaP effects on the larval attachment. The spat attachment on the collector was also significantly different among the cultures 2 weeks after the collector installment. The numbers of the spat attached on the collector were 14.3, 7.57, and 0.60 for 500 ppb spats.

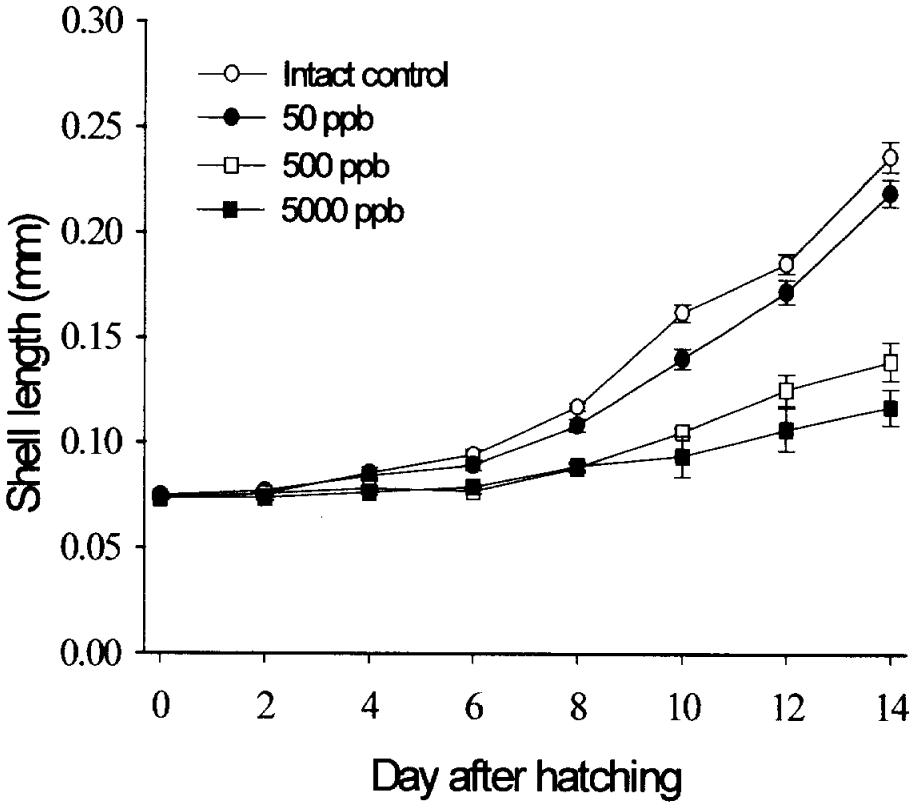

Fig. 7. Larval growth of *Crassostrea gigas* from different spawners stressed with different concentrations of benzo(a)pyrene for 28 days and then conditioned for maturation-induction without the chemical for another 28 days. For the BaP treatment to the oyster, concentrations of the chemical were exposed to the spawners by "algal vectoring method" as in Fig. 4 in which the spawners were fed 3 species of algal foods grown under 4 concentrations of BaP, 0, 50, 500, and 5000 ppb. Maturation of the spawners was gained by raising temperature to 25± 0.5℃ from the previous temperature regime (see Table 5). The culture of the oyster larvae continued until first appearance of an eye-spotted larva from any cultures. Vertical bar represents mean± standard error.

Table 11. Effects of benzo(a)pyrene-treated spawners on their larval growth and spat attachment

BaP conc.* Treated (ppb)	Hatching Rate (%)	Day to ESL[1] at 25℃	Survival (%) at control ESL	No at ESL (No collector)	No[2] spat attached 2-week after ESL
Control	88.5	14	20.5 (82,600**)	82,600 (82)	14.3
50	86.0	15	11.5 (46,100)	38,300 (38)	7.57
500	82.0	19	0.2 (9,100)	4,600 (5)	0.60
5000	19.0	-	-	-	-

* Benzo(a)pyrene concentration of vectoring algal culture.
[1]Stands for first appearance of eye-spotted larva.
[2]No. of spat attached on a shucked oyster shell installed as a collector in the larval culture on the basis of 1,000 larvae at ESL per each shell.
** Number in parenthesis represents larval survival at ESL.

IV. DISCUSSION

4.1 Gas chromatography-mass spectrometry analysis

The native standards of PAHs, including naphthalene, acenapthylene, acenaphthene, fluorene, phenannthrene, anthrancene, fluoranthene, pyrene, benzo(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, indeno(*1,2,-3-c,d*)pyrene, dibenzo(*a,h*)anthracene, and benzo(*g,h,i*)perylene were successfully dectected in the chromatogram of GC coupled with mass spectrometry at the retention times, around 10, 16.5, 17.5, 20, 24.5, 25, 30, 31, 36.5, 37, 41.5, 41.6, 42.5, 46.5, 47, and 48 minutes. Of them BaP appeared at 42.5 minutes. In GC/MS identification of BaP, the fragment at *m/z* 252 was the molecular ion with a ratio of M+1 to M, 21.4. The fragment pattern of BaP from samples was exactly same as that from standard. From these results, the BaP could be clearly identified and determined.

4.2 Algal role as a vector of benzo(a)pyrene to oyster

In the present study, algal foods for the oyster were pre-exposed to concentrations of BaP to use them as a vector of BaP to oyster. Normally, BaP has a low vapor pressure and high octanol-water partition coefficients (Krasnoschekova et al., 1992), suggesting that the chemical is rapidly absorbed by particulate matters and absorbed by living organisms. Kirso and Irha (1998) studied bioconcentration and transformation of the priority PAH, BaP, by several algae. In their flux budget study, they found that of all the BaP consumed, 89-99% was noticed in the biomass of the algae with insignificant parts in the solution and remainder of 4%, probably the

amount metabolized. Even though the transformation of BaP in the algae is species-specific and depends on the presence and activity of enzyme systems localized in the algal cells, the data from Kirso and Irha (1998) indicate the important role of algae in the biotransformation of the chemical in the solution.

Regarding the algae as a vector of BaP to the bivalve in the ecosystem, Okay et al., (2000) found that *Mytilus edulis* fed BaP-carrying algae accumulated significant amounts of the chemical in concentration-dependent way. Bjork and Gilek (1996) also studied the uptake and elimination of radio-leveled phenenthrene by the blue mussel *Mytilus edulis* at different algal concentrations, finding that differences in the concentration of particulate organic matter in the water did not only influence phenanthrene partitioning, but also the physiology of the mussel. These are supporting the availability of the BaP-carrying algae as a vector of the chemical to the oyster.

4.3 Benzo(a)pyrene bioaccumulation and depuration in the digestive gland

Water quality can be a critical factor affecting physiology of bivalves, therefore providing the animals with best water quality is always required in the physiological study of marine bivalves. To reduce the intrinsic stress from the culture itself in captivity, water quality in the culture tanks was maintained as best as it can be served in the culture system. The water temperature, salinity, expressed as a specific gravity, and water exchange given in the present culture system were normally favorable for the laboratory experiment for oysters.

An increasing variety of industrial, agricultural, and other chemicals is

entering the aquatic environment and being taken up to into the tissues of aquatic organisms, damaging the organisms physiologically (Livingstone et al., 2000). In reality, because the chemicals released into the environment rarely remain in the form, or at the location, of release, once introduced into the aquatic environments, they can affect most of the marine organisms living in the most parts of marine environment (Hodgson, 1997). Studied here were BaP bioaccumulation and biotransformation of the oyster exposed to one of the environmental pollutant PAHs, BaP.

In the present study, BaP-treated *C. gigas* with the chemical-carrying algae for 28 showed elevated concentrations of BaP in the oyster digestive gland. The concentrations of BaP of control remained low, lower than 5 ng/g dry weight. The chemical toxicants comprise chemicals of both long standing and more recent concern, including PAH, organochlorine pesticides (DDT, dieldrin), chlorophenols, polychlorobiphenyls (PCBs), hexachlorohexanes, hexachlorobenzenes, polychlorinated-dibenzo-*p*-dioxins, dibenzofurans, nitoaromatic and other heterocyclic compounds, organometallic compounds, organophosphate fertilizers, estrogenic compounds, and many metals (see Livingstone et al., 2000). In the present study, BaP concentrations in the control oysters contained no more than 5 ng/g in the digestive gland. Norena-Barroso et al. (1999) studied concentrations of PAHs in the oyster tissues from locations of oyster farming areas in the Gulf of Mexico to determine the BaP concentrations less than 10 ng/g, normally less than 5 ng/g.

Uptake of PAHs by bivalve is dependent on the bioabailability of the chemicals, normally chemical species and sediment and water concentrations of the chemical (Albert and Ravendra, 2000). In the present study, the BaP-carrying algae successfully rendered the chemical into the

oyster. When the oysters were exposed to BaP, the oysters significantly accumulated the chemicals in the digestive gland. For example, in the 4-time feeding experiment, the level increased sharply 172, 1071, and 5994 ng/g dry weight digestive gland for 50 ppb, 500 ppb, and 5000 ppb oysters, respectively. The increase by day 1 was clearly concentration-dependent. The increases continued by day 14 over the three cultures in a similar manner as the previous values, marking 5245, 21700, and 29971 for 50 ppb, 500 ppb, and 5000 ppb, respectively. The increase after day 21 differed among the cultures. For example, 50 ppb oysters continued to bio-accumulate the chemical by the end of the accumulation experiment, by showing 8026 ng/g digestive gland BaP by day 21 and 9866 ng/g digestive gland BaP ng/g by the end of the experiment. The concentration increase of 500 ppb culture was by 21 day, while that of 5000 ppb culture by 14 day. In most cases, it appears clear that the chemical transport can occur through food chain, although large amounts of the chemicals can be accumulated in the animal bodies via direct contact. The uptake of the chemicals can occur from sediments, suspended particles, water column, and food sources (Baumard et al., 1998a,b). Therefore, the bioaccumulation studied can be summarized that in the higher concentration the bioaccumulation of the chemical can not be removed by the physiological behavior, named depuration.

The failure of effective depuration in the oysters exposed to higher BaP concentrations could be due to physiological hindrance of metabolism. Different aquatic animals respond different species of xenobiotic chemicals; the metabolism of PAHs has been studied in several invertebrates, summarizing that the ability of invertebrates to biotransform PAHs is species specific and the range of the capacity is wide (Meador et al., 1995). Leppanen and Kukkonen (2000) studied fate of BaP in the fresh

water oligochaete and found that the relative proportion of parent BaP in tissue decreased to 60% after 336h, suggesting an active enzamatic activity to degrade BaP in the tissue. Similar results were found in the present study, where depurations were notable in the three experimental cultures, but not so effective in the higher concentrations. For example, the maximum BaP concentration of 50 ng/g culture was noticed by day 28. This figure, thereafter, started to decrease with the commencement of the depuration to 9069 ng/g by depuration day 7 and to 1191 by the end of the depuration. The levels in the depuration of 500 ppb and 5000 ppb cultures were 25123 and 29126 ng/g digestive gland BaP, respectively. These results suggest that oyster can do metabolite of BaP as was pointed out by the above authors only in the case that the concentration of the chemical exposure is only within the capacity of the animal.

Akcha et al. (1999) studied that the relationship between kinetics of BaP bioaccumulation and DNA binding in the mussel *M. galloprovincialis,* showing that the mesocosm system developed is suitable for the study of DNA adduct formation in mussel. BaP in feed is bioavailable for the organisms: for the dose applied, its accumulation and biotransformation in the mussel result in DNA binding of BaP metabolites.

Another possible explaination can be obtained from the finding of Akcha et al. (2000) who studied enzymatic biomarker measurement and study of DNA adduct formation in BaP-contaminated *M. galloprovincialis*. Actually the aim of this study was to improve the knowledge on the metabolic pathways involved in BaP activation and on the relationship between adduct levels and enzymatic biomarker activities. With this purpose, a model to assess pollutant exposure via food supply has been developed for the sentinel organism, *M. galloprovincialis*. These results support the proposal of BPH, AChE, DTD, and CAT activities as suitable

biomarkers of PAH exposure for these sentinel species. Large et al. (2002) found a close relationship between BaP concentration and CYP1A-immunopositive protein in *Mytilus edulis* exposed to different concentrations of the chemical. They found that CYP1A-immunopositive protein was significantly elevated from the untreated control in the mussel treated with higher levels of BaP. Cheung et al. (2001) studied that relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*.

In particular, it is still not adequately understood whether PAH may cause genetic damage in *Mytilus* spp. In laboratory or field conditions, and which molecular mechanism(s) may explain such detrimental effects in these aquatic invertebrates (Kurelec, 1987; Michel et al., 1994). Indeed, mixed function oxygenases (MFO) most likely exist in mussels (Livingstone and Farrar, 1984; Stegeman, 1985; Michel et al., 1994), and induction of chromosomal aberrations and micronuclei occurred in gill cells of *Mytilus galloprovincialis* (Lmk.) after treatment with BaP (Al-Sabti and Kurelec, 1985; Scarpato et al., 1990). On the other hand, only very weak evidence for DNA adducts induction was obtained after injection of mussels with ^{3}H-BaP (Marsh et al., 1992). In addition, the inability of mussels to metabolize PAH has often been suggested (Lee et al., 1972; Dunn and Stich, 1976; Valerio and D'Ambrosio, 1989).

Therefore, it is worth nothing that the accumulation of organic pollutants in mussels is determined by a dynamic balance between uptake and depuration processes which are, in turn, influenced by a dynamic equilibrium between pollutants in sediments, water, food particles and the organisms themselves (Fossato and Canzonier, 1976; Livingstone, 1991; Venier and Canova, 1996). However, considering that, in the present study,

the failure of effective depuration in the oysters exposed to higher BaP concentrations could be due to physiological hindrance of metabolism, probably from physical and physiological damage.

Several studies have examed the dynamics of the uptake and depuration of trace organic contaminants; however, the information found in the literature is confusing. As an example, Table 12 lists a number of studies published over the last two decades that report the depuration of different hydrocarbon mixtures by bivalves. The detailed explaination still needs further study.

Table 12. Results of different hydrocarbon uptake/depuration studies (data, modified from Sericano et al., 1996)

Bivalve	Exposure	Observation	Reference
Oystes	N°2 Fuel Oil (60 days)	Little depuration After 180 days	Blumer et al. (1970)
Oysters	N°2 Fuel Oil (49 days)	Nearly complete Depuration in 28 days	Stegeman and Teal (1973)
Oysters	Hanna Reef (48 days)	Significantly Depuration In 50 days	Sericano et al. (1996)
Oysters	Chronically Polluted	Nearly complete Depuration with BHL[a]= 4.4 days	Wormell (1979)
Oysters	PAHs (15 days)	Analytes below Detection limits after 4 days	Pittinger et al. (1985)
Mussels	PAHs (40 days)	Depuration with BHL between 14-30 days	Pruell et al. (1986)
Clams	PAHs (2 days)	No depuration In 45 days	Tanacredi and Cardenas (1991)
Clams	Chronically Polluted	Slight depuration After 120 days	Boehm and Quinn (1977)

[a]BHL, biological half-life

4.4 Benzo(a)pyrene effects on lysosomal membrane stability

Livingstone et al. (2000) summarized the development of biomarkers to detect the effects of pollutans where they explained that lysosomal alterations to cell injury can be conceptualized as increases or decreases in quanty of specfic lysosomal contents, rate of specific membrane fusion events, or permeability lysosomal membranes to specific substances. They also explained an events of lysosomes affected by pollutants in three: 1) the lysosomal membrane is damaged resulting in reduced lysosomal lysosomal hydrolase latency, 2) in *in vitro* studies, once the integrity of the limiting membrane is compromised, lysosomal contents leak into the cytosol, and 3) lysosomes become enlarged with an associated increase in fusion events. The lysosomal membrane stabilities expressed as a neural red retention are cell-specific. Petrovic et al. (2001) studied lysosomal membrane stability of the cells from *M. galloprovincialis* digestive gland in relatively non-polluted area to find about 30 minutes, while the lysosomal membrane stability of *M. edulis* bood cells were around 100 minutes (Lowe et al., 1995a).

In the present study, hemocytes were withdrawn from *C. gigas* exposed to different concentrations of BaP, and determined the lysosomal membrane properties of the oyster for a development of cellular biomarker to the xenobiotic chemical. The NRR of the xenobiotic BaP-free control lysosomes was 133 ± 6.06 (mean ± SD) minutes just after 10-day acclimation in captivity. It showed a trend decreasing along the progress of the experiment even if a little deviation from the trend was noticed; 123 ± 10.41 by week one, 103 ± 7.63 by week four, and 93 ± 5.77 minute by week eight. This explains the oysters have intrinsic stress from the captive life in the laboratory although the first significant decrease ($P<0.05$) was noticed by day 21. The figures from intact control were still bigger than

those from blood cells of European flat oyster, *Ostrea edulis*, in which the figures remained about 80 minutes (Hauton et al., 1998). In overall sense, the NRR was adversely proportional to concentration and duration of the chemical exposed. The decreasing trend was much evident in the oysters exposed to higher concentrations of the chemical. For example, the first significant decrease from control ($P<0.05$) was from day 21 for control, day 7 for 50, day 3 for 500, and day 1 for 5000 group. A further significant decrease of $P<0.01$ was found from day 3 for 5000 group, while from day 7 for 500 and day 21 for 50 group. No significant decrease of $P<0.01$ was noticed in the control during the exposure test. Interestingly, a recovering trend was observed during the depuration, and the trend was more prominent in the oysters exposed higher BaP concentrations. The NRR recovery in the oyster exposed to highest concentration from 35 to 45. This was significant, particularly considering that the intrinsic stress coming from captive culture in the laboratory was from 103 to 93 during the recovery in control.

It is quite reasonable to expect that there is a great potential for the increased stress in marine organisms due to anthropogenic pressures associated with the increased industrial activity in coastal zones. The critical issue, however, is not the presence itself of chemicals related to the stress potential in the marine environment, but the adverse effect of the chemicals attributed to the concentrations beyond compensatory ability the marine organisms may exert for sequestration, detoxification, or amelioration. In this sense, a concept of biomarker for the elucidation of physiological status of the concerned marine organisms has been taken into consideration although it has been principally employed in environmental toxicology in a variety of marine bivalves (Lowe et al., 1995 a,b; Bard, 2000; Domouhtsidou and Dimitriadis, 2001; Petrovic et al.,

2001; Chu et al., 2002). Borenfreund and Puerner (1984) found that the stressed cells do not take up as much of the supravital dye neutral red (3-amino-7-dimethylamino-2-methylphenazyne hydrochloride) as do intact control cells, probably due to their losing in cellular membrane stability. The finding led to a development of NRR assay by subsequent modification (Readers et al., 1989).

Determined is lysosomal membrane viability of the hemocyte of *C. gigas* exposed to different concentrations of BaP via NRR assay for a generation of cellular biomarker in the cultured species. All the hemocyte NRR time studied exhibited decreasing trends with time elapsed and concentrations of the chemical, being in a good agreement with the finding of Lowe et al. (1995a) who studied NRR time of mussel hemocyte exposed to fluoranthene. Similar results were also found in the previous records reported by researchers who used the assay for a generation of biomarker in bivalve molluscans stressed by source-different toxic chemicals (Lowe, 1988; Grundy et al., 1996; Ringwood et al., 1998; Petrovic et al., 2001). The results together with suggestions by other studies reveal potential availability of the assay as an early warning tool for the bivalve damage induced by ambient toxic chemicals.

Bivalve lysosomes function as a central site for sequestration and accumulation of toxic matters including metals and organic xenobiotics. The matters inducing environmental stressors are known to destabilize lysosomal membranes and injury is proportional to the magnitude of stress (Moore, 1985). Furthermore, elucidation of hemocyte lysosomal membrane stability from living cells is superior to tissue sections because the former will afford a better indication of animal condition (Lowe et al., 1992). Bivalve hemocytes can be obtained without sacrifice thus affording the opportunity of conducting non-destructive monitoring and the stability

of lysosomal membranes has been used as a diagnostic tool to measure mussel condition in the field populations exposed to different degrees of pollution (Nicholson, 1999). However, this is not without problem. In other words, the oyster has an intrinsic problem affecting the lysosomal membrane stability and hydrolytic enzyme activity in the natural population of oyster. Although our results produced by the measurement of NRR time can be a useful information in the generation of rational biomarker of environmental stresses, many factors might be working as a variable. A disruption of membrane stability can be one of the factors responsible for increased uptake of NR in the bivalve hemocyte.

PAHs are xenobiotic compounds relatively easily biodegradable by marine organisms, compared with highly persistant organic pollutants, such as PCBs and DDTs. Therefore, they do not tend to bioaccumulate in the body of organisms in quantities that reflect the exposure if the exposure is not heavy in the marine environment (van der Oost et al., 2003). Domouhtsidou and Dimitriadis (2001) also pointed out the importance of lysosomal membrane stability and the density of residual volume bodies in the generation of biomarker. Thus, it is probably expected that lysosomal responses to heavy metals and xenobiotics would yield different results with different cell type, even with different physiological status of the cell in a same cell type (Lowe et al., 1995a), recommending us paralleled studies of *in vivo* and *in vitro* using a same cell type in a same physiological status. Therefore, to make a scientifically sound NRR biomarker, it still requires quite a bit of further work, particularly on the intrinsic stress caused by factors the oysters encounter in their normal life.

4.5 Benzo(a)pyrene effects on hydrolytic enzyme activity

Nineteen hydrolytic enzyme activities of the hemocyte of *C. gigas* fed BaP-treated algae were studied, using API ZYM System. The control hemocytes of *C. gigas* showed enzyme activities of alkaline phosphatase, esterase (C4), acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, β -glucuronidase, and N-acetyl-β -glucosaminidase. The hydrolytic enzyme activities varied, but remained significant in all experimental groups throughout the experiment (data not shown). All the enzyme activities, however, failed to show some trends accordingly to the concentrations and durations of the chemical exposed except for activation of acid phosphatase and alkaline phosphatase which showed some potential available for the generation of enzymatic biomarker.

Oysters are lacking a specific immune response, but possessing various humoral and cellular factors, crucial in defense against pathogenic and nonpathogenic organisms. The overall immune competence of oysters will be responded to the any impacts on their function arising from pollutants. A number of xenobiotics are known to induce alterations in the bounding membrane of the lysosome, leading to destabilization (Moore and Lowe, 1985; Nott and Moore, 1987) and the subsequent release of the lysosomal hydrolases into the cytosol (Moore, 1976; Baccino, 1978). The release and activity of the lysosomal hydrolases vary from species to species (Suresh and Mohandas, 1990). The hemolymph hydrolytic enzyme activities of *C. gigas* determined by means of API ZYM system were evident in 7 species; alkaline phosphatase, esterase (C4), acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, β -glucuronidase, and N-acetyl-β -glucosaminidase. The enzyme activities revealed increasing but fluctuating tendency as the oyster was exposed to BaP,

probably due to happening of lysosomal membrane damage. Only two of the seven enzymes, acid phosphastase and alkaline phosphatase, represented some tendency in response to the chemical.

Unlike the changes in NRR time induced by BaP, the changes in hydrolytic enzyme activity were confusing in overall sense. In other words, the toxic chemical stimulated hydrolytic activity of the seven enzyme species, but the activity was not consistent enough to serve as data for generation of biomarker. This happened over all but alkaline phosphatase in which the enzyme activity increased with the increased chemical concentrations. Although the increased enzymatic activity is to be induced from the lysosomal membrane damage (Livingstone et al., 2000), the mechanism still remains unknown. While our results support the potential availability of NRR and, in some case, the hydrolytic enzyme activity as a biomarker to lysosomal membrane integrity, there is considerably less information on the effects of contaminants on the biochemical mechanism working on the lysosome membrane of *C. gigas* hemocyte. According to the earlier studies (Moore and Lowe, 1985; Moore et al., 1987; Suresh and Mohandas, 1990), xenobiotics are known to induce alterations in the bounding membrane of the lysosome and subsequent release of the lysosomal membrane hydrolases into the cytosol. Xenobiotics can also trigger the hypersynthesis of lysosomal hydrolases which can be subsequently released in the cytosolic compartment. Regarding the change in the enzymatic activity, in higher xenobiotic concentrations, the membrane destabilization is believed to take place in the early time, resulting in no more increase of enzyme activity (Suresh and Mohandas, 1990). Lysosomal enzyme activity of oyster under lower xenobioticp.` concentrations might be explained in a similar manner. In other words, when the BaP concentration is low the hypersynthesis of the lysosomal

hydrolases is not immediately inactivated by the chemical.

The membrane leakage is unlikely to occur as anything other than stress-induced factors, whereas elevation of enzyme activity can occur as a consequence of an increased metabolic demand (Lowe and Fossato, 2000). Hydrolytic enzyme activity is not specific to a single substrate, rather being triggered by diverse natural cellular process, and, in addition, changes in response to natural variations the animals can easily encounter such as temperature and salinity (Ringwood et al., 1998). More importantly, it also changes according to animal populations, seasons, and ambient pathogens (Carballal et al., 1997; Xue and Renault, 2000). Therefore, it is quite suggestive of considering the intrinsic stress the animal possesses in the generation of biomarker using both lysosomal membrane stability and hydrolytic enzyme activity.

4.6 Growth and survival of larvae from benzo(a)pyrene-treated spawners

In spite of lots of works on the BaP effects on the bivalve physiology (see Livingstone, 2000), informations are still lacking about how the BaP-stressed spawners affect their offsprings. In the present study the larval survival of *C. gigas* from different spawners under influence of different concentrations of BaP for 28 days and then under maturation-inducing condition without the chemical for another 28 days. BaP exposure to the spawners was achieved by "algal vectoring method" in which the spawners were fed 3 species of algal foods grown under 4 concentrations of BaP 0, 50, 500, and 5000 ppb. Maturation of the spawners was gained by raising temperature to 25 ± 0.5℃ from the previous temperature regime. The culture of the oyster larvae continued until first appearance of

an eye-spotted larva from any cultures. Normally DNA adduct is one of the major damage of oysters caused by BaP (Venier and Canova, 1996; Akcha et al., 1999; Akcha et al., 2000). A number of studies have also focused on the PAHs damage of bivalves, including pallial fluid buffering capacity of the marine mussel, *M. galloprovincialis* (Guerra-Rivas et al., 2002) and abnormal production of hsp70 (Cruiz-Rodriguez and Chu, 2002).

In the present study, the larval survival was also significantly affected by the adult exposure of BaP. The mortality of the oyster larvae from BaP-exposed spawners was affected by concentration their adults were exposed. Interestingly, mortality of larvae exposed to lower concentration was a little bit higher over control without statistical difference. However, the mortality by day 10 was much more significant, representing 47% by day 10 from previous measurement, 86% by day 8. The final survival rate of 50 ppb-larva was 14%, which was significantly low compared with control one ($P<0.05$), 21%. The difference of potential survival rate between the two larvae at the time of eye spot stage might be more significant, considering that none of the 50-ppb larvae reached eye spot stage by the time of day 14. The survival of 500 ppb-larvae was slightly lower than those from control and 50 ppb larvae for the first 6 days. However, this was not statistically significant. The first significant difference was found from day 8 where the survival rate of 500 ppb-larvae was 62%, quite low from control and 50 ppb-larvae, 80 and 83%, respectively. Thereafter the survivals declined sharply to make 27% by day 10, 14% by day 12, and 4% by day 14. These figures were significantly lower than those from control and 50 ppb-larvae. The larval survival of 5000 ppb group started to decline from day 4, finally reaching to 0.2% at the end of the experiment via 82, 66, 29, 11, and 4% by days 4,

6, 8, 10, and 12, respectively. The mass mortality in the 5000 ppb-larvae resulted in total crash before the larvae reached eye-spotted stage. Normally the oysters under the xenobiotics enfluence are additionally affected by the other environmental factors, including salinity and temperature (Niyogi et al., 2001), a variety of other pollutants and season (Weinstein 1995). Therefore, it is reasonable to understand the larval influence from the chemical together with these factors.

PAHs are also well-known to be pro-mutagens, causing genetic damage in *Mytilus* spp. In laboratory or field conditions, these are well documented in the molecular mechanism of invertebrates (Kurelec, 1987; Michel et al., 1994). Indeed, mixed function oxygenases (MFO) most likely exist in mussels (Livingstone and Farrar, 1984; Stegeman, 1985; Michel et al., 1994), and induction of chromosomal aberrations and micronuclei occurred in gill cells of *M. galloprovincialis* after treatment with BaP (Al-Sabti and Kurelec, 1985; Scarpato et al., 1990). On the other hand, only very weak evidence for DNA adducts induction was obtained after injection of mussels with ^{3}H-BaP (Marsh et al., 1992). In addition, the inability of mussels to metabolize PAH has often been suggested (Lee et al., 1972; Dunn and Stick, 1976). These are also important factors when the BaP effect on the larvae are concerned. However, our knowledge on the xenobiotic effects on the larval development or growth of oysters is quite a limited. The present findings of BaP effects on larval developments are reminiscent of DNA disorder of *C. gigas* larvae coming from the parent even though concrete studies are still in need.

IV. SUMMARY

It is well known that an increasing attention over last decades has been given on benzo(a)pyrene (BaP), one of polycyclic aromatic hydrocarbons (PAHs) being accumulated in the marine environments by natural and antropogenic combustion processes. PAHs may be present in the vapor phase, dissolved phase, micelle form, absorbed to dissolved or colloidal organic matter, absorbed to particles, incorporated into biota, or sedimentized to the bottom. However, because PAHs, like many other lipophilic organic environmental pollutants, have low vapor pressure and high octanol-water partition coefficients, most parts of BaP are partitioned into biota and sediment in the marine environment. This is particularly evident in the PAHs having higher numbers of fused rings. Therefore it is worth investigation of algae-mediated BaP effects on the bioaccumulation and the ensuing toxicity in marine bivalves.

Studied were the BaP bioaccumulation and depuration in the pooled digestive glands of adult Pacific oyster, *Crassostrea gigas* fed BaP-carrying algae, *Tetraselmis suecica, Isochrysis galbana,* and *Phaeodactylum triconutum,* using gas chromatography/mass spectrometry (GC/MS). The oysters were then conditioned to maturation for the production of gametes. Hatching rate of the fertilized eggs and larval growth, survival, and attachment were assessed to determine how the BaP-pretreated oyster spawners affect their larval growth. The BaP effects on the oysters were further determined in terms of lysosomal membrane stability and lysosomal hydrolytic enzyme activity to generate a cellular biomarker to the BaP.

The algae could be a good vector of BaP to the oysters. The oysters fed

BaP carrying algal foods bio-accumulated the chemical in a concentration-dependent manner for accumulation; $Y = - 0.0085x + 3.2299$ ($r^2 = 0.0105$) for control, $Y= 387.11x - 573.08$ ($r^2 = 0.9870$) for 50 ppb, $Y= 1035.2x + 3798.6$ ($r^2 = 0.8813$) for 500 ppb, and $Y= 831.95x + 12561$ ($r^2= 0.7084$). The depuration, however, was not clearly expressed for 500 and 5000 ppb oysters; $Y= -324.91x +19597$ ($r^2 = 0.9748$) for 50 ppb, $Y= -152.8x + 34590$ ($r^2 = 0.3294$), and $Y= -84.982x + 33632$ ($r^2 = 0.6487$).

The neutral red retention time (NRR) of the hemocyte showed a trend accordingly to BaP concentrations. Importantly, the oyster had some intrinsic stress which probably additionally affected the retention of the chemical. The decreasing trend was much evident in the oysters exposed to higher concentrations of the chemical.

The hemocytes of oyster showed high enzyme activities of alkaline phosphatase, esterase (C4), acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, β -glucuronidase, N-acetyl-β -glucosaminidase. All the enzyme activities, however, failed to show some trends accordingly to the concentrations and durations of the chemical exposed except for activation of acid phosphase and alkaline phosphatase which showed some potential available for the generable of enzymatic biomarker.

The larval survival of 500 ppb and 5000 ppb larvae significantly low, compared to control and 50 ppb larvae. The similar patterns were also found in the larval growth, hatching rate, and attachment rate.

The laval survival, growth, hatching rate and attachment rate of 50 ppb, 500 ppb, and 5000 ppb significantly low, compared to control.

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감사의 글

학부시절부터 많은 가르침을 주신 강주찬 교수님께 감사드립니다. 바쁘신 중에도 심사를 맡아 많은 격려와 조언을 해 주신 김기홍 교수님과 정현도 교수님께 감사드립니다. 그리고 많은 관심과 격려를 해 주신 박수일 교수님, 정준기 교수님, 허민도 교수님께 감사를 드립니다.

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논문을 마무리하면서 지난 2년을 되돌아보니 많은 분들이 힘이 되어 주셨습니다. 국립수산과학원의 김 윤 부장님과 민광식 과장님을 비롯한 양식생물과의 지영주 연구관님, 강용진 연구관님, 박민우 연구관님, 안철민 박사님, 김성연 박사님, 김대중 박사님, 이해영 박사님, 윤길하 박사님의 많은 관심에 감사 드립니다. 또한 도움과 충고를 아끼지 않으셨던 문효방 박사님, 이정호 박사님, 도정완 박사님, 김상수 박사님, 그리고 허영백 박사님께 진심으로 감사드립니다.

가까이서 항상 응원해 주시고 관심 가져주신 병리연구과의 연구원님들께 머리숙여 감사드립니다. 또한 선경언니와 윤정언니, 미라언니, 김종성 선생님께도 감사의 마음을 전합니다.

학부시절부터 많은 격려를 해주신 수권환경학 실험실 식구들, 정훈선배, 규석선배, 자근선배, 성길선배, 재원선배, 운기선배, 석우선배, 은영언니, 근의선배, 상규선배, 대국선배, 그리고, 두 후배 승엽과 유화에게 감사 드립니다.

항상 믿어준 친구들, **sea sound** 동기들에게 감사드리며, 마지막으로 부모님과 동생에게 작지만 이 영광을 돌립니다.