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Cellular Biomarker of Membrane Stability and Hydrolytic Enzyme Activity in the Hemocytes of Benzo(a)pyrene-exposed Pacific oyster, *Crassostrea gigas*

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The Pacific oysters, *Crassostrea gigas*, were stressed with different concentrations of benzo(a) pyrene and depurated to determine the hemocyte lysosomal membrane stability and hydrolytic enzymatic activity as a biomarker candidate to the chemical, using NRR (neutral red retention) and API ZYM System, respectively. The membrane damage measured as NRR decrease was significant with the increase of chemical concentration and exposure time ($P<0.05$), providing a possible tool for biomarker. Interestingly, the control showed intrinsic stress probably due to captive life in the laboratory, and a recovering trend was also found during the depuration. The benzo(a)pyrene-exposed oysters showed increased enzyme activities in alkaline phosphatase, esterase (C4), acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase, and N-acetyl-β-glucosaminidase. Of them, only two enzymes, acid phosphatase and alkaline phosphatase, showed some potential available for the generation of enzymatic biomarker in the oyster. The results are suggestive of the potential availability of the cellular and enzymatic properties as a biomarker. However, considering that a robust biomarker should be insensitive to natural stress coming from normal physiological variation, but sensitive to pollutants, a concept of intrinsic stress the animal possesses should be taken into consideration. This reflects the necessity of further research on the intrinsic stress affecting the cellular and enzymatic properties of the chemical-stressed oysters prior to using the data as a biomarker.

Key words: Lysosomal enzyme activity; Lysosomal membrane stability, Biomarker, Hemocyte, *Crassostrea gigas*

Introduction

Monitoring health of 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; Park et al., 1999). The methods suggested, however, are long term-based. Therefore, development of sensitive and easy-to-detect 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

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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).

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 been focused on the hemolymph role in the defence mechanisms 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; Toreilles et al., 1997; Xue and Renault, 2000).

Polycyclic aromatic hydrocarbons (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. Benzo(a)pyrene, 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).

The objective of the study is to assess the effects of the benzo(a)pyrene on the lysosomal membrane stability and the hydrolytic enzymatic activity of the cultured *C. gigas*. This might have some implication in the development of biomarker in the cultured *C. gigas* to benzo(a)pyrene, one of the most troublesome xenobiotics in the coastal waters, including oyster farming grounds.

Materials and Methods

Oysters and oyster culture

A total of 800 healthy spawners of the Pacific oyster, *C. gigas*, weighing 95.97 ± 21.47 g (mean $\pm$ 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 390 oysters, 90 oysters each, thereafter, were distributed into four 200 L flow-through aquaria (water volume, 120 L) for the benzo (a)pyrene exposure via algal food and depuration. Another 390 animals were evenly distributed to 10 rectangular bottles (water volume, 40 L) for the immersing exposure to benzo(a)pyrene.

Oyster exposure to benzo(a)pyrene via feeding and depuration

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, 400 to 500×10^5 cells mL^{-1} of *Isochrysis galbana* and 200 to 300×10^5 cells mL^{-1} of *Tetraselmis suecica* and *Phaeodactylum tricornutum* grown in the f/2 medium (Guillard and Ryther, 1962) with white light (PAR) at about 140 μmol m^{-2} s^{-1} were exposed to benzo(a)pyrene dissolved in acetone to make a serial concentrations of 0, 50, 500, 5000 ppb for 24 hr. For the control (0 ppb), just an acetone vehicle was added. Then, 20 L of the benzo(a)pyrene-exposed algae was supplied to each of the oyster culture to make final volume 100 L. Feedings were schemed in 30-minute duration and 8-hour interval with an alternative application. After feeding, the oysters were maintained in the flow-through manner with the water volume increased up to 120 L. Depuration of the chemical was achieved by feeding the chemical-free algae for 28 days. Seven animals from each experimental group

were bled from heart locating above/next to adductor muscle and pooled in a ample for the determination of NRR and hydrolytic enzyme activity by exposure days 0, 1, 3, 7, 14, 21, and 28 and by depuration day 28.

Oyster exposure to benzo(a)pyrene via immersion

Oysters were maintained in 10 different concentrations of benzo(a)pyrene (0, 1, 10, 25, 50, 100, 250, 500, 1000, 5000 ppb) without feeding for 14 days with an every two-day basis of water exchange. Seven animals from each experimental group were sacrificed by days 0, 3, 7, and 14 for the analysis of hydrolytic enzyme activity and lysosomal membrane stability.

NR retention Assay

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 mg 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.

API ZYM Test

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.

Results

NR retention

Hemocytes were withdrawn from *C. gigas* exposed to different concentrations of benzo(a)py-rene, and determined the lysosomal membrane pro-perties of the oyster for a development of cellular biomarker to the xenobiotic chemical. Table 1 shows cellular viabilities of the oyster hemocytes expressed as a 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

Table 1. Lysosomal membrane stability of *C. 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**
Control (0)	133 (6.06)	132 (7.64)	135 (5.00)	123 (10.41)	121 (7.64)	110^{+} (13.22)	103 (7.63)	93 (5.77)
50	-	128 (2.89)	132 (10.41)	107^{+} (20.21)	110 (8.66)	92^{++} (7.65)	83 (7.63)	82 (12.58)
500	-	127 (7.64)	85^{+} (18.03)	67^{++} (7.64)	70 (5.00)	68 (7.65)	57 (5.00)	55 (5.00)
5000	-	113^{+} (12.58)	63^{++} (15.28)	57 (2.89)	57 (5.77)	52 (10.41)	35 (13.23)	45 (5.00)

*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 *T. suecica, I. galbana,* and *P. tricornutum* preexposed 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).

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 benzo(a)pyrene 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.

Hydrolytic enzyme activity

Nineteen hydrolytic enzyme activities of the hemocyte of *C. gigas* fed benzo(a)pyrene-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.

Tables 2 and 3 exhibit the hydrolytic activities of acid phosphatase of oysters exposed to benzo(a) pyrene 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 2). The overall variation of acid phosphatase in the oyster immersed to the concentrations of benzo(a)pyrene was similar to that in the oyster fed benzo(a)prene-exposed algae (Table 3). The chemical did not affect the enzyme activity in the lower concentrations (1, 10, and 25 ppb) for a week. Unlike in the lower concentrations, the concentrations over 50 ppb showed a stimulating tendency from day 3. Once stimulated to highest level, the level continued throughout the experiment.

Tables 4 and 5 show the hydrolytic activities of alkaline phosphatase exposed to benzo(a)pyrene. Alkaline phosphatase activity appeared to be stimulated by the chemical concentrations. Higher concentration of benzo(a)pyrene stimulated the enzyme activity from the beginning of the experiment. The increased enzyme activity showed a decreasing tendency, thereafter particularly in the oysters ex-

Table 2. 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**
Control (0)	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

*Same as in Table 1.
**Same as in Table 1.
***Acid phosphatase activity determined by API ZYM System was represented as a level of nM of substrate hydrolyzed in the enzyme reaction, where, 1=5 nmol, 2=10 nmol, 3=20 nmol, 4=30 nmol, 540 nmol.

Table 3. Acid phosphatase activity in the hemolymph of *C. gigas* bathed in the benzo (a)pyrene solutions for 14 days

Day	BaP concentration*									
	0	1	10	25	50	100	250	500	1000	5000
0	2.0**	3.0	2.0	3.0	3.5	3.0	3.0	3.5	3.5	3.0
3	-	3.0	3.0	2.5	4.0	3.5	4.0	4.5	4.0	5.0
7	-	3.5	3.0	3.0	4.5	4.0	5.0	4.5	4.5	5.0
14	-	3.5	3.5	5.0	5.0	4.0	4.0	4.5	4.5	4.0

*Bathing concentration.
**Acid phosphatase activity is as in Table 2.

Table 4. 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**
Control (0)	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

*Same as in Table 1.
**Same as in Table 1.
***Alkaline phosphatase activity is as in Table 2.

Table 5. Alkaline phosphatase activity in the hemolymph of *C. gigas* bathed in the benzo(a)pyrene solutions for 14 days

Day	BaP concentration*									
	0	1	10	25	50	100	250	500	1000	5000
0	3.0**	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
3	2.5	2.0	3.0	2.5	2.0	3.5	3.0	4.5	5.0	4.5
7	2.5	2.5	2.5	2.0	2.5	4.0	5.0	3.5	4.5	3.5
14	2.0	2.0	2.5	2.5	3.0	3.5	3.0	4.5	3.5	1.5

*Bathing concentration.
**The alkaline phosphatase activity is as in Table 2.

posed to higher concentrations of the chemical. For example, the 500 and 5000 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.

Discussion

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., 1995a,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). We determined lysosomal membrane viability of hemocyte of *C. gigas* exposed to different concentrations of benzo(a)pyrene 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 fluoanthene. 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; Krishnakumar et al., 2000; 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. 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.

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 benzo(a)pyrene via bathing and feeding, 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, offering a potential as an enzymatic biomarker.

Unlike the changes in NRR time induced by benzo(a)pyrene, 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 applied both by feeding and bathing methods.

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 xenobiotic concentrations might be explained in a similar manner. In other words, when the benzo(a)pyrene concentration is low the hypersynthesis of the lysosomal hydrolases is not immediately inactivated by the chemical. This is reflected as continued increase in the alkaline phosphatase of the oysters exposed to lower concentration of benzo(a)pyrene in the present study (see Tables 4 and 5).

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 Fossa:o, 2000). Hydrolytic enzyme activity is not specif:c to a single substrate, rather being triggered by diverse natural cellular process, and, in addition, changes in response to natural variations the anima:s 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 stab:lity and hydrolytic enzyme activity.

References

Anderson, R.S. 1996. Interaction of *Perkinsus marinus* with humoral factors and hemocytes of *Crassostrea virginica.* J. Shellfish Res., 5, 127~134.

Baccino, F.M. 1978. Selected patterns of lysosomal response in hepatocytic injury. In *Biochemical Mechanisms of Liver Injury,* T.F. Slater, ed. Academic Press, New York, pp. 518~557.

Bachere, E. 1995. Knowledge and research prospects in marine mollusk and crustacean immunology. Aquaculture, 132, 17~32.

Bachere, E., D. Chagot and H. Grizel. 1988. Separation of *Crassostrea gigas* hemocytes by density gradient centrifugation and counterflow centrifugal elutriation. Dev. Comp. Immunol., 14, 261~168.

Bard, S.M. 2000. Multixenobiotic resistance as a cellular defence mechanism in aquatic organisms. Aquat. Toxicol., 48, 357~389.

Beckmann, N., M.P. Morse and C.M. Moore. 1992. Comparative study of phagocytosis in nomal and diseased hemocytes of bivalve molluscs *Mya arenaria.* J. Invertebr. Pathol., 59, 124~132.

Borenfreund, E. and J.A. Puerner. 1984. Simple quantitive procedure using monolayer cultures for cytotoxicity assays. J Tissue Cult. Meth., 9, 7~9.

Camus, L., E.E. Grosvic, J.F. Borseth, M.B. Jones and M.H. Depledge. 2000. Stability of lysosomal and cell membranes in haemocytes of the common mussel (*Mytilus edulis*): effect of low temperatures. Mar. Environ. Res., 50, 325~329.

Carballal, M, C. Lopez, C. Azevedo and A. Villalba. 1997. Enzymes involved in defence functions of hemocytes of mussel *Mytilus galloprovincialis.* J. Invertebr. Pathol., 70, 96~105.

Cheng, T.C. 1988. *In Vitro* effects of heavy metals on cellular defence mechanisms of *Crassostrea virginica:* Phagocytic and endocytic indices. J. Invertebr. Pathol., 51, 215~220.

Cheng, T.C. 1990. Effects of in vitro exposure of *Crassostrea virginica* to heavy metals of haemocyte viability and activity levels of lysosomal enzymes. In *Pathology in Marine Science,* F.O. Perkins and T.C. Cheng, eds. Academic Press, San Diego, USA., pp. 513~524.

Chu, F.-L.E., A.K. Volety, R.C. Hale and Y. Huang. 2002. Cellular responses and disease expression in oysters (*Crassostrea virginica*) exposed to suspended field - contaminated sediments. Mar. Environ. Res., 53, 17~35.

Domouhtsidou, G.P. and V.K. Dimitriadis. 2001. Lysosomal and lipid alterations in the digestive gland of mussels, *Mytilus galloprovincialis* (L.) as biomarkers of environmental stress. Environ. Pollut., 115, 123~137.

Dyrynda, E.A., R.K. Pipe and N.A. Ratcliffe. 1995. Host defence mechanisms in marine invertebrate larvae. Fish Shellfish Immunol., 5, 569~580.

Gallager, S.M. and R. Mann. 1986. Growth and Survival of larvae of *Mercenaria mercenaria* (L.) and *Crassostrea virginica* (Gmelin) relative to broodstock conditioning and lipid content of eggs. Aquaculture, 56, 105~121.

Grundy. M.M., N.A. Ratcliffe and M.N. Moore. 1996. Immune inhibition in marine mussels by polyclclic aromatic hydrocarbons. Mar. Environ. Res., 42, 187~190.

Guillard, R.R.L. and J.H. Ryther. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana and Detonula confervaceae Hustedt* (Cleve). Can. J. Microbiol., 8, 229~239.

Holland, D.L. 1978. Lipid reserves and energy metabolism in the larvae of benthic marine invertebrates. In *Biochemical and Biophysical Perspectives in Marine Biology,* P. L. Malins and J.R. Sargent, eds. Academic Press, London and New York, pp. 85~123.

Holm, N.P. and J. Shapiro. 1984. An examination of lipid reserves and the nutritional status of *Daphnia pulex* fed *Aphanizomenon flos-aquae.* Limnol. Oceanogra., 29, 1137~1140.

Kang, C.-K., M.S. Park, P.-Y. Lee, W-J. Choi and W-.C. Lee. 2000. Seasonal variations in condition, reproductive activity, and biochemical composition of the Pacific oyster, *Crassostrea gigas* (THUNBERG), in suspended culture in two coastal bays of Korea. J. Shellfish Res., 19, 771~778.

Kenchington, E.L.R. 1994. Spacial and temporal variation in adductor muscle RNA/DNA ratio in sea scallops (*Placo-pecten magellanicus*) in the Bay of Fundy, Canada. J. Shellfish Res., 13, 19~24.

Livingstone, D.R., J.K. Chipman, M.D. Lowe, C. Minier, C.L. Mitchelmore, M.N. Moore, L.D. Peters and R.K. Pipe. 2000. Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: revent molecular, genotixic, cellular and immunological studies on the common mussel (*Mytilus edulis* L.) and other mytilids. Int. J. Environ. Pollut., 13, 56~91.

Lopez, C., M.J. Carballal, C. Azevedo and A. Villalba. 1997. Differential phagocytic ability of the circulating haemocyte types of the carpet shell clam *Ruditapes decussates* (Mollusca: Bivalvia). Dis. Aquat. Org., 30, 209~215.

Lowe, D.M. 1988. Alterations in cellular structure of *Mytilus edulis* resulting from exposure to environmental contami-

nants under field and experimental conditions. Mar. Ecol. Prog. Ser., 46, 91~100.

Lowe, D.M. and V.U. Fossato. 2000. The influence of environmental contaminants on lysosomal activity in the digestive cells of mussels (*Mytilus galloprovincialis*) from the Venice Lagoon. Aquat. Toxicol., 48, 75~85.

Lowe, D.M., M.N. Moore and B.M. Evans. 1992. Contanminant impact on interations of molecular probes with lysosomes in lyving hepatocytes from dab *Limanda limanda*. Mar. Ecol. Prog. Ser., 91, 135~140.

Lowe, D.M., C. Soverchia and M.N. Moore. 1995a. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to flouranthene. Aquatic Toxicol., 33, 105~112.

Lowe, D.M., U. Valentono and M.H. Depledge. 1995b. Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an *in vitro* study. Mar. Ecol. Prog. Ser., 129, 189~196.

Moore, M.N. 1976. Cytochemical demonstration of latency of lysosomal hydrolases of digestive cells of the common mussel, *Mytilus edulis*, and changes induced by thermal stress. Cell Tissue Res., 175, 279~287.

Moore, M.N. 1985. Cellular responses to pollutants. Mar. Pollut. Bull., 16, 134~139.

Moore, C.A. and S.R. Gelder. 1985. Demonstration of lysosomal enzymes in hemocytes of *Mercenaria mercenaria* (Mollusca: Bivalvia). Trans. Am. Microsc. Soc., 104, 242 ~249.

Moore, M.N. and D.M. Lowe. 1985. Cytological and cytochemical measurements. In *The effects of Stress and Pollution on marine Animals*, B.L. Bayne, ed. Praeger Scientific, New York, pp. 46~74.

Moore, M.N., R.K. Pipe, S.V. Farrar, S. Thomson and P. Donkin. 1987. Lysosomal and microsomal responses to oil-derived hydrocarbons in *Littorina littorea*. In *Oceanic Processes in Marine Pollution*, J.R. Capuzzo and J.R. Kester, eds. ER Kriegger, Malabar, FL, USA, pp. 89~96.

NFRDI Report. 1997. Studies on the bad seed collection in oyster growing area and investigation of the new oyster seed collection area. National Fisheries Research and Development Institute, Pusan, Korea, pp. 12~26 (in Korean).

Nicholson, S. 1999. Cytological and physiological biomarker responses from green mussels, *Perna viridis* (L.) transplanted to contanminated sites in Hong Kong coastal waters. Mar. Pollut. Bull., 39, 261~268.

Nicholson, S. 2001. Ecocytological and toxicological responses to copper in *Perna viridis* (L.) (Bivalbia: Mytilidae) haemocyte lysosomal membranes. Chemosphere, 45, 399 ~407.

Nott, J.A. and M.N. Moore. 1987. Effects of polycyclic aromatic hydrocarbons on molluscan lysosomes and endoplasmic reticulum. Histochem. J., 19, 357~368.

Paon, L.A. and E.L.R. Kenchington. 1995. Changes in somatic and reproductive tissues during artificial conditioning of the sea scallop, *Placopecten magellanicus* (Gmellin, 1791). J. Shellfish Res., 14, 53~58.

Park, M.S., H.J. Lim, Q. Jo, J.S. Yoo and M. Jeon. 1999. Assessment of reproductive health in the wild seed oysters, *Crassostrea gigas*, from two locations in Korea. J. Shellfish Res., 18, 445~450.

Petrovic, S., B. Ozretic, M. Krajnovic-Ozretic and D. Bobinac. 2001. Lysosomal membrane stability and methallothioneins in digestive gland of mussels (*Mytilus galloprovincialis* Lam.) as biomarkers in a field study. Mar. Pollut. Bull., 42, 1373~1378.

Pieters, H., J.H. Kluytmans, W. Zurberg and I. Zandee. 1979. The influence of seasonal changes on energy metabolism in *Mytilus edulis* (L) I. Growth rate and biochemical composition in relation to environmental parameters and spawning. In *Cyclic Phenomena in Marine Plants and Animals*, E. Naylor and R.H. Hartnoll, eds. Pergamon Press, NY, USA. pp. 285~292.

Reader, S.J., V. Blackwell, R. O'Hara, R.H. Clothier, G. Griffin and M. Balls. 1989. A vital dye release method for assessing the short term cytotoxic effects of chemicals and formulations. ATLA, 17, 28~37.

Renwrantz, L., T. Yoshino, T.C. Cheng and K. Auld. 1979. Size determination of hemocytes from the American oyster, *Crassostrea virginica*, and the description of a phagocytosis mechanism. Zool. J. Physiol., 83, 1~12.

Ringwood, A.H., D.E. Conners and J. Hoguet. 1998. Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters *Crassostrea virginica*. Mar. Ecol. Prog. Ser., 166, 163~171.

Ruiz, C., M. Abad, F. Sedano, L.O. Garcia-Martin and J.L. Sanchez Lopes. 1992. Influence of seasonal environmental changes on the gamete production and biochemical composition of *Crassostrea gigas* (Thunberg) in suspended culture in EL Grove, Galicia, Spain. J. Exp. Mar. Biol. Ecol., 155, 249~262.

Suresh, K. and A. Mohandas. 1990. Hemolymph acid phosphatase activity pattern in copper-stressed bivalves. J. Invertebr. Pathol., 55, 118~125.

Sutherland, J.B., F. Rafic, A.A. Khan and C.E. Cerniglia. 1995. Mechanism of Polycyclic aromatic hydrocarbon degradation,. In *Microbial Transformation and Degration of Toxic Organic Chemicals*, L.Y. Yong and C.E. Cerniglia, ed, pp. 269~300. Wiley-Liss, New York, USA.

Torreilles, J., M.C. Guerin and P. Roch. 1997. Perioxidase-release associated with phagocytosis in *Mytilus galloprovincialis* haemocytes. Dev. Comp. Immunol., 21, 267~275.

Tripp, M.R. 1992. Phagocytosis by hemocytes of hard clam, *Mercenaria mercenaria*. J. Invertbr. Pathol., 59, 222~227.

Winstead, J.T. 1995. Digestive tubule atrophy in eastern oysters, *Crassostrea virginica* (Gmelin, 1791), exposed to salinity and starvation stress. J. Shellfish Res., 14, 105~111.

Xue, Q. and T. Renault. 2000. Enzymatic activities in European flat oyster, *Ostrea edulis*, and Pacific oyster, *Crassostrea gigas*, hemolymph. J. Invertebr. Pathol., 76, 155~163.