Journal of Environmental Biology
July 2008, 29(4) 507-512 (2008)
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Effects of the coastal sediment elutriates containing persistent organic pollutants (POPs) on early reproductive outputs of the Pacific oyster, *Crassostrea gigas*

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(Received: March 13, 2007; Revised received: December 14, 2007; Accepted: December 26, 2007)

Abstract: *We previously found that embryonic development of the bivalve species was highly vulnerable to xenobiotic chemicals, damaging the coastal ecosystem integrity. To further assess their potential damage to ecosystem, the xenobiotic composition of the sediment elutriates from two representative industrialized Korean coasts, Pohang and Ulsan, were determined with gas chromatography/mass spectrometry (GC/MS). The presumed critical dilution of the elutriate was then exposed to early life stages of the Pacific oyster (Crassostrea gigas), embryonic development and metamorphic stage to first spat, at which they were believably more vulnerable by the chemical exposure. The early life damage by the xenobiotic exposure was apparently significant by the significant degree of pollution. Here, we indicated their potential damages to the Pacific oyster.*

Key words: *POPs, Sediment elutriate, Xenobiotics, Early life stages, Crassostrea gigas, Sediment toxicity*
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Introduction

The widespread occurrence of hydrophobic persistent organic pollutants (POPs) in recent coastal sediments has been well documented (Baumard *et al*., 1999a; Hwang *et al*., 1999; Zitko, 2000). One of the alarming observations on the sediment POPs is that their accumulation is increasing even in the remote pristine arctic environment (Bustnes *et al*., 2000; Evenset *et al*., 2004, 2007). In the aquatic environments, sediments are not only a reservoir for the hydrophobic POPs, but also a source of the pollutants for aquatic lives (Long *et al*., 1998). Sediment analyses can document the presence of pollutants, but their potential impacts on the biota are not readily predictable because bioavailability is a dynamic component composed of complex physical, chemical and biological interactions (Knezovich *et al*., 1987; Landrum *et al*., 1992; Viganò *et al*., 2007).

The toxicity of POPs to marine lives differs from their chemical structures even in a given POP family. For instance, in polyaromatic hydrocarbons (PAHs), the higher molecular weights or the higher insoluble forms are less bioavailable, but more toxic. Therefore, information on POPs impacts obtained by sediment elutriate exposure might be more realistic than by a single or a combination of manufactured products.

Oyster embryos and larvae are currently used to assess the biological quality of coastal and estuarine seawaters and their sediments (Beiras and His, 1995; His *et al*., 1997; Geffard *et al*., 2002a,b; Geffard *et al*., 2004). The employment of the embryo and larva as a candidate for the environmental indicator renders several advantages because of their resistance to broad range of environmental conditions, rapid development and sensitivity to ambient contaminants. In this study, to assess the potential risk of the polluted sediments to marine ecosystem, the marine sediment elutriates from two representative industrialized coasts, Pohang and Ulsan, Korea, were analyzed for POPs and exposed to two vulnerable early life stages of the Pacific oyster, *Crassostrea gigas*.

Materials and Methods

Sampling sites: Pohang and Ulsan are representative industrialized cities, the former for steel industry and the latter for automobile and oil refinery industries in Korea. Because the two cities are located on the southern end of the east coast of the Korean Peninsula, they are under influence of a branch of the Kuroshio Extension which enters into Korean side of the East Sea (Sea of Japan). In spite of the geological benefit, the central parts of the two locations are relatively embayed.

Determination of sediment xenobiotics: Sediment samples (0-5 cm deep) were collected from 6 spots of Pohang Bay (P1–P6) and 5 spots of Ulsan Bay (U1–U5) with box core sampler and then kept frozen at -20°C until extraction. Detailed procedures of sample preparation for determination of polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-para-dioxins and dibenzofurans (PCDDs/DFs) and dioxin-like polychlorinated biphenyls (DLPCBs) have been described elsewhere (Moon *et al*., 2001, 2002, 2004). Sediment sample from Sockcho coast was also collected as an unpolluted reference site.

For PAHs analysis, sediments were extracted with Soxhlet apparatus using toluene (Ultra residue analysis, J.T. Baker, Phillipsburg, NJ, USA) for 24 hr after a spike of internal standards (ES 2044, Cambridge Isotope Laboratories, Inc., Andover, MA, USA). The extracts of samples were purified using an activated silica gel (Art No. 7734, 70-230 mesh, Merck, Darmstadt, Germany) column chromatography with successive eluants of hexane (Ultra

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Special Issue - **Marine Environmental Biology**
Guest Editor - **H.W. Shin, Korea**

residue analysis, J. T. Baker) and 15% methylene dichloride (Ultra residue analysis, J. T. Baker) in hexane. The second fraction was concentrated to less than 1 ml, and left at a room temperature for 1 to 2 days to evaporate to 100-200 µl. The residues were dissolved with 100 µl of *n*-nonane (Pesticide residue analysis, Fluka, St. Gallen, Switzerland) and determined for PAHs.

For the analysis of PCDDs/DFs and DLPCBs, sediments were extracted with Soxhlet apparatus with toluene for 24 hr after a spike of internal standards (EPA-1613 LCS; PCDDs/DFs and PCB-LCS-A; DLPCBs, Wellington Laboratories, Guelph, ON, Canada). The extracts were cleaned up on a multi-layer silica gel column chromatography containing $AgNO_3$-silica gel, H_2SO_4-silica gel and KOH-silica gel with 160 ml of hexane. The elutant fraction was concentrated to dryness and then determined for DLPCBs.

After the pre-cleaning with a multi-layer silica gel column chromatography, the elutant was purified using an activated alumina column chromatography with successive eluants of 60 ml of 3% methylene dichloride in hexane and 100 ml of 50% methylene dichloride in hexane. The second fraction was concentrated to less than 1 ml, and left at room temperature for 1 to 2 days to evaporate to dryness. The residues were dissolved with 20–50 µl of *n*-nonane and determined for PCDDs/DFs.

PAHs were determined using gas chromatography/mass spectrometry (GC/MS, Agilent 5973N, Agilent, Palo Alto, CA, USA) with DB-5MS capillary column (30 m length, 0.25 mm ID, 0.25 µm film thickness, J and W Scientific, Palo Alto, CA, USA). PCDDs/DFs and DLPCBs were analyzed with high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS, JMS 700D, JEOL, Tokyo, Japan). A SP-2331 capillary column (60 m length, 0.25 mm ID, 0.25 µm film thickness, Supelco, Bellefonte, PA, USA) and DB-5MS (60 m length, 0.25 mm ID, 0.25 µm film thickness, J and W Scientific) were used for the separation and detection of PCDDs/DFs. The capillary column used for the separation of DLPCBs was HT-8 (50 m length, 0.22 mm ID, 0.25 µm film thickness, SGE, Ringwood, VIC, Australia). The quantitative determination of PCDDs/DFs and DLPCBs was performed by a relative response factor (RRF) method obtained through standard solution injections.

Preparation of sediment elutriates: Elutriates were obtained following the method of Melzian (1990). The freeze- dried sediments were shaken mechanically in glass bottle filled with filtered seawater at a ratio of 1:4 (volume sediment:water) for 8 hr. Just after the mechanical shaking, the samples were centrifuged at 3,000 rpm for 10 min and the solution phase was vacuum-filtered to make a stock solution.

Elutriate effects on fertilization and embryonic development to D-shaped larvae: A total of 30 mature gonads from farmed oyster (20 for female, 10 for male) were taken by stripping method for fertilization and was maintained at laboratory condition (Park *et al.*, 2002) to obtain healthy eye-spotted pediveligers for the spat experiment. For the analyses of the elutriate effects on fertilization and embryonic development to D-shaped larvae, about 4,000 eggs were evenly distributed and fertilized in 13 microplates (6 well; culture volume, 10 ml) containing a 20% dilution of each stock solution from control (elutriate-free seawater), S, P1-P6 and U1-U5 sediments. In an hr after the fertilization, about 50 eggs were sampled from each well for a measurement of fertilization rate. The plates were then kept under total darkness for 26 hr to secure time long enough to have all the fertilized eggs metamorphosed to D-shaped larva. The wells were gently aerated by blowing surface of the well in every two hours. Fertilization and larval procedures were followed at (22 ±0.5°C). The culture experiment was replicated by 4 times.

The percent abnormality was calculated primarily based on the criteria employed by His *et al*. (1997). Abnormalities included were segmented eggs, normal or malformed embryos that failed to reach D-larval stage and D-larvae with either convex hinge, indented shell margins, incomplete shell or protruded mantle. A newly found abnormality criterion, D-shaped larva moving with its valves open continuously was additionally counted.

Elutriate effects on metamorphosis to spats: Cleaned oyster shells (3 x 3cm wide) were immersed into each of 13 elutriate dilutions (20% stock solution) for 24 hr. The chemical-carrying shells were then used as a substrate for larval attachment by suspending them in 4 oyster culture tanks (capacity, 120 l) containing 12 ind/ml of eye-spotted pedivelagers (eye-spotted rate, about 30%). Numbers of spats attached on the substrates were measured 2 and 3 weeks after the attachment.

Data analysis: All data were analyzed through one-way ANOVA using the statistical package SPSS, version 10. If an effect was significant, the difference between the means were analyzed by Tukey test for unplanned multiple comparisons of means at $p<0.05$ level of significance. Percent data were arcsine transformed before analysis, but non-transformed data were shown in figures.

Results and Discussion

Xenobiotic composition in the sediments and its critical dilution: The sediment compositions of xenobiotic pollutants from 13 study sites were determined. Total PAHs comprising NaP, AcPy, AcP, Flu, PhA, AnT, FluA, Pyr, BaA, Chr, BbF, BkF, BaP, InP, DbA, and BghiP from 12 study sites were 12.1, 1469.9, 5562.9, 10686.0, 1329.1, 809.0, 2403.4, 1720.0, 563.8, 681.8, 5130.1, and 1182.5 ng g^{-1} sediment, P2, P3, and U4 being the heavily polluted sites by the chemicals (Table 1). Total PCDDs/DFs represented by PCDDs, PCDFs, I-TEQ, ΣPCDDs and Σ PCDFs for the study sites from the 12 sites were 24.1, 1085.5, 322.1, 880.3, 201.5, 155.3, 477.2, 585.3, 432.4, 382.9, 348, and 484.2 pg g^{-1} sediment. Site P1 was most polluted by the chemicals, followed by sites P3 and U1 (Table 2). Total PCBs of PCB77, PCB81, PCB123, PCB118, PCB114, PCB105, PCB126, PCB167, PCB156, PCB157, PCB169, and PCB189 were undetectable, 0.71, 0.83, 2.82, 0.38, 0.23, 0.34, 19.29, 7.665, 22.645, 5.783, and 5.925 for the 13 sites, respectively. Overall,

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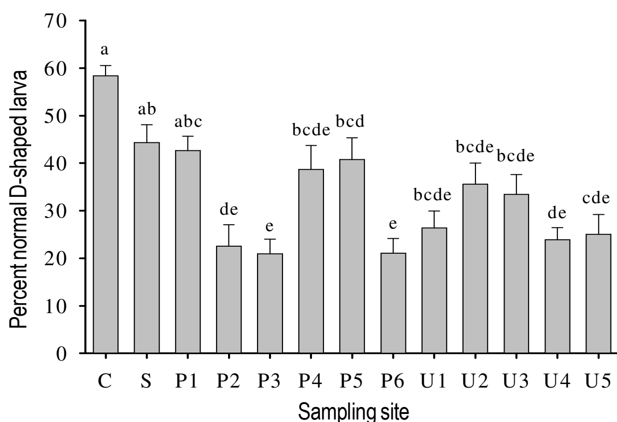

Fig. 1: Toxic effects of the Pohang (P1-P6) and Ulsan (U1-U5) sediment elutriates on embryonic development of the Pacific oyster, *Crassostrea gigas*. C, control as an untreated control; S, Sokcho as an unpolluted reference. Vertical bars stand for mean ± SE. Yields with no letter in common are significantly different on Tukey test, one-way ANOVA (p<0.05)

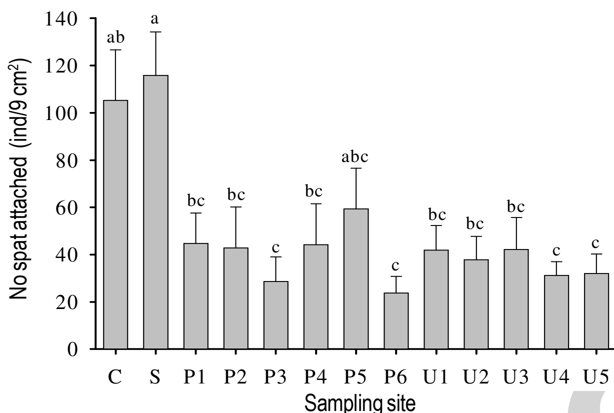

Fig. 2: Toxic effects of the Pohang (P1-P6) and Ulsan (U1-U5) sediment elutriates on larval settlement of the Pacific oyster, *Crassostrea gigas*. C, control as an untreated control; S, Sokcho as an unpolluted reference. Vertical bars stand for mean ± SE. Yields with no letter in common are significantly different on Tukey test, one-way ANOVA (p<0.05)

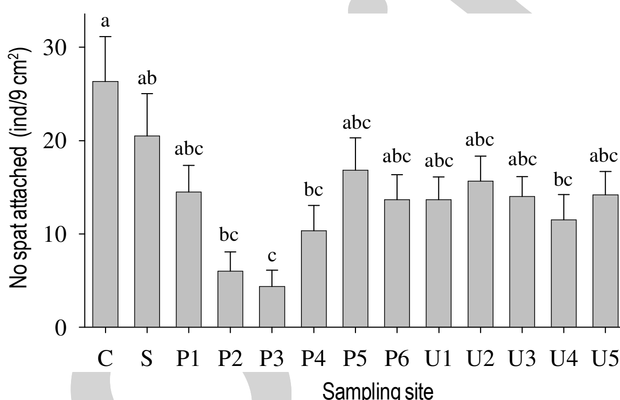

Fig. 3: Toxic effects of the Pohang (P1-P6) and Ulsan (U1-U5) sediment elutriates on larval settlement of the Pacific oyster, *Crassostrea gigas*. C, control as an untreated control; S, Sokcho as an unpolluted reference. Yields with no letter in common are significantly different on Tukey test, one-way ANOVA (p<0.05)

sediments of Ulsan contained higher magnitude of PCBs over those of Pohang (Table 3). Pohang and Ulsan are representative industrialized cities for steel, automobile, oil refinery, and other types of heavy industries in Korea. In our determination of the xenobiotic chemicals in the sediment, the pollutant concentrations of Pohang and Ulsan were as high as those of Jinhae, one of the most polluted locations in Korea.

Finding a test organism and its critical concentration for test pollutants makes it one of the key parameters in the laboratory toxicity study. The Pacific oyster toxicity bioassays have been fully recognized as reliable, sensitive, and ecologically important tools for biomonitoring coastal environments and to know the physiological status of the animal (Beiras and His, 1995; His *et al*., 1997; Geffard *et al*., 2002a,b; Geffard *et al*., 2004). Geffard *et al*. (2003) found 0.3 ng g^{-1} for total PAHs to be a critical concentration in the laboratory toxicity test for oyster embryos. Our previous studies confirmed that the critical concentration of the sediment elutriates representing the same range of the damage as found by Geffard *et al*. (2003) was a dilution around 20% elutriate stock solution (Jo *et al*., 2005a,b). A similar dilution factor was also employed in the present study.

Elutriate toxicity to embryonic development: Fertilization was not significantly influenced by the elutriate exposure. In all experimental groups including untreated control, the fertilization rates were around 85% (data not shown). However, the elutriate dilution from the polluted sediments influenced embryonic development. Fig. 1 shows achievement of the normal oyster larval development in the elutriates. The normality achievement for the control was nearly 60%, while the achievement was between 20 to 45% for experimental groups with most significant influence in the elutriates P3 and P6 followed by the elutriates P2 and U4. Only two elutriates S and P1 were comparable with control.

The potential of the increased stress in marine organisms due to the contamination of chemicals associated with increasing industrial development in coastal waters has demanded a careful monitoring of cellular and molecular biological responses and development of strategies to minimize the impacts. Besides the ecotoxicological approach, the xenobiotic pollution in aquaculture farm is also worth a deep consideration. However, in spite of our generalized acceptance of the chemical threat to all types of marine organisms, our knowledge on the subject is confined in a minor research theme. Sediments are not only a reservoir for contaminants, but also a source of toxicants for marine animals (Long *et al*., 1998). But sediment analyses can document the presence of contaminants, but their potential impacts on the biota are not readily predictable because bioavailability is a dynamic component composed of complex physical, chemical, and biological interactions. Therefore, employment of the sediment elutriate is suggestible for the toxicity test of the xenobiotics in the laboratory.

Elutriate toxicity to metamorphosis of eye-spotted larvae: The eye-spotted larvae were provided with substrates previously exposed to 20% of the sediment elutriates to understand the larval

Table - 1: Concentrations of PAHs (ng g^{-1} dry weight) in the sediments from the unpolluted reference and experiment sites

PAH	S	P1	P2	P3	P4	P5	P6	U1	U2	U3	U4	U5
NaP	0.1	6.4	72.1	176.6	4.5	18.8	0.4	0.9	0.4	0.3	2.3	1.2
AcPy	0.0	1.5	6.7	5.6	1.0	2.9	4.5	0.8	0.2	0.2	1.4	0.7
AcP	0.1	18.8	80.6	194.7	10.5	22.9	33.9	7.2	0.6	0.8	29.0	5.0
Flu	0.7	21.5	182.0	187.2	7.2	11.6	22.5	15.1	3.1	2.3	27.9	7.3
PhA	0.6	138.6	974.6	1249.4	64.3	68.7	172.0	132.6	36.1	35.0	340.8	77.1
AnT	0.1	26.9	178.2	242.3	12.8	15.7	39.1	39.3	8.2	8.4	53.7	16.9
FluA	1.7	184.7	734.8	1511.8	140.5	30.2	296.2	241.4	84.7	100.2	755.9	185.1
Pyr	2.1	199.2	72.0	160.4	157.1	100.4	339.1	314.4	84.6	115.3	868.2	220.9
BaA	0.6	111.0	523.9	1077.0	111.4	64.8	198.6	118.0	35.5	49.7	396.3	87.0
Chr	1.0	117.3	589.3	1217.7	134.2	78.4	227.5	199.4	56.8	71.4	526.4	126.8
BbF	1.7	200.7	706.1	1883.2	213.4	127.6	328.4	268.0	86.0	105.1	713.4	164.2
BkF	1.5	67.0	219.9	517.6	74.6	43.5	112.5	77.1	28.3	35.2	248.4	50.3
BaP	0.7	116.8	417.1	1033.9	104.2	53.8	211.5	65.4	34.8	39.6	377.3	68.8
InP	0.6	121.7	410.8	195.6	139.2	76.7	200.1	99.3	41.1	50.3	338.3	71.7
DbA	0.2	23.9	34.5	89.9	26.4	15.6	38.1	21.5	8.6	11.2	82.7	16.0
BghiP	0.5	113.9	360.2	943.3	128.0	77.7	179.0	119.8	54.7	56.8	368.2	83.4
ΣPAH	12.1	1469.9	5562.9	10686	1329.1	809.0	2403.4	1720	563.8	681.8	5130.1	1182.5

Table - 2: Concentrations of PCDDs/DFs (pg g^{-1} dry weight) in the sediments from the unpolluted reference and experiment sites

PCDD/DF	S	P1	P2	P3	P4	P5	P6	U1	U2	U3	U4	U5
PCDDs	0.1	3.9	1.5	3.7	0.6	0.6	1.5	1.3	0.6	1	0.9	1.2
PCDFs	0.1	12.1	3.2	8.6	1.9	1.5	4.5	3	1	1.5	1.5	2.1
I-TEQ*	0.2	16	4.8	12.3	2.5	2.1	6	4.4	1.6	2.5	2.4	3.3
ΣPCDDs	15.6	192.2	88.3	234.2	48	35.1	95.9	427.7	198.5	283.8	254.6	354.1
ΣPCDFs	8.6	893.2	233.8	646	153.5	120.3	381.3	158.2	76.8	99.1	93.4	130
ΣPCDD/DF	24.1	1085.5	322.1	880.3	201.5	155.3	477.2	585.3	432.4	382.9	348	484.2

* International toxicity equivalent

Table - 3: Concentrations of PCBs (pg g^{-1} dry weight) in the sediments from the unpolluted reference and experiment sites

PCB	S	P1	P2	P3	P4	P5	P6	U1	U2	U3	U4	U5
PCB77		0.04	0.03	0.14	0.01	0.01	0.01	0.38	0.300	0.368	0.239	0.292
PCB81		0.00	0.00	0.04	0.00	0.00	0.00	0.01	0.008	0.048	0.009	0.014
PCB123		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.001	0.005	0.001	0.001
PCB118		0.02	0.04	0.12	0.02	0.01	0.01	0.03	0.056	0.107	0.062	0.065
PCB114		0.01	0.01	0.03	0.00	0.00	0.00	0.01	0.027	0.152	0.042	0.039
PCB105		0.01	0.02	0.05	0.01	0.00	0.01	0.01	0.022	0.088	0.031	0.028
PCB126		0.41	0.35	1.50	0.18	0.11	0.20	18.55	5.256	7.606	3.093	3.442
PCB167		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.003	0.020	0.005	0.004
PCB156		0.04	0.08	0.17	0.03	0.02	0.02	0.07	0.236	1.438	0.296	0.268
PCB157		0.02	0.05	0.08	0.02	0.01	0.01	0.04	0.192	0.982	0.235	0.225
PCB169		0.15	0.25	0.67	0.11	0.06	0.08	0.19	1.548	11.765	1.752	1.530
PCB189		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.016	0.067	0.016	0.017
ΣDLPCB	nd	0.71	0.83	2.82	0.38	0.23	0.34	19.29	7.665	22.645	5.783	5.925

attaching performance on the chemical-carrying substrates. The substrates carrying the xenobiotic chemicals blocked the larval attachment on them. Fig. 2 exhibits the numbers of the oyster larvae attached on the substrates two weeks after attachment. The numbers of the control substrates by 2 weeks were about 105 ind/9cm^2. Most of the experimental groups failed to have as many spats attached on the substrates as control. The failures to attach were particularly significant in sediment elutriates P3, P6, U4, and U5 ($p<0.05$). The number of spats on P3, P6, U4, and U5 substrates were more or less 30 ind/9cm^2. The rest except for an unpolluted reference sediment (S) which had a spat density about 110 ind/9cm^2 ranged 40 to 60 ind/9cm^2.

Fig. 2 shows the numbers of the oyster larvae attached on the substrates after 3 weeks attachment. The spat numbers on the substrates by week 3 markedly decreased from those of the previous

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week. For instance, the control spat number by week 2, 105 ind/ $9cm^2$, became to about 25 ind/$9cm^2$ by week 3. The most significant reduction was noticed in P3 where only 5 individual spats on average were persisted on the substrate ($p<0.05$). The spats numbers on P2, P4 and U4 were also reduced from control ($p<0.05$), while rest of the elutriates were comparable with control. Unlike the elevated number measured by week 2, the number on unpolluted reference elitriate by weeks 3 was lowered than the control without a statistical significance.

In the present study, embryonic development of *C. gigas* was significantly affected by the sediment elutriate. Xenobiotic chemicals can damage marine organisms directly by disrupting cellular pathways and indirectly via metabolites that are extremely toxic. BaP as a representative of PAHs, for instance, is metabolized by phase I-enzymes to benzo(a) pyrene-7,8-diol,-9,10-epoxide as the ultimate carcinogen, causing DNA-adduct formation with possible mutations during DNA replication or DNA strand-break induction (Harreus *et al*., 2004). The damage can be resulted in loss of viability in variety of physiological process. However, as was found in our previous study (Jo *et al*., 2005a,b; Choy *et al*., 2007), the damage of early life of the oyster observed in the present study also appeared much more sensitive than the rest of the total life. Adults bivalves have an established defense mechanism termed phases I to III (Kurelec and Pivcevic, 1991; Kurelec, 1992; Bard, 2000). The embryos of oyster and mussel also show P-gp-like activity with xenobiotic resistance(McFadzen *et al*., 1999). However, although phase 0 defense system is present in the unfertilized eggs, its activity starts some time after fertilization (McFadzen *et al*., 1999). Therefore, the larval sensitivity to the chemicals over the critical point found in the present study might be due to the chemical exposure before fertilization.

The present study has some implications in ecotoxicology and aquaculture business. Xenobiotic damage study by sediment elutriate exposure might bring a realistic information. In case of PAHs, for example, the high molecular weight PAHs are found to possess mutagenic, carcinogenic and/or teratogenic characteristics (Babson *et al*., 1986; Kanaly and Harayama, 2000), whereas the low molecular weight PAHs are known to exert acutely toxic effects such as narcosis (Swartz *et al*., 1990). Another ecotoxicological implication of the elutriate test is a synergistic effect of the mixed contamination of priority xenobiotics. Schmidt *et al*. (2005) found that a single dose of some xenobiotic compound did not affect the body growth of a fish, while a synergistic effect was apparent when two or more compound mixed were provided. This explanation is reasonable considering the xenobiotics employed in our study are from sediment elutriate.

The Pacific oyster occupies major proportion of the farmed production in Korea. The Blue Belts in Tongyoung, Korea, is one of the most productive locations for Pacific oyster in the world. Along the Blue Belts production of the oyster has decreased since 1987 due to local failures of good quality seed collection probably attributed to appearance of unhealthy broodstocks (NFRD, 1997; Park *et al*., 1999, Kang *et al*., 2000). The continued monitoring exhibited a possible relationship between occurrence of unhealthy seeds and progressive pollution in the locations (Park *et al*., 1999; Jo *et al*., 2002; Choy *et al*., 2003; Choy *et al*., 2007). A similar trend was also observed in the present study at Pohang and Ulsan where pollution is progressive. There is a growing body of evidence suggesting that pollutants in the global waters are progressive and certain xenobiotics may pose a greater hazard to aquatic organisms than previously demonstrated in laboratory because of their increased toxic potential under ultraviolet light (Peachey and Crosby, 1996; Lyons *et al*., 2002).

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