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Effects of Sediment Elutriates on the Early Reproductive Outputs in the Pacific oyster, *Crassostrea gigas*

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This is a subsequent study to our previous finding that Pacific oysters, *Crassostrea gigas*, gained a so-called upper plateau concentration, around 30,000 ng/g dry weight digestive gland for benzo(a)pyrene, showed reproductive behavior but produced their ensuing reproductive outputs damaged. A serial dilution of sediment elutriates from Jinhae Bay, Korea, where pollution was progressive, were exposed to gametes of the Pacific oyster for 0, 5, 10, 20, 30, and 60 min to detail the pollutant effects on very young specimens. There was an apparent critical dilution over which adverse effects are evident. This was 10% of the present sediment elutriate, corresponding to 0.3 ng/g on the basis of total polycyclic aromatic hydrocarbons (PAHs) for the oyster. Within the dilution the embryonic development was not influenced by the duration of exposure to its gamete stage. At higher dilutions over the critical dilution, occurrence of abnormality increased dependent on the pollutant dilution and the duration of exposure. Similar trends were also found in larval mortality. However, overall, the chemical toxicity was more significant to morphogenesis than to survival, suggesting a potential recruitment of the pollutants-induced abnormal larvae in the wild population to threaten the population integrity.

Key words: Sediment elutriate, POPs, Toxicity, Embryogenesis, *Crassostrea gigas*

Introduction

It is well known that a wide variety of toxic xenobiotics are present in global oceans. These include natural products as well as compounds of anthropogenic origins. 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 aglobal sense. For example, persistent organic and inorganic pollutants have been documented even in remote areas such as polar oceanic regions and deep seas (Stegeman et al., 1986; Muir et al., 1988; Mason and Fitzgerald, 1990; Ballschmiter et al., 1997; Stegeman et al., 2001). Therefore, the xenobiotic pollution in aquaculture farms is worth deep consideration. This is because they are classified as a priority pollutant, a group of compounds selected on the basis of their known or suspected carcinogenicity, teratogenicity or acute toxicity to aquatic organisms (Livingstone et al., 1990; Cavalieri et al., 1993a, 1993b; Bigger et al., 1994; Maccubbin, 1994; Venier and Canova, 1996; Akcha et al., 2000; Law et al., 2002; White, 2002).

Bivalves are especially vulnerable to ambient xenobiotic concentrations because of their filtering activity of organic matter including phytoplanktons, into which a considerable amount of the hydrophobic chemicals partition. However, they are able to survive and perform reproductive behavior in polluted waters (Chu et al., 2003). This means that they have their own pathways to metabolize or eliminate the chemicals that they are exposed (Stegeman, 1985; Kurelec and Pivcevic, 1991; Mitchelmore et al., 1998; Cheung et al., 2001; Boutetet al., 2004). One of the powerful cellular defense mechanisms the bivalves have is a system based on the multixenobiotic

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resistance (MXR) commonly due to expression of transmembrane P-glycoprotein (P-gp) which actively transports a wide variety of structurally and functionally diverse xenobiotics (Kurelec and Pivcevic, 1991; Kurelec, 1992; Bard, 2000). Xenobiotics metabolizing enzymes such as phases I (hydroxylation via cytochromes P450) and II (glutathione-S-transferases catalized in conjugation with glutathione) can be considered as another line of defense. Unlike our knowledge on the adult metabolism of the xenobiotic pollutants, embryonic metabolism of the pollutants has rarely been reported. In the study of pollutant toxicity finding a critical concentration of a pollutant is one of the preliminary approaches. This is particularly meaningful when a metabolic approach is excluded.

It is known that *Crassostrea gigas* exposed to upper plateau concentration of BaP achieves adult reproductive success but produces reproductive outputs damaged (personal communication). Interestingly, earlier stages of the reproductive output of the Pacific oyster were more resistant against the exposure. Here, we found a critical dilution of a polluted sediment elutriate and detailed how the elutriates over the critical dilution affected lives of the gametes and embryos of the oyster.

Materials and Methods

Oyster and maturation condition

Healthy immature spawners of the Pacific oyster, *C. gigas*, weighing 92.5±18.3 g (mean±SD) total weight, were collected from a local oyster farm in Tongyoung, Korea. On arrival at the NFRDI Shellfish Laboratory, the oysters were roughly cleaned by removing the epifaunae from the shells, and then acclimated in aquaria under flowing filtered water for 2 weeks prior to commencement of the experiment. The oysters in captivity were supplied with pasted algal foods of *Isochrysis galbana*, *Tetraselmis suecica*, *Phaeodactylum tricornutum*, and *Chaetoceros gracilisin* an alternative way supplemented with occasional live food *I. galbana* and *T. suecica* to maintain a nutritional balance.

Determination of sediment xenobiotics

Polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (DLPCBs) in sediments were determined as sediment xenobiotics in this study. Surface sediments (0-5 cm depth) were sampled from a location of Jinhae Bay as a polluted area and from a location in the Sokcho area as a reference site. The samples were collected with a box core sampler and then kept frozen at -20℃ until extraction. Detailed procedures of sample preparation for determination of PAHs, PCDDs/DFs and DLPCBs have been described in Moon et al. (2001, 2002, 2004).

For PAHs analysis, sediments were extracted with Soxhlet apparatus with toluene (Baker, USA) for 24 hr, after the spike of internal standards (ES 2044, Cambridge Isotope Laboratories Inc., USA). The extracts of samples were purified using an activated silica gel (Art No. 7734, 70-230 mesh, Merck, USA) column chromatography with successive eluants of *n*-hexane (Baker, USA) and 15% methylene dichloride (Cica-Merck, USA) in *n*-hexane. The second fraction was concentrated to less than 1 mL, and left at room temperature for 1 to 2 days to evaporate to 100-200 μL. The residue was dissolved with 100 μL of *n*-nonane (Fluka, USA) and determined for PAHs.

For the analysis of PCDDs/DFs and DLPCBs, sediments were extracted with Soxhlet apparatus with toluene for 24 hr, after the spike of internal standards (EPA-1613 LCS; PCDDs/DFs and PCB-LCS-A; DLPCBs, Wellington Laboratories, Canada). The extracts were cleaned 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 *n*-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 *n*-hexane and 100 mL of 50% methylene dichloride in in *n*-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 residue was dissolved with 20-50 μL of *n*-nonane and determined for PCDDs/DFs.

PAHs were determined using gas chromatography/mass spectrometery (GC/MS) with a DB-5MS capillary column (30 m×0.25 mm ID, 0.25 μm film thickness, J&W Scientific, USA). PCDDs/DFs and DLPCBs were analyzed with high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). A SP-2331 capillary column (60 m×0.25 mm ID, 0.25 μm film thickness, Supelco) and DB-5MS (60 m×0.25 mm ID, 0.25 μm film

thickness, J&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×0.22 mm ID, 0.25 μm film thickness, SGE). The quantitative determination of PCDDs/DFs and DLPCBs was performed by a relative response factor (RRF) method obtained through standard solution injections. Analytical details and instrument parameters for PAHs, PCDDs/DFs and DLPCBs have been presented by Moon et al. (2001, 2002, 2004).

Preparation of sediment elutriates

Elutriates were obtained based on a modification of the Melzian method (1990). The freeze-dried sediments were shaken mechanically in a 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.

Gamete exposure to sediment elutriate

Gametes from both sexes fully conditioned to maturation were taken by stripping (Park et al., 2002). Each gamete solution wasallocated to beakers containing different dilutions of sediment elutriates, 0 (seawater vehicle only), 5, 10, 20, 30, 50, and 100%. The gametes from each dilution were fertilized in the 24-well microplate 0, 5, 10, 20, 30, and 60 hr after exposure. The plates were then kept under total darkness for 26 hr to secure a duration long enough to have all the fertilized eggs metamorphosed to D-shaped larva. All the procedures were done in a room where the temperature was fixed to 22 (±0.5)℃. The cultures were replicated 4 times.

Measurement of embryonic success

The percent of abnormality was calculated primarily based on the criteria employed by His et al. (1997). Included were segmented eggs, normal or malformed embryos that failed to reach D-shaped larvae and D-shaped larvae with either a convex hinge, indented shell margins, an incomplete shell or a protruded mantle. A newly found abnormality criterion, for instance, D-shaped larva moving with its valves open continuously was additionally counted. The survival rate was calculated by a count of all the survivors to the total of the survivors and the dead multiplied by 100.

Statistics

The statistical analysis was done by a student *t*-test.

Results

Sediment concentrations of xenobiotic pollutants The sediment compositions of xenobiotic pollutants from the reference and the study sites were expressed in Tables 1 and 2. The composition of the major organic pollutant in the reference sediment was very low with an entire quantity of 4.04 ng/g dry weight for PAHs, 11.9 pg/g dry weight for PCDDs/DFs, and

Table 1. Concentrations of major xenobiotic pollutants in the sediment from reference site, Sokcho, Korea

PAHs	Concentration[a]	PCDDs/DFs	Concentration[b]	DLPCBs	Concentration[c]
Naphthalene	0.15	Tetra-CDDs	0.44	PCB 77	-[d]
Acenaphthalene-a	0.002	Penta-CDDs	0.37	PCB 81	-
Acenaphthene-	0.28	Hexa-CDDs	0.60	PCB 123	-
Fluorene-	0.14	Hepta-CDDs	0.95	PCB 118	-
Phenanthrene-	0.23	Octa-CDD	6.15	PCB 114	-
Anthracene-	0.04	Tetra-CDFs	0.63	PCB 105	-
Fluoranthene-	0.60	Penta-CDFs	0.87	PCB 126	-
Pyrene-	1.42	Hexa-CDFs	0.68	PCB 167	-
Benzo(a)anthracene-	0.05	Hepta-CDFs	0.60	PCB 156	-
Chrysene-	0.20	Octa-CDF	0.55	PCB 157	-
Benzo(b)fluoranthene-	0.21			PCB 169	-
Benzo(k)fluoranthene-	0.15			PCB 189	-
Benzo(a)pyrene-	0.15				-
Indeno(1,2,3-c,d)pyrene	0.17				-
Dibenzo(a,h)anthracene	0.04				-
Benzo(g,h,i)perylene	0.21				-
ΣPAH	4.04	ΣPCDD/DF	11.9	ΣDLPCB	-

[a]ng/g dry weight, [b]pg/g dry weight, [c]pg-TEQ/g dry weight, [d]not available.

Table 2. Concentrations of major xenobiotic pollutants in the sediment from Jinhae Bay, Korea

PAHs	Concentration[a]	PCDDs/DFs	Concentration[b]	DLPCBs	Concentration[c]
Naphthalene	0.25	Tetra-CDDs	67.6	PCB 77	0.00787
Acenaphthalene0.00787	0.87	Penta-CDDs	61.6	PCB 81	0.00015
Acenaphthene0.00015	1.00	Hexa-CDDs	84.4	PCB 123	0.00008
Fluorene0.00008	18.99	Hepta-CDDs	95.4	PCB 118	0.00365
Phenanthrene0.00365	17.20	Octa-CDD	313	PCB 114	0.00077
Anthracene0.00077	6.87	Tetra-CDFs	116	PCB 105	0.00128
Fluoranthene0.00128	31.22	Penta-CDFs	134	PCB 126	0.10316
Pyrene0.10316	39.65	Hexa-CDFs	143	PCB 167	0.00007
Benzo(a)anthracene0.00007	18.84	Hepta-CDFs	112	PCB 156	0.00592
Chrysene0.00592	22.36	Octa-CDF	70.4	PCB 157	0.00234
Benzo(b)fluoranthene0.00234	26.33			PCB 169	0.01350
Benzo(k)fluoranthene0.01350	20.26			PCB 189	0.00017
Benzo(a)pyrene0.00017	24.11				
Indeno(1,2,3-c,d)pyrene	62.04				
Dibenzo(a,h)anthracene	13.41				
Benzo(g,h,i)perylene	59.68				
ΣPAH	363	ΣPCDD/DF	1,198	ΣDLPCB	0.139

[a]ng/g dry weight, [b]pg/g dry weight, [c]pg-TEQ/g dry weight.

negligible for DLPCBs. The total organic pollutants from the study sediment was 363 ng/g dry weight for PAHs, 1,198 pg/g dry weight for PCDDs/DFs, 0.139 pg-TEQ/g dry weight for DLPCBs.

Elutriate toxicity to normal development

Eggs and sperm of *C. gigas* were exposed to 0, 5, 10, 20, 50, and 100% of the sediment elutriates for 0, 5, 10, 20, 30, and 60 min, respectively. After the exposure, fertilization was conducted to study the toxic effects of sediment elutriates on the embryonic development of *C. gigas* (top of Fig. 1). The control solution induced more than 99% normal growth of the embryos. The figure showed a decreasing trend with an increasing percent of sediment elutriate. However, it was not statistically significant up to 10% sediment elutriate added throughout all the durations of exposure. This means that the sediment elutriate was not statistically toxic to embryonic development regardless of the exposure duration within 60 min, provided it comprised less than 10% of the solution.

A significant difference was first noticed in the embryos exposed to 20% sediment elutriate ($P<0.01$). In this concentration, the exposure duration became a factor affecting the abnormal development of the oyster embryos. The embryos exposed to the concentration for 5 min showed about 90% of normal development which was not statistically different from those exposed to lower concentrations. However, the embryos exposed to the concentration longer than 5 min revealed 80% or less than 80% normal development. This concentration- and duration-dependent toxicity was more significant in exposing concentrations higher than 50% ($P<0.01$).

Elutriate toxicity to larval survival

Eggs and sperm of *C. gigas* exposed to 0, 5, 10, 20, 50, and 100% of the sediment elutriates for 0, 5, 10, 20, 30, and 60 min, respectively, were fertilized, and the toxic profiles to the embryos were expressed in terms of the survival of D-shaped larvae (bottom of Fig. 1). Overall, the larval survival was less affected by the concentration and duration of the elutriate treated than by larval abnormality. Like the abnormality, the elutriate proportions up to 10% did not affect the larval survival in a statistical way. At the proportion of 20%, larval survival was not constantly affected by the concentration, nor by the duration of the exposure. The elutriate proportion by which embryonic development was critically affected was over 50%. At this concentration the larval survival was significantly lowered from the previous group ($P<0.01$). However, the survival of the embryos fluctuated shortly after the elutriate proportion. The survival of the D-shaped larvae exposed to 50% elutriate for 60 min was around 40%. Interestingly, the embryonic development was evident in the total sediment elutriate. At this concentration the adverse effects of the elutriate was duration-dependent.

Discussion

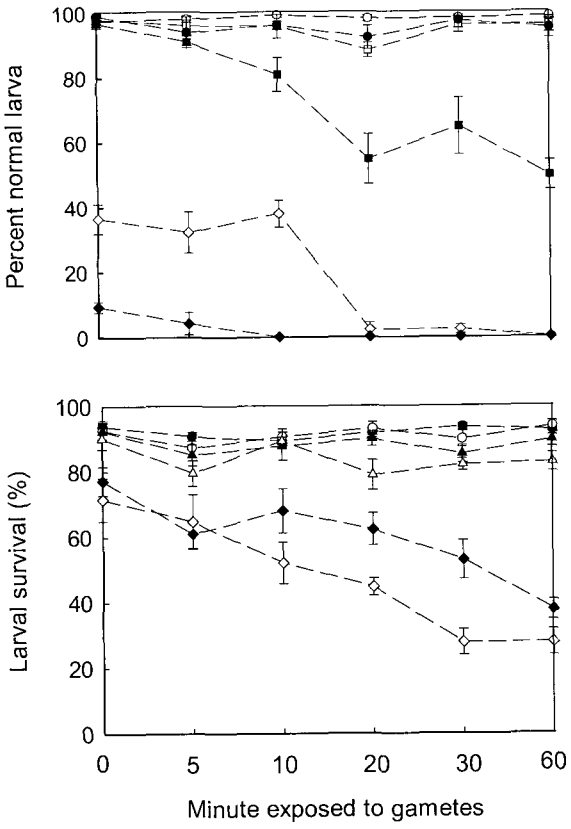

Fig. 1. Toxic effects of the Jinhae sediment elutriates on the early developmental events of the Pacific oyster, *Crassostrea gigas*. Occurrence rates of normal D-shaped larva (top) and survival (bottom) in a serial dilution of the elutriates after gametes exposure to the dilutions for 0, 5, 10, 20, 30, and 60 min. Symbols represent cultures containing different dilutions of the elutriates; vacant circle for elutriate-free control, solid circle for 5%, vacant rectangle for 10%, solid rectangle for 20%, vacant diamond for 50%, and solid diamond for 100% elutriate. Vertical bars stand for mean ± SE.

The sediment used in this study was contaminated. For the determination of the pollution grade, we compared the sediment with aso-called uncontaminated reference. In the comparison, the composition of the major organic pollutants in the reference sediment was very low with an entire quantity of 4.04 ng/g dry weight for PAHs, 11.9 pg/g dry weight for PCDDs/DFs, and negligible for DLPCBs. The total organic pollutants from the study sediment was 363 ng/g dry weight for PAHs, 1,198 pg/g dry weight for PCDDs/DFs, 0.139 pg-TEQ/g dry weight for DLPCBs (Tables 1 and 2).

The preparation of sediment elutriate is routinely achieved by taking an upper phase after a settling down period of 8 hours after physical shaking (Melzian, 1990). In the present study, the sediment elutriate was prepared by centrifuging the sediment mixtures fully shaken and then by immediately vacuuming them. This method was laborious, but found to render better withdrawal by eliminating repartition factors after the physical shaking. Methodologically, it is important because most of the priority organic pollutants, 5-ringed compounds for PAHs, for instance, show a low vapor pressure and high octanol-water partition coefficients (Krasnoschekova et al., 1992) which probably enable them to repartition to the sediment after being extracted. This was supported by Geffard et al. (2003) who found infinitesimal amounts of chemicals in the elutriate withdrawn from upper phase of mixtures after physical shakings.

In the present study, embryos of *C. gigaswere* employed as a reference for the embryo toxicity test. The oyster toxicity bioassays have been fully recognized as reliable, sensitive, and ecologically important tools for biomonitoring coastal environments and the physiological status of the animal (Beiras and His, 1995; His et al., 1997; Geffard et al., 2002a, 2002b; Geffard et al., 2004). The present study revealed a persistence of embryos against a substantial amount of the chemicals exposed. A dilution of 10% elutriate appeared to be a critical toxic concentration for the embryonic development. Abnormalities were significantly increased beyond the dilution, suggesting the dilution to be a critical point. Our finding, that the elutriate dilutions up to 10% did not affect morphogenesis of the oyster larvae regardless the exposing durations, supported this suggestion.

It is unlikely the adult oysters, where normal reproductive behavior was evident in the previous body burden of around 10,000 ng/g benzo(a)pyrene (personal communication), were effected by an early larval life that was sensitive to the chemical. The critical body burden in the *C. gigas* larvae was known to be around 0.3 ng/g for total PAHs, above which malformation was observed (Geffard et al., 2003). The sensitivity difference between the adults and the larvae might be due to differences in metabolic abilities of the chemicals. The critical body burden of an organic pollutant varies with chemical species and the metabolic ability of the pollutant that it is exposed to. Bivalves have a powerful cellular defense mechanism by achieving a multixenobiotic resistance commonly due to activation of transmembrane P-glycoprotein which actively transports a

wide variety of structurally and functionally diverse xenobiotic chemicals (Kurelec and Pivcevic, 1991; Kurelec, 1992; Bard, 2000). The embryos of oysters (*Crassostrea virginica*) and mussels (*Mytilus edulis*) also show P-gp like activity (Keppler, 1997; McFadzen et al., 1999). However, although P-gp is present in the unfertilized eggs, the activity of the glycoprotein starts 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.

Our finding that the sediment elutriate was more toxic to morphogenesis than to survival has some ecological meaning. One expected ecological damage is a potential recruitment of the pollutants-induced abnormal larvae in the wild population to threaten the population integrity. The pollutant damage also might be problematic in aquaculture. This is because, besides a potential hazardousness to consumers, the embryonic accumulation of the pollutants whether from maternal transfer or ambient waters, might be a potential factor threatening the wild seed collection for the oyster.

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