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Stress Expression by the Maternally Transferred Xenobiotic Pollutants in the Reproductive Outputs of the Pacific Oyster, *Crassostrea gigas*

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We previously pointed out that the polluted sediment elutriate manifestly affected the early events of reproductive outputs in the Pacific oysters, *Crassostrea gigas*. A serial dilution of priority xenobiotic sediment elutriates determined by gas chromatography/mass spectrometry (GC/MS) were exposed to gametes of the oyster with different stress burdens to detail the maternal stress transfer to its reproductive outputs. There was an apparent critical concentration over which survival and morphogenesis were significantly affected with more profound damage in morphogenesis. The critical concentration which drives mortality and abnormal morphogenesis of the larvae corresponded to a dilution between 10 and 20% of our elutriate. The adverse effects of the early lives by the xenobiotic exposure over the critical concentration were magnified by the maternal stress from the exposed benzo(a)pyrene (BaP), one of the priority polyaromatic hydrocarbons (PAHs) during the maturation condition. These results indicate that maternal transfer of the xenobiotic compounds from oysters living in the contaminated location might represent a significant adverse effect to their larval population of wild seeds.

Keywords: Sediment elutriate, Xenobiotics, Maternal transfer, Reproductive outputs, *Crassostrea gigas*

Introduction

It is well known that a wide variety of toxic xenobiotics are present in the global coasts. 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 is more generalized contamination 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 seas (Stegeman et al., 1986; Muir et al., 1988; Mason and Fitzgerald, 1990; Ballschmiter et al., 1997; Stegeman et al., 2001). The ubiquity of the chemicals in the coastal waters has been a recent research topic among the ecotoxicologists. 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).

The potential for the increased stress in marine organisms due to the chemical pressures associated with increasing 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 item. Sediments are not only a reservoir for contaminants, but also a source of toxicants for marine animals. 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, an employment of the sediment elutriate is suggestible for the toxicity test of the xenobiotics in the laboratory.

Oyster embryos and larvae are currently used to assess the biological quality of coastal estuarine seawaters and their sediment (Beiras and His, 1995; His et al., 1997; Geffard et al.,

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2002a, b; Geffard et al., 2003, 2004; Jo et al., 2005). 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. The embryonic and larval damages of *Crassostrea gigas* by the xenobiotic pollutants can be research concerns of aquaculturists. One of the concerns is the potential influence of the chemicals on the aquaculture success, including wild seed collection of the oyster. Previously, we found that the embryonic and larval lives of the oyster were significantly influenced by the elutriate of the sediment from polluted sites. Here, we detail the influence by introducing a maternal stress parameter in it.

Materials and Methods

Preparation of benzo(a)pyrene-vectoring algae

Four algal species, *Isochrysis galbana*, *Tetraselmis suecica*, *Phaeodactylum tricornutum*, and *Chaetoceros gracilis* were cultured in 3 L round flasks containing f/2 medium (Guillard and Ryther, 1962) with white light (PAR) at about 140 μmol m^{-2} s^{-1} and 24L:0D cycle as inocula. Algae at mid-logarithmic growth phase were added to 30 L round chambers (culture volume, 20 L) to make final cell densities, 400 to 600×10^5 cells mL^{-1} for *I. galbana* and 300 to 500×10^5 cells mL^{-1} for *T. suecica*, *P. tricornutum*, and *Chaetoceros gracilis* The cultures were exposed to 4 BaP concentrations, 0 (acetone vehicle only), 50, 500, and 5000 ng/g in the f/2 medium at 20 ± 0.5°C for an hour.

Oyster exposure to benzo(a)pyrene

The immature *C. gigas* were supplied for 2 months with algae exposed to 4 BaP concentrations, 0 (control), 50, 500, and 5000 ng/g (named, hereafter, control, 50, 500, and 5000 ng/g oyster, respectively). Oysters were basically supplied with *I. galbana* and *T. suecica* alternatively with occasional *P. tricornutum* and *C. gracilis* for nutritional balance. The feeding scheme was 2 to 3 times a day. Prior to every feeding, the flowing water was stopped and then added 20 L BaP-carrying algae to make total water volume of 120 L. Each feeding was allowed for 20 minutes. After the feeding, the flowing water was resumed until next feeding. Water flowing rate after feeding was 500 L/hr. The culture waters used were the sand- and charcoal-filtered. Water temperatures were in a range of 10-15 ± 0.5°C.

Test sediment elutriates

The powdered sediments from Jinhae Bay, Korea, where pollution was progressed were elutriated by Melzian's method (1990) and then preserved in total darkness at 4 after GC/MASS analysis for priority xenobiotic compounds in the sediment. Because the preserved elutriates were used 2 days after the elutriation for our previous experiment (Jo et al., 2005), they were not further determined for the chemical compositions. Four priority xenobiotic chemicals previously determined were polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (DLPCBs. All the sediments were treated and extracted by the method described by Moon et al. (2001, 2002, 2004).

PAHs were determined using gas chromatography/mass spectrometery (GC/MS) with DB-5MS capillary column (30 m length, 0.25 mm ID, 0.25 μm film thickness, J&W Scientific). PCDDs/DFs and DLPCBs were analyzed with high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). A SP-2331 capillary column (60 m length, 0.25 mm ID, 0.25 μm film thickness, Supelco) and DB-5MS (60 m length, 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 length, 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). Sediment elutriates from Sokcho, Gangwon, Korea were also determined in ways described above for a reference elutriate.

Gaméte exposure to sediment elutriate

Gametes from fully maturation-conditioned oysters were taken by stripping way (Park et al., 2002). Each gamete solution from each oyster were allocated to 5 L 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 hours after exposure. The plates were then kept under total darkness for 26 hours to secure time long enough to have all the fertilized eggs metamorphosed to D-shaped larva. All the procedures were done in a room where temperature was fixed to 22 ± 0.5°C. The cultures were rep-

licated by 4 times.

Measurement of embryonic success

The percent normality was calculated by the method of Jo et al. (2005) that was primarily modified from 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 convex hinge, indented shell margins, incomplete shell or 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 survived to total of the survived and the dead mutiplied by 100.

Statistics

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

Results

Xenobiotic composition in the sediments

The sediment compositions of xenobiotic pollutants from reference and study sites were determined by means of GC/MS analyses. The reference compositions were: total PAH quantity of 4.04 ng/g dry weight (naphthalene 0.15, acenaphthalene 0.002, acenaphthene 0.28, eluorene 0.14, phenanthrene 0.23, anthracene 0.04, fluoranthene 0.60, pyrene 1.42, benzo(a)anthracene 0.05, chrysene 0.20, benzo(b)fluoranthene 0.21, benzo(k)fluoranthene 0.15, benzo(a)pyrene 0.15, indeno (1,2,3-c,d)pyrene 0.17, dibenzo(a,h)anthracene 0.04, and benzo (g,h,i)perylene 0.21), total PCDDs/DFs of 11.90 pg/g dry (tetra-CDDs 0.44, penta-CDDs 0.37, hexa-CDDs 0.60, hepta-CDDs 0.95, octa-CDD 6.15, tetra-CDFs 0.63, penta-CDFs 0.87, hexa-CDFs 0.68, hepta-CDFs 0.60, and octa-CDF 0.55), and negligible. The total organic pollutants from the study sediments were: 363 ng/g dry weight for PAHs (Naphthalene 0.25, acenaphthalene 0.87, acenaphthene 1.00, fluorine 18.99, phenanthrene 17.20, anthracene 6.87, fluoranthene 31.22, pyrene 39.65, benzo(a)anthracene 18.84, chrysene 22.36, benzo(b)fluoranthene 26.33, benzo(k)fluoranthene 20.26, benzo (a)pyrene 24.11, indeno(1,2,3-c,d)pyrene 62.04, dibenzo(a,h) anthracene 13.41, and benzo(g,h,i)perylene 59.68), 1198 pg/g dry weight for PCDDs/DFs (tetra-CDDs 67.6, penta-CDDs 61.6, hexa-CDDs 84.4, Hepta-CDDs 95.4, octa-CDD 313, tetra-CDFs 116, penta-CDFs 134, hexa-CDFs 143, hepta-CDFs 112, and octa-CDF 70.4), and 0.139 pg-TEQ/g dry weight for DLPCBs (*PCB 77* 0.00787, *PCB 81* 0.00015, *PCB 123* 0.00008, *PCB 118* 0.00365, *PCB 114* 0.00077, *PCB 105* 0.00128, *PCB 126* 0.10316, *PCB 167* 0.00007, *PCB 156* 0.00592, *PCB 157* 0.00234, *PCB 169* 0.01350, and *PCB 189* 0.00017).

Elutriate toxicity to normal development

Left figures in Fig. 1 show the normal larval development against the chemical dilution and maternally oriented BaP stress. The embryonic development from the intact oysters achieved more than 99% normality (control of left figures in Fig. 1). The percent 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 the durations except for dilution 10% at 20 minutes exposure, where the normality was significantly lower than others ($P<0.01$). In other words, sediment elutriate was not statistically toxic to embryonic development regardless the exposing duration within 60 minutes, provided it was exposed to less than 10% in the solution. The oyster embryos were vulnerable to 20% sediment elutriate. It was particularly significant when their gametes were exposed to the chemical dilution over 20 minutes ($P<0.01$); a drastic percent of the abnormal larvae appeared from the point. The embryos grown under 50% or lower dilution showed extremely low normality regardless the times their gametes were exposed to the dilutions.

The embryonic development of 50 ng/g oysters (50 ng/g of left figures in Fig. 1) showed similar trend as that of control, only differing in decreased frequency of normal larvae. For example, the embryonic development grown under low dilutions after 60 minutes gamete exposure gained percent normal development between 68.6 and 76.8. These values were noticeably lowed, compared with control values (over 87.4).

The bottom 2 of left figures in Fig. 1 show the embryonic development of 500 ng/g and 5000 ng/g oysters. One of the striking differences of the two figures from the previous ones was in the drastically lowed occurrence of normal larvae; none of the larvae from the 500 ng/g oysters showed the normality over 25%. This was much more evident in the embryos of 5000 ng/g oysters.

Elutriate toxicity to larval survival

Five oyster groups were separately stressed during the reproducing period by feeding algae grown 0, 50, 500, and 5000 ng/g BaP solution. The eggs and sperm of *C. gigas*

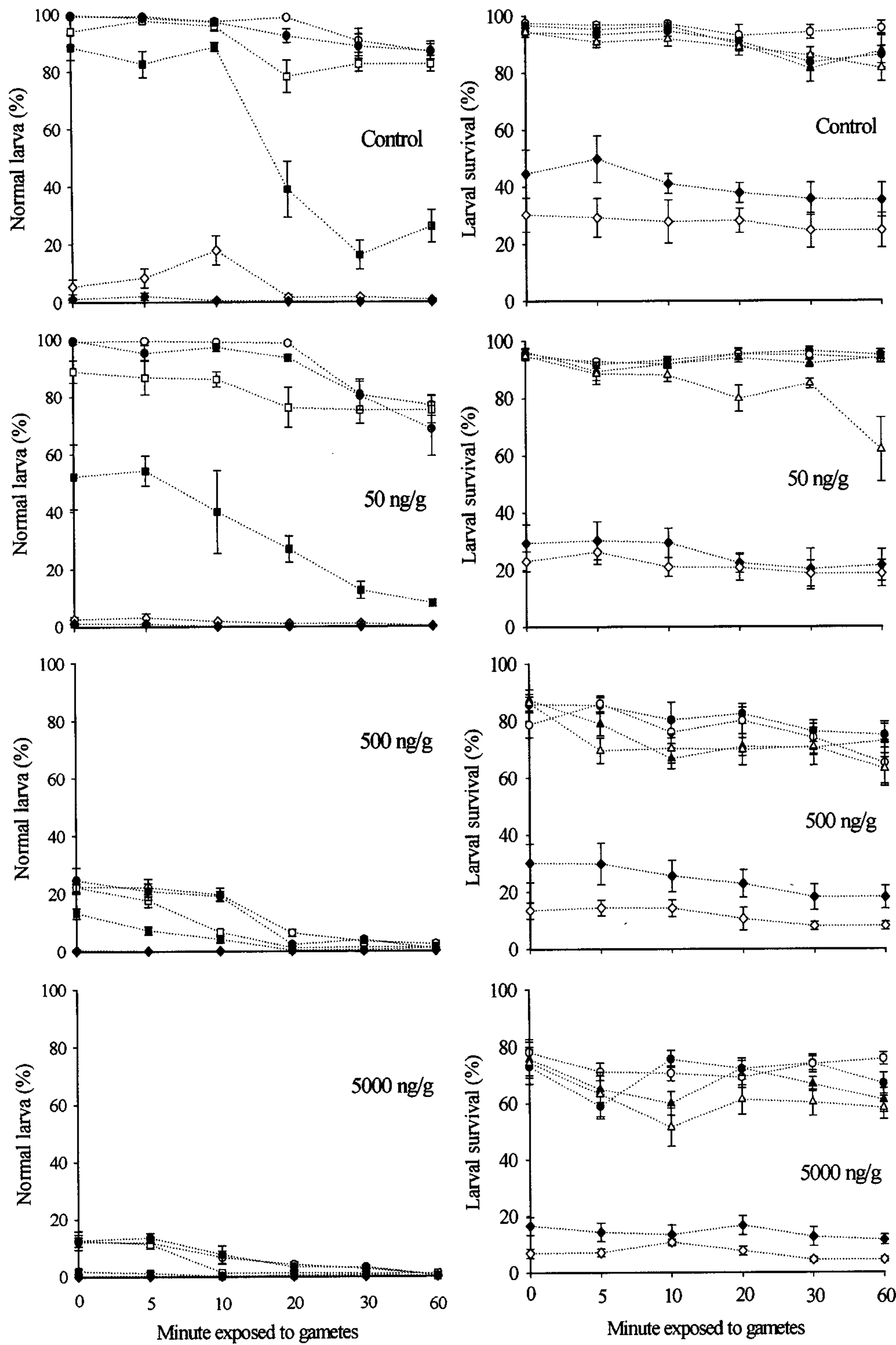

Fig. 1. Toxic effects of the Jinhae sediment elutriates on the early developmental events of Pacific oyster, *Crassostrea gigas* stressed by the exposure of toenzo(a)pyrene. Measured are normal D-shaped larva and survival after the following procedure. Freshly gained eggs and sperm of the oyster were exposed to a serial dilution of sediment elutriates for 0, 5, 10, 20, 30, and 60 minutes, respectively, and fertilized and incubated to D-shaped larva for each dilution. The incubation continued for 26 hrs at 22±0.5 °C to provide time enough to metamorphose to D-shaped larva. Left row refers normalities from spawners conditioned in BaP concentrations: top for control, the 2nd top for 50 ng/g oysters, 3rd top for 500 ng/g oyster, and bottom for 5000 ng/g oysters. Right row refers D-shaped larval survival from spawners conditioned in BaP concentrations: top for control, the 2nd top for 50 ng/g oysters, 3rd top for 500 ng/g oyster, and bottom for 5000 ng/g oysters. Symbols represent cultures containing different percents of sediment elutriate; 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.

exposed to 0, 5, 10, 20, 50, and 100% of the sediment elutriates for 0, 5, 10, 20, 30, and 60 minutes, respectively, were fertilized, and the toxic profiles to the embryos were expressed in terms of D-larval survival (right figures in Figure 1). Overall, the larval survival was less affected by the concentration and duration of the elutriate treated than larval normality. Like the normality, the elutriate proportions up to 20% did not affect the control larval survival in a statistical way. The D-larval survival from 50 ng/g oysters showed a similar but decreased tendency as that from control with slight difference at dilution 20%. At the dilution, larval survival was not constantly affected by the concentration, only did the value at 60 minutes gamete exposure differed from the rest of the durations in a significant way ($P<0.01$). This tendency was also found in the D-larval survival from 500 ng/g and 5000 ng/g oysters. Interestingly, unlike the tendency of normal larval occurrence, the tendency of larval survival continued through the degree of the maternal BaP stress.

Discussion

The study sediment characterized by 363 ng/g dry weight for PAHs, 1,198 pg/g dry weight for PCDDs/DFs, and 0.139 pg-TEQ/g dry weight for DLPCBs, and reference sediment contained 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 were extreme enough to be employed as tools for a toxicity test. This was supported by our previous study in which developmental damages of *C. gigas* embryos was in a way dependent on a serial concentration of the elutriates (Jo et al., 2005). Our successful achievement of the oyster damage difference between reference and study sediments also rendered the usefulness of the modified elutriation procedure from Melzian's method (1990).

In the present study, embryonic survival of *C. gigas* was significantly affected by the chemical exposure. 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 (Mitchelmore et al., 1998; Boutet et al., 2004; Harréus 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., 2005), the damage of early life of the oyster observed in the present study appeared much more sensitive than the rest of the total life. Adults bivalves have an established defense mechanism termed phases I to III (Stegeman, 1985; Kurelec and Pivcevic, 1991; Kurelec, 1992; Bard, 2000). The embryos of oyster and mussel also show P-gp-like activity (Keppler, 1997; McFadzen et al., 1999). However, although phase 0 defense system is present in the unfertilized eggs, its activity 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.

Another possible explanation of the sensitivity is a synergistic effect of the mixed containment 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.

Finding a species-specific critical concentration of xenobiotic toxicants makes one of the key parameters in the toxicity study. The oyster toxicity bioassays have been fully recognized as reliable, sensitive, and ecologically important tools for biomonitoring coastal environments and physiological status of the animal (Beiras and His, 1995; His et al., 1997; Geffard et al., 2002a, b; Geffard et al., 2004). The critical concentration of the present study expressed in terms of elutriate dilution was in a same range as that of our previous study (Jo et al., 2005). The concentration was some dilution between 10 and 20%, representing around 0.3 ng/g for total PAHs in the sediment elutriate (Geffard et al., 2003).

We could conclude that the stress expression by the chemical exposure over the critical concentration was more significant in the abnormality than survival. At the same time the trend was clearer when the reproductive outputs were from stressed parents. One of the expected problems is a potential recruitment of the abnormal larvae in wild population to threaten the population integrity. This might have an implication in aquaculture success. The embryonic damage by the xenobiotics might be a potential factor threatening the wild seed collection. For adults, the perceptions and signaling of the xenobiotic chemicals through cellular pathway of the farmed organisms inevitably leads to changes in reproductive behavior and endogenous processes (Fingerman and

Nagabhushanam, 1997), damaging reproductive progress of the farmed animals (Voltz et al., 2002) and maternal transfer to their reproductive outputs.

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