

# Seawater Polluted with Highly Concentrated Polycyclic Aromatic Hydrocarbons Suppresses Osteoblastic Activity in the Scales of Goldfish, *Carassius auratus*

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**Seawater polluted with highly concentrated polycyclic aromatic hydrocarbons suppresses osteoblastic activity in the scales of goldfish, *Carassius auratus***

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## **ABSTRACT**

We have developed an original in vitro bioassay using teleost scale that has osteoclasts, osteoblasts, and bone matrix as each marker: alkaline phosphatase (ALP) for osteoblasts and tartrate-resistant acid phosphatase (TRAP) for osteoclasts. Using this scale in vitro bioassay, we examined the effects of seawater polluted with highly concentrated polycyclic aromatic hydrocarbons (PAHs) and nitro-polycyclic aromatic hydrocarbons (NPAHs) on osteoblastic and osteoclastic activities in the present study. Polluted seawater was collected from 2 sites (the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea). Total levels of PAHs in the seawater from the Alexandria and Suez Canal sites were 1364.59 and 992.56 ng/l, respectively. We can detect NPAHs in both seawater samples. Total levels of NPAHs were detected in the seawater of the Alexandria site (12.749 ng/l) and the Suez Canal site (3.914 ng/l). Each sample of polluted seawater was added into culture medium at dilution rates of 50, 100, and 500 and incubated with the goldfish scales for 6 hrs. Thereafter, ALP and TRAP activities were measured. As a result, ALP activity was significantly suppressed by both polluted seawater samples diluted at least 500 times, although TRAP activity did not change. In addition, mRNA expressions of osteoblastic markers (ALP, osteocalcin, and the receptor activator of the NF- $\kappa$ B ligand) decreased significantly, as did the ALP enzyme activity. Actually, ALP activity decreased with selected PAHs and NPAHs treatments. We conclude that seawater polluted with highly concentrated PAHs and NPAHs influenced bone metabolism in teleosts.

**Keywords:** polycyclic aromatic hydrocarbons; nitro-polycyclic aromatic hydrocarbons; osteoblasts; osteoclasts; fish scales; bone metabolism; goldfish; bioassay

## INTRODUCTION

Oil spills on the sea surface are seen relatively often. Observed oil spills correlate very well with the major shipping routes (Brekke and Solberg, 2005). In addition, accidental oil spills have occurred. Big oil spills from the Deepwater Horizon and sunken oil tanker ships (the Exxon Valdez and the Nakhodka) directly caused oil pollution in the marine environment and affected marine organisms (Bue et al., 1998; Heintz et al., 2000; Hayakawa et al., 2006; de Soysa et al., 2012). Oil contamination can seriously impact the marine environment toxicologically. Polycyclic aromatic hydrocarbons (PAHs) are toxic substances in oil (Hayakawa et al., 2006). For a long time (more than 14 years) after big oil spill from the Exxon Valdez, the toxicity of PAHs originating from spilled oil has been observed in numerous marine animals (Peterson, 2003). In fish, which are representative of aquatic animals, PAHs have been reported to cause bone deformities in Pacific herring, pink salmon, and sea bass (Barron et al., 2004; Danion et al., 2011). Therefore, more attention should be given to the metabolism of fish bone.

The teleost scale is calcified tissue that contains osteoblasts, osteoclasts, and the two layers of bone matrix, i.e., a bony layer, which is a thin, well-calcified external layer, and a fibrillary layer, which is a thick, partially calcified layer (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007; Azuma et al., 2007; Ohira et al., 2007). The bone matrix, which includes type I collagen (Zylberberg et al., 1992), osteocalcin (Nishimoto et al., 1992), and hydroxyapatite (Onozato et al., 1979), is present in scale as well as in mammalian bone. Furthermore, it is known that teleost scale regenerates after being removed. We previously reported that the osteogenesis in regenerating scales was very similar to that in calvarial bone (mammalian head bone) (Yoshikubo et al., 2005).

Osteoblastic activity in the regenerating scale was considerably higher than that in normal scale (Yoshikubo et al., 2005; Suzuki et al., 2009). The response of osteoblasts to estrogen was higher in regenerating scales than in normal scales (Yoshikubo et al., 2005). In regenerating scales, osteoclastic activity was at the same level as osteoblastic activity and sensitively responded to gravity loading (Suzuki et al., 2009). These facts indicate that regenerating scale can be utilized as a model for teleost bone to analyze the influence of PAHs on osteoblasts and osteoclasts.

For the purpose of analyzing marine pollution from oil spills, in the present study, we used our scale in vitro bioassay system to examine the influence on osteoblasts and osteoclasts of polluted seawater samples from the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea.

## **MATERIALS AND METHODS**

### *Animals*

Goldfish (*Carassius auratus*), purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan), were artificially fertilized from female and male goldfish (20–30 g) in the Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology. Fish were fed a commercial pellet diet for puffer fish every morning and were maintained in fresh water at 26°C. Growing fish 12–15 cm in body length were moved to Noto Marine Laboratory in Kanazawa University and used for in vitro experiments analyzing cell activity and mRNA expression.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University.

*Sampling sites and analysis of PAHs and nitro-polycyclic aromatic hydrocarbons (NPAHs) in seawater samples*

Surface water was collected from the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea (Figure 1). There are very many shipping lanes in these sea areas. The sampling, extraction, and analysis of PAHs and nitro-polycyclic aromatic hydrocarbons (NPAHs) were performed as follows.

Water samples were collected using narrow-neck glass 2-liter bottles (previously washed with dichloromethane). One hundred ml (5%) of methanol was added to each sample. The sample was filtered by using a glass fiber filter (GC-50, 0.45- $\mu$ m pore size, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and then the filtrate was filtered again by using a 3M Empore solid phase extraction disk (C18,  $\phi$ 47 mm, 3M Company, Saint Paul, MN, USA). The 3M disks were ultrasonically twice extracted with dichloromethane and then the solution was filtered through a glass filter paper. After addition of 100- $\mu$ l dimethyl sulfoxide (DMSO), the filtrate was concentrated. The residue containing 100- $\mu$ l DMSO was dissolved in 900- $\mu$ l of ethanol. The final sample solution was filtered through a membrane filter (HLC-DISK13, 0.45- $\mu$ m pore size, Kanto Chemical Co., Inc., Tokyo, Japan). Other conditions were the same as in our previous reports (Tang et al., 2005a, 2005b; Hattori et al., 2007; Nassar et al., 2011).

We analyzed fifteen PAHs using HPLC with fluorescence detection. The system consisted of two HPLC pumps (LC-10A, Shimadzu, Kyoto, Japan), a fluorescence detector (RF-10A,

Shimadzu), a system controller (SCL-10A, Shimadzu), an integrator (Chromatopac C-R7Ae, Shimadzu), a degasser (DGU-14A, Shimadzu), an auto sample injector (SIL-10A, Shimadzu), a column oven (CTO-10AS, Shimadzu), a guard column (Inertsil ODS-P, 4.0 i.d. × 10 mm, GL Sciences Inc., Tokyo, Japan), and an analytical column (Inertsil ODS-P, 4.6 i.d. × 250 mm, GL Sciences Inc., Tokyo, Japan). The mobile phase was a mixture of acetonitrile and water with a gradient concentration mode of acetonitrile. The flow rate was 1.0 ml/min. The fluorescence time program detector was set to detect at the optimum excitation and emission wavelength for each PAH.

NPAHs were analyzed by HPLC with chemiluminescence detection with several modifications in accordance with our laboratory detection method (Tang et al., 2009; Nassar et al., 2012, 2015). The HPLC system consisted of two analytical columns (both Cosmosil 5C18-MS, 4.6 i.d. × 10 mm, Nacalai Tesque, Kyoto, Japan), two mobile phase pumps (LC-10A, Shimadzu, Kyoto, Japan), a chemiluminescence reagent solution pump (DMX-2000, Sanuki, Tokyo, Japan), a chemiluminescence detector (CLD-10A, Shimadzu, Kyoto, Japan), a system controller (SCL-10A, Shimadzu, Kyoto, Japan), a chromatopac integrator (C-R4A, Shimadzu, Kyoto, Japan), a degasser (DGU-14A, Shimadzu, Kyoto, Japan), an auto sample injector (SIL-10A, Shimadzu, Kyoto, Japan), a column oven (CTO-10AC, Shimadzu, Kyoto, Japan), and a guard column (Cosmosil 5C18-MS, 4.6 i.d. × 10 mm, Nacalai Tesque, Kyoto, Japan). The mobile phase was imidazole–perchloric acid buffer (pH 7.6) : acetonitrile (1:1) at a flow rate of 1.0 ml/min. The post-column chemiluminescence reagent solution was an acetonitrile solution containing 0.02 mM *bis*(2,4,6-trichlorophenyl) oxalate and 15 mM hydrogen peroxide at a flow rate of 1.0 ml/min.

The validity of the above-mentioned method was already confirmed in our previously published papers, which illustrated recovery variations of 87 to 104%; limits of detection

(S/N = 3) varied from 0.25 to  $1.5 \times 10^{-15}$  mol, and limits of quantification (S/N = 10) varied from  $10^{-15}$  to  $10^{-12}$  mol (over two orders) and showed good linearity ( $r^2 \geq 0.899$ ) (Tang et al., 2005a; Hattori et al., 2007; Nassar et al., 2011).

*Effects of polluted-seawater samples on scale osteoblastic and osteoclastic activities using cultured scales of goldfish*

Goldfish were anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc., St. Louis, MO, USA), and the normally developed scales on the body were removed to allow the regeneration of scales. On day 14, goldfish were anesthetized again, and the regenerating scales were removed. Using the removed regenerating scales, we examined the influences of seawater samples on the osteoblasts and osteoclasts with alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) as markers because, in mammals, the effects of hormones and some bioactive substances on osteoblasts and osteoclasts have been investigated using ALP and TRAP as respective markers (Vaes, 1988; Dimai et al., 1998; Suda et al., 1999). These scales were incubated for 6 hrs in Leibovitz's L-15 medium (Phenol Red-free, Invitrogen, Grand Island, NY, USA) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals, Inc., Aurora, OH, USA) supplemented with polluted seawater (diluted 50, 100, and 500 times with L-15 medium). The influences of polluted seawater on osteoblasts and osteoclasts were compared with that of artificial seawater (Allen seawater: NaCl 3%,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.358%,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  0.272%,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.06%, KCl 0.039%,  $\text{NaHCO}_3$  0.01%; Suzuki et al., 1992) as a non-polluted seawater.



From one goldfish, 24 scales from the left or right side were utilized. The 24 scales used in the present study were used as follows: (1) 8 scales for ALP analysis with 500-times dilution, (2) 8 scales for ALP analysis with 100-times dilution, (3) 8 scales for ALP analysis with 50-times dilution. The respective mean for ALP (obtained from 8 individual scales of one goldfish) was compared with that of the right side (control group: diluted artificial seawater samples). After incubation, ALP activities were measured using the same methods previously described (Suzuki et al., 2009). The results are shown as the means  $\pm$  SEM (n = 10). In the case of TRAP, the same experiment was done repeatedly using 10 individual goldfish.

The methods for measuring ALP and TRAP activities were as follows. An aliquot of 100  $\mu$ l of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM MgCl<sub>2</sub>) or an acid buffer (0.1 M sodium acetate, including 20 mM tartrate, pH 5.3) was added to each well. Then, each scale was put into its own well in a 96-well microplate. This microplate was immediately frozen at -80°C and then kept at -20°C until analysis. An aliquot of 100  $\mu$ l of 20 mM para-nitrophenyl phosphate in an acid or alkaline buffer was then added to each well of melted solution in the microplate. This plate was incubated at 23°C for 20 min, while being shaken. After incubation, the reaction was stopped by adding 50  $\mu$ l of 3 N NaOH. One hundred fifty  $\mu$ l of a colored solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of para-nitrophenol (pNP) produced using a standard curve for pNP.

After measuring both ALP and TRAP activities, the scales were measured with Image J. Afterward, ALP and TRAP activities were normalized to the surface area (mm<sup>2</sup>) of each scale (Suzuki et al., 2009).

*Changes in osteoblastic and osteoclastic marker mRNA expressions in polluted seawater-treated goldfish scales*

Scales were collected from goldfish anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich). To examine changes in osteoblastic (ALP, osteocalcin, and the receptor activator of nuclear factor  $\kappa$ B ligand: RANKL) and osteoclastic (cathepsin K, vacuole-ATPase, and matrix metalloproteinase-9: MMP-9) marker mRNAs that responded to polluted seawater, these scales were incubated for 6 hrs in L-15 medium (Phenol Red-free, Invitrogen) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals) that included polluted seawater or artificial seawater samples (each diluted 50 times). After incubation, the scales were frozen at -80 °C for mRNA analysis.

Total RNAs were prepared from goldfish scales using a total RNA isolation kit for fibrous tissue (Qiagen GmbH, Hilden, Germany). Complementary DNA synthesis was performed using a kit (Qiagen GmbH). Gene-specific primers for ALP (sense: 5'-TGGACACAGCGGTGAGGAAA-3'; antisense: 5'-GTGGGCATATGCTGCACTCG-3') (Thamamongood et al., 2012), osteocalcin (sense: 5'-ATGCCTGAGCGCAGGTCTTC-3'; antisense: 5'-CACAGGCCAGGTTTGCTTCA-3') (Thamamongood et al., 2012), RANKL (sense: 5'-CGAGTGTGGCGATTTTGTG -3'; antisense: 5'-ATGGGCGTCTTGATTGGAAG-3') (AB894120), cathepsin K (sense: 5'-TGGGAGGGCTGGAAACTCAC-3'; antisense: 5'-CATGAGCCGCATGAACCTTG-3') (AB236969), vacuole-ATPase (sense: 5'-ACACCGCTTGCTGCTTTCTTTC-3'; antisense: 5'-ACCAGTGTGGAGCAGAACTTG-3') (AB894122), and MMP-9 (sense: 5'-

GCTTCTGCCCCAGTGAGCTT-3’; antisense: 5’-GTGGAGCACCAGCGATACCC-3’) (AB889498) were used. The amplification of EF1 $\alpha$  cDNA using a primer set (sense: 5’-ATTGTTGCTGGTGGTGGTGG-3’; antisense: 5’-GGCACTGACTTCCTTGGTGA-3’) (AB979720) was performed. The PCR amplification was analyzed by real-time PCR apparatus (Mx3000p, Agilent Technologies, Santa Clara, CA, USA) (Suzuki et al., 2011a). The annealing temperature of ALP, osteocalcin, RANKL, vacuole-ATPase, cathepsin K, MMP-9, and EF1 $\alpha$  was 60°C. The ALP, osteocalcin, RANKL, vacuole-ATPase, cathepsin K, and MMP-9 mRNA levels were normalized to the EF1 $\alpha$  mRNA level.

#### *Effects of PAHs and NPAHs on scale osteoblastic activity using cultured scales of goldfish*

In both the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea, highly concentrated PAHs (naphthalene and acenaphthene) and NPAHs (2-nitrofluorene and 3-nitrobenzanthrone) were selected. The influence of these chemicals on ALP activity in scales was examined to confirm the toxicity of PAHs and NPAHs on fish bone metabolism. The concentrations of PAHs (naphthalene and acenaphthene) and NPAHs (2-nitrofluorene and 3-nitrobenzanthrone) were 6 ng/l and 40 pg/l, respectively. The concentrations of these chemicals used in this experiment corresponded to approximately 50-times dilutions of polluted seawater, as indicated in Table 1.

The preparation and incubation of regenerating goldfish scales were described above. After 6 hrs of incubation supplemented with PAHs and NPAHs, ALP activity was measured and normalized to the surface area (mm<sup>2</sup>) of each scale (Suzuki et al., 2009).

### *Statistical analysis*

All results are expressed as the means  $\pm$  SE. The statistical significance between the control and experimental groups was assessed by paired *t*-test. In all cases, the selected significance level was  $P < 0.05$ .

## **RESULTS**

### *Analysis of PAHs and NPAHs in the Alexandria and Suez Canal sites*

Polluted seawater was collected from 2 sites (the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea). The results are indicated in Table 1. In the seawater of the Alexandria and Suez Canal sites, the levels of total PAHs were 1364.59 and 992.56 ng/l, respectively. In addition, we detected NPAHs in both seawater samples. Total levels of NPAHs were detected in the seawater of the Alexandria site (12.749 ng/l) and the Suez Canal site (3.914 ng/l).

### *Effects of seawater samples on scale osteoblastic and osteoclastic activities using the cultured scales of goldfish*

Each polluted seawater sample was added to culture medium diluted 50, 100, or 500 times and incubated with the goldfish scales for 6 hrs. Thereafter, ALP and TRAP activities were measured. The diluted seawater from both sites significantly suppressed ALP activity (500-time dilution:  $P < 0.05$ ; 100-time dilution:  $P < 0.05$ ; 50-time dilution:  $P < 0.01$ ) as compared with diluted artificial seawater (Fig. 2).

On the other hand, polluted seawater from both sites did not influence TRAP activity at least present conditions (Fig. 3).

*The influences of polluted seawater in osteoblastic and osteoclastic marker mRNA expressions*

Osteoblastic and osteoclastic marker results are indicated in Figs. 4 and 5, respectively.

At 6 hrs of incubation after treatment of polluted seawater from the Alexandria and Suez Canal sites, the mRNA expressions of osteoblastic markers (ALP, osteocalcin, and the receptor activator of the NF- $\kappa$ B ligand: RANKL) significantly ( $P < 0.05$ ) decreased, as did the ALP enzyme activity, although polluted seawater from both sites did not influence the expression of osteoclastic marker (cathepsin K, vacuole-ATPase, and MMP-9) mRNA expression at least present conditions.

*Effects of PAHs and NPAHs on scale osteoblastic activity using cultured scales of goldfish*

In both the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea, the high concentrated-PAHs (naphthalene and acenaphthene) and -NPAHs (2-nitrofluorene and 3-nitrobenzanthrone) were selected. The influences of these chemicals on scales ALP activity were examined in the present study.

With the addition of each chemical, ALP activity in the scales of goldfish decreased (Table 2). Furthermore, ALP activity in the scales of goldfish was significantly suppressed with acenaphthene treatment (Table 2).

## DISCUSSION

In the present study, we found that polluted seawater from Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea affected the osteoblastic activity of goldfish scales. In addition, osteoblastic markers such as ALP, osteocalcin, and RANKL mRNA expression were suppressed by the polluted seawater. We believe that one of the toxic causative agents is PAH because bone deformity was reportedly caused in fresh water teleosts (zebrafish and Medaka) (Billiard et al., 2006; Farwell et al., 2006) as well as seawater teleosts (Pacific herring, pink salmon, and sea bass) (Barron et al., 2004; Danion et al., 2011) by PAHs.

Benzo[*a*]pyrene (BaP) is a kind of PAH well known as a toxic substance and carcinogen (Kizu et al., 2003; Moffat et al., 2015). Epidemiological and experimental animal studies associate exposure to BaP with an increased risk of several forms of cancer, including tumors in the forestomach, oral cavity, liver, and lung (Moffat et al., 2015). Furthermore, we can detect NPAHs, such as 1-nitropyrene, 3-nitrobenzanthrone, and 6-nitrochrysene, in samples from the Alexandria and Suez Canal sites. It has been reported that 1-nitropyrene, 3-nitrobenzanthrone, and 6-nitrochrysene are toxic compounds (Hayakawa et al., 1997; Mitchell and Thomassen, 1990; Taga et al., 2005; Huang et al., 2014). Also, 1-nitropyrene and 6-nitrochrysene were detected in the bodies of trout collected from Lake Michigan (Huang et al., 2014). In consideration of above-mentioned toxicity of PAHs and NPAHs, we examined the influences of highly concentrated PAHs (naphthalene and acenaphthene) and NPAHs (2-nitrofluorene and 3-nitrobenzanthrone) on the osteoblastic activity of scales. As a result, we found that acenaphthene was toxic to osteoblastic activity in goldfish scales. Furthermore, with the addition of naphthalene, 2-nitrofluorene, and 3-nitrobenzanthrone, osteoblastic

activity tended to decrease. Thus, we concluded that polluted seawater suppressed osteoblastic activity in the scales of goldfish through the additive and/or synergistic actions of these PAHs and NPAHs.

Our *in vitro* bioassay system can detect the activities of both scale osteoblasts and osteoclasts with TRAP and ALP as markers (Persson et al., 1995; Suzuki et al., 2007; de Vrieze et al., 2010). The enzyme activity in one scale can be detected by transferring it into a 96-well microplate and directly incubating it with the substrate in each well. Using this *in vitro* system, the effects of endocrine disruptors—such as bisphenol-A (Suzuki and Hattori, 2003), tributyltin (Suzuki et al., 2006), and polychlorinated biphenyl (Yachiguchi et al., 2014a)—and heavy metals (i.e., cadmium and organic mercury) (Suzuki et al., 2004; Suzuki et al., 2011b; Yachiguchi et al., 2014a) on osteoblasts and osteoclasts have been measured. In the case of cadmium, its concentration (even at  $10^{-13}$  M) influenced osteoclastic activity in the scale (Suzuki et al., 2004). Also, even  $10^{-10}$  M tributyltin significantly inhibited osteoblastic activity. Furthermore, polychlorinated biphenyl 118 influenced osteoclastic activity at a concentration of 0.025 ppm (around  $10^{-8}$  to  $10^{-7}$  M) (Yachiguchi et al., 2014b). In the present study, we can sensitively evaluate the effect of seawater polluted by PAHs and NPAHs on osteoblasts for short incubation times. Thus, our bioassay system is very useful for evaluating the effect of environmental pollutants on bone metabolism.

In Sardinia of Western Mediterranean Sea, total concentrations of PAHs ranged from 0.272 to 1.392 ng/l with a mean value of 0.623 ng/l (Marrucci et al., 2013). PAH levels in Sardinia were lower than those in the Japan Sea (8.5 ng/l) (Chizhova et al., 2013). In the seawater of the Alexandria site, however, a high level of PAHs (1364.59 ng/l) was detected. PAHs derived from ship oil are more likely to be a cause of oil pollution because oil spills correlated very well with the major shipping routes in marine environments (Brekke and

Solberg, 2005). Thus, we will examine the toxic influence of PAHs and NPAHs on the bone metabolism of fish in the polluted marine area and call for the prevention of PAH pollution from ship oil.

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## FIGURE LEGENDS

Fig. 1. Locations of sampling sites

Point 1: The Alexandria site on the Mediterranean Sea; Point 2: the Suez Canal site on the Red Sea

Fig. 2. Effect of polluted seawater on ALP activity in cultured scales incubated for 6 hrs

\* and \*\* indicate statistically significant differences at  $P < 0.05$  and  $P < 0.01$ , respectively, from the values in the control scales.  $n = 10$  samples; one sample from one fish.

Fig. 3. Effect of polluted seawater on TRAP activity in cultured scales incubated for 6 hrs

There is no significant difference between experimental and control scales at least present conditions.  $n = 10$  samples; one sample from one fish.

Fig. 4. Expression analysis of osteoblastic markers: ALP (A), Osteocalcin (B), and RANKL

(C) mRNAs in scales treated with polluted seawater incubated for 6 hrs. ALP, Osteocalcin, and RANKL mRNA levels were normalized to the EF-1 $\alpha$  mRNA level. The value of the ordinates indicates the relative ratios of ALP/EF-1 $\alpha$  (A), Osteocalcin/ EF-1 $\alpha$  (B), and RANKL/ EF-1 $\alpha$  (C), respectively. \* indicates a statistically significant difference at  $P < 0.05$  from the values in the control scales.  $n = 8$  samples; one sample from one fish.



Fig. 5. Expression analysis of osteoclastic markers: Cathepsin K (A), Vacuole-ATPase (B), and Matrix Metalloproteinase-9 (MMP-9) (C) mRNAs in scales treated with polluted seawater incubated for 6 hrs. Cathepsin K, Vacuole-ATPase, and MMP-9 mRNA levels were normalized to the EF-1 $\alpha$  mRNA level. The value of the ordinates indicates the relative ratios of Cathepsin K/EF-1 $\alpha$  (A), Vacuole-ATPase/ EF-1 $\alpha$  (B), and MMP-9/ EF-1 $\alpha$  (C), respectively. n = 8 samples; one sample from one fish.

#### **TABLE CAPTION**

Table 1 PAH and NPAH concentrations in the seawater of both Alexandria and Suez Canal sites

Table 2 Effect of PAHs (6 ng/l) and NPAHs (40 pg/l) on ALP activity in cultured scales incubated for 6 hrs