VOLUME 3 ISSUE 2 2023

ISSN 2583-1690

INTERNATIONAL JOURNAL OF BIOLOGICAL AND ENVIRONMENTAL INVESTIGATIONS

Forum for Biological and Environmental Sciences
Published by Saran Publications, India

International Journal of Biological and Environmental Investigations
Contents available at Journals Home Page: www.ijbei.net
Editor-in-Chief: Prof. Ajai Kumar Srivastav
Managing Editors: Dr. Abhishek Kumar & Dr. Abhai K. Srivastava
Published by: Saran Publications, Gorakhpur, India

ISSN: 2583-1690

Analysis of Seawater Toxicity at Five Concentrated Seawater Discharge Sites in Korea Using a Goldfish Scale-Based *In Vitro* Bioassay System

Hong Chun-Sang[1], Kuroda Kouhei[2], Sato Masayuki[2], Hatano Kaito[2], Watanabe Kazuki[3], Hirayama Jun[3], Sakatoku Akihiro[4], Toyota Kenji[1], Rafiuddin Muhammad Ahya[5], Matsubara Hajime[5], Honda Masato[6], Srivastav Ajai K.[7], Lee Sungjong[1] and Suzuki Nobuo[2*]

[1]Hankuk University of Foreign Studies, Yongin, Gyeonggi-do 17035, Korea
[2]Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Ogi, Noto-cho, Ishikawa 927-0553, Japan
[3]Department of Clinical Engineering, Faculty of Health Sciences, Komatsu University, Komatsu, Ishikawa 923-0961, Japan
[4]School of Science, Academic Assembly, University of Toyama, Gofuku, Toyama 930-8555, Japan
[5]Noto Center for Fisheries Science and Technology, Kanazawa University, Ossaka, Noto-cho, Ishikawa 927-0552, Japan
[6]Botanical Garden, Institute of Nature and Environmental Technology, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, Japan
[7]Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur 273 009, India

Corresponding Author

Received: 2nd July, 2023; **Accepted:** 9th August, 2023; **Published online:** 13th August, 2023

https://doi.org/10.33745/ijbei.2023.v03i02.001

Abstract: This study examined seawater toxicity at five concentrated seawater discharge sites in Korea using a goldfish scale-based *in vitro* bioassay system to examine the toxic effects of waste seawater from seawater desalination on aquatic animals. Goldfish were anesthetized and normal scales were removed to allow for scale regeneration. On day 14, the goldfish were anesthetized again, and the regenerating scales were removed. We then examined the influence of discharged seawater samples on osteoclasts and osteoblasts in regenerating scales using tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) as markers of osteoclast and osteoblast. Scales were first incubated for 6 h in Leibovitz's L-15 medium supplemented with seawater samples (diluted 10 times with L-15 medium). The effect of exposure to seawater on osteoclasts and osteoblasts was then assessed and compared to surface seawater samples sourced from Tsukumo bay (Noto Peninsula, Ishikawa Prefecture, Japan) as a control. We then measured TRAP and ALP activities in all samples from five seawater discharge sites. Our results showed that the diluted seawater samples from the five discharge sites did not influence TRAP and ALP activity as compared with diluted control seawater samples. Therefore, the results of this scale-based bioassay system permit us to conclude that discharged seawater samples did not exert toxic effects under the present conditions.

Keywords: Goldfish scales, Osteoblasts, Osteoclasts, Seawater desalination facility, Waste seawater

Citation: Hong Chun-Sang, Kuroda Kouhei, Sato Masayuki, Hatano Kaito, Watanabe Kazuki, Hirayama Jun, Sakatoku Akihiro, Toyota Kenji, Rafiuddin Muhammad Ahya, Matsubara Hajime, Honda Masato, Srivastav Ajai K., Lee Sungjong and Suzuki Nobuo: Analysis of seawater toxicity at five concentrated seawater discharge sites in Korea using a goldfish scale-based *in vitro* bioassay system. Intern. J. Biol. Environ. Invest. 3(2): 1-8, 2023.

https://doi.org/10.33745/ijbei.2023.v03i02.001

Introduction

To protect against water shortage, the Gijang seawater desalination facility (Fig. 1) was completed in August 2014. It was designed to supply 1.5 million households in the Busan area with fresh water. Since marine areas near this facility contain active fish farms, the impact of the desalinization facility on the marine environment has been assessed *via* diffusion modelling of waste seawater. In addition, a preliminary assessment of the marine toxicity risk of waste seawater was recently conducted (Lee *et al.*, 2017). However, despite attempts to improve its environmental impact, the facility was shut down in January 2018, since it was only 11 km away from the Kori Nuclear Power Plant, and faced considerable opposition from residents and environmental groups concerned about radioactive contamination.

In preparation for the facility to become operational, the present study was performed to further investigate the concentration of waste in seawater that was purified using reverse osmosis membranes for desalination. To do so, we conducted a bioassay using fish scales (i.e., used as bone models) (Suzuki *et al.*, 2000; Suzuki and Hattori, 2002) to investigate the effect of desalinated seawater on the marine ecosystem. This assessment is critical, since some pollutants such as polycyclic aromatic hydrocarbons (PAHs) and heavy metals have been found to cause abnormal bone metabolism in fish (Malekpouri *et al.*, 2011; Fernández *et al.*, 2018; Suzuki *et al.*, 2022; Tarasco *et al.*, 2022).

Fish scales are calcified organs that are important for calcium storage. In fish, and especially freshwater fish inhabiting low calcium environments, plasma calcium levels are maintained at approximately 10 mg/dl using calcium stored in the scales (Suzuki *et al.*, 2008; Suzuki *et al.*, 2016a). Moreover, fish scales are equipped with both bone-resorptive cells (osteoclasts) as well as bone-forming cells (osteoblasts) (Suzuki *et al.*, 2008; Suzuki *et al.*, 2016a; Hirayama *et al.*, 2023). Given the physical properties and biological significance of fish scales, we have developed a scale-based *in vitro* bioassay system to evaluate seawater pollution (Suzuki *et al.*, 2016b; Zanaty *et al.*, 2019). In a previous study, we used this system to test seawater from the Suez Canal that was contaminated with PAHs (Suzuki *et al.*, 2016b). In that study, we found that osteoblastic activity was reduced when seawater diluted 500 times was added to the culture medium. However, we did not observe a difference in osteoclastic activity under these conditions (Suzuki *et al.*, 2016b).

With this background, the purpose of the present study was to use the fish scale bioassay system to estimate the effect of waste seawater discharged from the Gijang seawater desalination facility on fish bones.

Materials and Methods

Animals:

Male goldfish (*Carassius auratus*) (n = 6) were purchased from a commercial fish supplier (Higashikawa Fish Farm, Yamatokoriyama, Japan). These fish were used for the scale *in vitro* assay (n = 6 for osteoclastic activity; n = 6 for osteoblastic activity).

This study was conducted in strict accordance with the ethical guidelines of Kanazawa University. All experiments were performed under anesthesia to minimize pain and discomfort to animal subjects.

Effect of waste seawater on scale osteoclastic and osteoblastic activity:

Surface seawater samples were collected from five locations near the waste seawater discharge site of the Gijang seawater desalination facility (Table 1, Fig. 2) in October 2016. The effect of waste

Fig. 1: Gijang seawater desalination facility.

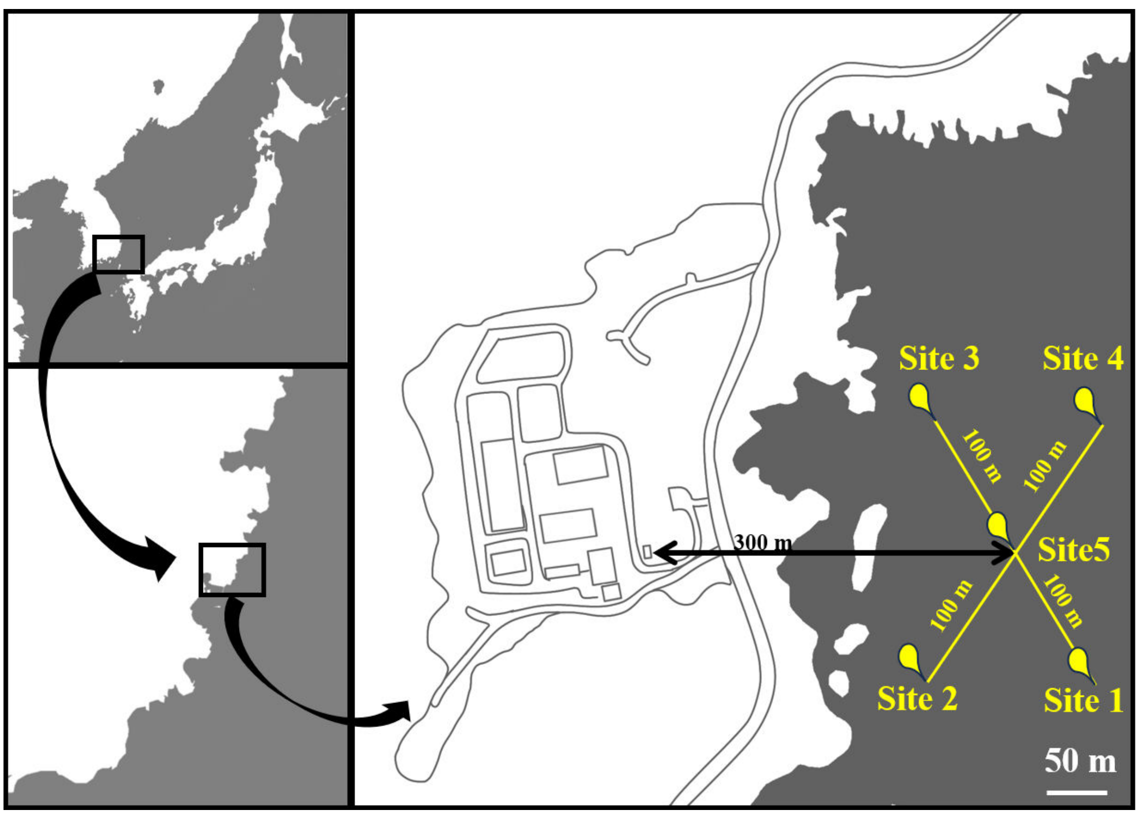

Fig. 2: Sample site locations. Site 5 is near the wastewater discharge outlet. In addition, seawater samples were also taken from other points (i.e., Sites 1, 2, 3, and 4) within 100 m of Site 5.

Table 1: Latitude and longitude of sampling sites

Sampling sites	Latitude	Longitude
1	35°13'37.08''N	129°14'29.47''E
2	35°13'30.63''N	129°14'37.53''E
3	35°13'30.64''N	129°14'29.44''E
4	35°13'37.12''N	129°14'37.35''E
5	35°13'34.68''N	129°14'32.54''E

seawater samples on the osteoclasts and osteoblasts of goldfish scales was then examined.

For the bioassay, goldfish were first anesthetized using 0.03% ethyl 3–aminobenzoate-methanesulfonic acid salt (Sigma–Aldrich, Inc., St. Louis, MO, USA). Then, normally developed scales from both sides of the body were removed to permit scale regeneration. Fourteen days after scale removal, goldfish were anesthetized again so that the regenerating scales could be collected. The regenerating scales were then used to examine the effect of different seawater samples on osteoclasts and osteoblasts using tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) as markers for osteoclast and osteoblast, respectively (Suzuki *et al.*, 2000; Suzuki and Hattori, 2002). To do so, collected scales were added, one per well to the plate 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 seawater samples (diluted 10 times in L-15 medium) and then incubated for 6 h. The effect of seawater sample exposure on osteoblasts and osteoclasts was then compared with exposure to a surface seawater control sample collected from Tsukumo bay (Noto Peninsula, Ishikawa Prefecture, Japan).

For analyses of TRAP or ALP activities, we prepared 5 sites for collecting scales (analytical sites) on each of right and left sides of one goldfish' body. Twelve scales were collected from each of the analytical sites. Six of them were tested for TRAP activity and the remaining 6 scales were tested for ALP activity. The average value of the TRAP or ALP activities of the 6 scales was considered the values of TRAP or ALP activities on each of analytical sites. For each goldfish, the scales from the 5 left analytical sites were treated with waste seawater for 6 h (experimental groups; n = 5), while those from the 5 right sides of the body were untreated (the control groups; n = 5). Six goldfish were used for the analyses of TRAP or ALP activities. Accordingly, the sample numbers for each of experimental and control groups were 30 in both analyses of TRAP and ALP activities. Measurement of TRAP and ALP activities was conducted as previously described (Suzuki *et al.*, 2009).

The method for measuring TRAP and ALP activities was as follows. After the medium in each well was aspirated with 8 series of pipettes, each scale was washed with phosphate buffer solution. Then, an aliquot of 100 µl acid buffer (0.1 M sodium acetate, including 20 mM tartrate, adjusted to pH 5.3) or alkaline buffer (100 mM Tris-HCl, adjusted to pH 9.5; 1 mM $MgCl_2$) was added to each well of a 96-well plate. This microplate was then immediately frozen at −80°C and kept at −20°C until further analysis. Later, the plate was thawed and an aliquot of 100 µl of 20 mM para-nitrophenyl phosphate in an acid or alkaline buffer was added to each well of the microplate. The plate was then incubated at 23°C for 20 min under shaking conditions. After

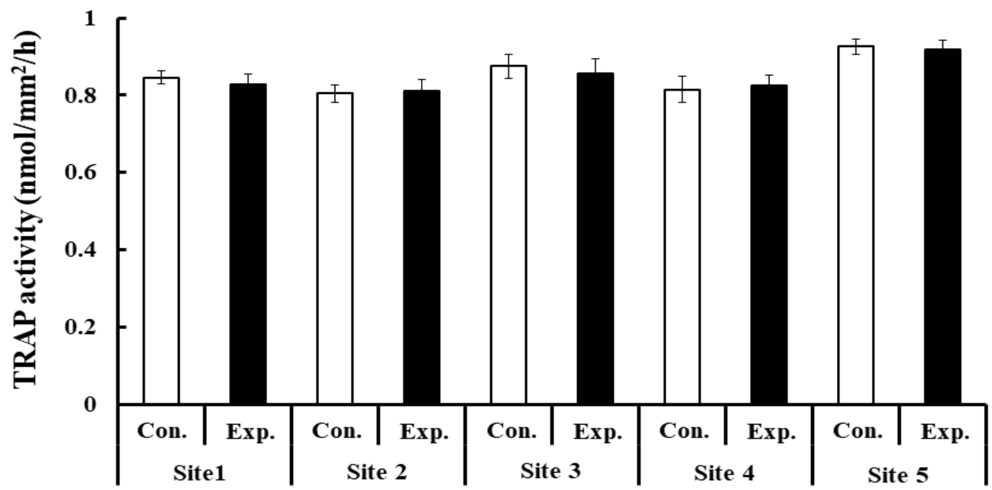

Fig. 3: Influence of waste seawater samples on scale osteoclastic activity as measured using the fish scale bioassay system. We found no effect of waste seawater on osteoclasts under the present conditions. All results (n = 6) are expressed as mean ± standard error. Cont.: control group; Exp.: Experimental group.

incubation, the reaction was stopped by adding 50 µl of 3N NaOH. Next, 150 µl of a colored solution was transferred to the wells of the plate, and the absorbance of each well was measured at 405 nm. These absorbance values were then converted into the amount of para-nitrophenol (pNP) produced using a standard curve for pNP. After measuring both TRAP and ALP activities, the area of each scale was measured using NIH ImageJ. Finally, TRAP and ALP activities were normalized to the surface area (mm^2) and enzyme reaction time (hour) of each scale (Suzuki *et al.*, 2009).

Statistical analyses:

All results are expressed as mean ± SEM (n = 6). The statistical significance of differences in mean values was assessed using paired *t*–tests, and the significance level selected was $p < 0.05$.

Results

Effect of waste seawater samples on scale osteoclastic activity:

Data regarding osteoclastic activity are indicated in Figure 3. We evaluated the effect of waste seawater samples on osteoclasts using scales from six goldfish. Our results showed that waste seawater did not influence osteoclast activity in any of the individuals under the present conditions.

Effect of waste seawater samples on scale osteoblastic activity:

Data regarding osteoblastic activity are indicated in Figure 4. As for the osteoclasts, we found that waste seawater did not affect the osteoblasts under the present conditions.

Discussion

The present study indicated that waste seawater discharged from the seawater desalination facility was non-toxic to osteoclasts and osteoblasts sourced from goldfish scales.

Goldfish scales are known to be highly sensitive to environmental contaminants. For example, the osteoclasts of goldfish have been found to be highly sensitive to heavy metals such as cadmium (Cd) and gadolinium (Gd) (Suzuki *et al.*, 2004; Suzuki *et al.*, 2019). Cd at concentrations as low as 10^{-13} M suppressed TRAP activity following 6 h of incubation (Suzuki *et al.*, 2004). Similarly, osteoclast activity was found to be reduced at concentrations of Gd as low as 10^{-13} M Gd under similar incubation conditions (Suzuki *et*

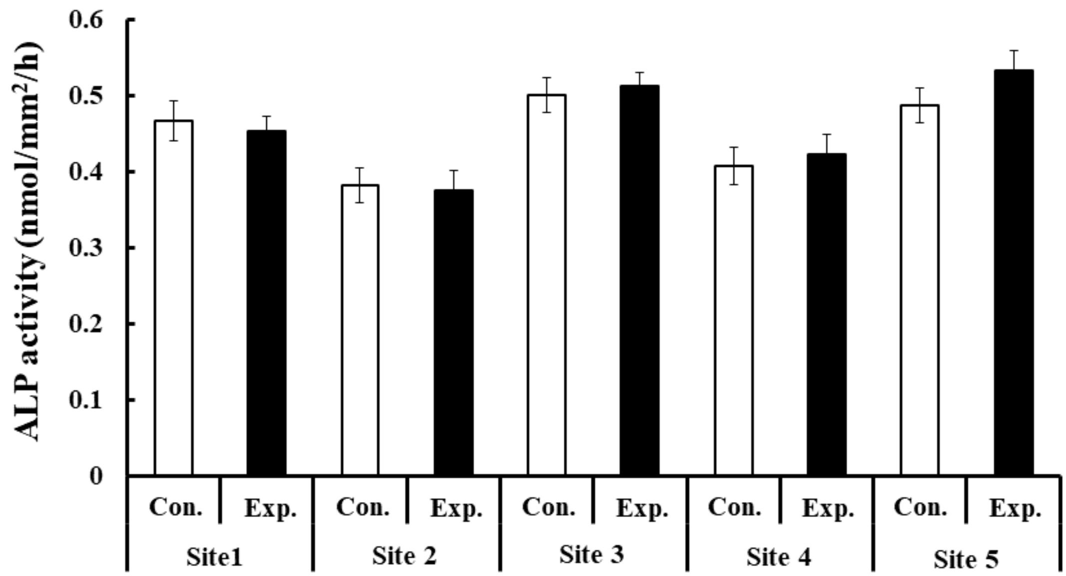

Fig. 4: Influence of waste seawater samples on scale osteoblastic activity as measured using the fish scale bioassay system. We found no effect of waste seawater on osteoblasts under the present conditions. All results (n = 6) are expressed as mean ± standard error. Cont.: control group; Exp.: Experimental group.

al., 2019). Moreover, in another study of mercury, methylmercury (MeHg) was found to be more toxic to goldfish scales than inorganic mercury (InHg) (Suzuki *et al.*, 2004; Suzuki *et al.*, 2011). Thus, after 6 h of incubation, MeHg (10^{-8} M) treatments significantly suppressed TRAP activity (Suzuki *et al.*, 2004), while InHg exhibited similar inhibition at concentrations ranging from 10^{-5} to 10^{-3} M under similar incubation conditions (Suzuki *et al.*, 2011). In addition to the PAHs described in the Introduction, goldfish scales are also known to be highly sensitive to the marine pollutant tributyltin (TBT), an aquatic environmental pollutant originally used as a biocide in anti-fouling paint (Suzuki *et al.*, 2006). In the same study, we also found that ALP activity significantly decreased from the control values following treatment with TBT treatments (i.e., at concentrations from 10^{-10} to 10^{-5} M) after 6 h of incubation (Suzuki *et al.*, 2006).

As described above, heavy metals such as Cd, Gd, MeHg, and InHg are known to exert negative effects on osteoclasts. In contrast, PAHs and TBTs decrease osteoblast activity even after as little as 6 h of incubation. In this study, we found that waste seawater samples showed no toxic effects identified by the fish scale bioassay system. Therefore, we conclude that these waste solutions do not contain toxic quantities of any of these compounds. Our results indicate that the seawater desalination facility may exert only a minimal impact on the marine environment, and the resumption of its original purpose can be resumed in the future.

Conclusion

We examined the potential toxic influence of waste-concentrated seawater from a Korean seawater desalination facility on the aquatic animals. Specifically, we used an *in vitro* goldfish scale-based bioassay system to examine the toxicity of waste seawater sourced from five sites near where waste seawater is discharged to the seashore. We found no differences between experimental and control groups, and we therefore conclude that waste seawater samples do not exert toxic effects under the present conditions.

Acknowledgements

This research was supported by a grant (No. 16IFIPB089908-03) from the Plant Research Program funded by the Ministry of Land, Infrastructure, and Transport of the Republic of Korea. This work was also partly supported by a cooperative research program of the Institute of Nature and Environmental Technology, Kanazawa University (Acceptance Nos. 23004, 23028, and 23029). In addition, this work was also supported by the Korea Environment Industry and Technology Institute (KEITI) *via* the Industrial Facilities and Infrastructure Research Program, which is funded by the Korean Ministry of Environment (MOE) (Grant No. RE201901109) as well as the foreign studies research fund (2023) of Hankuk University.

References

Fernández I, Gavaia PJ, Laizé V and Cancela ML. (2018) Fish as a model to assess chemical toxicity in bone. Aquat Toxicol. 194: 208-226.

Hirayama J, Hattori A, Takahashi A, Furusawa Y, Tabuchi Y, Shibata M, Nagamatsu A, Yano S, Maruyama Y, Matsubara H, Sekiguchi T and Suzuki N. (2023) Physiological consequences of space flight, including abnormal bone metabolism, space radiation injury, and circadian clock dysregulation: Implications of melatonin use and regulation as a countermeasure. J Pineal Res. 74: e12834.

Lee S, Ha H, Hong J, Kang G, and Hong C. (2017) Toxicity identification and evaluation for the effluent from a nonmetallic mineral mining facility in Korea using *D. magna*. Toxicol Ind Health 33: 681-686.

Malekpouri P, Moshtaghie AA, Kazemian M and Soltani M. (2011) Protective effect of zinc on related parameters to bone metabolism in common carp fish (*Cyprinus carpio* L.) intoxified with cadmium. Fish Physiol Biochem. 37: 187-196.

Suzuki N and Hattori A. (2002) Melatonin suppresses osteoclastic and osteoblastic activities in the scales of goldfish. J Pineal Res. 33: 253-258.

Suzuki N, Honda M, Sato M, Yoshitake S, Kawabe K, Tabuchi Y, Omote T, Sekiguchi T, Furusawa Y, Toriba A, Tang N, Shimasaki Y, Nagato EG, Zhang L, Srivastav Ajai K, Amornsakun T, Kitani Y, Matsubara H, Yazawa T, Hirayama J, Hattori A, Oshima Y and Hayakawa K. (2022) Hydroxylated benzo[*c*]phenanthrene metabolites cause osteoblast apoptosis and skeletal abnormalities in fish. Ecotoxicol Environ Saf. 234: 113401.

Suzuki N, Kitamura K and Hattori A. (2016a) Fish scale is a suitable model for analyzing determinants of skeletal fragility in type 2 diabetes. Endocrine. 54: 575–577.

Suzuki N, Kitamura K, Omori K, Nemoto T, Satoh Y, Tabata MJ, Ikegame M, Yamamoto T, Ijiri K, Furusawa Y, Kondo T, Takasaki I, Tabuchi Y, Wada S, Shimizu N, Sasayama Y, Endo M, Takeuchi T, Nara M, Somei M, Maruyama Y, Hayakawa K, Shimazu T, Shigeto Y, Yano S and Hattori A. (2009) Response of osteoblasts and osteoclasts in regenerating scales to gravity loading. Biol Sci Space 23: 211-217.

Suzuki N, Sato M, Nassar HF, Abdel-Gawad FKh, Bassem SM, Yachiguchi K, Tabuchi Y, Endo M, Sekiguchi T, Urata M, Hattori A, Mishima H, Shimasaki Y, Oshima Y, Hong CS, Makino F, Tang N, Toriba A and Hayakawa K. (2016b) Seawater polluted with highly concentrated polycyclic aromatic hydrocarbons suppresses osteoblastic activity in the scales of goldfish, *Carassius auratus*. Zool Sci. 33: 407-413.

Suzuki N, Somei M, Seki A, Reiter RJ and Hattori A. (2008) Novel bromomelatonin derivatives as potentially effective drugs to treat bone diseases. J Pineal Res. 45: 229-234.

Suzuki N, Suzuki T and Kurokawa T. (2000) Suppression of osteoclastic activities by calcitonin in the scales of goldfish (freshwater teleost) and nibbler (seawater teleost). Peptides 21: 115-124.

Suzuki N, Tabata MJ, Kambegawa A, Srivastav Ajai K, Shimada A, Takeda H, Kobayashi M, Wada S, Katsumata T and Hattori A. (2006) Tributyltin inhibits osteoblastic activity and disrupts calcium metabolism through an increase in plasma calcium and calcitonin levels in teleosts. Life Sci. 78: 2533-2541.

Suzuki N, Watanabe K, Sekimoto A, Urata M, Zanaty MI, Sekiguchi T, Kitani Y, Matsubara H, Srivastav Ajai K and Hattori A. (2019) Gadolinium at low concentration suppresses both osteoclastic and osteoblastic activities in the scales of goldfish. Am J Environ Sci. 15: 137-144.

Suzuki N, Yamamoto M, Watanabe K, Kambegawa A and Hattori A. (2004) Both mercury and cadmium directly influence calcium homeostasis resulting from the suppression of scale bone cells: The scale is a good model for the evaluation of heavy metals in bone metabolism. J Bone Miner Metab. 22: 439-446.

Suzuki N, Yachiguchi K, Hayakawa K, Omori K, Takada K, Tabata JM, Kitamura K, Endo M, Wada S, Srivastav Ajai K, Chowdhury VS, Oshima Y and Hattori A. (2011) Effects of inorganic mercury on osteoclasts and osteoblasts of the goldfish scales *in vitro*. J Fac Agr Kyushu Univ. 56: 47-51.

Tarasco M, Gavaia PJ, Bensimon-Brito A, Cordelières FP, Santos T, Martins G, de Castro DT, Silva N, Cabrita E, Bebianno MJ, Stainier DYR, Cancela ML and Laizé V. (2022) Effects of pristine or contaminated polyethylene microplastics on zebrafish development. Chemosphere 303: 135198.

Zanaty MI, Nassar HF, Mahmoud HM, Abdel-Gawad FK, Sato M, Yachiguchi K, Shimizu N, Hayakawa K, Hattori A, Mukai K, Shimasaki Y, Oshima Y and Suzuki N. (2019) Influence of polluted sea water in the Red Sea on the osteoclasts and osteoblasts of goldfish, *Carassius auratus*. J Fac Agr Kyushu Univ. 64: 287-291.