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Bioluminescence Inhibition Test (*Vibrio fischeri*) for Surface Sediments from Wastewater Treatment Plant Effluent Outfall Area

Minkyu Choi, Yeonsu Park, Hyo-Bang Moon, Sang-Pil Yoon[1], Rae-Hong Jung[1], Jun Yu†, and Hee-Gu Choi

Marine Environment Research Division, National Fisheries Research and Development Institute (NFRDI), Busan 619-705, Korea
[1]Coastal Wetland Research Institute, NFRDI, Gunsan 573-882, Korea

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A toxicity of 20 sediment samples from wastewater treatment plant (WWTP) effluent outfall area was performed using bioluminescence inhibition test (*Vibrio fischeries*). Sediment toxicity showed an increase with exposure time to sediment extracts. The effective concentration 50%, EC50, of sediments at 30 min of exposure time ranged from 0.014 to 0.148 mg/mL. Sediment toxicity decreased with increasing distance from WWTP effluent outfall, suggesting that WWTP effluent would contribute to sediment toxicity. In comparison with critical values by other studies, significant toxicity is widespread in the outfall area. The sediment toxicity also showed good correlations with chemical levels and macrobenthic community structure previously reported in the sampling locations. These suggest that bioluminescent test is a useful screening method for sediment toxicity prediction.

Key words: Wastewater treatment plant, Sediments, EC50, chemical levels, benthic community

1. Introduction

Bioluminescence bacteria bioassay is a screening tool for a variety of toxicity testing applications. It is sensitive, reproducible, cost effective, and easy to operate without ethical problems, and requires only 5-30 min for toxicity prediction.[1-3] Its results have been compared with those of other conventional bioassays, with good correlation in many cases.[2-5] The bioluminescence bacteria bioassay has shown to be the most sensitive to contaminated marine sediments, in comparison to several other bioassays.[6-10]

Bioluminescence bacteria testing has been widely used for ecotoxicological assays since 1979, and standardized internationally (ASTM D-5660, ISO 11348, DIN 38412-34). In many countries, this assay was used for the quality assessment of marine sediments in coastal areas.[6,7,11-13] In Korea, it was standardized by MOMAF.[14] Many applications on evaluating toxicity of sewage sludge,[15] soils treated with sewage sludge,[15,16] effluent from dye industry,[17] and trace metals.[18] However, it has little been reported about screening for the estuarines and marine sediments, which provide habitat, feeding, and breeding areas for a number of benthic and infaunal organisms.

Discharge of wastewaters to the marine environment through outfalls of wastewater treatment plants (WWTPs) are considered as an important source of organic and estrogenic contaminants in coastal environments worldwide.[19,20] In 2005, there were 294 WWTPs in Korea, the treating capacity of which was approximately 22.5 million tons/day. These WWTPs produced approximately 2.6 million tons of sludge, most of which (about 78%) was released into the marine environment. Approximately 19% of WWTP effluent produced was directly discharged into the marine

†To whom correspondence should be addressed.
Tel: 051-720-2531, Fax: 051-720-2515, E-mail: jyu@nfrdi.go.kr

environment through outfalls.[21] The Korean Ministry of Environment has regulated the discharge of various toxic chemicals in wastewaters by using only the water quality-based control approach. However, previous studies have shown that WWTP discharges contribute to heavy contamination and risks for organisms in an aquatic ecosystem.[22-24]

To our knowledge, the present study is the first report on assessing the quality of surface sediments collected from WWTP effluent outfall area in Korea using bioluminescence inhibition test (*Vibrio fischeries*). We also compared the toxicity to previous results regarding toxic organic pollutants and macrobenthic community structure.[24]

2. Materials and Methods

2.1. Description of WWTP

The WWTP, situated at Duckdong in Masan city, was established in 1994. The plant treats 260,000 ton/day of wastewater and discharges the effluent into the Masan Bay through an underground pipeline.[25] The type of wastewater treated by this plant includes domestic (1 million inhabitants) and industrial effluent. Most of the wastewater treated in this plant is domestic effluent (90%); less than 10% is from industrial activities.[25] The plant uses sedimentation reservoirs and an activated sludge treatment process to purify the wastewater.

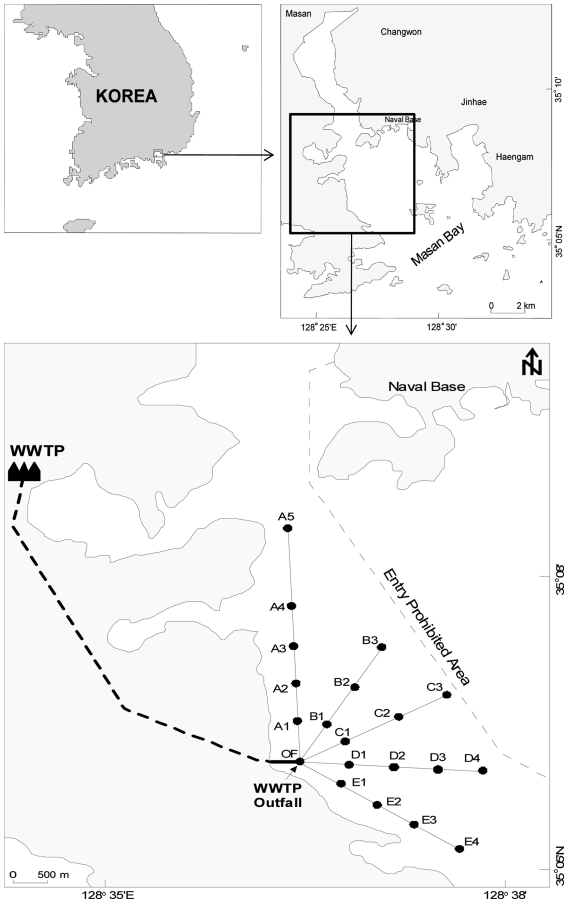

Fig. 1. Sampling locations of surface sediments collected near the WWTP effluent outfall area in Masan Bay, Korea.

2.2. Sample collection

Twenty sampling points were selected based on the prediction of the movement of effluent from the WWTP released into the bay (Fig. 1). We divided the area into five transects (A to E lines), originating from the center of the outfall (Station OF). In this study, we did not consider the pollution by Naval Base, which is located northern part of the bay from the WWTP outfall. Marine sediments were sampled in February 2005 using a 0.05 m^2 van Veen grab sampler. Surface sediment (0-4 cm) were wrapped in aluminum foil and then immediately frozen in a refrigerator on the research vessel. The samples were transported to the laboratory where they were kept in a freezer at –20°C until further analysis.

2.3. Bioluminescence test of *V. fischeri*

Bioluminescence testing was conducted on organic solvent extracts of sediments, based on the standard method of MOMAF[14] with some modifications. Marine sediments were freeze-dried and sieved through a 2 mm sieve. A 1-2 g portion of freeze-dried sediment was placed in a 50 mL Teflon centrifuge tube with a Teflon cap. The sample was extracted twice by mechanical shaking for 1 h with 20 mL of 50% dichloromethane (ultra residue analysis; J.T. Baker, USA) in chloroform (ultra residue analysis; J.T. Baker, USA), and then was centrifuged at 3000 rpm for 15 min and filtrated to minimize effects of suspended particles. All extracts were combined and concentrated by Turbo

Vap LV (Caliper Life Science Inc., USA). Solvent exchanges to the less-toxic dimethyl sulfoxide (ACS reagent, DMSO; Aldrich, USA) were performed. DMSO is a polar solvent that dissolves both polar and non-polar compounds, being suitable for bioassays due to its low to toxicity. Testing was conducted with a N-TOX (Model 200; Neoenbiz Inc., Korea) using the organic solvent solubilization method. Organic extracts, prepared with DMSO, were previously diluted in dilution solution (DW 200; Neoenbiz Inc., Korea) to give a final concentration of 1%. Then, basic test protocol was performed with four 1:2 serial dilutions. The determination of the toxicity was done at 0, 15, and 30 minutes contact in all tests.

Potassium dichromate and 3,5-dichlorophenol solutions as reference chemicals were provided by Dr. Lee in Neoenbiz Inc. They were run for every fresh vial of bacteria to ensure the validity of all tests. Lypophilised *vibrio fischeri* bacteria and all bioluminescence reagents were obtained from Neoenbiz Inc. A log-linear model was used to calculate the Effective Concentration 50% (EC50) with 95% confidence limits. Results of EC50 have been expressed as mg dry sediment/mL aqueous extract. Sediment reference index (SRI) was used to compare the degree of the sediment toxicity between sites. A SRI was calculated by dividing EC50 of negative control sample by EC50 of test samples. The EC50 of washed seasand (Fisher Scientific, USA) was measured as a negative control sample, based on the same experimental procedure to sediment samples, which was 17.096 mg/mL.

3. Results and Discussion

3.1. Sediment toxicity

EC50 values of sediments from WWTP effluent

Table 1. Toxicity data of sediments from WWTP effluent outfall area in Masan Bay, Korea

	EC50 (mg/mL)[a]			EC50 (uL/mL)[b]	SRI[c]
Station	0 min	15 min	30 min	30 min	30 min
OF	5.56	0.021	0.015	0.076	1119
A1	4.93	0.032	0.025	0.123	696
A2	5.35	0.042	0.033	0.163	524
A3	5.07	0.090	0.066	0.329	260
A4	4.87	0.049	0.032	0.159	538
A5	41.9	0.067	0.062	0.311	275
B1	37.4	0.015	0.014	0.068	1250
B2	42.9	0.064	0.058	0.289	296
B3	5.32	0.051	0.046	0.229	372
C1	5.25	0.042	0.029	0.145	591
C2	5.58	0.071	0.057	0.284	301
C3	4.60	0.094	0.069	0.345	248
D1	55.9	0.016	0.014	0.069	1240
D2	37.1	0.029	0.026	0.130	656
D3	5.08	0.074	0.055	0.273	313
D4	33.1	0.160	0.148	0.741	115
E1	4.96	0.028	0.023	0.117	732
E2	30.9	0.059	0.041	0.203	422
E3	5.47	0.123	0.094	0.468	183
E4	5.47	0.115	0.095	0.476	180
Min	4.60	0.015	0.014	0.068	115
Max	55.9	0.160	0.148	0.741	1250
Median	5.47	0.055	0.043	0.216	397
Mean	17.3	0.062	0.050	0.250	515
Std	17.6	0.039	0.034	0.168	

a. The concentration unit is mg sediment per mL aqueous solution; b. Concentration unit is μL DMSO per mL aqueous solution; c. Sediment Reference Index.

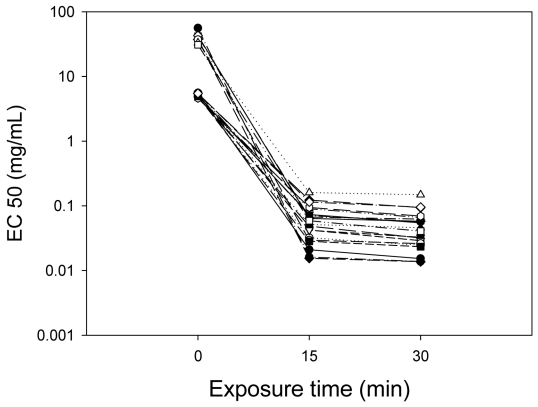

Fig. 2. The response of bioluminescence inhibition to sediment extracts at 0, 15, and 30 min of exposure time.

outfall area are summarized in Table 1. The EC50 is the sediment concentration that reduces bioluminescence by 50% relative to water controls and decreases with increasing sediment toxicity. Bioluminescence inhibition in all samples was increased at steep slopes between 0 min and 15 min of exposure time to samples, and at gentle slopes to 30 min (Fig. 2), It indicates that bioluminescence bacteria mostly responds within 15 min. This pattern is a typical response of the bacteria after exposure to organic contaminants,[13] and therefore the sediments of sampling locations were expected to considerable amounts of organic contaminants.

The EC50 values of sediments at 30 min of exposure time ranged from 0.014 to 0.148 mg/mL. SRI ranged from 115 to 1250, indicating that sediment toxicities in this study are 115 to 1250 times higher than that in the negative control sample. The greatest sediment toxicity was found at B1 and D1 (EC50=0.014) and the next highest was at OF (EC50=0.015), which have 1000 times higher toxicity than that in the negative control sample. Higher sediment toxicities were found at stations adjacent to WWTP effluent outfall, and then sediment toxicity decreased with increasing distance from WWTP effluent outfall (Fig. 3). This suggests that WWTP effluent causes sediment toxicity.

The results of the toxicity test can be classified as toxic samples to compare to critical values reported by other studies. Long[7] suggested a critical value, EC50< 0.51 mg/mL, which is the 80% lower prediction limit of the NOAA Microtox database (n=1013). Bombardier and Bermingham[26] also reported four stages of EC50 dilution levels (expressed as μL DMSO per mL aqueous solution); non-toxic (≫1 μL/mL), marginally toxic (0.1-0.9 μL/mL), moderately toxic (0.01-0.09 μL/mL), and highly toxic (<0.01 μL/mL). In the present study, all data were much lower than 0.51 mg/mL, and 15% of 20 samples was classified as "moderately toxic" and 85% was as "marginally toxic (85%)", respectively. These results indicate that significant toxicity is widespread around WWTP effluent area.

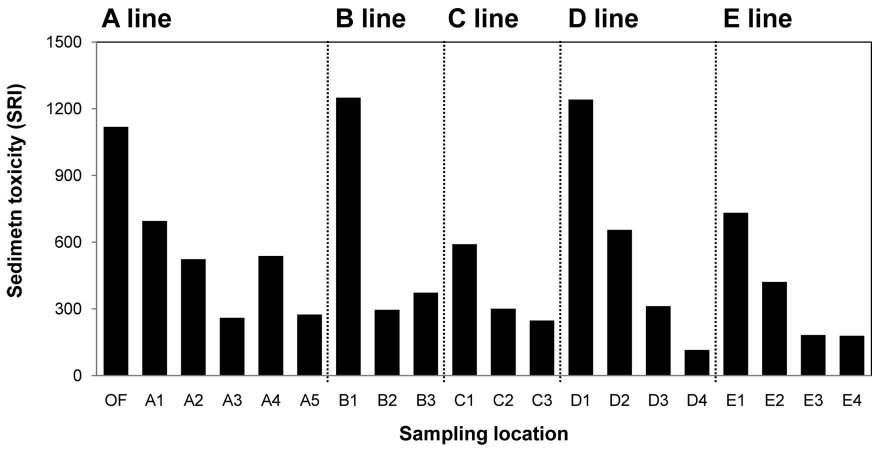

Fig. 3. Distribution of sediment toxicity (Sediment Reference Index, SRI) near the WWTP effluent outfall area in Masan Bay, Korea.

EC50 of sediments in this study was were compared to those in other locations or countries (Table 2). The EC50 in WWTP effluent outfall area is comparable to that in Masan Harbor sediments, [27] which considerable anthropogenic contaminants are discharged by maritime and industrial activities. It suggests that WWTP effluents are an important contributor in sediment contamination.The EC50 in the present study revealed higher toxicity levels than those in Irish marine sediments (>1000 mg/mL),[13] Po River, Italy (0.46-36.80 mg/mL),[28] Ebro River, Spain (0.04-12.96 mg/mL),[3] and Waukegan Harbor, USA (0.42-14.50 mg/mL).[11] However, EC50 values of up to 0.0002 mg/mL were recorded in Barcelona Harbor, Spain, which is heavily contaminated by intensive marine and industrial activities.[12]

Table 2. Comparison of sediment toxicity using bioluminescence inhibition test with concentrations reported from other studies

Locations	EC50	References
WWTP effluent outfall area	0.014-0.148	This study
Masan Bay, Korea	0.014-0.089	Choi ***et al.***[27]
Shihwa Lake, Korea	0.018-0.061	Choi ***et al.***[27]
Ulsan Bay, Korea	0.066-1.126	Choi ***et al.***[27]
Irish marine sediments	>1000	Macken ***et al.***[13]
Waukegan Harbor, USA	0.42-14.50	Kemble ***et al.***[11]
Lake Geneva, USA	0.008-3.42	Pardos ***et al.***[29]
Barcelona Harbor, Spain	0.0002-0.0044	Martínez-Lladò ***et al.***[12]
Ebro River, Spain	0.04-12.96	Ocampo-Duque ***et al.***[3]
Po River, Italy	0.46-36.80	Viganò ***et al.***[28]

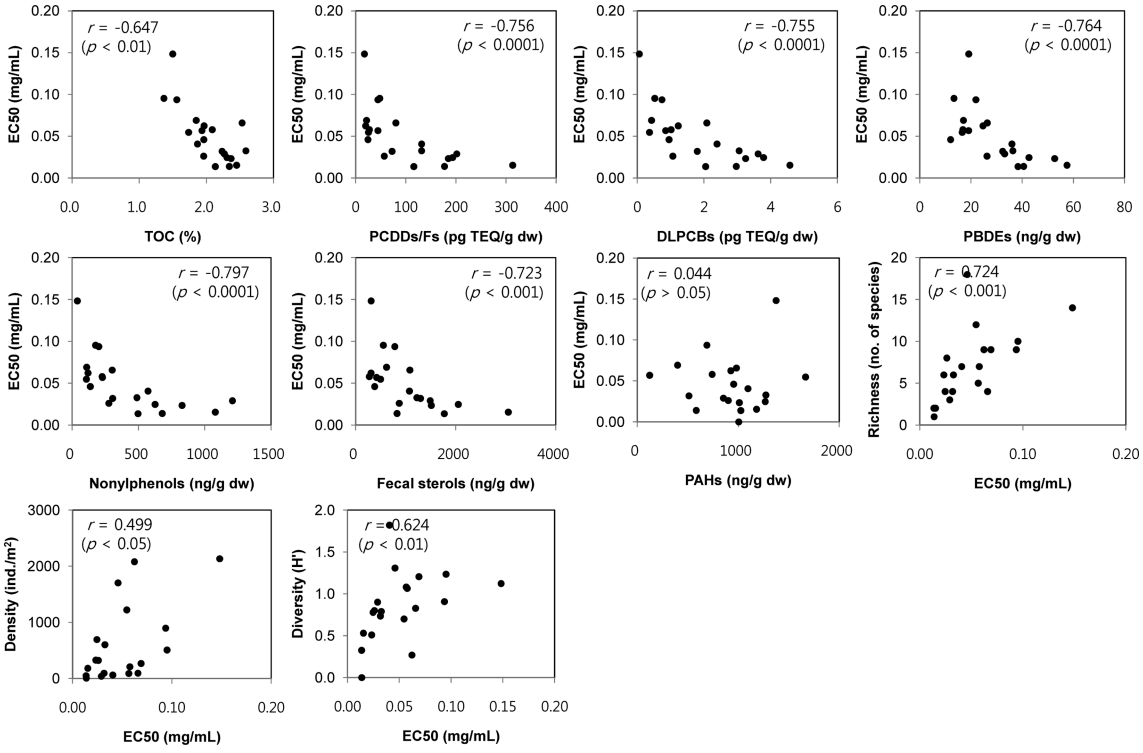

Fig. 4. Spearman rank correlation of sediment toxicity with chemical levels and benthic community in WWTP effluent outfall area.

3.2. Relationship between toxicity response and sediment contamination

The results of Spearman rank correlation analysis of sediment toxicity (EC50) with levels of chemical components and with biotic indices of macrobenthic community reported by Moon *et al.*[24] are shown in Fig. 4. EC50 was negatively correlated with TOC content, PCDDs/DFs, dioxin like PCBs (DLPCBs), PBDEs, nonyphenols (NPs), and fecal sterols, suggesting that organic rich sediments containing toxic organic chemicals increase sediment toxicity. Highly significant correlations of EC50 were found with PCDDs/DFs, DLPCBs, PBDEs and NPs ($p < 0.0001$), which are mostly associated with industrial effluents and showed apparent decline trends with increasing distance from the WWTP outfall. EC50 showed little correlation with PAH concentrations, which is affected by WWTP effluent and different sources such as oil spills and/or combustion processess.[24] EC50 was positively correlated with biotic indices such as the number of species, density, and diversity. It indicates that environmental disturbance by high toxic organic contaminants and high sediment toxicity can affect the benthic community. High correlation of EC50 revealed with the number of species ($p < 0.001$), and diversity and density were moderately correlated with EC50 ($p < 0.05$).

Correspondence analysis (CA) was performed, in order to characterize contaminated areas by WWTP effluent outfall. The results of CA based on chemical parameters, sediment toxicity, and benthic community at sampling locations are shown in Fig. 5. Axis I and II accounted for 65.5% and 11.7% of variance, respectively. Axis I represents contamination and toxicity responses, and reveals high (negative) and low (positive). Two groups were classified according to sampling locations and parameters. The first group comprised the locations closer to WWTP outfall (OF, A1, B1, C1, D1, and E1) and some locations (A2-A4, D2, and E2), characterized by severe pollution by chemicals, while the second group comprised the locations further from WWTP outfall, characterized by relatively low sediment toxicity and abundant benthic community. The spatial distribution on the map of the WWTP effluent outfall area can also provide information on transfer directions and effect ranges of WWTP effluents in the marine environment.

4. Conclusion

Bioluminescence inhibition test was an easy and fast screening method for sediment samples, and the test results in the present study were comparable to those by those from instrumental analysis and biological responses. WWTP effluent outfall area was influenced

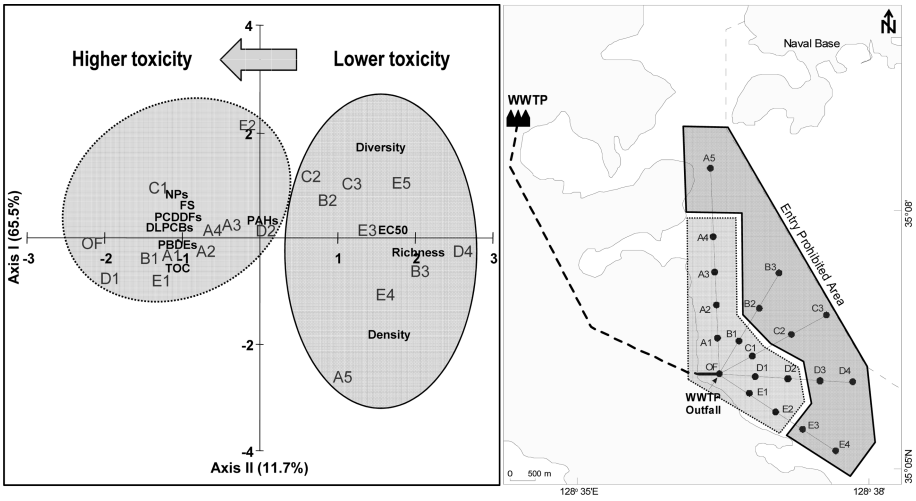

Fig. 5. Correspondence analysis plot (left) for scores according to chemical and biological parameters, and its spatial distribution (right) on the map of the WWTP effluent outfall area.

by the discharges, as evidenced by distribution of chemicals and biotic indices as well as sediment toxicity. Therefore, bioluminescence bioassay test will be able to provide complementary information on the degree of contamination along with chemical analysis and biological response.

Acknowledgments

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