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Ecotoxicological Evaluation of Sewage Sludge Using Bioluminescent Marine Bacteria and Rotifer

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Abstract – Bioassay using the marine bacteria, *Vibrio fischeri* and rotifer, *Brachionus plicatilis*, and chemical analyses were conducted to assess the toxicity of the various sewage sludges, one of the major ocean dumped materials in the Yellow Sea of Korea. Sludge elutriates extracted by filtered seawater were used to estimate the ecotoxicity of the sludge. Chemical characterization included the analyses of organic contents, heavy metals, and persistent organic pollutants in sludge. Bacterial bioluminescent inhibition (15 min), rotifer mortality (24 hr) and rotifer population growth inhibition (48 hr) assay were conducted to estimate the sludge toxicity. EC50 15 min (inhibition concentration of bioluminescence after 15 minutes exposed) values by Microtox® bioassay clearly revealed different toxicity levels depending on the sludge sources. Highest toxicity for the bacteria was found with the sludge extract from dyeing waste and followed by industrial waste, livestock waste, and leather processing waste. Clear toxic effects on the bacteria were not found in the sludge extract from filtration bed sludge and rural sewage sludge. Consistent with Microtox® results, rotifer neonate mortality and population growth inhibition test also showed highest toxicity in dyeing waste and low in filtration bed and rural sewage sludge. High concentrations of persistent organic pollutants (POPs) and heavy metals were measured in the samples from the industrial wastes, leather processing plant waste sludge, and urban sewage sludge. However, there was no significant correlation between pollutant concentration levels and the toxicity values of the sludge. This suggests that the ecotoxicity in addition to the chemical analyses of various sludge samples must be estimated before release of potential harmful waste in the natural environment as part of an ecological risk assessment.

Key words – sewage sludge, marine bioluminescent bacteria, rotifer, bioassay, toxicity, *Vibrio fischeri*, *Brachionus plicatilis*, Microtox

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1. Introduction

Environmental toxicology or ecotoxicology deals with the potentially harmful effects on organisms of countless man-made chemicals and wastes released into the biosphere. Chemical analyses alone do not estimate toxic effects because of:

a) unknown toxic substances, b) interaction between the substances (additive, synergistic, antagonistic etc), c) toxic effects after degradation and transformation of products, d) no insight on the quantity of the bioavailable chemicals, e) no insight on the effects on biota. These problems can be solved by bioassay of environmental samples.

The Microtox® system is a screening tool used for a variety of toxicity testing applications. The advantages of this bioassay are simplicity, sensitivity, repeatability, and precision, when compared to the chemical analysis (Robinson 1988; APHA *et al.* 1985). The Microtox® assay uses freeze dried luminescent bacteria *Vibrio fischeri* as the test organism. The bacteria's light-producing mechanism is tied to the metabolic processes of the cell (Azur Environmental 2005). If these processes are altered or damaged by a toxic substance, a reduction in light output results. The bioassay is based on inhibition of luminescence of the bacterium detecting these changes in light output (SDI 2002). This method has been widely used for ecotoxicology assays since 1979 (Boluda *et al.* 2002; Cotou *et al.* 2002; Bogaerts *et al.* 2001; Choi and Meier 2001; Doherty 2001; Amoros *et. al.* 2000; Pardos *et al.* 2000; Cleveland *et al.* 1997; Del Valls *et al.* 1997; Sweet and Meier 1997; Gaggi *et al.* 1995; Oanh and Bengtsson 1995; Gaelli *et al.* 1994; Ankley *et al.* 1989) and standardized internationally (ASTM D-5660, ISO 11348, DIN 38412-34).

Also, marine rotifer *Brachionus plicatilis* is a commonly

used toxicity test species due to its cosmopolitan distribution, important trophic roles in the marine ecosystem (Wallace and Snell 1991; Nogrady *et al.* 1993), short generation time (about 24 hrs), easy culture in small space (Fukusho 1983; Korunuma and Fukusho 1987; Fielder *et al.* 2000), and wide salinity adaptability (Komis 1992). This species is found in salt-water ponds, estuaries, and lagoons in Korean waters (Hur and Park 1996a). Also, there are various studies on cyst production and mass production for fish culture (Park and Hur 1996a; Hur and Park 1996b; Park and Hur 1996b; Park and Hur 1996c; Park *et al.* 1999). Standard methods for toxicity testing using the rotifers (*B. plicatilis* for marine and *B. calyciflorus* for freshwater) have been established (Guerra 2001; Preston and Snell 2001a,b; Preston *et. al.* 2000; ASTM 1996; Snell and Persoone 1989).

Ocean-dumping of waste generated on land had been carried out for several years by industrialized countries before international rules to prevent marine pollution when the London Convention 1972 came into force in 1974. During 1992-1994, the total annual quantity dumped rose from 12.5 to 16.25 million tons (MOMAF 2004) and this may result in potential environmental problems.

In this study, toxicity of the sludge extract is estimated with bioluminescent marine bacteria and rotifer, and the bioassay results are compared with the chemical properties of the sludge. These multiple approaches with bioassays and chemical analyses may provide useful methods for estimating the environmental toxicity of sludge dumping in Korea.

2. Materials and Methods

Sludge samples were collected from the 11 sewage plants encompassing urban sewage, industrial waste, rural sewage, and livestock waste. One part of each sludge sample was freeze-dried for chemical analysis and another part was stored in the dark at 4°C for less than a week prior to the bioassay experiment. The sludge samples were mixed at a ratio of 1:10 (sludge: filtered seawater (0.45 μm, 30 psu)), then the mixture was shaken for 12h, and allowed to settle before recuperating the supernatant (elutriate). To estimate the chemical properties of each sludge sample, heavy metals (Co, Hg, Cd, Pb, As, Cu, Zn, Cr) and PCBs (PCB 52+101+138+180), PAHs (Polycyclic Aromatic Hydrocarbons = Naphthalene + Phenanthrene + Anthracene +Fluoranthene + Benzo(a)anthracene + Benzo(a)pyrne), COD, pH, water contents, ignition loss (IL), particulate organic carbon (POC), particulate organic nitrogen (PON), phenol, oil and grease, cyanides (CN) were measured (APHA *et al.* 1995). The details can be found in the MOMAF report (2004). The above values were used for principal component analysis and correlation to identify the relationship between chemical properties and bioassay endpoints by the sludge samples.

Table 1. Test conditions for the definitive acute toxicity test with the marine rotifer *Brachionus plicatilis* neonates

Parameters	Conditions
Test type	static non-renewal
Duration	24h
Endpoint	mortality
Temperature	25±1°C
Dilution water	filtered seawater/DI water
Photoperiod	none (dark)
Test chamber size	12 ml
Test solution volume	10 ml
Age of test animals	neonates
Number of animals per chamber	10
Number of replicates per concentration	3

Microtox® bioassay (*Vibrio fischeri* NRRL B-11177) was conducted following the standard methods of MOMAF (1998) and the supplier provided user's manual (Strategic Diagnostics Inc. Delaware, USA). A twenty-four hour rotifer mortality test was conducted using Rotoxkit M (Microbiotests Inc., Belgium) following ASTM procedures (1996) (Table 1). Also, the rotifer population growth rate (PGR) was estimated using a parthenogenic female. Five females with eggs were allocated into each well of 6 well plates and incubated for 48 hours in the dark. The number of rotifer individuals was counted after 48 hours and the PGR (population growth rates) was calculated as the following; $r = (\ln N_t - \ln N_o)/t$ (r = PGR, N_t = number of individuals at time t, N_o = initial population density, t = hour). Then, population growth inhibition rates were estimated by the comparison between the PGR at each concentration and control level. Eleven sludge extracts were tested with 6 dilutions (0, 6.25, 12.5 25, 50, 100%) and 3 replicates for each dilution.

Two reference Microtox® tests using zinc sulfate were conducted to identify the sensitivity of the bacteria and revealed the EC50 15 min = 7.99 mg/L and 6.04 mg/L, respectively. These values were within the EC50 15 min ranges (3.0-10.0 mg/L) provided by the supplier of the product (Fig. 1). Rotifer PGR at control levels were also satisfied with the ASTM standard (r>0.7) (Fig. 2). All the toxicity values for rotifer assay were estimated by trimmed Spearman-Karber methods using the TOXCAL program (Tidepool Scientific Software, USA) and Microtox® program by SDI (Strategic Diagnostics Inc., USA) for 50% bioluminescent inhibition calculation (EC50s).

3. Results and Discussion

The physicochemical properties of sludge

All the chemical concentration values in the sludge

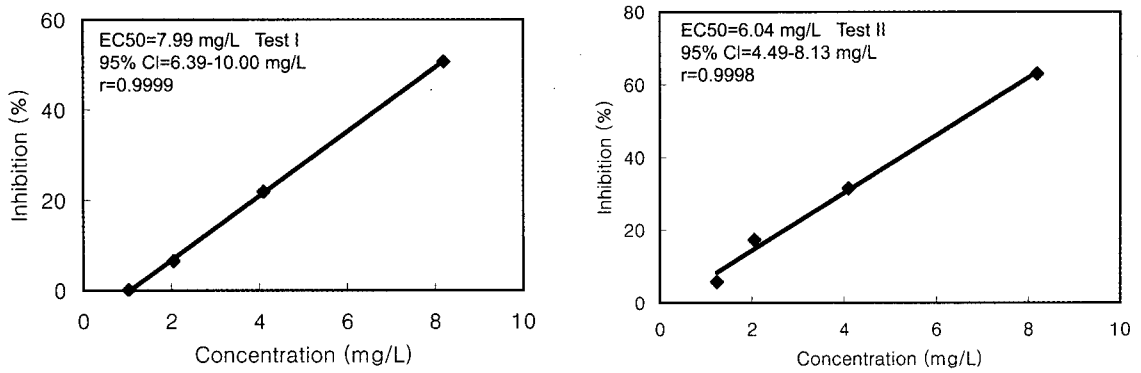

Fig. 1. Microtox® bioluminescent inhibition test using zinc sulfate as reference test material.

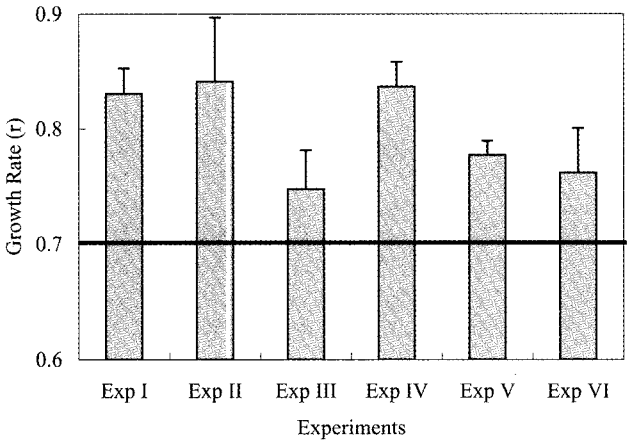

Fig. 2. Population growth rates (PGR/hr) of the marine rotifer *Brachionus plicatilis* at control conditions.

samples are provided in Table 2 and 3. The concentration ranges of chemical parameters in sewage sludge were significantly higher than those found in the natural marine sediment and even in the sediment at the ocean dumping site located in the East Sea of Korea (Table 4). Sludges from the industrial waste, urban sewage, and livestock waste plants contained relatively high concentrations of pollutants, especially heavy metals; high chromium in sludge from the leather processing plant, high phenol from livestock waste, high zinc in industrial sewage, high mercury in urban sewage, etc. Hence, the sludge from the industrial waste and urban sewage were located on the 1/4 and 2/4 space, which correlated with heavy metals and CN in the two-dimensional principal component plot (Fig. 3). The concentration values and details for the physicochemical properties of each sludge sample can be found in the MOMAF report (2004).

Toxicity assay of sludge samples

Microtox toxicity values as EC50 15 min (50% bioluminescent inhibition concentration compared with control level after 15 min exposed) of sludge extract ranged from 21.8% to over 100%. The highest toxicity levels were found in the sludge extract from the dyeing waste plant and the lowest from the rural sewage plant and filtration bed. Generally, extracts from the industrial waste and livestock farm waste revealed high toxicity in the marine bacteria, *Vibrio fischeri* (Fig. 4, Table 5). The rotifer mortality bioassay also revealed the highest toxicity in the extract from

Table 2. Physicochemical properties of sludges

Sludge source / parameters	COD mg/g dry	pH	water content %	IL %	POC %	PON %	phenol ppm dry	Oil/grease ppm	CN ppm
Dyeing waste (DW)	238.24	5.95	72.11	50.21	24.55	3.73	0.00	34645.3	0.87
Industrial waste (IW)	272.23	7.27	83.75	60.38	27.76	5.94	0.00	22955.9	0.60
Livestock waste (LW)	-	6.50	78.39	63.16	26.82	6.51	58.40	40950.2	0.16
Leather waste (LT)	292.97	7.40	63.58	56.06	31.58	5.54	0.00	88510.4	0.10
Urban sewage (US)	377.05	6.03	82.00	72.40	37.13	5.80	0.00	64449.7	0.40
Food waste (FW)	353.08	6.29	89.86	71.24	31.49	5.07	4.13	19740.9	0.07
Textile waste (TW)	148.35	8.04	80.63	39.43	14.91	2.12	0.00	20305.4	0.10
Mixed sewage (MX)	146.59	7.82	72.15	37.13	14.04	2.92	0.00	22670.0	1.70
Industrial sewage (IS)	234.21	7.10	78.05	51.13	22.61	4.28	0.00	34426.5	0.30
Filtration bed sludge (FS)	69.16	6.49	67.30	21.08	3.11	1.26	1.39	-	0.10
Rural sewage (RS)	227.25	7.37	65.00	48.85	21.37	4.00	0.00	24432.7	0.50

Table 3. Heavy metals and persistent organic pollutants found in sludge (unit = ppm)

Sludge source / parameters	Co	Hg	Cd	Pb	As	Cu	Zn	Cr	PAHs	Total organic P	PCBs
Dyeing waste	7.9	0.3	5.8	1.9	6.4	9.1	459.0	1.9	549.0	2144.1	15.8
Industrial waste	16.5	1.2	100.5	892.1	68.9	3728.8	4778.0	168.8	5768.9	4765.7	20.7
Livestock waste	5.1	0.0	0.9	7.6	2.6	928.2	2009.9	14.7	665.0	1240.4	.
Leather waste	7.2	0.0	0.0	7.8	1.7	21.6	171.1	24053.7	4080.7	3163.4	.
Urban sewage	7.8	107.1	30.9	356.7	10.3	1044.0	1945.9	82.9	3709.0	2013.1	59.2
Food waste	7.8	0.0	0.5	23.1	1.2	100.0	227.0	95.0	4640.3	1672.7	.
Textile waste	7.1	0.9	0.1	13.6	2.1	161.8	703.1	283.2	1662.0	3050.2	19.3
Mixed sewage	14.2	1.0	2.2	88.7	10.7	365.7	807.3	68.4	3176.9	1797.2	12.7
Industrial sewage	90.9	1.4	27.0	1007.6	21.6	1839.1	20784.0	4220.5	2718.0	6210.5	21874.4
Filtration bed	13.8	0.0	0.2	43.3	27.4	33.2	166.6	56.6	1487.5	.	.
Rural sewage	12.6	3.1	2.1	80.5	8.8	259.5	970.0	52.8	2059.8	2338.5	4.6

Table 4. Comparison of chemical properties in marine sediment and sewage sludge

Parameters	Sites and sources	Average (ranges)	References
COD (mg/g dry)	Sewage sludge	235.9 (69.1-377.1)	this study
	West coast of Korea	4.8 (0.5-13.2)	MOMAF, 2002
	South coast of Korea	17.6 (0.9-56.2)	MOMAF, 2002
	Ocean dumping site (East Sea)	-	
POC (%)	Sewage sludge	23.2 (3.1-37.1)	this study
	West coast of Korea	0.42 (0.06-2.92)	MOMAF, 2002
	South coast of Korea	0.75 (0.10-2.28)	MOMAF, 2002
	Ocean dumping site (East Sea)	2.4 (2.3-4.1)	MOMAF, 2005
PON (%)	Sewage sludge	4.3 (1.3-6.5)	this study
	West coast of Korea	-	
	South coast of Korea	0.15 (0.02-0.29)	MOMAF, 2002
	Ocean dumping site (East Sea)	0.29 (0.23-0.46)	MOMAF, 2005
IL (%)	Sewage sludge	51.9 (21.1-72.4)	this study
	West coast of Korea	2.9 (0.3-6.3)	MOMAF, 2002
	South coast of Korea	5.6 (0.8-10.5)	MOMAF, 2002
	Ocean dumping site (East Sea)	-	
Copper (ppm dry)	Sewage sludge	771.9 (9.1-3,728.8)	this study
	West coast of Korea	11.5 (3.7-21.4)	NFRDI (Unpub)
	South coast of Korea	20.0 (2.4-36.9)	MOMAF, 2002
	Ocean dumping site (East Sea)	61.8 (32.0-151.0)	MOMAF, 2005
Lead (ppm dry)	Sewage sludge	229.4 (1.9-1007.6)	this study
	West coast of Korea	24.7 (13.2-52.0)	NFRDI (Unpub)
	South coast of Korea	12.1 (1.2-22.5)	MOMAF, 2002
	Ocean dumping site (East Sea)	84.2 (44.0-297.0)	MOMAF, 2005
Zinc (ppm dry)	Sewage sludge	3002.0 (166.6-20,784.0)	this study
	West coast of Korea	76.0 (24.3-113.9)	NFRDI (Unpub)
	South coast of Korea	97.2 (25.4-154.5)	MOMAF, 2002
	Ocean dumping site (East Sea)	236.0 (124.0-640.0)	MOMAF, 2005
Cadmium (ppm dry)	Sewage sludge	15.5 (0.0-100.5)	this study
	West coast of Korea	0.10 (0.03-0.46)	NFRDI (Unpub)
	South coast of Korea	0.11 (0.01-0.29)	MOMAF, 2002
	Ocean dumping site (East Sea)	1.27 (0.25-9.90)	MOMAF, 2005

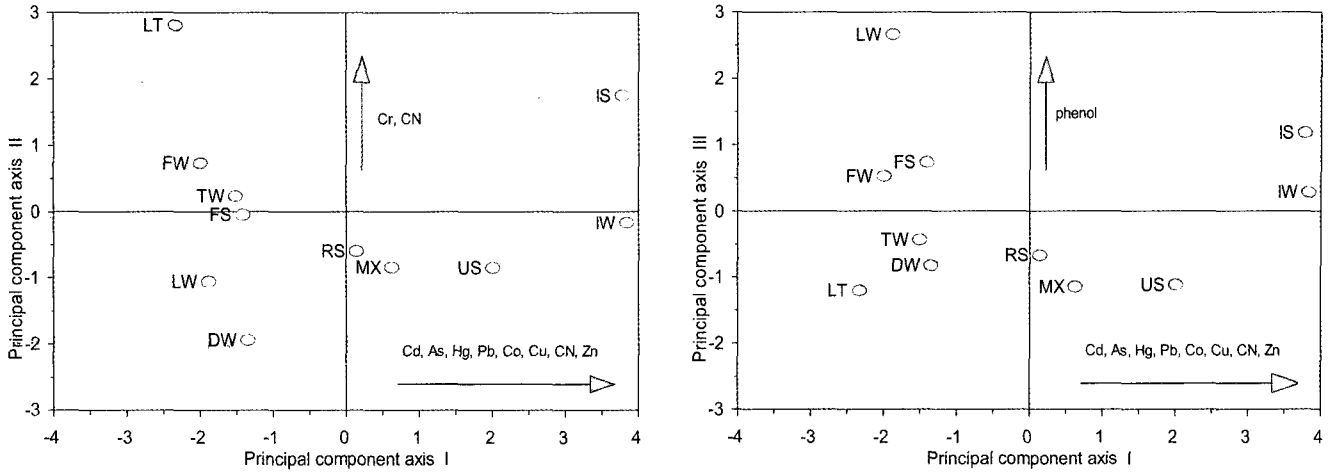

Fig. 3. Principal component ordination of 11 sludge samples according to physicochemical properties. Abbreviations refer to Table 2.

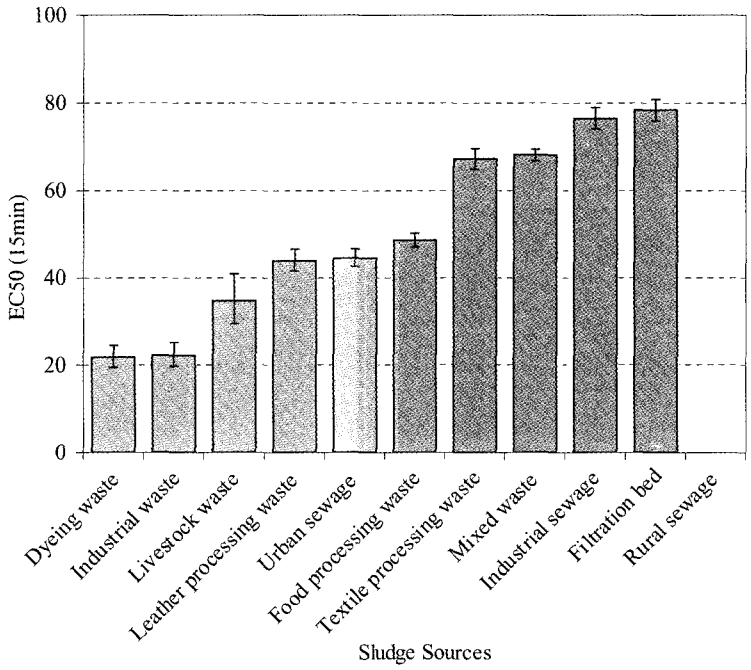

Fig. 4. EC50s as bioluminescent inhibition estimated by Microtox® bioassay for sludge extracts. Error bars indicate 95% confidence interval. Rural sewage showed no toxicity and EC50 was not estimated.

dyeing waste (24hr LC50=2.9%) and industrial waste (24hr LC50=37.7%) (Fig. 5, Table 5). By the rotifer PGR, high toxicity was also found with the sludge from dyeing waste, mixed sludge (industrial and domestic), and leather processing waste (Fig. 6). There was no acute toxicity in the other sludges for rotifer neonate mortality and rotifer PGR. Rotifer mortality by sludge extract showed a threshold effect, where mortality occurred over the certain concentration. For example, neonate mortality sharply increased to over 50% of the sludge extract.

The rotifer mortality (LC50) was not as sensitive as the Microtox® bioassay. Both toxicity tests showed the highest toxicity in dyeing waste even with relatively lower concentrations of pollutants (Table 2 and 3). This indicated that the bioassay would be a useful tool for bio-screening of toxicity which can be overlooked by the screening based on the chemical analysis alone.

Correlations between physicochemical parameters and EC50 for the marine bacteria, *Vibrio fischeri*, or LC50 for marine rotifer were not significant in any combinations,

Table 5. Comparison of toxicity end points estimated by various species

Sludge sources	Bacteria	Rotifer	Sea urchin*		Amphipod*	Sea weed**
	Bioluminescent	Mortality	Fertilization	Development	Mortality	Sporulation rates
	EC50(%)	LC50(%)	EC50(%)	EC50(%)	LC50(%)	EC50(%)
Dyeing waste	21.84	2.9	1.82	0.024	3.35	12.88
Industrial waste	22.31	37.7	0.02	0.004	0.81	10.66
Livestock waste	34.78	95.7	5.34	0.040	27.79	12.28
Leather waste	44.02	80.9	0.16	0.014	3.50	13.48
Urban sewage	44.62	74.9	0.17	0.005	3.09	9.66
Food waste	48.71	88.5	1.65	0.005	1.42	10.97
Textile waste	67.25	>100.0	8.06	0.088	15.78	61.52
Mixed sewage	68.13	>100.0	0.25	0.022	10.39	11.15
Industrial sewage	76.50	>100.0	0.32	0.003	2.33	6.56
Filtration bed	78.41	67.7	5.49	0.113	20.78	29.29
Rural sewage	>100.00	64.0	1.33	0.033	12.04	13.17

* Sea urchin and amphipod toxicity data are sited from MOMAF (2004) produced by Dr. Lee and Choi at KORDI.
** Seaweed toxicity data are provided by Dr. Han at Incheon University.

Table 6. Spearman's rank correlation analysis between bioassay end points (EC50 and LC50) and chemical properties. Top numbers indicate correlation coefficients, the middle calculated probabilities, and the bottom the number of observations. None of the combinations are significant at $\alpha = 0.05$ level

Chemicals Parameters	Phenol	CN	Co	Hg	Cd	Pb	As	Cu	Zn	Cr	PAHs	OP	PCBs
Microtox bioassay	-0.037	-0.178	0.340	0.200	-0.188	0.527	0.176	0.176	0.018	0.309	0.050	0.100	0.357
	0.919	0.623	0.336	0.579	0.603	0.117	0.627	0.627	0.960	0.385	0.898	0.798	0.432
	10	10	10	10	10	10	10	10	10	10	9	9	7
Rotifer bioassay	-0.037	-0.417	-0.190	-0.176	0.169	-0.236	0.176	-0.285	0.345	0.370	-0.233	-0.017	0.017
	0.919	0.265	0.599	0.626	0.641	0.511	0.627	0.425	0.328	0.293	0.546	0.966	0.879
	10	10	10	10	10	10	10	10	10	10	9	9	7

representing the fact that a single chemical analysis cannot be a good estimation of sludge toxicity (Table 6). Also, there was no significant correlation between Microtox® and rotifer bioassay either ($r = 0.41$, $p > 0.05$), even where the highest toxicity was found in the dyeing waste for both tests.

Concentration-response curves revealed that the response of bioluminescent bacteria on the sludge extract was consistent and produced very reliable results for toxicity testing. General sensitivity of Microtox® was higher than that of rotifer mortality and growth inhibition, but lower than that of the sea urchin (*Strongylocentrotus intermedius*) fertilization and development bioassay, and seaweed (*Ulva pertusa*) sporulation rate assay using the same sludge samples (Table 5). However, Scheers *et al.* (2002) reported the sensitivity of Microtox bioassay was comparable with the fish cell line bioassay using cultured fathead minnows (*Pimephales promelas*). Rotifer PGR were less sensitive than the neonate mortality test. This suggests that toxicity end points from the younger stages or critical periods of the life cycle such as fertilization and development can be more sensitive and reliable compared with the response of adults. As a study for ocean dumped sewage toxicity estimation, Costello and Read (1994) reported that the average 96h LC50 for sewage sludges ranged from 20% wet volume of sludge in seawater for polychaetes (*Malacoceros fuliginosus* and *Scolelepis squamata*) to 0.0003% for shrimp larvae (*Crangon crangon*). They also found that one third of the tests reviewed showed minimum toxic concentrations of less than or equal to 0.1% using their indoor mesocosm. In general, dilution after dumping at sea is less than 0.1% (1 in 1000) within 30 min and 0.01% within 1h (Costello and Read 1994) and therefore, acute toxicity is unlikely to occur at ecologically significant or detectable levels at dump sites.

In this study, the physicochemical properties provided were from the whole sludge rather than extracts. Therefore, the direct comparison between toxicity of sludge extract and chemical properties in the sludge may not be suitable. Perez *et al.* (2001) reported that EC50 values for Microtox® by PAHs in sludge extracts ranged from 159 to 750 mg/kg. In this study, much higher ranges of PAHs

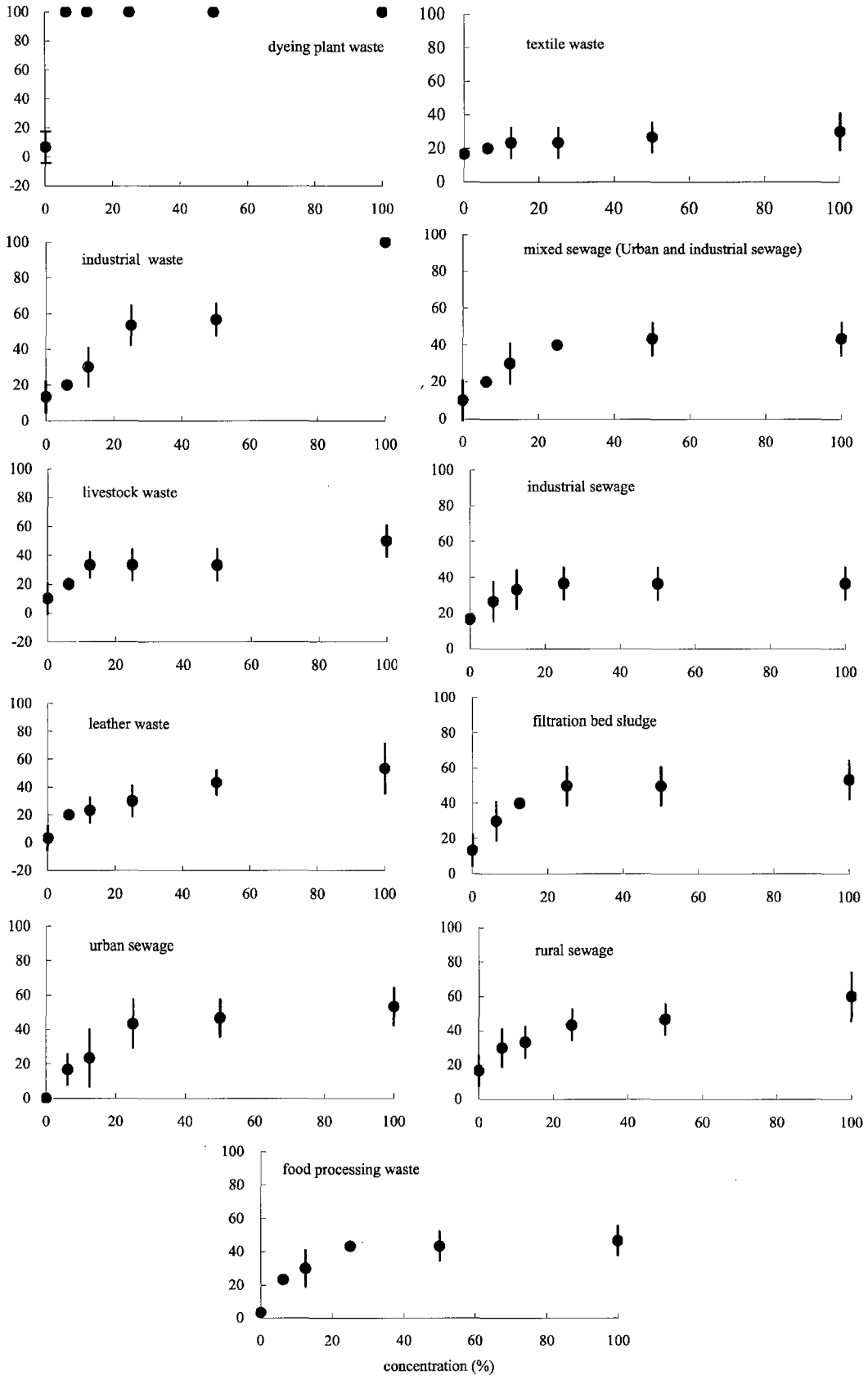

Fig. 5. Rotifer neonate mortality exposed to the dilution of sewage sludge extracts in seawater.

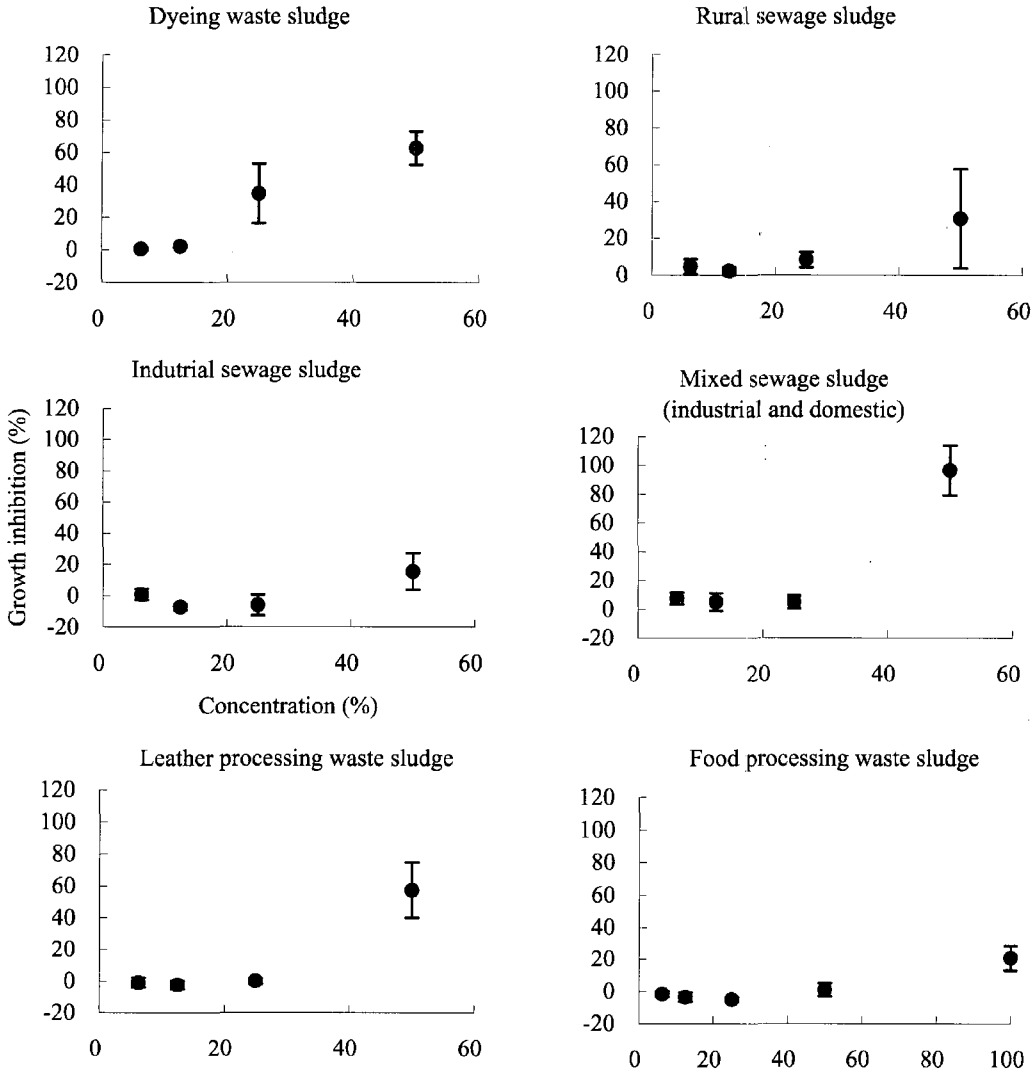

Fig. 6. Population growth inhibition of the marine rotifer exposed to the sewage sludge extracted in seawater. The other sewage sludges as on Fig. 5 had no toxic effects on the rotifer population growth inhibition.

(665-4, 640 mg/kg) were found in the sludge samples. Based on this observation, the sludge extracts are likely to be very toxic to marine organisms and especially heavy metals and POPs are significantly higher in industrial waste sludge, which may need more processing for toxicity reduction prior to being dumped in the ocean.

Two bioassays in this study are a part of a series of marine ecotoxicological evaluation standard methods which are being established at National Fisheries Research and Development Institute (NFRDI) by 2006 with a set of 6 methods using bacteria, phytoplankton, zooplankton, small fish, benthic amphipod, and seaweed. Once the standard methods are established all the chemicals and ocean dumping wastes are screened in advance using the methods as an environmental ecological risk assessment tool.

As the above results indicate, toxicity evaluation of sewage sludges by physicochemical characterization only cannot be an appropriate method without biological evaluation in terms of the ecological risk assessment. Accordingly, multiple approaches such as ecotoxicological evaluations in addition to the chemical analyses must be applied to estimate the environmental impact of sewage sludge released into the natural environment.

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