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Implications of Chemical-Based Effluent Regulations in Assessing DNA Damage in Fathead Minnows *(Pimephales promelas)* When Exposed to Metal Plating Wastewater

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The application of chemical-based regulation on effluents has limitations because of their diverse chemical components, complicated interactions between contaminants, and the lack of information on the bioavailability of constituents (U.S.EPA 1991). In Korea, like in most developing countries, environmental regulations for the protection of the aquatic biota from discharges are based only on this chemical-based approach. The Permissible Wastewater Discharge Standards (PWDS) are the current legislation in Korea. The discharge limits are set for several heavy metals (e.g. Cr, Fe, Zn, Cu, Cd, Hg, As, Pb, etc.), a few organics (phenol, PCB, trichloroethylene, tetrachloroethylene, etc.) and some physicochemical parameters (pH, temperature, etc.). Among these pollutants, the trace metals are important because they might adversely affect the indigenous aquatic biota even at low levels. Genetic toxicity of trace metal has been a major concern (Barron and Adelman 1984; Ciccarelli and Wetterhahn 1985; Parrott and Sprague 1993; Beyersmann and Hartwig 1994; Hartwig *et al.* 1994). Metals readily bind to phosphate groups and to heterocyclic bases of the DNA and, hence not only change the stability, but also hinder the normal functioning of the DNA (Eichhom *et al.* 1970). The resulting consequences of metal-induced DNA damage include mutagenesis, carcinogenesis, and/or teratogenesis (Jones and Parry 1992).

Many researchers have demonstrated genotoxic effects of various metal species on aquatic organisms (Barron and Adelman 1984; Knowles and McKee 1987; Parrott and Sprague 1993; Theodorakis *et al.* 1994; Black *et al.* 1996). However, no studies have been carried out that investigated how well the chemical-based regulations for genotoxic materials protect aquatic organisms when exposed to a complex matrix found in wastewater. In this study, the utility of the chemical-based effluent regulations in protecting aquatic biota from genotoxic impairment was investigated. Metal plating wastewater was utilized for this evaluation, as it contains various metal contaminants that were known to be genotoxic. The genetic impact on fathead minnows was evaluated with a fish DNA damage assay employing an agarose gel electrophoresis technique. A previous study showed that this DNA assay exhibited a good correlation with both acute and chronic toxicity tests using the same fish species (Choi *et al.* 2000). The chemical parameters determined in the plating wastes encompassed various pollutants that

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are presently controlled by the PWDS of Korea. Hence, the objective of this study was to investigate the agreement between chemical-based regulatory measures and genetic toxicity that was observed in fathead minnows.

MATERIALS AND METHODS

Moderately hard water (MHW) was prepared in accordance with the U.S. EPA guidelines (1993). It was used for the maintenance of test organisms, for dilution in preparation of exposure concentrations for the fish DNA damage assay, and also served as the control. Eleven metal plating wastewater samples were collected on four separate sampling events from two plating facilities located in Michigan, USA. The samples represented untreated wastewater, partially treated process water and final effluent. The purpose for taking samples from these three different process points was to have a broad range of toxicant concentrations. Samples were collected in 19-L containers and transported, at 4°C to the Institute of Environmental and Industrial Health Laboratory (Ann Arbor, MI, USA). Upon arrival, a portion was removed for physicochemical analyses and the remaining sample was utilized in the fish DNA damage assay. The samples were analyzed for total residual chlorine and pH to minimize toxicity masking effects. If residual chlorine was found, it was oxidized with sodium thiosulfate. Samples with extreme pH were adjusted to a range between 7 and 8.

The samples were analyzed for various pollutants listed in the PWDS of Korea in accordance with either U.S.EPA analytical standard methods or American Public Health Association Standard Methods (1992). The determined parameters included BOD_5, COD, total suspended solids (TSS), cyanide, and various metal species. Dissolved oxygen, alkalinity, hardness, conductivity, and ammonia were also measured. Ammonia, residual chlorine and pH were analyzed by respective ion specific electrodes. For hexavalent chromium and cyanide, spectrophotometry was employed (EPA 7196 and EPA 355.2, respectively). Mercury was evaluated with a cold vapor atomic absorption spectrophotometer (EPA 7470A), and the remaining metal species that included Cd, Cr, Cu, Fe, Pb and Zn were analyzed utilizing inductively coupled argon plasma atomic emission spectroscopy (EPA 6010).

Six to seven eight-day-old fathead minnows (Source: Aquatox, Hot Springs, AR, USA) were exposed to a series of five or more metal process water concentrations and a control at 20 °C for two hours. After the exposure, all fish were sacrificed and the DNA was extracted with sodium dodecyl sulfate, phenol and chloroform. The fish DNA extract was applied to an electrophoresis system (Bio-Rad® Sub-gel Electrophoresis System: Bio-Rad Laboratories, Hercules, CA, USA). Pictures of the DNA bands were taken with the IS-1000 Digital Image System (Alpha Innotech, San Leandro, CA, USA), and the ImageQuaNT software of the STORM System (Molecular Dynamics, Sunnyvale, CA, USA) was employed to quantify each DNA band. The cellular DNA band represented the undamaged fraction of DNA, and it was chosen as an indicator to evaluate genotoxic impact. The calf thymus DNA was used as a standard and a dose-dependent increasing pattern was

ascertained with the cellular DNA bands. The detailed procedure of this DNA assay was presented elsewhere (Lan 1998).

The relative fish DNA concentrations from the cellular band were utilized to compute the median effective concentrations (EC50s) using TableCurve 2D software (version 4, Jandel Scientific, San Rafael, CA, USA). For the Fisher's Exact Test and the test of agreement, SPSS (version 7, SPSS, Chicago, IL, USA) was employed.

RESULTS AND DISCUSSION

The physicochemical constituents, and the median effective concentrations (EC50s) obtained from the DNA damage assay within the samples are summarized in Table 1. Sample ID 1 contained high levels of Zn and Cu, which exceeded the LC50 values for the test organism (Broderius and Smith 1979; Munkittrick and Power 1991), and showed the most severe DNA damage. However it is interesting to note that no genotoxic effect was observed from sample ID 9, which had the highest levels of Ni and hexavalent Cr. Also the Cu level was higher than the LC50 value in sample ID 9 (Munkittrick and Power 1991). The fact that the Cu, Cr and Ni toxicities are reduced by hardness (Pickering and Henderson 1966; Pickering 1980; U.S.EPA 1986) and fathead minnows are quite tolerant to hexavalent Cr and Ni compared to other test organisms might partly explain this observation. It has been documented that hardness can reduce metal toxicity by forming metallic hydroxides and/or metallic carbonates, and therefore reduce toxic metal concentrations from biological binding sites (Newman 1995). In this regard, that genotoxic impairment observed with the sample ID 10, of which hardness was among the lowest, might be considered plausible.

For comparison, the concentration of each pollutant was standardized by the maximum permissible limit and this information was graphed. The current Korean regulation has varying limits that depend upon the area and volume of discharge; however, the most broadly applied standards were chosen in this case. The standard levels (in mg/L) used for this comparison were as follows: BOD_5 (80) COD (90) total suspended solids (80) Cd (0.1) total Cr (2) hexavalent Cr (0.5), Cu (3), Fe (10), Pb (1), Zn (5) Pb (0.005), and CN (1) (Korean Ministry of Environment 1998). Figure 1 depicts the relationship between chemical-based regulatory compliance and the corresponding EC50 values from the DNA assay. The left Y-axis represents the standardized contamination level, and therefore any value exceeding 1 would be out of compliance with the regulation. The right Y-axis denotes the resulting toxicity endpoint (EC50) of the DNA assay. The samples having an EC50 value at or below 100 % were regarded as being genotoxic.

A two by two contingency table was developed according to the compliance to the regulation and genotoxicity of each sample. On the data presented in Table 2, the Fisher's Exact Test was performed to determine if genotoxicity was dependent

Table 1. The physicochemical components analyzed from designated metal plating wastewater samples (concentration in mg/L, unless otherwise noted), and the corresponding median effective concentration derived from the fish DNA damage assay[a]

ID	Physicochemical Parameters													Fish DNA Damage Assay EC50 (%)
	BOD	COD	TSS	Cr (total)	Cr^{6+}	Cu	Fe	Ni	Zn	NH_3	Alkali-nity[b]	Hard-ness[b]	Specific conductivity[c]	
1	93	620	156.6	14.7	0.18	12.8	17.0	1.86	81.6	32.3	132	340	5380	1.9
2	106	470	4.7	3.1	2.81	0.32	0.07	0.06	0.35	34.2	96	180	1880	31.1
3	7	55	29.4	65.0	62.1	1.60	2.66	55.3	0.65	0.6	164	NA	570	8.7
4	20	50	5.5	0.29	<0.01	<0.05	0.06	0.30	<0.05	0.5	2	1.2	1460	46.8
5	<2	<5	6.3	<0.05	<0.01	<0.05	1.34	0.07	<0.05	0.3	15	240	1640	>100
6	<2	<5	35	0.31	<0.01	0.36	8.59	0.70	0.44	<0.1	132	288	1060	>100
7	4.8	5	47.8	0.40	<0.05	0.23	10.4	1.33	2.19	<0.1	140	248	1530	>100
8	<2	<5	4.8	<0.05	<0.05	<0.05	1.48	0.13	<0.05	<0.1	130	232	2160	>100
9	<2	<5	56.3	103.6	99.0	3.15	6.15	10.0	<0.5	<0.1	98	952	615	>100
10	2.5	110	2.1	0.21	<3.0	<0.05	0.28	0.08	<0.05	5.2	158	12	9050	23.2
11	<2	5	4.9	0.13	<0.005	<0.05	1.31	0.10	<0.05	<0.1	168	220	1510	>100

[a]Cd, Pb, Hg, and CN were not detected. Detection limit for Cd and Pb, 0.05 mg/L; Hg, 0.0002 mg/L; CN, 0.01 mg/L.
[b]mg/L as $CaCO_3$
[c]μmhos/cm

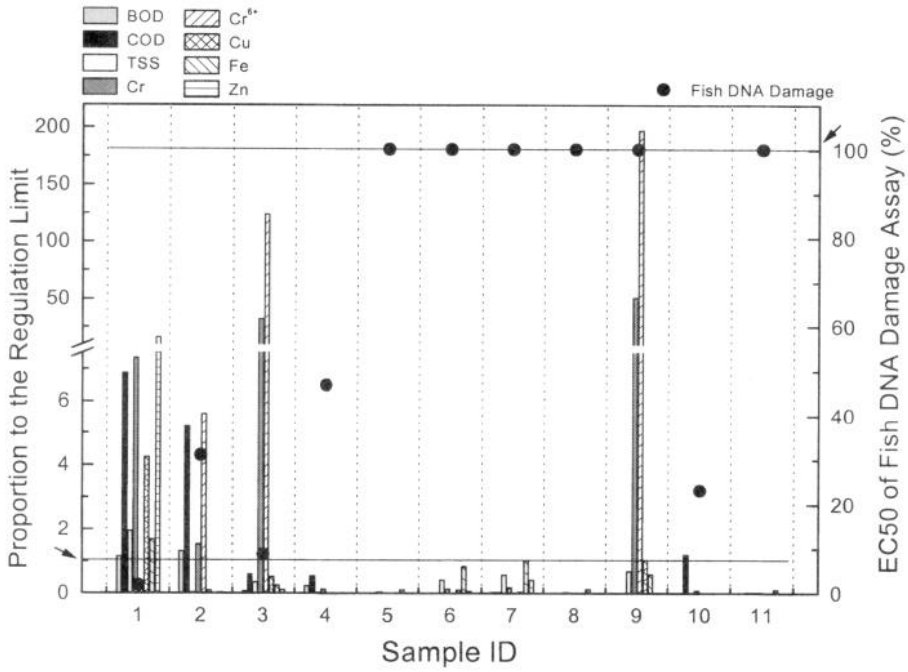

Figure 1. The relationship between regulated pollutant concentrations and EC50 derived from fish DNA damage assay. Cd, Pb, Hg and CN were not detected from the samples. Detection limit for Cd and Pb, 0.05 mg/L; Hg, 0.0002 mg/L; CN, 0.01 mg/L. Data points on the line of 100% indicate equal to or greater than a 100% of dilution.

Table 2. Two by two contingency table of regulation compliance and the fish DNA damage toxicity result

Genotoxicity[a]	Regulation Compliance		Total
	No	Yes	
Present	4	1	5
Non–present	2	4	6
Total	6	5	11

[a]The samples exhibiting an EC50 value of the fish DNA damage assay at or below 100% were regarded as “genotoxicity-present”. Regulation compliance was determined according to the current Korean Permissible Wastewater Discharge Standards (1998).

on the regulation compliance. The result indicated an independency between the regulation compliance and genotoxicity ($p>0.10$), which indicated no correspondence. In addition, a chance-corrected measurement of agreement was conducted between chemical and genotoxicological observations (Fleiss 1981). The kappa value served as an index of the degree of agreement between the compliance to the regulation and genotoxicity to fish, and the resulting calculation of 0.46 suggested a weak agreement. Based on these results, the sole reliance on a chemical-based regulation does not seem to be sufficient to predict genetic toxicity of effluents, especially when considering metal-rich plating wastewater. It should, however be noted that there might have been some unidentified genotoxic agents present in these samples that may have resulted in DNA impairment. This possibility in turn does demonstrate a shortcoming of the current chemical-based regulations.

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