A New Assay for Evaluating Hepatotoxicity and Cytotoxicity Using LDL-Uptake Activity of Liver Cells

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A rapid and sensitive bioassay for detecting cytotoxicity was developed in this study to be used in evaluating many kinds of chemicals. This assay, based on the LDL (low density lipoprotein)-uptake activity of human hepatoblastoma cells, Hep G2, can evaluate cytotoxicity for 48 h with high sensitivity and selectivity using a 96 well plate and a fluorescent plate reader. We evaluate the toxicity of 230 kinds of chemicals and formulate the dose response data by a simple mathematical equation. The toxicity parameters derived by the formulation had some correlations in terms of chemical groups, which were classified as aromatics, organics, metals, and so on.

Key words —— LDL-uptake activity, cytotoxicity, hepatotoxicity, environmental pollutant

INTRODUCTION

Deterioration in the quality of environmental water, including drinking-water sources and river water, is becoming more clearly recognized,[1] and this tendency is expected to continue in the future. The effect of this deterioration on human health is likewise expected to become a more serious problem in the near future. The toxicity of the pollutants found in environmental water is not yet systematically understood, and the effect of each potential toxicant and the integrated effect of plural contaminants are yet to be classified. *In vitro* bioassay has been considered as a promising method for evaluating the effect of such toxicity on human.[2] Compared to *in vitro* bacterial bioassay, bioassay using mammalian cell lines is expected to be more readily applicable to human and to more reliably estimate the toxic effect on humans.

To estimate the effects of toxicity on human health, animal tests seem to be the most suitable method, since they would show the total effects on the whole body; but these require much money and time. In terms of acute toxicity, bioassay using cultured cells would also provide a good estimation. Also, MEIC (Multi-center Evaluation of *In vitro* Cytotoxicity) projects have investigated many kinds of bioassays using selected compounds, and have shown similarities and differences among bioassays and the human effects of acute toxicity.[3] However, it is difficult to estimate levels of toxicity to humans, since the target toxicants are many kinds of chemicals and their total effects on the human body should be estimated. This makes it important to evaluate the organ-specific toxicity of many kinds of chemicals.

The liver is one of the main organs that metabolizes toxicants and is important in the estimation of toxicity.[4] If this system could be easily applied to on-site estimation of environmental water, it could become a novel bioassay, although improvement would be needed. Since the liver is one of the most important organs for both medical and manufacturing purposes, many attempts to evaluate hepatotoxicity have been made;[5] most methods, however, take a long time and the process involved in evaluating toxicity is difficult. Evaluation of the toxicity of many kinds of chemicals or environmental water samples requires development of a rapid bioassay that is sensitive enough to detect the hepatotoxicity of the samples.

A project to establish the application of a bioassay to overall evaluation of a toxicant's effect on humans and the ecology was organized by Utsumi *et al.* in collaboration with 18 institutes.[6] Large sets of bioassay data on chemicals have accumulated in the literature. Such data should be organized and formulated to make the use of these chemicals in controlling toxicity and managing environmental waters more effective and practical.

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We have developed a rapid and sensitive bioassay method based on the LDL (low density lipoprotein)-uptake and metabolic activities of human cells.[6,7] Since LDL is one of the most important nutrients for mammalian cells, the LDL uptake rate is essentially very high.[8,9] The amount and rate of LDL uptake when cells are exposed to toxicants are interesting as a novel index for the hepatotoxicity of environmental pollutants, since the activity of LDL-uptake can be found strongly in hepatocytes.[8] In this study, we developed a means of rapidly and sensitively evaluating hepatotoxicity based on the metabolic changes of human hepatoma Hep G2 cells. The hepatotoxicity of 230 kinds of chemicals was evaluated and their dose response curves were formulated by a simple equation to evaluate their quantitative toxicity.

MATERIALS AND METHODS

Cell Culture ——

Medium and Cells: DMEM (Dulbecco's Modified Eagle Medium, Nissui Pharm., Tokyo, Japan) supplemented with 20 mM HEPES (*N*-2-hydroxyethylpypelazine-*N*-ethansulfonic acid, Dojindo Lab., Kumamoto, Japan), 10% fetal bovine serum (FBS), 100 units penicillin /ml and 100 μg streptomycin / ml were used for the cell culture. Hep G2 cell (human hepatocarcinoma cell; obtained from the Japanese Cancer Research Bank) was used.[10] This cell has many receptors on its membrane for uptaking various substrates involving LDL.[11]

LDL: The procedure for preparing FITC-labeled LDL followed the method of Week *et al.*[12] by using FITC labeling kits (American Qualex, San Clemente, CA, U.S.A.). It was further purified with a 10 ml column of Sephadex™G25M (Pharmacia Biotech AB, Uppsala, Sweden). The relation between the LDL concentration and fluorescence strength was appropriate for this purpose ($R^2 = 0.987$).

To detect the cell activities, 20 *m*g/ml of LDL (BioPur AG, Bubendorf, Switzerland) conjugated with FITC (fluorescein isothiocyanate) was added to serum-free medium.

Cell Culture: Cells were inoculated in 96 well plates (Sumilon, Tokyo) at an initial cell density of 1.0×10^5 cells/cm^2 (70%-confluent) in serum free medium, at 24 h before the toxicity measurement. The cells were loaded with toxicants or water samples with labeled LDL for 4 or 48 h in an incubator (37°C, 5% CO_2).

Assay ——

LDL-Uptake Activity Assay (LDL Assay): After 48 h of exposure, the culture medium was removed, and the cell monolayer was washed 3 times with PBS to remove free LDL. Then, the cells were soaked in 1N NaOH. The cell membranes were destroyed by repeated pipetting and the accumulated LDL was released and dissolved. The amounts of LDL uptaken and accumulated in the cells were measured with a microplate fluorometer (Cambridge Technologies, Inc., U.K.). The excitation and emission wavelengths were set to 490 nm and 520 nm, respectively. The fluorescence strength of 520 nm was proportional to the amount of the total cell-associated LDL, which was shown to be the sum of LDL bound to the receptors and internalized by the cells.[13] To detect the LDL bound to the receptors, the culture was carried out at 4°C to prevent LDL from being internalized by the cell. A cell-free well in each plate was used for determining the blank absorbance, and this blank value was subtracted from the absorbance of each cell-containing well. Milli-Q (prepared by Milli-Q II, Millipore Co., Bedford, MA, U.S.A.) water was used as the negative control.

Cell Survival Assay (AP Assay): We used the acid phosphatase (AP) assay to measure cell growth, because this method can count the living cell number rapidly and easily.[14] The cells were inoculated in 96 well plates (Sumilon) at an initial cell density of 1.0×10^5 cells/cm^2 in a medium with 10% FBS containing various toxicants. After 2 d of culture, the culture medium was removed, the cells were rinsed with 100 μl PBS/well and soaked in sodium acetate buffer (pH 5.5) containing 0.037 g/l *p*-nitrophenylphosphate (Sigma, St. Louis, U.S.A.) and one drop of Triton X-100 (Wako, Osaka, Japan). After 2 h of incubation at 37°C, the absorbance at 405 nm developed in each well was measured with a microplate reader (MPR A4i, TOSOH Co., Tokyo, Japan). The absorbance was proportional to the living cell number ($R^2 > 0.998$).

Formulation ——

Objectives and Advantages of Formulation: Various kinds of bioassay data for chemicals can be found in the literature. However, quantitative toxicity studies of the environmental samples or environmental pollutants are too poor to allow development of a methodology that can evaluate the human risk from the bioassay data. Particularly, very few reports quantitatively refer to the overall toxicity due to the various chemicals contained in environmental water. It is necessary to organize this bioassay data for

use in practical applications of toxicity control and management of environmental waters.

In addition, to extrapolate the experimental range of exposure levels, some mathematical expression about response to dose should be available. A few reports have evaluated the risk of carcinogenicity expressed at very low concentration ranges.[15] However, these methods have been used only in the pharmacological field, for instance in developing a new anti-cancer drug.[16] There have been few studies developed for environmental risk management. Since environmental waters are contaminated by various chemicals, possibly including some which are unknown, these methodologies would be efficient at evaluating the total human risk.

Various Models for Describing Dose Response Curve: A number of quantitative theories of cytotoxicity attempt to relate the cell activity or the cell survival (response) to the intensity of exposure to the chemical (dose).[17] It is generally said that the basic dose–response method is the one-hit or linear model. This model is based on the concept that a tumor can be induced by a single receptor that has been exposed to a single quantum or effective dose unit of a chemical.[18] The multihit model is considered as an extrapolation of the one-hit model.[19,20] Generalization of the multihit model has given rise to the multistage model of carcinogenesis, where it is assumed that cancer originates as a malignant cell, which is initiated by a series of somatic-like mutations occurring in finite steps. It is also assumed that each mutational stage can be depicted as a Poisson process in which the transition rate is approximately linear with dose rate.[21] The log-probit model has been used extensively in the bioassay of dichotomous responses. This model assumes that each animal has its own threshold dose, below which no response occurs and above which a tumor is produced by exposure to a chemical. The log-probit model assumes that the distribution of log dose thresholds is Gaussian.[18] The logistic model, like the probit model, leads to a sigmoidal dose response curve, symmetrical about the 50% response curve. It approaches zero response as dose decreases more gradually than does the probit curve. The practical implication of this characteristic is that the logistic model leads to a lower NOAEL (no observed adverse effect level) than the probit model, even when both models are equally descriptive of the data in the observable range.[19] The Weibul and Gompertz models also lead to a sigmoidal curve.

Curve Fitting: To describe the dose response curves by a simple mathematical equation, the logistic curve expression given by Equation (1) was employed.

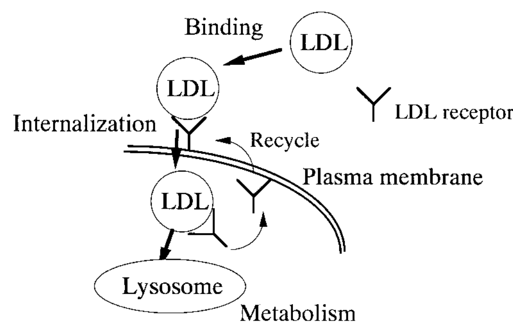

Fig. 1. Schematic Drawing of LDL Uptaken into Cells

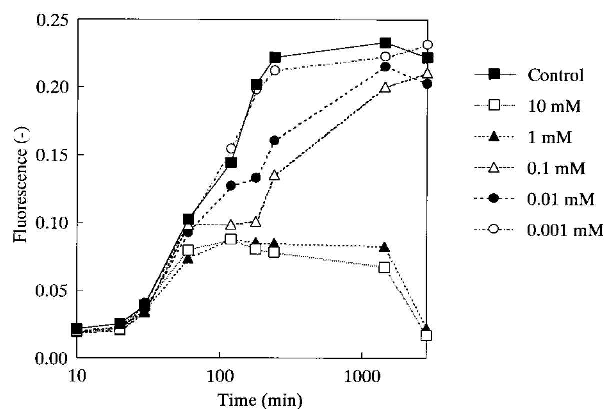

Fig. 2. Changes in LDL Uptake When Exposed to As_2O_3

$$y = \frac{1}{1 + \exp\left(\frac{x-m}{s}\right)} \tag{1}$$

where x is the logarithm of chemical concentration (mM), m is the logarithm of the concentration of the chemical (mM) reducing the cell survival or activity 50% (= ED_{50}), and s is the dispersion factor. The correlation coefficients (R^2) between experimental data and the formulated curve were also calculated. The curve fitting and the calculation were carried out by the Simplex method with the DeltaGraph (ver. 4.0.5, SPSS, Chicago, IL, U.S.A.) using Macintosh computer (PowerBook G3, Apple, U.S.A.).

RESULTS AND DISCUSSION

Fundamental Characteristics of LDL-Uptake Activity Assay

Changes in LDL uptaken in the cells adminis-

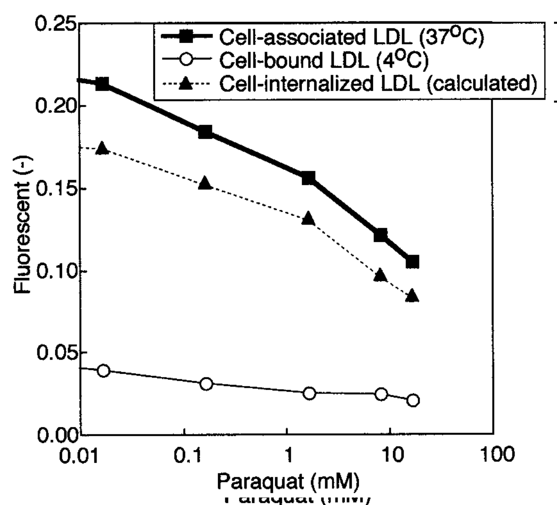

Fig. 3. Dose–Response curves of Cell-Associated, -Bound and -Internalized LDL Administered Paraquat

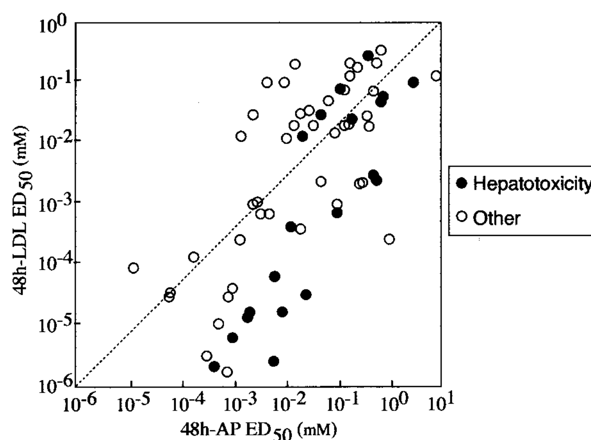

Fig. 4. Comparison of ED_{50} Values Obtained by LDL and AP Assays

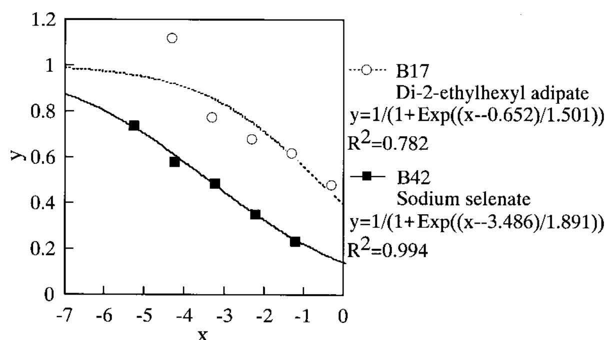

Fig. 5. Examples of Curve Fitting

tered As_2O_3 are shown in Fig. 2. The earliest appearance of toxic effects was found within 120 min, and the effect became larger and larger until 240 min. In the subsequent experiments of this work, the uptake activities were evaluated by the amount of LDL uptaken after at least 4 h of exposure. In this work, since the LDL-uptake activity assay was used to detect hepatotoxicity, the exposure time was set to 48 h.

The dose–response curves of cell-associated, -bound and -internalized LDL are shown in Fig. 3, when paraquat was administered. The dependency of the cell-bound LDL amount on the paraquat concentration was negligible, and no toxicity could be detected by this index. On the other hand, the dependency of the cell-associated LDL amount was considerably high. This was simply because the absolute fluorescence strength by cell-bound LDL was so small that the effects of toxicants were not observed. Therefore, in the subsequent experiments of this work, the amount of cell-associated LDL (37°C) was measured and used for the toxicity evaluation.

Selectivity and Sensitivity

Of 230 chemicals tested, significant toxicity was detected for 148 and 133 of them by LDL and AP assays, respectively. The ED_{50} values were compared with those obtained by 48 h-AP assay. The results are shown in Fig. 4, where no clear correlations are found. This may be simply because the endpoints of toxicity evaluated are quite different between LDL and AP assays. Hepatotoxic chemicals like glyoxal or formaldehyde might have a strong effect on LDL-uptake activity, since uptaking LDL is a hepatocyte specific function.[8] Thus, the toxicity index of the LDL-uptake activity assay is superior in detecting hepatotoxicity. In terms of basal cytotoxicity, the sensitivity of the LDL-uptake activity assay is almost the same as that of cell survival assay. The ED_{50} values obtained by LDL assay were smaller than those obtained by AP assay. In summary, the bioassay proposed in this work is believed to be suitable for detecting the hepatotoxicity of various chemicals.

Correlation Between Experimental Data and Formulated Curves

The resultant dose response curves were expressed by a simple mathematical equation, since the description of bioassay data by mathematical equations is needed when the data are utilized to control toxicity and manage environmental waters.

The resultant dose response curves were fitted

by Eq. (1) and the parameters, m and s, were determined. Examples of curve fitting are shown in Fig. 5 for di-2-ethylhexyl adipate (B17) sodium selenate (B42). Plots represent the experimental data and the lines are the formulated curves. Sodium selenate was the best and di-2-ehylhexyl adipate was the worst example in term of correlation coefficients (R^2). A few chemicals showed no toxicity, such as diphenylamine (B21). Sodium selenate may act like a hormone to promote animal cell growth at a low concentration in medium.[23] In some chemicals that have toxicity even at a low concentration, Eq. (1) could not well formulate the dose response curves. The curve of 2,4,5-trichlorophenol (A26) was also not well fitted by Eq. (1), because cell survival at a low concentration range was over the control value. Although Eq. (1) cannot express values over unity, it can be employed for this purpose, since the relative cell activity/survival is usually below unity in toxicity assays. Therefore, only the curve fittings of relative cell activity/survival were carried out for values below 1.

Table 2 summarizes m, s, and R^2 values obtained for 230 chemicals. Some chemicals, for example potassium dichromate (B39), had no toxicity detected by our assay. The toxicity of others, such as benzo(*a*)pyrene (A02) and tributyltin chloride (A25), could not be detected in the tested concentration ranges. This may be because tributyltin chloride has a weak toxicity to cultured cells.[24] However, most chemicals showing some degree of toxicity gave a correlation coefficient, R^2, of over 0.8. Equation (1) thus formulates well the dose response curves derived by our assay.

Relationships between m and s are shown in Fig. 6, where chemicals are classified into groups of organics, aromatics, metals, and others. Clear positive correlations between s and log m can be found as shown by the solid lines in Fig. 6. Although a positive correlation between m and s were found for most chemicals, a clear negative correlation was found for aromatic chemicals. This may be because phenols have toxic actions to cultured cells that differ from those of other organic chemicals.[25] This approach would be useful in the future for estimating toxicity particularly to chemicals on the basis of molecular structure, physicochemical properties, and so on, with the aid of computer-based analysis.

In summary, the toxicities of various chemicals or river water were rapidly and sensitively evaluated by the change in LDL-uptake activity of Hep G2 cells. Further investigation will be needed especially regarding the response property for detecting toxicity of individual chemicals from their mechanism of toxicity expression. In addition, formulation of the dose response curves for 255 chemicals by a simple mathematical equation having two parameters (*i.e.*, an ED_{50} value and a curve shape) was carried out, and the formulation was generally successful with some exceptions. It is concluded

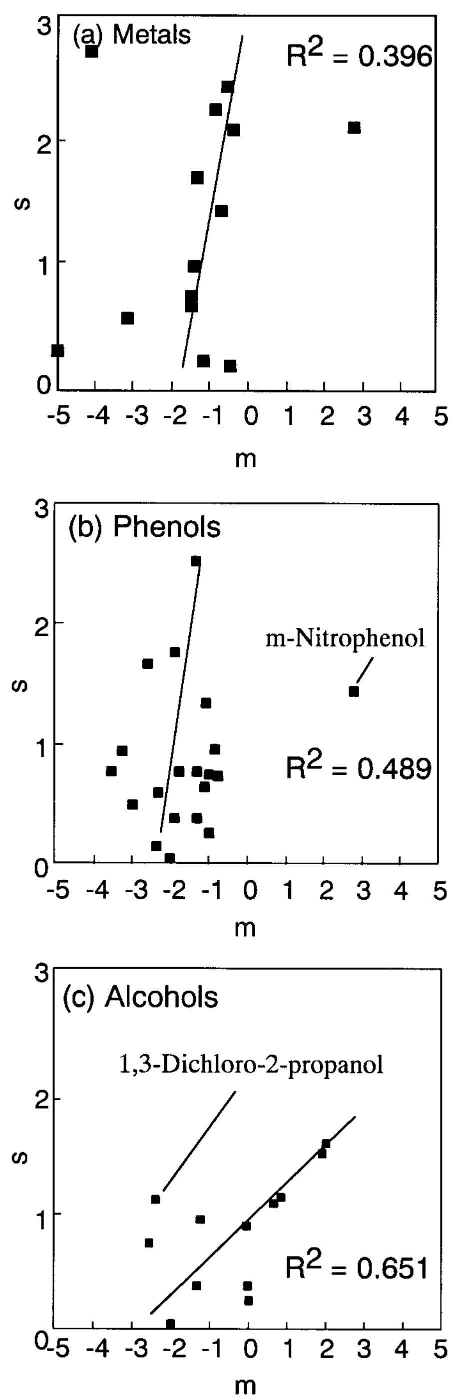

Fig. 6. Plots of s vs m

Table 1. List of Chemicals Used in This Study

No.	substance	CAS No.	M.W.	No.	substance	CAS No.	M.W.
A01	2-Aminoanthracene	613-13-8	193.2	B19	N,N-Dimethylformamide	1968.12.2	73.09
A02	Benzo(a)pyrene	50-32-8	252.3	B20	1,4-Dioxane	123-91-1	88.1
A03	Bis-phenol-A	1980.5.7	228.28	B21	Diphenylamine	122-39-4	169.22
A04	Catechol	120-80-9	110.11	B22	Epichlorohydrin	106-89-8	92.53
A05	2-Chloro-1,1,2-	310-71-4	162.54	B23	Ethylene glycol	110-80-5	90.12
	trifluoroethyl ethyl ether				monoethyl ether		
A06	Di-2-ethylhexyl phthalate	117-81-7	390.6	B24	Hexachloro-1,3-butadiene	87-68-3	260.76
A07	2,5-Dichlorophenol	583-78-8	163	B25	1,2,3,4,5,6-Hexachloro	58-89-9	290.85
A08	2,4-Dichlorophenoxy	94-75-7	221.04		cyclohexane		
	acetic acid			B26	Isophorone	78-59-1	138.21
A09	Diethylstilbestrol	56-53-1	268.34	B27	Malathion	121-75-5	330.36
A10	b-Estradiol-17-acetate	1743-60-8	314.4	B28	Maneb	12427-38-2	265.3
A11	17-a-Ethynylestradiol	57-63-6	296.39	B29	Manzeb	8018.1.7	265.3
A12	Formaldehyde	50-00-0	30.03	B30	2-Mercaptobenzothiazole	149-30-4	167.25
A13	Glyoxal	4405-13-4	210.1	B31	2-Mercaptoimidazoline	96-45-7	102.17
A14	Lead nitrate	10099-74-8	331.23	B32	Monochloroacetic acid	1979.11.8	94.5
A15	Menadione	58-27-5	172.17	B33	Nickel(II)chloride	7718-54-9	129.61
A16	3-Methylcholanthrene	56-49-5	268.34	B34	Nitrilotriacetic acid	139-13-9	191.14
A17	Methylmercury Chloride	115-09-3	251.08	B35	Nitrobenzene	98-95-3	123.11
A18	1-Nitropyrene	5522-43-0	247.2	B36	N-Nitrosodimethylamine	62-75-9	74.08
A19	4-Nitroquinoline-N-oxide	56-57-5	190.1	B37	N-Nitrosodiphenylamine	86-30-6	198.23
A20	p-Nonylphenol	104-40-5	220.35	B38	2-Phenylene diamine	95-54-5	108.14
A21	Pentachlorophenol	87-86-5	266.34	B39	Potassium dichromate (VI)	7778-50-9	294.21
A22	Sodium arsenite	7784-46-5	129.91	B40	Simazine	122-34-9	201.67
A23	Thiobencarb	28249-77-6	257.78	B41	Sodium molybdate	10102-40-6	205.92
A24	Thiuram	137-26-8	240.44	B42	Sodium selenate	13410-01-0	188.94
A25	Tributyltin chloride	1461-22-9	325.49	B43	Thiourea	62-56-6	76.12
A26	2,4,5-Trichlorophenol	95-95-4	197.45	B44	Tributyl phosphate	126-73-8	266.32
A27	Trp-P-2	72254-58-1	257.29	B45	2,4,5-Trichloro	93-76-5	255.49
A28	Paraquat	1910-42-5	257.16		phenoxyacetic acid		
A29	Cucumechinoside D	125640-33-7		B46	Triethylenetetramine	112-24-3	146.23
A30	Marthasteroside A1	89383-05-1		B47	Trifluralin	1582-09-8	335.29
B01	Acetaldehyde	75-07-0	44.05	B48	Triphenyltin(IV) chloride	639-58-7	385.46
B02	Acrylamide	1979.6.1	71.08	B49	Tris(2-chloroethyl)	115-96-8	285.49
B03	Alachlor	15972-60-8	269.77		phosphate		
B04	Aniline	62-53-3	93.12	B50	Vinylacetic acid	625-38-7	86.1
B05	Antimony(III)chloride	10025-91-9	228.13	C01	Aflatoxin B1	1162-65-8	312.28
B06	Benzophenone	119-61-9	182.21	C02	2-Aminoethanol	141-43-5	61.08
B07	Biphenyl	92-52-4	154.2	C03	m-Aminophenol	591-27-5	109.12
B08	Boric acid	10043-35-3	61.84	C04	3-Amino-1H-1,2,4-triazole	61-82-5	84.08
B09	Cadmium chloride	10108-64-2	183.32	C05	Ziram	137-30-4	305.82
B10	4-Chloronitrobenzene	100-00-5	157.56	C06	1,2-Benzanthracene	56-55-3	228.28
B11	4-Chlorotoluene	106-43-4	126.58	C07	Benzo[b]fluoranthene	205-99-2	252.32
B12	1,2-Dibromo-3-	1996.12.8	236.36	C08	Benzo[e]pyrene	192-97-2	252.3
	chloropropane			C09	Benzoic acid	65-85-0	122.12
B13	2,6-Di-t-butyl-	128-37-0	220.34	C10	Bis(2-chloroethyl) Ether	111-44-4	143.02
	4-methylphenol			C11	Bromodichloromethane	75-27-4	163.83
B14	1,4-Dichlorobenzene	106-46-7	147.01	C12	n-Butylbenzene	104-51-8	134.21
B15	Dicyclohexylamine	101-83-7	181.31	C13	p-t-Butylbenzoic acid	98-73-7	178.23
B16	Dicyclopentadiene	77-73-6	132.21	C14	Captans	133-06-2	300.57
B17	Di-2-ethylhexyl adipate	103-23-1	370.58	C15	p-Chlorophenol	106-48-9	128.56
B18	Diethyl sulfate	64-67-5	154.19	C16	Copper (2) sulfate	7758-99-8	249.68

Table 1. Continued

No.	substance	CAS No.	M.W.	No.	substance	CAS No.	M.W.
C17	p-Cresol	106-44-5	108.13	D19	1,2,4-Trichlorobenzene	120-82-1	181.46
C18	Cycrohexyl amine	108-91-8	99.17	D20	N,N-Dimethylaniline	121-69-7	121.18
C19	DDVP	62-73-7	220.98	D21	Adipic acid	124-04-9	146.14
C20	1,2:5,6-Dibenzanthracene	53-70-3	278.33	D22	Chlorodibromethane	124-48-1	208.28
C21	Dibutyl phthalate	84-74-2	278.34	D23	Dimethyl phthalate	131-11-3	194.19
C22	2,4-Dichlorophenol	120-83-2	163.01	D24	n-Butyl acrylate	141-32-2	128.17
C23	1,3-Dichloro-2-propanol	96-23-1	128.99	D25	1-Nonanol	143-08-8	144.26
C24	Diethyl phthalate	84-66-2	222.24	D26	Diethylbenzene, mixture	25340-17-4	134.22
C25	1,8-Dinitropyrene	42397-65-9	292.25	D27	2,4-Dinitrophenol	51-28-5	184.11
C26	EDTA 2NA	60-00-4	292.24	D28	o-Dinitrobenzene	528-29-0	168.11
C27	Ethyl benzene	100-41-4	106.16	D29	2,4-Dichloroaniline	554-00-7	162.02
C28	MeIQx	77500-04-0	213.24	D30	m-Nitrophenol	554-84-7	139.11
C29	Melamine	108-78-1	126.13	D31	2,6-Dimethylnapthalene	581-42-0	156.23
C30	Mercury (2) chloride	7487-94-7	271.52	D32	4-Chloro-3-methylphenol	59-50-7	142.58
C31	Methomyl	16752-77-5	162.2	D33	1-Butanol	71-36-3	74.12
C32	Methoxychlor	72-43-5	345.65	D34	Bromoform	75-25-2	252.77
C33	2-Methylpyridine	109-06-8	93.12	D35	2,2-Bis(3,5-dibromo-4 -hydroxyphenyl)propane	79-94-7	543.87
C34	Molinate	2212-67-1	187.31				
C35	Morpholine	110-91-8	87.12	D36	Antraquinone	84-65-1	208.2
C36	NAC	63-25-2	201.22	D37	1,2,3-Trichlorobenzene	87-61-6	181.46
C37	NIP	1836-75-5	284.1	D38	2,4,6-Trichlorophenol	1988.6.2	197.46
C38	2-Nitrofluorene	607-57-8	211.22	D39	o-Chloronitrobenzene	88-73-3	157.56
C39	p-Nitrotoluene	99-99-0	137.14	D40	o-Nitrophenol	88-75-5	139.11
C40	MEP	122-14-5	277.25	D41	3-Nitrofluoranthene	892-21-7	247.25
C41	Phenol	108-95-2	94.11	D42	N-Phenyl-1-naphthylamine	90-30-2	219.29
C42	PhIP Hydrochloride	105650-23-5	224.26	D43	Naphthalene	91-20-3	128.16
C43	Potassium cyanide	151-50-8	65.11	D44	o-Dichlorobenzene	95-50-1	147.01
C44	Simetryne	1014-70-6	213.32	D45	o-Toluidine	95-53-4	107.15
C45	Sodium lauryl sulfate(SDS)	151-21-3	288.38	D46	2,4-Diaminotoluene	95-80-7	122.17
C46	Thallium(I)chloride	7791-12-0	239.85	D47	2,5-Dichloroaniline	95-82-9	162.02
C47	1,1,1,2-Tetrachloroethane	630-20-6	167.85	D48	1-Chloro-2,4-nitrobenzene	97-00-7	202.56
C48	p-Toluenesulfoneamide	70-55-3	171.22	D49	2,4-Dinitroaniline	1997.2.9	183.12
C49	TPN	1897-45-6	265.89	D50	Cumene	98-82-8	120.19
C50	Vinclozolin	50471-44-8	286.11	E01	Kelthane	115-32-2	370.47
D01	Benzylalcohol	100-51-6	108.13	E02	Hexachlorophene	70-30-4	406.92
D02	Benzaldehyde	100-52-7	106.12	E03	Permethorin	52645-53-1	391.29
D03	N-Methylaniline	100-61-8	107.15	E04	Tris(butoxyethyljphosphate	78-51-3	398.48
D04	Hydroxyl ammonium sulfate	10039-54-0	164.14	E05	p-Bromophenol	106-41-2	173.02
D05	Diphenylmethane	101-81-5	168.23	E06	o-Tolidine	119-93-7	212.28
D06	Dibenzyl ether	103-50-4	198.25	E07	$2,2',2''$-Nitrilotriethanol	102-71-6	149.19
D07	N-Ethylaniline	103-69-5	121.18	E08	Pyrene	129-00-0	202.24
D08	Bis(2-ethylhexyl)amine	106-20-7	241.46	E09	Quinoline	91-22-5	129.15
D09	4-Chloroaniline	106-47-8	127.57	E10	1,2-Epoxyethylbenzene	1996.9.3	120.15
D10	1,2-Dibromoethane	106-93-4	187.88	E11	Ethyl carbamate	51-79-6	89.09
D11	Ethylene glycol	107-21-1	62.07	E12	Triethylamine	121-44-8	101.19
D12	Chlorobenzene	108-90-7	112.56	E13	1,2,3-Trichloropropane	96-18-4	147.43
D13	Diethylene glycol	111-46-6	106.12	E14	α-Methylstyrene	98-83-9	118.18
D14	n-Decyl alcohol	112-30-1	158.28	E15	Fthalide	27355-22-2	271.9
D15	Decabromodiphenyl ether	1163-19-5	943.1	E16	1-Methylnaphthalene	90-12-0	142.2
D16	2-Aminoanthraquinone	117-79-3	223.23	E17	Phenylhydrazine	100-63-0	108.14
D17	2,4,6-Tribromophenol	118-79-6	330.83	E18	Tetrachloroethylene	127-18-4	165.85
D18	Anthracene	120-12-7	178.22	E19	Aplysiaterpenoid A	116836-80-7	273.98

Table 1. Continued

No.	substance	CAS No.	M.W.	No.	substance	CAS No.	M.W.
E20	Thiophanat-methyl	23564-05-8	342.4	E36	N-Nitrosodiethylamine	55-18-5	102.14
E21	1,3-Dichloropropene	542-75-6	110.98	E37	Benzo[k]fluoranthene	207-08-9	252.32
E22	Microcystin RR	111755-37-4	1009.53	E38	PAP	2597.3.7	320.37
E23	Toluene	108-88-3	92.13	E39	IBP	26087-47-8	288.35
E24	Hydroquinone	123-31-9	110.11	E40	Cyclohexanol	108-93-0	100.16
E25	MPP	55-38-9	278.34	E41	EPN	2104-64-5	323.31
E26	Dimethoate	60-51-5	229.28	E42	PCNB	82-68-8	295.36
E27	Acetamde	60-35-5	59.07	E43	Tetraethylenepentamine	112-57-2	189.31
E28	Acephate	30560-19-1	183.16	E44	EDDP	17109-49-8	310.36
E29	Cyclohexanone	108-94-1	98.14	E45	Styrene monomer	100-42-5	104.14
E30	DCPA	709-98-8	218.09	E46	Coumestrol	479-13-0	268.21
E31	Bifenox	42576-02-3	342.14	E47	Terephthalic acid	100-21-0	166.13
E32	Diazinon	333-41-5	304.36	E48	2-Methyl-1-propanol	78-83-1	74.12
E33	BPMC	3766-81-2	207.27	E49	Resorcinol	108-46-3	110.11
E34	Benzo[ghi]perylene	191-24-2	276.34	E50	1,6-Dinitropyrene	42397-64-8	292.25
E35	N-Phenyl-2-naphthylamine	135-88-6	219.29				

Table 2. Parameters, m and s, and Correlation Coefficients Derived from the Formulation (48 h-LDL)

Substance	m	s	R^2	Substance	m	s	R^2	Substance	m	s	R^2
A01	−2.546	0.892	0.674	B02	−1.138	1.003	0.945	B33	−0.004	2.418	0.979
A02	−4.151	2.752	0.927	B03				B34	−0.103	5.658	0.317
A03	−5.604	2.224	0.961	B04	−3.048	1.677	0.936	B35	−0.419	0.535	0.687
A04	−2.578	1.653	0.892	B05	−1.72	5.776	0.941	B36	−1.065	0.795	0.193
A05	−2.29	0.92	0.905	B06	−2.278	5.762	0.829	B37	−2.905	0.764	0.152
A06	−2.089	0.586	0.823	B07	−1.171	1.47	0.887	B38	−9.911	0.194	0.267
A07	−2.332	0.141	0.731	B08	−2.294	1.248	0.95	B39	−1.899	0.804	0.975
A08	−2.965	0.887	0.962	B09	−1.449	5.615	0.988	B40	−0.256	1.293	0.894
A09	−1.878	0.376	0.511	B10	−9.733	2.472	0.268	B41			
A10	−1.307	0.375	0.279	B11	3.419	1.532	0.149	B42	3.486	1.891	0.994
A11	−1.989	0.032	0.242	B12	1.663	7.281	0.439	B43			
A12	−1.794	1.067	0.979	B13	6.204	2.648	0.346	B44	−1.274	0.974	0.979
A13	−2.417	1.012	0.899	B14	2.107	6.592	0.037	B45	0.998	0.046	0.432
A14	−1.753	1.368	0.981	B15	1.998	7.574	0.296	B46	−1.518	0.969	0.416
A15	−1.003	0.377	0.427	B16	1.179	1.891	0.976	B47	1.681	1.144	0.992
A16	−4.034	0.425	0.914	B17	−6.52	1.502	0.782	B48	−2.312	1.061	0.925
A17	−2.116	0.442	0.771	B18	−1.329	1.449	0.835	B49	−0.564	0.246	0.999
A18	−4.047	0.659	0.863	B19	−1.17	2.454	0.917	B50	−5.663	1.23	0.158
A19	−3.41	0.649	0.832	B20	−1.42	7.531	0.808	C01			
A20	−3.25	0.935	0.967	B21	−2.043	2.75	0.22	C02			
A21	−3.523	0.759	0.937	B22			0.285	C03			
A22	−2.219	1.251	0.934	B23	−0.014	0.376	0	C04			
A23	−3.521	1.172	0.932	B24			0.183	C05	−1.507	1.724	0.981
A24	−4.929	0.934	0.978	B25	−2.889	1.308	0.407	C06			
A25	−2.041	0.575	0.475	B26	−57.28	22.6	0.05	C07			
A26	−2.308	0.582	0.535	B27	−7.751	4.967	0.299	C08			
A27	−3.923	0.728	0.917	B28				C09	−5.637	1.582	0.94
A28	−2.713	0.889	0.987	B29				C10	−5.044	5.473	0.992
A29	−2.304	1.151	0.834	B30	−6.935	3.488	0.513	C11			
A30	−2.375	1.123	0.832	B31	−1.214	3.543	0.941	C12	−1.29	1.849	0.971
B01	−1.293	1.002	0.848	B32	−1.122	1.956	0.806	C13	−7.705	3.459	0.988

Table 2. Continued

Substance	m	s	R^2	Substance	m	s	R^2	Substance	m	s	R^2
C14				D11				E07			
C15				D12				E08			
C16	−1.115	2.26	0.959	D13	2.031	1.606		E09			
C17	−1.859	1.762	0.804	D14				E10			
C18	−1.529	1.995	0.798	D15				E11			
C20	−1.163	1.927	0.84	D16	−0.667	0.996	0.825	E12			
C21	−0.812	1.927	0.972	D17	−0.837	0.94	0.898	E13			
C22	−1.32	2.523	0.997	D18			0.78	E14			
C23	−2.55	0.741	0.906	D19				E15			
C24				D20				E16			
C25				D21	−0.808	1.136	0.515	E17	0.601	0.799	0.92
C26				D22				E18			
C27	−0.444	2.067	0.9	D23	0.724	0.986		E19			
C28	−2.719	2.146	0.88	D24	4.588	2.869		E20	−0.976	0.244	0.967
C29				D25	1.937	1.517		E21			
C30	−0.787	2.441	0.971	D26				E22			
C31				D27	−1.284	0.757	0.977	E23			
C32				D28	−1.903	0.745	0.888	E24	−0.976	0.244	0.99
C33	−1.651	2.457	0.943	D29	0.02	0.66		E25			
C34	−1.668	0.89	0.784	D30	2.803	1.429	0.936	E26			
C35				D31				E27	0.073	0.392	0.992
C36	−2.368	2.034	0.969	D32	−0.738	0.725	0.673	E28	4.174	1.916	0.525
C37	−2.617	3.204	0.989	D33	0.68	1.095		E29			
C38	−1.626	1.862	0.933	D34				E30			
C39				D35	−1.74	0.758	0.988	E31			
C40	−1.097	1.738	0.832	D36				E32			
C41	−1.04	1.326	0.73	D37	0.02	0.858	0.932	E33	−0.636	0.717	0.983
C42				D38	−1.083	0.634	0.676	E34	−1.874	0.374	0.979
C43	−0.651	2.096	0.931	D39	1.971	2.283		E35	−0.454	0.593	0.986
C44	−3.927	2.512	0.94	D40	−0.973	0.729	0.903	E36	26.752	7.144	0.069
C45	−0.389	1.627	0.95	D41	1.679			E37			
C46				D42	−1.695	0.444	0.965	E38			
C47				D43	0.489	1.067	0.61	E39			
C48	−2.454	1.762	0.948	D44	0.559	1.723	0.634	E40	0.017	0.251	0.955
C49				D45	0.379	1.145	0.701	E41			
C50				D46				E42			
D01	−1.23	0.947	0.969	D47				E43			
D02	−1.127	1.254	0.833	D48	−1.887	0.72	0.995	E44			
D03				D49	−0.441	0.926	0.99	E45			
D04	0.574	1.744	0.635	D50				E46			
D05	0.483	1.247	0.971	E01	0.858	1.145	0.988	E47	−0.14	0.983	0.983
D06	3.907	2.181	0.555	E02	−2.983	0.48	0.994	E48	−0.045	0.9	0.992
D07	2.309	2.079	0.412	E03				E49			
D08	1.516	1.813	0.321	E04	−1.187	0.028	0.296	E50	2.623	1.984	0.782
D09	0.706	1.554	0.948	E05							
D10				E06	−0.465	0.221	0.978				

that the methodology developed in this work can be the first step toward development of an instrumental bioassay needed for monitoring environmental water in the near future.

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