Review Article

Integrated Biomonitoring of Dioxin-like Compounds for Waste Management and Environment

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Abstract: Many of the biological and toxic responses of polychlorinated dioxins and furans (PCDD/Fs) have been shown to be mediated through the aryl hydrocarbon receptor (AhR). Several bioanalytical methods have been developed to analyze Ah receptor agonists in cost-and-time effective way such as CALUX-bioassay (chemical-activated luciferase gene expression). This bioassay can detect the sum of dioxin-like activity (including unknown substances) in complex waste and environmental samples. The present paper gives a description of the bioanalytical method and application examples to analyze dioxin-like compounds in waste management samples of thermal/chemical dechlorination processes. The current detection limit of the CALUX in rat hepatoma H4IIE recombinant cells is less than 1 pM TEQ (0.06 pg TEQ/well). The CALUX bioassay is considered to be useful tools for the rapid and integrated monitoring of environmental emission and residual samples as a pre-screening or in combination to chemical analysis.

Key words: Dioxin, Bioassay, Ah receptor, CALUX, Waste management, Incineration fly ash, Thermal dechlorination, PCB, Chemical treatment

Introduction

Dioxin-like compounds such as PCDD/Fs and PCBs show similar properties and display a wide variety of toxic effects in mammals, birds and fishes. Among the toxic effects observed as a result of exposure are immunotoxicity, carcinogenicity, metabolic changes, endocrine disruption and even death¹⁾. The mechanism of action has been extensively studied over the past decades. The binding to the Ah receptor offers the biochemical analyst now several new technologies to analyse the dioxin-like activity of these compounds quantitatively (e.g. *in vitro* luciferase assays). The total sum of toxic equivalents (TEQ) of all kind of dioxin-like compounds can be measured. These new technologies have

been used already in several screening and biomonitoring programmes. Advantages are the extremely high sensitivity, rapid, easy clean-up/work-up, small sample size and reduced cost compared to instrumental methods. They have been already applied on a wide variety of matrices such as pure chemicals, food/feed and environmental samples.

Dioxin-like compounds have two types of their origin. PCBs have been intentionally manufactured and PCDD/Fs have been occurred as unwanted by-products in chemicals or formed in thermal processes. These persistent toxic substances (PTS) are now found in all kind of reservoirs e.g. contaminated landfills, sediments/soils and tend to be accumulated in biota. Therefore, PTS destruction processes have been developed as fly ash dechlorination and waste PCB destruction. This paper covered CALUX bioassay protocol, relative responses of dioxin-like compounds and some applications of biomonitoring during PTS destruction processes.

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CALUX Bioassay Protocol and Relative Responses (REPs) of PCDD/Fs and PCBs

The EROD (7-ethoxyresorufin *O*-deethylase) activity, which is one function of the cytochrome P-450 monooxygenase enzyme has been widely used for the detection of PHAHs (polyhalogenated aromatic hydrocarbons) in environmental samples. Because EROD can measure the catalytic activity (conversion of 7-ethoxyresorufin to resorufin) of the CYP1A1 isozyme induction which is the most prominent pathway through the aryl hydrocarbon receptor (AhR)-gene battery system. Also EROD activity can be measured both *in vivo* and *in vitro*. *In vivo* EROD assay has been conducted as a biomarker of exposure to PHAHs. Liver microsome containing CYP1A1 is extracted from animals such as rat, mouse, fish and wild bird, and their EROD activity can be measured²⁻⁸. *In vitro* micro-EROD assays have been simplified using culture cells such as H4IIE rat hepatoma cell line, mouse Hepa 1c1c7 cell line, the PLHC-1 fish hepatoma cell line, rainbow trout liver cell line RTL-W1 and human hepatoma HepG2 cells in 96-well microplates for the purpose more related to analysis of PHAHs⁹⁻¹⁹. For *in vitro* assay, induction of EROD activity caused by test samples can be compared with the induction curve of TCDD standard to determine biological TEQ (EROD-TEQ) values in samples.

EROD bioassay, however, has the following drawbacks²⁰⁻²²:
—*in vivo* seasonal-, sex-, age-, diet-dependent fluctuations in induction

—low enzyme stability after death of animal (*in vivo*)
—*in vitro* substrate inhibition at greater ligand concentrations
—*in vitro* long exposure time (requires 72 h for obtaining sufficient induction)

To circumvent the shortcomings of *in vitro* EROD, CALUX assay has been successfully developed based on AhR-mediated firefly (*Photinus pyralis*) luciferase gene expression²²⁻²⁶. A vector containing the luciferase gene under transcriptional control of DREs (dioxin responsive element) isolated from the 5'-flanking region of the mouse P450 1A1 gene, was stably transfected into hepatoma cell lines, including mouse (Hepalclc7) and rat (H4IIE) cell lines. The increase of luciferase activity is dependent on exposed TCDD concentration and comparable to that of the endogenous CYP1A1 activity. The primary improvement of the CALUX assay compared to the EROD assay is that increase of chemical concentrations do not cause decrease in the response of luciferase activity. Additionally, assay sensitivity to TCDD could be further improved compared to EROD, that is the current detection limit of the CALUX in rat hepatoma H4IIE

Fig. 1. Flowchart of the CALUX bioassay.

recombinant cells is less than 1 pM TEQ (0.06 pg TEQ/well) and the dose-response curve saturates at TCDD concentrations greater than 300 pM. Linear working range for the CALUX in H4IIE recombinant cells was also better than for the EROD in H4IIE wild type cells. Thus, the CALUX has been applied to human blood, human milk, cow milk, recycled paper and sediment, *etc.* as a promising risk assessment tool for dioxin-like compounds^{22, 25-33}. Hereafter, “CALUX” means the DR-CALUX® (Dioxin Responsive-Chemical Activated LUciferase eXpression) using rat hepatoma H4IIE recombinant cells and “EROD” means the micro-EROD method using rat hepatoma H4IIE C3/T cell line in this paper.

The assay procedure of CALUX bioassay conducted in our institute is shown in Fig. 1. The cells were plated in plastic 96-well plates in 0.1 ml α -Minimal Essential Medium supplemented with 10% Fetal Bovine Serum per well. Following 24-hour incubation, the cells with at least 95% confluency were dosed by adding 0.1 ml of the above medium supplemented with the chemical or extract to be tested dissolved in DMSO (0.4%). Basically after 24-hour treatment, the dose medium was removed and the exposed wells were filled with 0.1 ml phosphate buffer solution (PBS)

Table 1. EROD & CALUX activities of PCDDs/DFs and PCBs

Congener	EROD-TEF(H4IIE cells)					CALUX-TEF (H4IIE-luc cells)			TEF/WHO ^j
	Safe ^a & Sawyer ^b	Clemons ^c	Schrenk ^d & Schmitz ^e	Tillitt ^f	Our Study ^g	Sanderson ^h	Bovee ⁱ	Our Study ^g	(Humans/ mammals)
Dioxins									
2,3,7,8-TetraCDD	1	1	1	1	1	1	1	1	1
1,2,3,7,8-PentaCDD	0.01	1.1	0.181	0.42	0.61	0.79	0.49	0.54	1
1,2,3,4,7,8-HexaCDD	0.045	0.5	0.184	0.08	0.14			0.30	0.1
1,2,3,6,7,8-HexaCDD		0.2	0.043	0.02	0.15		0.068	0.14	0.1
1,2,3,7,8,9-HexaCDD			0.061	0.03	0.049			0.066	0.1
1,2,3,4,6,7,8-HeptaCDD		0.1	0.047	0.02	0.034			0.046	0.01
OctaCDD	3 × 10 ⁻⁴		3.5 × 10 ⁻⁴	5 × 10 ⁻⁴				4.7 × 10 ⁻⁴	0.0001
Furans									
2,3,7,8-TetraCDF	0.09	0.03	0.4	0.2	0.24			0.32	0.1
1,2,3,7,8-PentaCDF	0.06	0.2	0.03	0.2	0.21			0.21	0.05
2,3,4,7,8-PentaCDF	1.38	0.4	0.6	1.4	0.39	0.69	0.34	0.50	0.5
1,2,3,4,7,8-HexaCDF	0.49	0.3	0.2	0.02	0.15			0.13	0.1
1,2,3,6,7,8-HexaCDF	0.148		0.06	0.06	0.046			0.039	0.1
1,2,3,7,8,9-HexaCDF					0.12			0.11	0.1
2,3,4,6,7,8-HexaCDF	0.03		0.1	0.3	0.11			0.18	0.1
1,2,3,4,6,7,8-HeptaCDF				0.3	0.018			0.029	0.01
1,2,3,4,7,8,9-HeptaCDF				0.02	0.021			0.041	0.01
OctaCDF								0.0065	0.0001
Non-ortho PCBs									
3,3',4,4'-TetraCB (77) ^k	9 × 10 ⁻⁴	8 × 10 ⁻⁴	1 × 10 ⁻⁴	2 × 10 ⁻⁵	4.5 × 10 ⁻⁴	7.1 × 10 ⁻⁴		0.0013	0.0001
3,4,4',5-TetraCB (81)	4 × 10 ⁻⁵	0.007		0.002		0.0027		0.0042	0.0001
3,3',4,4',5-PentaCB (126)	0.32	0.1	0.2	0.02	0.046	0.017	0.065	0.067	0.1
3,3',4,4',5,5'-HexaCB (169)	0.003	0.001	0.003	5 × 10 ⁻⁴	0.0022	5.5 × 10 ⁻⁴	0.0015	0.0034	0.01
Mono-ortho PCBs									
2,3,3',4,4'-PentaCB (105)	0.0015	2.5 × 10 ⁻⁵	1 × 10 ⁻⁵	8 × 10 ⁻⁶	9.8 × 10 ⁻⁶		2 × 10 ⁻⁶	1.2 × 10 ⁻⁵	0.0001
2,3,4,4',5-PentaCB (114)	3 × 10 ⁻⁴		7 × 10 ⁻⁵	<1 × 10 ⁻⁶	3.2 × 10 ⁻⁵			4.8 × 10 ⁻⁵	0.0005
2,3',4,4',5-PentaCB (118)	2 × 10 ⁻⁵	1 × 10 ⁻⁵	4 × 10 ⁻⁶	4 × 10 ⁻⁷	1.0 × 10 ⁻⁵		4.9 × 10 ⁻⁶		0.0001
2',3,4,4',5-PentaCB (123)	1 × 10 ⁻⁴		1 × 10 ⁻⁴	1 × 10 ⁻⁵	1.4 × 10 ⁻⁵			2.4 × 10 ⁻⁵	0.0001
2,3,3',4,4',5-HexaCB (156)	2 × 10 ⁻⁴	5 × 10 ⁻⁵	6 × 10 ⁻⁵	6 × 10 ⁻⁵	9.9 × 10 ⁻⁵		3.8 × 10 ⁻⁵	2.1 × 10 ⁻⁴	0.0005
2,3,3',4,4',5'-HexaCB (157)	2 × 10 ⁻⁵		5 × 10 ⁻⁵	2 × 10 ⁻⁵	4.2 × 10 ⁻⁵			8.0 × 10 ⁻⁵	0.0005
2,3',4,4',5,5'-HexaCB (167)	9 × 10 ⁻⁶		8 × 10 ⁻⁶	9 × 10 ⁻⁶				8.2 × 10 ⁻⁶	0.00001
2,3,3',4,4',5,5'-HeptaCB (189)			<1 × 10 ⁻⁶	1 × 10 ⁻⁵				6.7 × 10 ⁻⁶	0.0001

^aSafe *et al.*, 1986/1989^{34, 2)}, ^bSawyer *et al.*, 1982⁹⁾, ^cClemons *et al.*, 1994/1996^{16, 35)}, ^dSchrenk *et al.*, 1991³⁶⁾, ^eSchmitz *et al.*, 1995³⁷⁾, ^fTillitt *et al.*, 1996³⁸⁾, ^gBehnisch *et al.*, 2002³⁹⁾, ^hSanderson *et al.*, 1996²⁶⁾, ⁱBovee *et al.*, 1998³¹⁾, ^jVan den Berg *et al.*, 1998¹⁾, ^kInternational Union of Pure Applied Chemistry number.

containing 1 mM calcium and magnesium ions. PBS was renewed and 0.1 ml luciferase assay substrate (PerkinElmer) was added per well. 30 minutes after the addition of assay substrate, luciferase activity was measured by a luminometer for 10 seconds per well. CALUX assay procedure itself requires 3 days.

CALUX-TEF values expressing AhR-ligand activity for single compounds were calculated by comparing the EC₅₀ values of 2,3,7,8-TCDD and the tested chemical. The standard curve was fitted using a cumulative fit function.

CALUX-TEQ values for the extracted samples were obtained from their dilutions so that their luciferase activities were in the reproducible lower part of the linear range corresponding to 1–4 pM in TCDD.

The dioxin-like potency of a single compound is expressed as EROD- or CALUX- TEF (toxicity equivalent factor), that is relative potency (REP) to TCDD in terms of EC₅₀. The reported EROD- and CALUX-TEF values for PCDD/Fs and Co-PCBs are shown in Table 1^{1, 2, 9, 16, 26, 34–39)}. EROD- or CALUX- TEFs were close to WHO-TEFs for PCDD/Fs and

Fig. 2. Project overview of "destruction processes of dioxins/PCB and their bioassay monitoring".

the ratio between bioassay-TEF and WHO-TEF were 0.4–5 with a few exceptions. Co-PCB congeners showed comparatively lower EROD- or CALUX-TEFs than expected based on the WHO-TEF values (the ratio between bioassay-TEF and WHO-TEF values were 0.01–0.7), while bioassay-TEF for PCB #77 showed one order of magnitude higher than the WHO-TEF value. Our result shows a good agreement between bioassay-TEF values obtained by EROD and CALUX indicating that these two bioassays may have similar response to PCDD/Fs and Co-PCBs.

Research Project on Bio-monitoring for Destruction Processes of Dioxin-like Compounds

The authors have put forward the project research "development of dioxin/PCB destruction process and their bioassay monitoring" supported in 2000–2003 by a Grant-in-aid for the Development of Innovative Technologies from

the Ministry of Education, Culture, Sports, Science and Technology of Japan. Research framework comprises of three following themes as shown in Fig. 2:

- Development of bioassay methods including suitable sample pre-treatment for dioxin-like compounds
- Application of bioassay to the monitoring during destruction process of fly ashes from municipal solid waste incineration
- Analysis of PCB destruction mechanism and application of bioassay to the monitoring during chemical destruction processes of waste PCBs

In this research, EROD and CALUX methods have been positively applied to the monitoring of destruction process of wastes such as fly ashes and PCB oil on which we have little toxicological information on their behavior during the process. Monitoring of such wastes containing PCDD/Fs and Co-PCBs and their treated byproducts and assessing their risk to the environment or human health by looking at individual compounds are not feasible. Our objectives for

Fig. 3. Chemical-TEQ and bioassay-TEQ of several untreated and treated ashes.

the practical use of bioassays are the two points below;

- From a toxicology-directed viewpoint, EROD and CALUX bioassays where dioxin-like effects are directly estimated can provide important information on the predicted overall dioxin-like potency of the complex mixture of samples. Those bioassays can be more useful if combined with instrumental analytical approaches in terms with Toxicity Identification and Evaluation (TIE). In conducting TIE approaches, bioassays are associated with sample fractionation and instrumental analysis to elucidate the compounds contributed to dioxin-like toxicity in the sample.
- From a standpoint based on the necessity of rapid screening, bioassays can be a promising tool. If a certain correlation can be found between bioassay index (e.g., bioassay-TEQ) and chemical concentrations (e.g., I-TEQ, WHO-TEQ) in the tested sample matrices, rapid and costless bioassay can provide significant information or may be possibly replaced/ complemented with instrumental analysis for screening samples with significant levels of dioxin-like compounds. The latest findings on this project research were abstracted below.

Bioassay Application for Municipal Solid Waste Residues

To investigate the dechlorination of fly ashes during a

low temperature treatment under oxygen deficiency conditions (thermo-catalytic treatment or Hagenmaier process) six fly ashes from six different incineration plants were treated in a laboratory experiment (300°C, 30 min) or in an actual plant (400°C, 120 min). The aim of this study was to confirm the decrease of the I-TEQ (international toxicity equivalency of PCDD/Fs and Co-PCBs) and for the first time also the decrease in the sum of dioxin-like toxicity (bioassay-TEQ) measured by EROD and CALUX⁴⁰⁾.

I-TEQ and bioassay-TEQ values in untreated and treated fly ashes of municipal solid waste incineration were obtained as shown in Fig. 3. The here described combination of bioassay and chemical analysis resulted in I-TEQ, EROD- and CALUX-TEQ values of untreated fly ashes for plants A-D in a range between 2.1–12 ng-I-TEQ/g, 2.1–18 ng-EROD-TEQ/g and 2.6–59 ng- CALUX-TEQ/g, respectively. These values are in the same range as the values previously reported⁴¹⁾. Bioassay-TEQ values for the untreated fly ashes (n=6) were on average maximal 3-times higher than I-TEQs, indicating the importance of PCDD/Fs for the sum of dioxin-like toxicity. Analysis of six untreated fly ashes revealed excellent correlation between bioassay-TEQ and I-TEQ (EROD: $r^2=0.94$; CALUX: $r^2=0.92$). Including also treated fly ash samples (total sum of samples: n=18), the correlation coefficient increases in case of the EROD bioassay ($r^2=0.96$), while decreases in case of the CALUX assay ($r^2=0.72$). This

could maybe explained due to the different kinetics (CALUX: 24 h versus EROD: 72 h) and the therefore different metabolic capacities of the cells. Similar bioassay-TEQ/Chemical-TEQ ratio values were already reported for studies measuring ash samples from various sources using cell based bioassays such as the H4IIE-EROD^{41–44}) or CALUX using mouse hepatoma cells⁴⁵) (for review see literature^{46, 41}).

The I-TEQ/EROD-TEQ/CALUX-TEQ values of four untreated fly ash could be decreased in the plants from a mean value of 5.4 (chemical analysis)/6.9 (EROD)/19 (CALUX) ng TEQ/g to finally a level of 0.02/0.02/0.52 ng TEQ/g at maximum after the dechlorination treatment. This resulted in treatment efficiencies (TE %) based on I-TEQs, EROD-and CALUX-TEQ values for ashes treated in plant A, B and D of more than 99%. It could be shown that the dechlorination/hydrogenation (Hagenmaier process) treatment of fly ashes reduces I-TEQ and bioassay-TEQ values of the untreated sample up to 99%. Comparing the I-TEQ and bioassay-TEQ levels from the tested six fly ashes indicated only a small amount of unknown dioxin-like compounds (such as brominated dioxin-like compounds) occurred.

The findings so far on the fly ash thermo-catalytic treatment have confirmed that concentrations of PCDD/Fs decreased, but this doesn't mean that the characterization of final products or the decomposition mechanism of fly ashes have been confirmed. Therefore, the findings have not given proper answers to the concerns whether other uneasy by-products could be formed by the dechlorination, even if the concentration of PCDD/Fs is certainly declined. New findings to answer the questions are as follows, 1) the decomposition rate of bioassay-TEQ is obtained to be almost equivalent to the PCDD/Fs by chemical analysis. 2) while I-TEQ concentration by low temperature treatment under the condition of 400°C, 120 minutes is 0.01–0.1 ng TEQ/g,

it is confirmed that bioassay-TEQ is 0.02–0.5 ng TEQ/g. 3) Although the bioassay-TEQ/chemical-TEQ ratio value was about a few times, there is no striking difference of the proportion before and after the treatment. Consequently, it is proved that a large amount of Ah receptor agonists except PCDD/Fs cannot be formed as by-products after the dechlorination of fly ashes.

Similar bioassay-TEQ/I-TEQ ratios between treated and untreated ashes indicates that equal amounts of dioxin-like compounds known as PCDD/Fs and coplanar PCB and unknown dioxin-like compounds are decontaminated in the here described dechlorination/hydrogenation process. Both here used rat hepatoma H4IIE cell based bioassays are available for monitoring the reduction of overall dioxin-like toxicity through the here described decomposition performance.

Chemical Destruction Methods of Waste PCBs and their Integrated Monitoring

Bioassay analysis can be also a promising tool to measure the reduction of PCBs and other undesirable by-products such as PCDD/Fs during chemical destruction processes. The described study here focuses on mineral insulating oil and capacitor oil containing PCBs and their chemically treated samples⁴⁷.

In conducting this research, mineral insulating oil contains quite high amounts (several % wt) of polyaromatic hydrocarbons (PAHs) originated from mineral oil. The AhR agonist activity of PAHs might be overwhelming and could lead to a misinterpretation of the activity of PCBs. Therefore, clean-up procedures for separating PCBs from mineral oil matrix were validated and evaluated. Silica gel/sulfuric acid (44%) reflux treatment was established and used as a

Table 2. CALUX-TEQ values (µg/kg) and induction strength for actual waste PCB (treated) samples (results for each fraction of four clean-up methods)

(Treated) waste PCB samples	Pd/C treated PCB-1		Pd/C treated PCB-2		PCB insulation oil	
	Bio-TEQ	Induction	Bio-TEQ	Induction	Bio-TEQ	Induction
Pretreatment type						
Method A (DMSO/n-hexane extraction)	0.78	39	0.35	27	2,200	62
Method B (A+ silica gel-H ₂ SO ₄ (22%) reflux)	0.36	14	0.45	25	3,100	60
Method C (A+ silica gel-H ₂ SO ₄ (44%) reflux)	NM	NM	NM	NM	11	49
Method D (C+ activated carbon dispersed silica gel)	<0.1	–	0.31	42	1.0	77
PCB content (µg/kg)	<100		5,200		6,900	
I-TEQ (µg-TEQ/kg)	0.00072		0.11		0.30	
Bio-TEQ (method D)/I-TEQ	–		2.8		3.3	

Key: NM, not measured; induction, induction strength (% TCDD max).

complete removal method of PAHs in samples prior to CALUX. PCBs themselves are AhR agonists and antagonists in complex mixtures. 2–4 ortho PCBs have been reported to act as antagonists which mask the AhR binding activity of agonists of 0–1 ortho PCBs^{48–51}. “Agonistic” 0–1 ortho planar PCBs and PCDD/DFs have been fractionated as an attempt by using activated carbon dispersed silica gel column. Then the obtained CALUX results with/without the carbon column treatment were compared.

To ascertain the usefulness of the proposed clean-up method, fractions of the three actual PCB (treated) wastes were sampled at each level of clean-up procedures, replaced with DMSO and assayed using CALUX (Table 2). Overestimation (>1,000 fold of interference) of bioassay-TEQ for Pd/C (Palladium/carbon catalyst) treated PCB capacitor oil-1 and PCB insulating oil samples (method A fractions) was remarkably improved by completing the proposed pre-treatment (method D fractions). Finally, the ratio between bioassay-TEQ and I-TEQ values for Pd/C treated PCB capacitor oil-2 and PCB insulating oil became 2.8 and 3.3, respectively. Bioassay-TEQ value for Pd/C treated PCB capacitor oil-1 became under the determination limit.

To check the assay sensitivity to mineral oil samples, PCB-containing mineral oil of various concentrations from 0.5 ppm to 50 ppm in total PCBs was prepared by diluting Kanechlor-400 based capacitor oil diluted with the oil. Fractions obtained by both of the reflux pretreatment and additional carbon column fractionation were tested by the CALUX. The CALUX data showed good agreement with I-TEQ values for all the tested range of PCB samples in both fractions of reflux and additional carbon column treatment. The ratio of CALUX-TEQ divided by I-TEQ was all in the range of 1–3. CALUX can theoretically quantify the TEQ up to 0.01 ppb (10 pg-TEQ/g) level on the adopted scale of sample clean-up.

Open Questions on Future Biomonitoring Design

The use of the CALUX system here described for the detection of Ah receptor agonist has many advantages. It is easier, more reliable, less time consuming, and fewer inhibition effects has been reported. Therefore, this bioassay could be a valuable new tool for rapid screening of complex environmental pollutants and waste management samples. The wide possibility of application makes this tool very useful for biomonitoring studies as well as screening of industrial chemicals or environmental control processes. But, in the

future, this system should also have several quality criteria like the chemical analysis, which still has to be evaluated and applied on round robin studies^{52, 53}. There is an urgent need to evaluate which clean-up should be used with or without non-dioxin-like compounds such as PAHs and which compounds should be tested for their dioxin-like potency. In the future, it would be desirable to lessen the necessary amounts of samples and simplify the process of pretreatment within a range which quantitative determination can be maintained. In addition, false negative ratio, which means true value by chemical analysis shows control value and over whereas bioassay value indicates less than control value, is required to be minimized. The applicable uses of bioassay method can be expanded not only to grasp the actual toxic overviews of industrial chemicals and environmental pollutions, but as this research mentioned, to manage processes such as observing toxicity change and confirming the destruction status. While appropriate validations are conducted continuously, it is expected to be applied practically.

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