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Phytotoxicity comparison of organic contaminants and heavy metals using *Chlorella vulgaris*

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ABSTRACT

The green alga *Chlorella vulgaris* (*C. vulgaris*) is proposed as a test species for the phytotoxicity assessment of organic contaminants and heavy metals. The relationship between concentration and inhibition was established from the phytotoxic effects on *C. vulgaris* over a long-term exposure of 72 h. The concentration for 50% of maximal effect ($EC_{50,metal}$) for Cr, Cd, Hg, Cu, Ni, Zn, and Pb was 0.11, 0.13, 0.22, 0.24, 0.28, 1.13, and 5.69 mg/L, respectively. On the other hand, the $EC_{50,TOC}$ total organic carbon (TOC) for trichloroisocyanuric acid (TCCA), 1-naphthylamine, sodium dodecylbenzene sulfonate, Ciprofloxacin, Acetaminophen, phenol was 0.02, 1.50, 5.99, 17.23, 55.79, and 236 mg/L, respectively. For raw sewage and secondary effluent from a domestic wastewater treatment plant (WWTP), $EC_{50,TOC}$ of 53.88 and 70.94 mg/L was obtained by concentrating organic matter through reverse osmosis. This result suggests that WWTPs reduce not only the organic compounds concentration, but also the phytotoxicity of wastewaters. Through a combined analysis of the concentrations of organic compounds and heavy metals, and the phytotoxicity of two typical industrial wastewaters, it was ascertained that the phytotoxicities of coking wastewater and brewery wastewaters were mainly caused by the organic contaminants. Nonetheless, the phytotoxicity of coking wastewater (toxicity index of $EC_{50,\%} = 0.37\%$) was significantly higher than that of brewery wastewater ($EC_{50,\%} = 32\%$). This difference in phytotoxicity may be because the coking wastewater contains more toxic organic contaminants ($EC_{50,TOC} = 32.95$ mg/L) than the brewery wastewater ($EC_{50,TOC} = 258.4$ mg/L).

Keywords: Phytotoxicity; *C. vulgaris*; Heavy metal; Organic contaminants; Wastewater

1. Introduction

The aquatic environment is often exposed to various pollutants including heavy metals and organic compounds owing to increasing amounts of industrial and/or agricultural wastes. Of the pollutants that may have toxic effects on the aquatic ecosystem, organic compounds and heavy metals are often the main components. The wastewater from domestic sources usually contains high proportions of organic compounds mainly contributed by detergents, although the heavy metal concentrations are low [1,2]. On the contrary, the wastewater from industrial sources may contain high concentration of both the heavy metals and the organic compounds [3–5]. In addition to the

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concentration of specific substances, an understanding of the overall impact of these substances on the water environment has become increasingly important [6].

It is well known that bioassays are a valuable tool for comprehensively assessing the toxic effect of pollutants in wastewaters [7]. Various organisms, including primary producers (e.g. alga), primary consumers (e.g. flea), secondary consumers (e.g. fish), and decomposers (e.g. bacteria), have been applied for characterizing biotoxicity at different trophic levels in aquatic ecosystems [8,9]. These tests can provide relevant information for improving the technique that ensures reduced potential hazards of contaminants to aquatic ecosystems [7]. Microalgae are sensitive indicators of environmental change and, as a basis of most freshwater and marine ecosystems, are widely used in the assessment of risk and development of environmental regulations for the heavy metals and organic compounds [10]. Moreover, a test involving the use of alga as the test organism is easy to perform and offers a fast response to the wastewaters. In addition, alga has a higher sensitivity than other organisms in the aquatic ecosystem probably because alga is a primary producer [11–13]. *Chlorella vulgaris*, an alga in freshwater, has a high sensitivity to toxicants [14], and is easily cultured in the laboratory.

In most cases, wastewater contains not only organic compounds but also heavy metals that exhibit a combined toxicity. Therefore, to manage wastewater properly, there is a need to identify the kind of substances that have a greater contribution to phytotoxicity. In this study, an alga growth inhibition test was conducted for known organic compounds and heavy metals in wastewater. Based on both the chemical analyses and *C. vulgaris*, wastewaters from domestic and industrial sources were evaluated.

2. Materials and methods

2.1. Sample collection

2.1.1. Selection of organic compounds and heavy metals

Six typical organic compounds and seven typical heavy metals were selected during the testing period. The organic compounds included phenols (phenol), disinfector (Trichloroisocyanuric acid (TCCA)), amines (1-naphthylamine), medication (acetaminophen, Ciprofloxacin (CPFX)), and surfactants (Sodium dodecylbenzene sulfonate (SDBS)). The heavy metals were copper, chromium, cadmium, lead, zinc, nickel, and mercury. As shown in Table 1, all the materials are of analytical grade and all the experiments were performed in triplicate.

2.1.2. Sampling and pretreatment of wastewaters

Wastewater samples were collected from a domestic wastewater treatment plant (WWTP) in Xi'an, China, where an oxidation ditch process was applied. The influent samples were collected after the grit chamber, and the effluent was collected from the outlet of the secondary settler before the disinfection was conducted. Brewery wastewater was collected from a beer production plant, and coking wastewater was collected from a coking plant. Brown glass bottles were used as sample containers. After collection, the samples were immediately filtered through a 0.45 μm mixed cellulose ester membrane to remove suspended matter. The filtered samples were kept in a refrigerator at 4℃ until use. All the analyses were completed within 24 h after sampling. The 0.45 μm mixed cellulose ester membranes were boiled in Milli-Q water before use in order to remove the organic matter from the membranes.

The low concentration samples were concentrated using a MSM-2008 RO Membrane Separation Unit (Shanghai Mosu Scientific Equipment Corporation, China) with an RO-1812 spiral RO membrane in order to enrich the organic substances in the samples and to improve the efficiency of organic fractionation. The RO setting was washed by 0.1–0.2% NaOH before and after use. The concentration ratio was evaluated by comparing the TOC concentration of the original sample and that of the concentrated solution. An organic carbon analyzer (TOC-V_{CPH}, Shimadzu) was used to measure the total organic carbon (TOC) concentration. UV absorbance at 254 nm (UV_{254}) value was detected by a UV spectrophotometer (UV-1650PC, Shimadzu). A coupled plasma-mass spectrometry instrument (ICP-MS, Elan Drce, PE, USA) was used to measure the total soluble heavy metal concentrations after the wastewater samples were filtered through 0.22 μm mixed cellulose ester membranes.

2.2. Alga growth inhibition test

2.2.1. Culture conditions

The algal growth inhibition tests were carried out according to the standard method of OECD 201 and analysis of the water and wastewater monitoring methods (fourth edition) [15,16]. Microalgae *C. vulgaris* as an experimental species were purchased from the wildlife species library—quality freshwater algae of Chinese Academy of Sciences Library (FACHB-1227), and were cultured in sterile BG11 medium (pH adjusted to 7.5 ± 0.1) at 25 ± 1℃ under 12 h light at 4000L× – 8000L× followed by 12 h in the dark in an

Table 1
Phytotoxicity and characteristics of organic matters, heavy metals, and domestic wastewater

Category	Item	Chemical formula	Molecular weight	Model applied	SD	R^2	$EC_{50,TOC}$ (mg/L)	$EC_{50,metal}$ (mg/L)	SC (mg/L)
Organic compounds	Phenol	C_6H_6O	94.11	Exponential	3.1112	0.930	236	–	19.5
	TCCA	$C_3N_3O_3Cl_3$	232.41	Linear	0.0011	0.933	0.02	–	0.008
	1-naphthylamine	$C_{10}H_9N$	143	Exponential	0.1463	0.917	1.50	–	0.089
	Acetaminophen	$C_8H_9NO_2$	151.16	Linear	4.4362	0.951	55.79	–	3.2
	CPFX	$C_{17}H_{18}FN_3O_3{\cdot}HCl{\cdot}H_2O$	367.8	Exponential	0.4310	0.920	17.23	–	1.94
	SDBS	$C_{18}H_{29}NaO_3S$	348.48	Exponential	0.0636	0.993	5.99	–	0.585
Domestic wastewater	Raw sewage	–	–	Linear	0.2495	0.974	53.88	–	–
	Secondary effluent	–	–	Linear	0.2067	0.973	70.94	–	–
Heavy metals	Zinc	$ZnSO_4{\cdot}7H_2O$	287.56	Exponential	0.0218	0.975	–	1.13	0.049
	Copper	$CuSO_4{\cdot}5H_2O$	249.69	Exponential	0.0071	0.937	–	0.24	0.014
	Cadmium	$CdCl_2{\cdot}2.5H_2O$	228.36	Linear	0.0037	0.992	–	0.13	0.002
	Chromium	$K_2Cr_2O_7$	294.18	Logistic	0.0027	0.985	–	0.11	0.006
	Lead	$Pb(NO_3)_2$	331.21	Exponential	0.1922	0.930	–	5.69	0.269
	Mercury	$HgCl_2$	271.5	Linear	0.0010	0.968	–	0.22	0.012
	Nickel	$Ni(NO_3)_2{\cdot}6H_2O$	290.8	Linear	0.0054	0.957	–	0.28	0.009

MGC-100P constant light incubator. The cultures were agitated manually three times daily by hand to homogeneously distribute the algae in solution and to prevent the algae from sticking to the flask.

2.2.2. Experimental design

We set up one blank control medium and five different test concentrations of samples (chemicals and wastewaters) with the medium after pre-experiment. After the algal incubator had reached a steady state, algal liquid and samples were mixed in the triangular bottle at 1:1 (v/v). The density of algal cells was determined by using a blood counting chamber on a microscope (Nikon-Eclitse-90i) at 0, 24, 48, 72, and 96 h, respectively. The concentration of a sample to cause 50% growth rate reduction was defined as the effective concentration (EC_{50}), which was originally obtained from the dose–response curve (where concentration could be the mass concentration of a metal ion or organic compound, the sample concentration times or the percentage of sample dilution), and then expressed as $EC_{50,\ TOC}$ for the organic compounds in terms of the corresponding TOC concentration (mg/L), and $EC_{50,metal}$ for the metals in terms of the corresponding metal concentration (mg/L), or $EC_{50,\%}$ for the industrial wastewaters in terms of the corresponding percentage of sample dilution.

The growth rate and inhibition values were evaluated as shown below:

$$\mu = \frac{\ln_{Nn} - \ln_{N1}}{t_n - t_1} \tag{1}$$

where μ—growth rate; N_n—the density of algal cells on the Nth time; N_1—the density of algal cells on the first time; t_n—the Nth time of counting; and t_1—the first time of counting.

$$E = \frac{\mu_0 - \mu}{\mu_0} \times 100\% \tag{2}$$

where E—inhibition (%); μ_0—the average growth of all blanks; and μ—the average growth of the parallel samples.

The safe concentration (SC) of toxicant:

$$SC = E_b C_{50,96h} \times 0.1 \tag{3}$$

3. Results and discussions

3.1. Phytotoxicity of typical organic compounds and domestic wastewaters

Fig. 1 shows the concentration–inhibition relationships of the six organic compounds and two wastewaters from the WWTP. The experimental data well fitted the correlative curves and lines. The EC_{50} values (as mass concentrations for the organic compounds and sample concentration times for the domestic sewage and secondary effluent) were obtained as shown on each of the graphs (Fig. 1). Different toxins had different mechanisms of action on *C. vulgaris*. The relationship between the concentration of toxicants including TCCA and Acetaminophen and their inhibition were best fitted to the linear equation, suggesting that the inhibition increases with increase in the concentration of these substances. Meanwhile, the concentration–inhibition relationship of phenol, 1-naphthylamine, CPFX, and SDBS was best fitted to an exponential equation. At lower concentrations (below the EC_{50} value), phenol, 1-naphthylamine, CPFX, and SDBS stimulated algae growth, while the inhibition was increased following the increasing concentrations of the toxicants.

To find a common base for comparing the phytotoxicity due to organic substances, the EC_{50} values were transferred to $EC_{50,TOC}$ values based on the measured TOC concentrations (Table 1). For the six organic compounds, the $EC_{50,TOC}$ values ranging between 0.02 and 236 mg/L, and the toxicity was in the order of TCCA > 1-naphthylamine > SDBS > CPFX > Acetaminophen > Phenol. The introduction of *C. vulgaris* bioassay allowed reclassifying the six organic compounds into four toxicity levels: "non-toxic" ($EC_{50} > 100$ mg/L), which consisted of phenol;

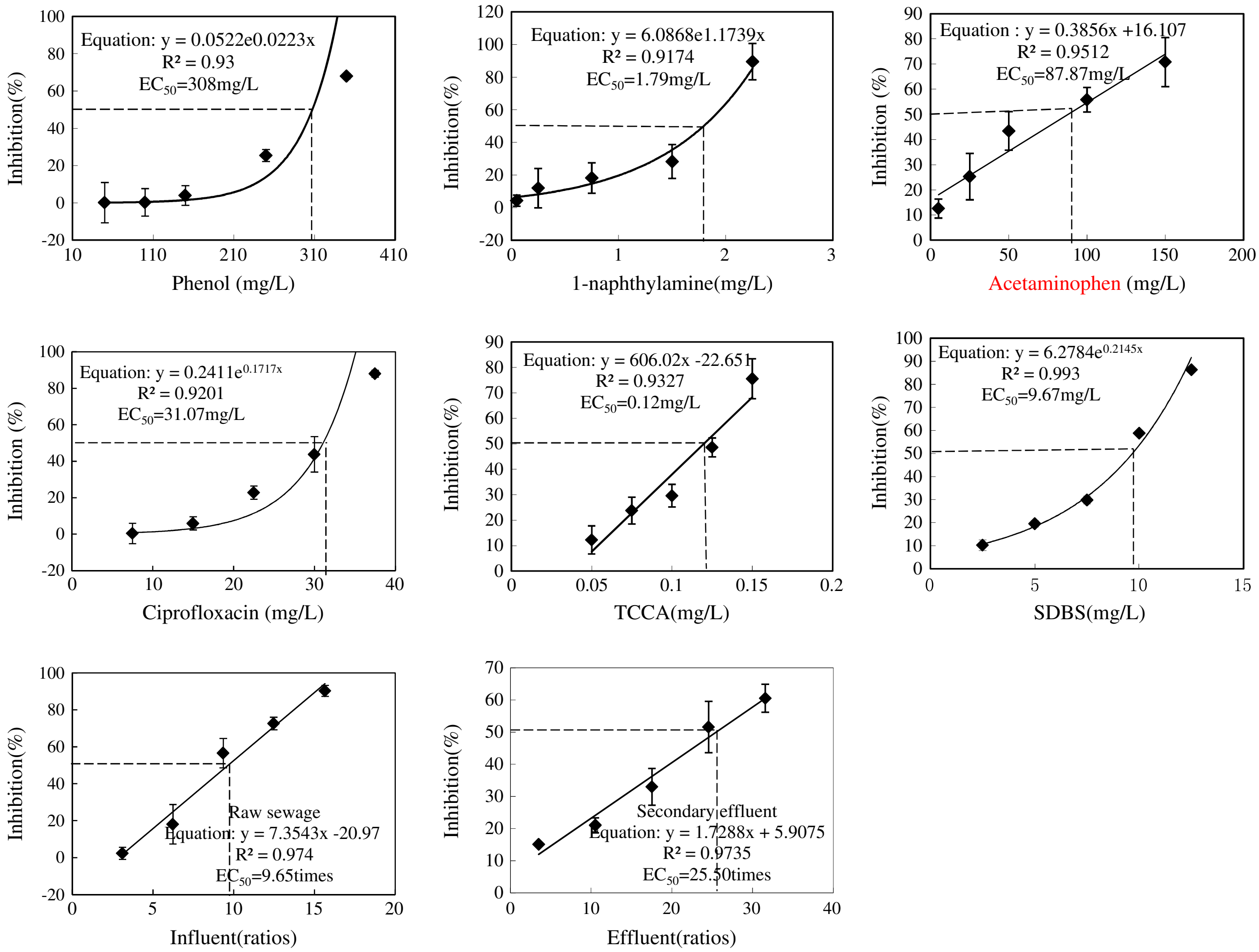

Fig. 1. The dose–response curve of typical organic compounds and domestic wastewaters.

“harmful to aquatic organisms” ($10\ \text{mg/L} < EC_{50} < 100\ \text{mg/L}$), which consisted of Acetaminophen and CPFX; “toxic” ($1\ \text{mg/L} < EC_{50} < 10\ \text{mg/L}$), which consisted of SDBS, and “very toxic” ($EC_{50} < 1\ \text{mg/L}$), which consisted of 1-naphthylamine and TCCA [16]. The SCs of the six organic compounds were found to be 0.008 mg/L for TCCA, 0.089 mg/L for 1-naphthylamine, 0.585 mg/L for SDBS, 1.94 mg/L for CPFX, 3.2 mg/L for Acetaminophen, and 19.5 mg/L for Phenol in Table 1. So, the concentrations of these organic compounds in water must be lower than the SC. TCCA as a disinfector and an oxidant is often used to disinfect and bleach, which forms hypochlorous acid (HClO) and hypochlorite ions (ClO^{-}). HClO is significantly more effective as a biocide and oxidant than hypochlorite ions, which could seriously damage the membranes and the cell structure of alga [17–19]. Besides, TCCA could produce oxygen-free radicals that inhibit the composition of chlorophyll in algae or damage the thylakoid membrane structure in chlorophyll to inhibit the growth of algal cells [20]. So, the toxic effect of TCCA cannot be ignored in domestic wastewater. Furthermore, the volume of surfactants present in detergents used in domestic applications, such as SDBS, cannot be ignored in domestic wastewater either. On the other hand, the concentrations of phenol and the two medications (Acetaminophen, CPFX) are often at very low μg/L levels in domestic wastewater, which are far lower than the safety concentration [21] and therefore, cannot cause significant phytotoxicity.

The EC_{50} of the domestic wastewaters increased from 9.65 times to 25.50 times following treatment by the oxidation ditch process (Fig. 1). This significant reduction might be mainly due to the effective removal of organic matters in terms of TOC or COD. Moreover, the $EC_{50,\text{TOC}}$ was increased from 53.88 mg/L in the raw sewage to 70.94 mg/L in the secondary effluent, suggesting that the organic matter remaining in the raw sewage might be of higher toxicity than in the secondary effluent. As SDBS often exists in raw sewage with concentrations up to several mg/L [22], coupled with their $EC_{50,\text{TOC}}$ value, could contribute significantly to the toxicity of the raw sewage. However, quantitative removal in the biological treatment plants is reflected by low alkyl sulfate concentrations measured in the effluents of WWTPs (mostly below 10 μg/L) [23]. Therefore, in secondary effluents, the levels are far below the concentration required to cause significant phytotoxicity comparing with the $EC_{50,\text{TOC}}$ value. Since TCCA is disinfected in WWTPs, other metabolites may cause the toxicity in the secondary effluents.

3.2. Phytotoxicity of typical heavy metals

Fig. 2 shows the relationship between concentration and inhibition of the seven heavy metals. From the correlation curves or lines that best fit the experimental data, the $EC_{50,\text{metal}}$ values are obtained in the range of 0.11–5.69 mg/L. The phytotoxicity was in the order of $Cr^{6+} > Cd^{2+} > Hg^{2+} > Cu^{2+} > Ni^{2+} > Zn^{2+} > Pb^{2+}$. According to the category of toxicity for standard substances proposed by the European Union, a chemical can be considered “very toxic” if EC_{50} is less than 1 mg/L and “toxic” if EC_{50} is within 1–10 mg/L [24]. If these criteria are deducted, in the seven heavy metals, cadmium, chromium, mercury, copper, and nickel are very toxic, whereas zinc and lead are toxic to hydrophytes (primary producers). Many heavy metal ions have a direct influence on various physiological and biochemical processes of microalgae, such as photosynthesis, growth inhibition, membrane injury, respiration, and nutrient uptake. [25]. In addition, Table 1 shows that the SC of the seven heavy metals are 0.002 mg/L (Cd), 0.006 mg/L (Cr), 0.012 mg/L (Hg), 0.014 mg/L (Cu), 0.009 mg/L (Ni), 0.049 mg/L (Zn), and 0.269 mg/L (Pb). Notably, the concentration of Cd, Cr, and Ni must be under several μg/L in water.

Only the concentration–inhibition relationship of Cr^{6+} fitted the logistic equation. This may be related to their mechanisms of action on *C. vulgaris*. Within a very low concentration range, chromium could cause the inhibition to increase quickly. Such a response can be attributed to interference with the previously-established DNA damage caused by Cr in yeast and other organisms or that Cr exposure provokes errors in mRNA translation [26]. So, the concentration of chromium in wastewater discharged into the environment needs to be strictly controlled. Otherwise, it will have a serious impact on the aquatic organisms. Meanwhile, the concentration–inhibition relationship of Pb^{2+}, Cu^{2+}, and Zn^{2+} fitted the exponential equation. At lower concentration, Pb^{2+}, Cu^{2+}, and Zn^{2+} can enhance the growth of algae, but the inhibition starts to increase following the increase in concentration. When the inhibition exceeded 50%, it tended to rise sharply, probably due to the destruction of the chloroplast and death of alga cell at high concentrations of the heavy metals [25].

3.3. Phytotoxicity of industrial wastewaters

Fig. 3 shows that $EC_{50,\%}$ of the brewery and coking wastewaters was 32 and 0.37%, respectively. There is an exponential relationship between inhibition and

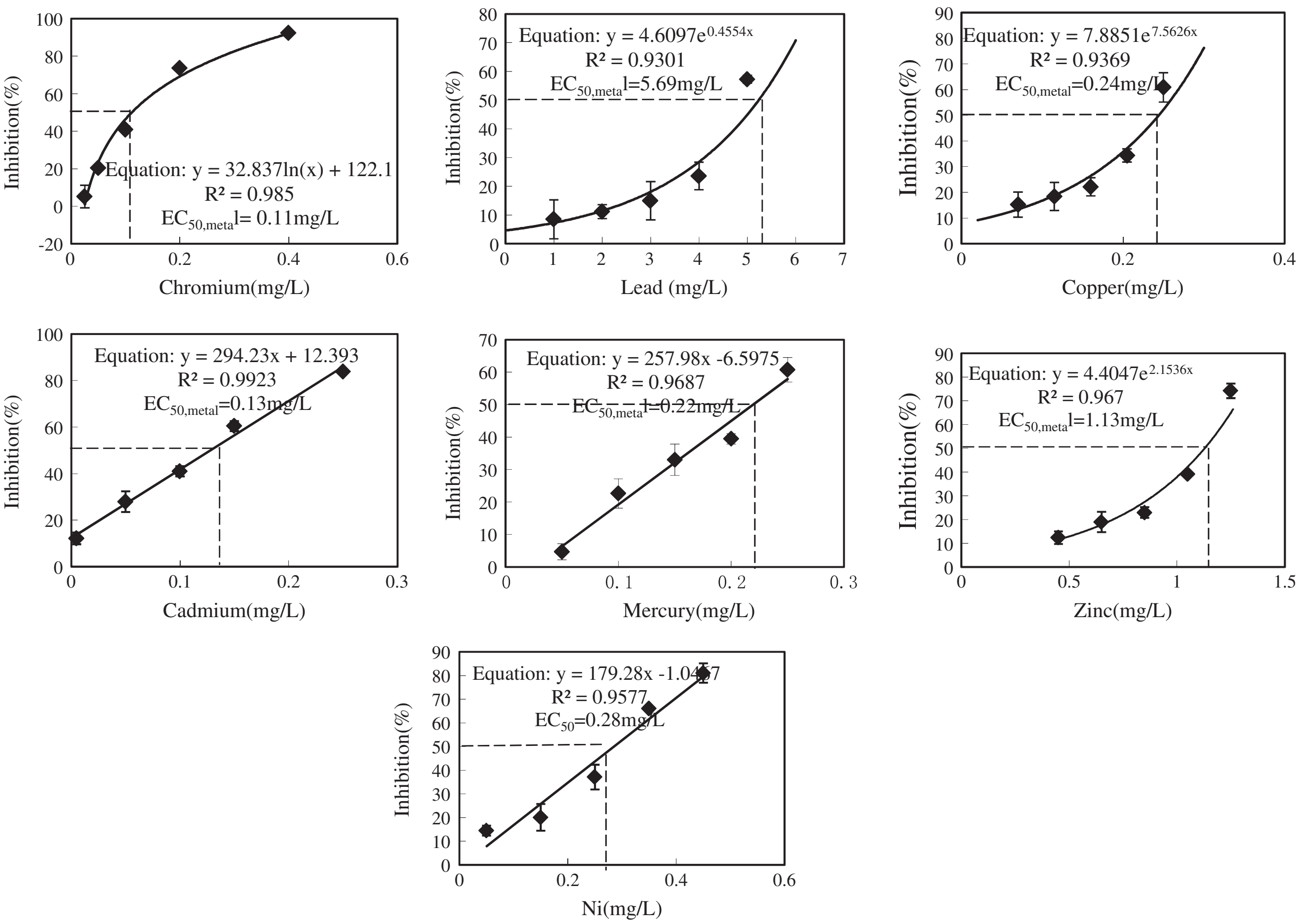

Fig. 2. The dose–response curve of heavy metals.

coking wastewater and a linear relationship between inhibition and brewery wastewater. In the coking wastewater, Table 2 shows that the concentrations of heavy metals were far less than the $EC_{50,metal}$ values except Zn concentrations, which exceeded its $EC_{50,metal}$ value (1.85 vs. 1.13 mg/L) and that of Cr (0.2 vs. 0.11 mg/L). However, the real Zn and Cr concentration after dilution to obtain the $EC_{50,\%}$ was only 6.85 and 0.74 μg/L, the levels were insufficient to show any toxic effect on green algae. However, the concentrations of heavy metals in the coking wastewater were higher than the SC except for Hg and Pb. From

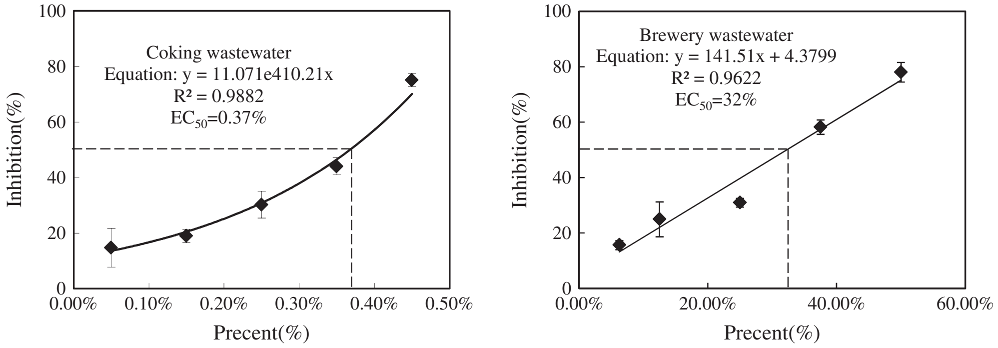

Fig. 3. Concentration–inhibition relationships of industrial wastewaters.

Table 2
Chemical analysis and phytotoxicity indices of industrial wastewaters

Item	Coking wastewater	Brewery wastewater
TOC (mg/L)	8,900	857
COD (mg/L)	18,569	4,813
UV_{254} (1/m)	1.620	5.01
pH	8.16	7.03
$EC_{50,\%}$	0.37%	32%
Cr (μg/L)	200.11	5.9
Ni (μg/L)	73.28	1.82
Cu (μg/L)	92.03	0.02
Zn (μg/L)	1,850	11.32
Cd (μg/L)	2.60	0.19
Pb (μg/L)	89.30	1.37
Hg (μg/L)	6.56	0.14

its original TOC of 8,900 mg/L, the TOC after dilution was calculated to be 32.93 mg/L, which corresponded to a harmful level for aquatic organisms according to the $EC_{50,TOC}$ values for the organic compounds (Table 1). As a result, the high phytotoxicity probably resulted from the toxic organic substances contained in the coking wastewater. Other previous studies have shown that coking wastewater contains metals, phenols, cyanides, polycyclic aromatic compounds, and nitrogen-, oxygen-, and sulfur-containing heterocyclic compounds [27,28], which could have negative impacts on aquatic organisms in the receiving environment [29].

For the brewery wastewater, the concentrations of heavy metals were at a lower level in comparison with their $EC_{50,metal}$ values and were lower than safety concentration. However, the organic substances contained in the brewery wastewater had aromatic properties, indicated by the high UV_{254} value, which may contribute to its toxicity to green algae. Generally, brewery effluents are composed of carbohydrates, ethanol, proteins, lactic acid, malic acid, phenols, propyleneglycol, vitamins, lipids, yeast, caramel, and malt as well as inorganic ions such as chloride, sulfate, phosphate, and carbonate [30].

4. Conclusions

Algae are ecologically important organisms in the aquatic food chain and are an abundant bioresource in aquatic systems. Through algae growth inhibition tests with *C. vulgaris*, the phytotoxicity of six typical organic compounds, and seven typical heavy metals were evaluated. Findings indicate that the phytotoxicity of organic compounds in raw sewage could be significantly reduced along with the removal of organics by the conventional biological wastewater treatment. The $EC_{50,TOC}$ was found to increase from 53.88 mg/L in the raw sewage to 70.94 mg/L in the secondary effluent, suggesting that the residual organic substances in the treated effluent were less toxic than in the raw sewage. For industrial wastewater, both organic substances and heavy metals may lead to phytotoxicity. By combining *C. vulgaris* tests with chemical analysis, different contributions of the two categories of substances were identified in the wastewaters from different sources. The properties of organic substances and the type and concentration of heavy metals in comparison with their individual toxic levels are important factors that affect the overall phytotoxicity of the water samples. This finding can deepen our understanding of the usefulness and effectiveness of phytotoxicity assessment using green alga *C. vulgaris*. In most cases, individual toxic substances in wastewaters may be difficult to detect accurately. Nonetheless, a comprehensive index—phytotoxicity by green alga could assess the overall impact of pollutants in the wastewater on aquatic ecosystems.

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