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Toxicological Effects of Chlorophenols to Green Algae in Case of Coexisting Humic Substances

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Abstract

Chlorophenols are well known as various pesticide, herbicide, wood preservative and defoliant. Whereas, they are also known as pollutants having strong toxicity to aquatic organisms such as phytoplankton. Nowadays, they have been discussed about effect of pesticides to the health of human beings, but less to ecosystem. The toxicity of pollutants such as heavy metals and HOPs (Hydrophobic Organic Pollutants) is influenced by coexisting dissolved organic matter (DOM). Majority of DOM consists of humic substances such as fluvic acid and humic acid (HA) derived from microbiological metabolisms in soil environment. The objective of this study is to elucidate the effect of coexisting HA to the toxicity of chlorophenols to *Chlorella*. Algal growth inhibition (AGI) test was chosen to examine the toxicity of chlorophenols as growth inhibition rate following the OECD guideline 201. *Chlorella* was selected to test organism as the representative of phytoplankton. Chlorophenols including 2,4,6-trichlorophenol (2,4,6-TCP), 2,4-dichlorophenol (2,4-DCP) and *p*-chlorophenol (*P*-CP), and pentachlorophenol (PCP) were examined. Several different concentrations (100, 50, 25, 5, 0.5 ppm) of chlorophenols were exposed to *Chlorella*. The cell density was measured by absorbance measurement (λ=690 nm) at 24 h and 72 h of administered time. Results of no coexisting and coexisting 10 ppm HA were compared. It was predicted that coexisting HA decreases the toxicity of chlorophenols because of adsorption. Whereas, some enhancement of toxicity was observed at several concentrations in case of 2,4,6-TCP. It was assumed that HA acts as mediator of chlorophenols to the inside of phytoplankton cells, then the toxicity was enhanced. In recent years, interactions of HA to metabolisms of organisms have been studied in fact. This study suggests that HA could be enhancer of toxicity at water environment. Interesting Effects of coexistence of HA to the toxicity of chlorophenols including 2,4,6-DCP and *p* -CP were also observed.

Keywords: Humic acid; Chlorophenols; Algal growth inhibition test; Adsorption

Introduction

With the development of chemical synthetic technology, an enormous number of chemical substances have been produced. The newly registered chemicals include various pesticides, herbicides and fungicides. In both 2011 and 2012, nearly 6 billion pounds pesticides were used throughout the world [1]. Chlorophenols are known as not only pesticides but also contaminants contained in industrial wastewater and having strong toxicity to aquatic organisms (fish such as zebrafish, rainbow trout and zooplankton such as *Daphnia magna*) [2,3]. Most of the chlorophenols are released into water environment, with very little entering the air [4]. 2,4,6-TCP and 2,4-DCP, one of the chlorophenols exist in the effluent of the process of bleaching paper pulp [5,6]. *p* -CP has been used to germicide [4]. They all have carcinogenicity to human body and persistent in long terms in water environment [7,8]. Thus, chlorophenols are designated to important toxicants in many countries [9,10]. Nonetheless, there is few study about toxicology of chlorophenols in water environment.

The toxicity of contaminants in the environment are influenced by various factors such as pH, water hardness and dissolved organic matter (DOM) [11-13]. In this study, Humic substances (HS) including fluvic acid (FA) and humic Acid (HA) were focused.

HS composes from 60 to 80% of non-living organic matter in nature [14]. HS are natural organic compounds synthesized by microorganism's metabolic modification such as decomposition and condensation of dead bodies in soil environment [15]. HS are abundantly present in soil and water environment [16,17]. In recent years, it is reported that HA effects the speciation of pollutants, microorganism's metabolisms and pollutants toxicity [17-19]. HA contains hydrophilic parts such as carboxyl and phenolic hydroxyl group but also hydrophobic groups such as aromatic rings and carbon chains in its structure [17,18,20]. Amphiphilic property of HA controls not only its adsorption capability of ionic substances such as heavy metals and organic compounds, but the bioavailability of the toxicants [21,22].

In recent years, the hydrological models of flood are improved, and the knowledge of relationship between a forest and river water is obtained gradually [23-26]. It is known that the disturbance of river sediment including HA and harmful substances is caused by flood. It is expected that microscopic point of view (such as toxicological effects of chlorophenols to water organisms) and macroscopic point of view (such as hydrological knowledges) conduct the new way of toxicity prediction model and environmental assessment.

It is possible that chlorophenols discharged from industrial wastewater and HA originated from soil cause an interaction and reduces the toxicity to aquatic organisms in the natural environment. The elucidation of the influence on the toxicity due to the interaction between HA and hydrophobic organic pollutants could give an important

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knowledge to the toxicity prediction model and could lead to more accurate and inexpensive risk assessment of chemicals. The purpose of this study is to elucidate the toxicological effects of chlorophenols to Green Algae in case of Coexisting HA. Green algae, *Chlorella* was used to test organism as representative of phytoplankton. The growth inhibition curves and logistic curves were applied to experimental data. 2,4,6-TCP, 2,4-DCP and *p* -CP were exposed to *Chlorella* in case of with and without HA.

Materials and Methods

Chemicals

2,4,6-Trichlorophenol (97% purity, Wako 1st Grade), 2,4-Dichlorophenol (90% purity, Practical Grade), *p* -Chlorophenol (98% purity, Wako 1st Grade), 1 mol/L NaOH Solution (1 N Sodium Hydroxide Solution, Factor (20°C)=1.001, for Volumetric Analysis), 1 mol/L HCl Solution (1 N Hydrochloric Acid, Factor (20°C)=1.000, for Volumetric Analysis), Humic Acid (Practical Grade) and MOPS (3-Morpholinopropanesulfonic acid, 99%) as a Good's Buffer were purchased from the FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan).

Experimental procedure of algal growth inhibition test

Calibration curve of *Chlorella*: From the absorbance measurement of *Chlorella* suspension, λ_{max}=690 nm was determined as maximum absorbance wavelength of *Chlorella* cells. This wavelength value close to 663 nm means the existence of Chlorophyll a [27]. Analyzing the correlation between the *Chlorella* cell density and λ_{max} absorbance, Calibration curve of *Chlorella* cell density was prepared. Cell density was measured by the counting chamber (Fuchs Rosenthal, 0, 200 mm Tiefe Depth Prodondeur, HIRSCHMANN).

Precultivation: Subcultured *Chlorella* was used to precultivation in this study. Then it was preserved in the AAP (Algal Assay Procedure) medium (Table 1) coexisting good buffer 0.75 g/L MOPS and cultivated in 1 L glass bottle [28]. Light intensity was 3000 lux. The solvent of AAP medium stock and MOPS was Milli-Q water. *Chlorella* confirmed in a logarithmic growth phase was used to the algal growth inhibition (AGI) test. This *Chlorella* sample as the test organism, 0.75 g/L MOPS-AAP medium mixture as solvent were used throughout this study.

Algal growth inhibition test

This AGI test was following OECD guidelines 201 [29]. *Chlorella* suspension was collected from precultivation medium by centrifugation at 10 minutes, 1500 rpm (KUBOTA, KN-70). Chlorophenols were solved in test medium and stirred for more than 30 minutes with magnetic stirrer in shading and sealing condition. Solving chlorophenols completely, 100 mg/L (ppm) chlorophenols stock solutions were prepared. Diluting chlorophenols stock solutions, several different concentrations (100, 50, 25, 5, 0.5 ppm) of chlorophenols solutions were prepared. In case of HA coexisting experiment, 1.0 g/L HA stock solution was prepared using ultrasonic irradiation and 1 mol/L NaOH solution to solve solid HA, then 1.0 g/L HA stock solution was diluted to 10 ppm with chlorophenols stock solutions. After dropping *Chlorella* suspension to the test reagents, the pH of reagents was controlled by pH 7.5 using 1 mol/L NaOH solution and 1 mol/L HCl solution. The 100 mL Erlenmeyer flasks sealing with silicon plug were selected to test vessels. In each test and control groups, three vessels were prepared (6 concentrations including control × 3 vessels). The vessels were filled with 30 mL test reagents. The vessels were illuminated in a light growth chamber with 2000 lux light intensity and controlled at 24 ± 1°C (OHM ELECTRIC CO., LTD BOX COOL Thermal Control System). The vessels were fixed on shaker (IWAKI Universal Shaker SHK-U3, EYELA NTS-1300) and shaken at 110 rpm. The administered duration of AGI test was 72 hours.

Nutrient	Concentration (mol.L^{-1})	Nutrient	Concentration (mol.L^{-1})
$NaNO_3$	3.00 × 10^{-4}	$MnCl_2$-4H_2O	1.34 × 10^{-6}
K2HPO_4-3H_2O	4.38 × 10^{-6}	$ZnCl_2$	2.42 × 10^{-8}
$MgCl_2$-6H_2O	2.88 × 10^{-5}	$CoCl_2$-6H_2O	3.23 × 10^{-9}
$MgSO_4$-7H_2O	6.00 × 10^{-5}	$CuCl_2$-2H_2O	5.61 × 10^{-11}
$CaCl_2$-2H_2O	3.09 × 10^{-5}	Na_2MoO_4-2H_2O	2.99 × 10^{-8}
$NaHCO_3$	1.77 × 10^{-4}	$FeCl_3$-6H_2O	3.52 × 10^{-7}
H_3BO_3	3.00 × 10^{-6}	Na_2EDTA-2H_2O	7.98 × 10^{-7}

Table 1: The Components of AAP medium.

Measurement of *Chlorella* cell density

At 0, 48 and 72 hours proceeded, absorbance of test reagents was measured at 690 nm (HITACHI U-0080D). The *Chlorella* cell density was determined by calibration curve. The pH of test reagents was also measured (Lutron PH-201) at 0, 48 and 72 hours.

Evaluation of toxicity

The toxicity of chemical is evaluated by several indices such as EC_{50}, Growth Inhibition Rate, LOAEL and so on. Generally, growth inhibition rate is used to evaluate toxicity in case of AGI test. This evaluation method is adapted to the case as the logarithmic increase in the biomass [29]. Algal growth inhibition is derived from the growth rate constant of *Chlorella*. The growth rate constant is given by Equation (1):

$$\mu = \frac{\ln Nn - \ln N_0}{T} \quad (1)$$

where, T [-] stands for the elapsed time of AGI test, N_n [cells mL^{-1}] stands for the cell density of *Chlorella* at 48 and 72 hours elapsed, N_0 [cells mL^{-1}] stands for the cell density of *Chlorella* at initial condition, μ [hour^{-1}] stands for the growth rate constant of *Chlorella*.

The growth inhibition rate of *Chlorella* is given by Equation (2).

$$I_\mu = \frac{\mu_c - \mu_T}{\mu_c} \times 100 \quad (2)$$

where, μ_c [hour^{-1}] stands for the average of growth rate constant in terms of control, μ_T [hour^{-1}] stands for the average of growth rate constant in terms of each group, Iμ [-] stands for the growth inhibition rate of *Chlorella*. Growth inhibition curves of *Chlorella* in case of each chlorophenol were given by the growth inhibition rate.

Curve fitting with logistic equation

It is necessary for applying logistic equation to normalize the growth inhibition rate. On this chapter, another way of calculating growth inhibition rate such as Eq. (2) was applied. The growth inhibition rate calculated by the number of the algal cells at endpoint is given by Equation (3).

$$y = \frac{N_c - N_T}{N_c} \times 100 \quad (3)$$

where, N_T [cells mL^{-1}] is the cell density of *Chlorella* at 72 hours elapsed, N_c [cells mL^{-1}] is the cell density of negative control at 72 hours elapsed.

To obtain EC_{50} from the dose response curves by a simple mathematical equation, the logistic curve expression given by Equation (4) was employed.

$$y = 1 - \left(\frac{1}{1 + \left(\frac{x}{EC_{50}} \right)^{\beta}} \right) \quad (4)$$

where, x [ppm] is the chemical concentration, EC_{50} [ppm] is the algal cell survival or activity 50%, β [-] is the coefficient determining the form of the curves and y [-] is the growth inhibition rate. The coefficient of determination (R^2) between experimental data and the formulated curve were also calculated. The curve fitting and the calculation were carried out by the Generalized Reduced Gradient method with the EXCEL (Microsoft® Excel® 2016 MSO 32 bit, Microsoft Corporation, USA) using Surface Pro (Microsoft Corporation, USA).

Langmuir equation

To evaluate the affinity between absorbate and absorbent such as chlorophenols. and HA, Langmuir equation given by Equation (5) was employed.

$$Q = \frac{Q_{max} KC(1-\alpha)}{1 + KC(1-\alpha)} \quad (5)$$

where, Q_{max} [mol/kg] is the saturated amount of adsorption, Q [mol/kg] is the amount of adsorption, K [L/mol] is the adsorption equilibrium constant, C [mol/L] is the equilibrium concentration, α [-] the degree of ionization of chlorophenols.

Results and Discussion

Growth inhibition curves of chlorophenols in case of absence and existence of HA

Figure 1 shows the relationship between the concentration of chlorophenols and the growth inhibition rate.

Each plot shows the average value of nine samples. In case of *p* -CP, some growth enhancement was observed at 0.5 and 5 ppm. Sufficiently thin toxicant often gives better growth of organisms. On this experiment, growth enhancement was regarded as no growth inhibition. In Figure 1, from the relationship between the concentration of chlorophenols and the growth inhibition rate corresponded, the effects of HA depend on the species of chlorophenols. For 2,4,6-TCP, an enhancement of toxicity was observed at a relatively low concentration: 25 ppm, whereas the reduction of toxicity was observed at 100 ppm. For 2,4-DCP, the significant difference of growth inhibition rate between with and without HA was not observed at low concentration group. In contrast, an enhancement of toxicity was observed at 50 ppm and 100 ppm in case of coexisting 10 ppm HA. On the other hand, *p* -CP showed a decrease in toxicity only at 100 ppm, and no difference in the other concentration was observed. The toxicity difference of chlorophenols was considered divided into low concentration (0.5, 5, 25 and 50 ppm) and high concentration (100 ppm). When the concentration of chlorophenols was low, it was observed that the toxicity was in the order of *p* -CP<2,4,6-TCP<2,4-DCP. It is known that the increase of the number of Cl- (chloro-) groups gives the increase of the toxicity of agricultural chemicals. However, the results in this study did not agree with that order [30]. The reason was considered to the influence of electrostatic interaction. The structure, pKa (acid dissociation constant) and logP (partition coefficient) of chlorophenols were shown in Table 2 [31,32].

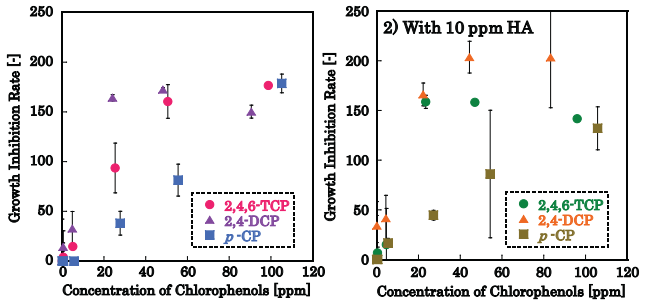


Figure 1: Growth inhibition curves of chlorophenols in case of 1) HA absent and 2) HA coexistence.

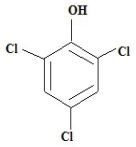
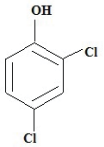
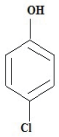
Species	2,4,6-TCP	2,4-DCP	*p*-CP
Structure			
pKa	5.99	7.89	9.41
Logp	3.69	3.06	2.39

Table 2: Details of chlorophenols.

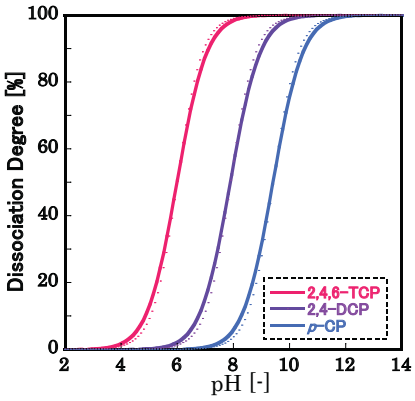


Figure 2: pH dependence of proton dissociation degree of each chlorophenols.

The pH dependence of the proton dissociation degree of chlorophenols was shown in Figure 2. Proton dissociation curves were derived by Henderson-Hasselbalch equation. Figure 2 showed the most of the 2,4,6-TCP dissociates protons of the hydroxyl group and it is ionized at pH 7.5 which is the test medium pH. On the other hand, about half of 2,4-DCP dissociates proton and it is ionized. Most of *p* -CP is not ionized because of the lack of proton dissociation. The ionization of chlorophenols induces the charge to negative charges. It is generally known that phytoplankton cell surfaces such as *Chlorella* are negatively charged [33]. From the viewpoint of bioavailability, the negative charge of chlorophenols promoted the electrostatic repulsion between chemical species and cells. Most of 2,4,6-TCP was ionized at pH 7.5, and it is expected that bioavailability was greatly reduced from electrostatic repulsion. Because approximately half of 2,4-DCP were ionized, bioavailability of 2,4-DCP was expected to decrease slightly. This decrease in bioavailability due to electrostatic repulsion was thought to have reversed growth inhibition rates at 2,4,6-TCP and 2,4-DCP. Although it was thought that *p* -CP was not expected to reduce the bioavailability by ionization, it was expected that the inversion

of the growth inhibition rate did not occur because the toxicity was sufficiently small compared to 2,4-DCP. When the concentration of chlorophenols was high, the growth inhibition rate of chlorophenols showed almost same levels. This could be explained by two points. Firstly, 2,4,6-TCP existed sufficiently as much as strongly toxic even if most of 2,4,6-TCP was ionized. Secondly, because the bioavailability of *p* -CP was not influenced by ionization, the toxicity of *p* -CP showed dose-response like increase. Since half of the 2,4-DCP were ionized and the bioavailability was decreased, it was predicted that a proportional increase of toxicity such as *p* -CP would not occur easily in case of 2,4-DCP. For further explanation of the mechanism of toxicity development, further experiments are needed.

Figures 1 and 2 also shows the growth inhibition curves of chlorophenols in case of 10 ppm HA coexisting. Some growth enhancement was also observed at 0.5 ppm pf *p* - CP. This growth enhancement was also regarded as no growth inhibition. Toxicity of chlorophenols was considered as low concentration (0.5, 5, 25 and 50 ppm) and high concentration (100 ppm) as in the non-coexistence of HA. For the low concentration group, it was observed that the toxicity was in the order of *p* -CP<2,4,6-TCP<2,4-DCP similarly as in the non-coexistence of HA. Comparing the growth inhibition rate curves of each, the difference of growth inhibition rate between 2,4,6-TCP and 2,4-DCP at 25 ppm was small, but the difference was large at 50 ppm. Subsequently, when the concentration of chlorophenols was high, the growth inhibition rate of 2,4-DCP was the largest, and there was almost no difference in growth inhibition rate of 2,4,6-TCP and *p* -CP. Based on the above results, the effect of HA on the growth inhibition rate in each chlorophenol was investigated.

For 2,4,6-TCP, Figure 1 shows that the coexisting HA increased the growth inhibition rate at 25 ppm, but decreased at 100 ppm. The increase of growth inhibition rate was reversed to decrease around 50 ppm. The increase of toxicity at 25 ppm is assumed that an increase in the toxicity associated with the internal uptake mechanism of HA. Some research shows that low molecular weight HA is possible to be easily taken up into cells [34,35]. In this experiment, an ultrasonic irradiator was used to dissolve HA in the process of preparing HA stock solution. This is an operation to generate partial cleavage of the HA skeleton in order to make dissolution in an alkaline solution more effective.

Because of this operation, it is reasonable that low molecular weight HA is sufficiently present under the present test conditions [36]. HA has a diffused electric double layer called Donnan phase, where negatively charged chemical species are captured [37]. The binding of ionized 2,4,6-TCP into Donnan phase is easily occurred. In fact, a previous study shows partially ionized chlorophenols adsorb onto HA [38]. 2,4,6-TCP bound into the Donnan phase would be taken into the cell in the process of internalization of HA. However, the growth inhibition rate did not increase at 100 ppm. This is explained by the HA deposition effect on the cell wall surface. A previous study reported that HS including HA and FA accumulate on the surface of *Chlorella* cells [16]. Since HA is normally charged to a negative charge, HA deposited on the cell surface causes electrostatic repulsion with ionized 2,4,6-TCP. Accumulating HA onto the algal surfaces could enhance the electrostatic repulsion between chemical species and cell surface, and decrease bioavailability [39]. The effect of negative charge repulsion on the surface of *Chlorella* cells is enhanced by the accumulation effect of HA on the cell surface, and the bioavailability of 2,4,6-TCP is also expected to decrease. Internalization of 2,4,6-TCP is also conceivable, the toxicity is probably rather reduced due to the combined effect of 2,4,6-TCP captured in the Donnan phase of HA in the bulk solution.

In terms of the growth inhibition curves of 2,4-DCP in the absence of HA and coexistence of 10 ppm HA, the difference in the growth inhibition curve due to the coexistence of 10 ppm HA was small or insignificant at relatively low concentration (0.5, 5 and 25 ppm). At high concentration (50 and 100 ppm), an increase of toxicity was observed due to coexistence of HA. As mentioned above, about half of 2,4-DCP is ionized at pH 7.5. It is assumed that a half of ionized 2,4-DCP is bound into Donnan phase of HA and another half of non-ionized 2,4-DCP is directly adsorbed to the aromatic moiety or ethylene moiety of HA. Previous study shows both electrostatic interactions and hydrophobic interactions are driving forces of adsorption between HA and chlorophenols [38]. Since proton adsorption and desorption in chlorophenol is explained by chemical equilibrium such as repeating protonation and deprotonation. 2,4-DCP is thought to be loose constrained around HA, therefore the effect of HA accumulated on the surface of *Chlorella* cell wall and the effect of reducing bioavailability due to negative charge repulsion effect on cell wall surface could be small. In addition, it is possible that internalization of HA loosly constraining 2,4-DCP to *Chlorella* cells increases the toxicity of 2,4-DCP similar to 2,4,6-TCP.

Next, in terms of the growth inhibition curves of *p* -CP in the absence of HA and coexistence of 10 ppm HA, the coexistence of 10 ppm HA slightly increased in the growth inhibition rate in the low concentration groups, and the growth inhibition rate decreased in the high concentration group at 100 ppm. It was considered that *p* -CP was hardly ionized at pH 7.5 and directly adsorbed with HA by hydrophobic interaction. Desorption of protons such as 2,4-DCP did not occur, and *p* -CP strongly adsorbed onto HA compared to other chlorophenols. At a low concentration groups (0.5, 5, 25 and 50 ppm), most of *p* -CP is easily internalized due to the effect of low-molecular-weight HA because of strong adsorption, and the growth inhibition rate is expected to be increased. However, unlike 2,4-DCP, since *p* -CP strongly adsorbs onto HA, the effect of increasing bioavailability to cells is small. As a result, the change in growth inhibition rate is expected to be small. At a high concentration (100 ppm), HA present in the solution as DOM constrained *p* -CP, suggesting that bioavailability reduction effect was noticeable.

Previous studies have reported that HA itself has various metabolic interference. For example, a low molecular weight HS having a molecular weight of 3.5 kDa or less easily permeates the membrane and is internalized into cells [40]. In addition, reactive oxygen species in cells increase with the time of exposure to HS, and HS increase active nitrogen species [5]. Particularly, taking it into account that HS changes the activity of bio transportation enzyme and behaves as hormone of nematode, it is natural to regard HS as xenobiotic substances [34]. As a particularly important finding, the previous study discussed that the multixenobiotic resistance (MXR) pump is interacted by HS and changed the activity of MXR [34]. This indicates that HS have a huge impact on bioavailability. For example, it has been suggested that HS exposed at the same time promotes bioaccumulation of chemical substances [35]. Also, the effect to metabolisms depends on the concentration of HA. When coexisting HA is 30 ppm or less, toxicity of triclosan (preservative) to diatoms was alleviated, but if it was 40 ppm or more, the toxicity rather increased [41]. It is assumed that the toxicity effect of HA such as decrease and increase likely depends on the interaction with the target chemical species and the species of organism. It is known that the physiological effect of HS also depends on the generation environment, concentration, molecular weight [40].

Relationship between adsorption equilibrium constant and EC_{50}

Table 3 shows the EC_{50} derived from the curve fitting by logistic curve and their coefficient of determination. From the optimization by Generalized Reduced Gradient method, β was determined as 0.966. The logistic curve was fitted well with experimental data from R^2. To clarify the relationship between K_{AV} and EC_{50}, logarithm of the EC_{50} was calculated by the contraction based on the amount of substances. The adsorption equilibrium constants (K_{AV}) were obtained the data from previous study and calculated by the Eq. (5) [38].

K_{AV} was obtained from the average value of adsorption equilibrium constants at pH 5, 6, 7. This table shows that the toxicity of chlorophenols was in the order of 2,4,6-TCP>2,4-DCP>*p*-CP in case of both the absence and coexistence of HA. The presence of HA decreased the EC_{50} and took smaller value than the absence of HA. From the comparison between absence and presence of HA, it is suggested that HA has a capability of toxicity enhancement at low concentration of chlorophenols. Figure 3 shows the relationship between logarithm of the EC_{50} value of each chlorophenol and the adsorption equilibrium constant of chlorophenols to HA.

There was an inverse proportional relationship between EC_{50} and the adsorption equilibrium constant in the absence of HA and coexistence of HA. The Langmuir formula treats all the adsorption sites equally and postulates the adsorption equilibrium state. When chlorophenols develop toxicity, it is necessary to reach the cell surface once. Because of this chlorophenols pathway, bioavailability is closely related to adsorption to the cell surface. It is assumed that the adsorption behavior of chlorophenols on the cell surface related to the EC_{50}. The correlation between the adsorption equilibrium constant of chlorophenols to HA and EC_{50} suggests that there is similarity between

Condition		2,4,6-TCP	2,4,6-DCP	p-CP
Without HA	EC_{50} [ppm]	26.2	26.9	148
	Log_{10} (EC_{50}) [-]	-3.88	-3.78	-2.94
	R^2 [-]	0.976	0.892	0.982
With 10 ppm HA	EC_{50} [ppm]	14.4	18.1	66.8
	Log_{10} (EC_{50}) [-]	-4.14	-3.95	-3.28
	R^2 [-]	0.920	0.954	0.981

Table 3: EC_{50} of each chlorophenols and R^2 of curve fitting.

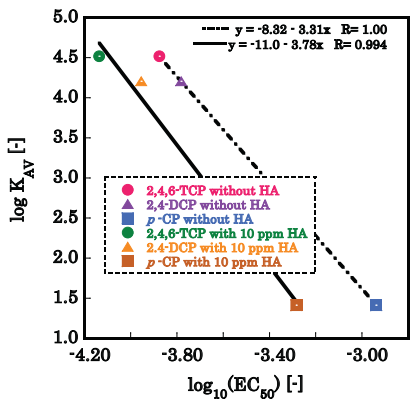


Figure 3: The relationship between logarithm of the EC_{50} value of each chlorophenol and the adsorption equilibrium constant of chlorophenols.

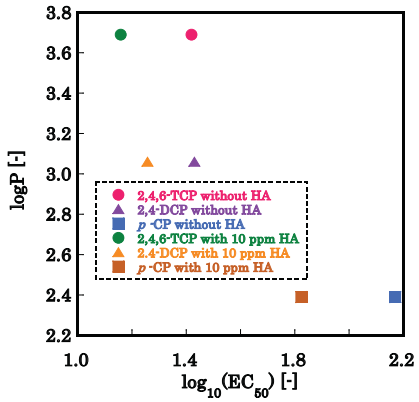


Figure 4: The relationship between EC_{50} of chlorophenols and partition coefficient.

adsorption of chlorophenols onto HA and adsorption of chlorophenols on the cell surface. The same tendency was observed in case of HA coexisting. In addition, it is suggested that HA behaves as a carrier of chlorophenols and toxicity enhancement was observed mentioned above. Figure 4 shows the relationship between logarithm of the EC_{50} of chlorophenols and partition coefficient (logP).

Partition coefficient is one of the hydrophobicity index. Figure 4 shows that there was an inverse proportional relationship between logarithm of the EC_{50} and partition coefficient (logP). That relationship indicates the high hydrophobicity of chlorophenols gives strong toxicity to *Chlorella*. This relationship is explained from the two reasons. Firstly, the hydrophobicity of chemicals means the capability of permeation to the algal cells. High hydrophobicity causes high bioavailability. Secondly, the hydrophobicity of chlorophenols increases along with the increase of the chloro- (Cl-) groups number. Number of Cl- (chloro-) (Cl-) groups directly connects to the toxicity to plants [30].

Conclusion

In this study, to investigate the toxicological effects of chlorophenols to green algae in case of coexisting HS, growth inhibition test of chlorophenols using *Chlorella* was carried out. Increase of toxicity was observed at 25 ppm 2,4,6-TCP in case of HA coexisting, and toxicity decreased at 100 ppm. On the other hand, for 2,4-DCP, the influence of HA to toxicity was not observed in the low concentration groups, but an increase of toxicity was observed in the high concentration groups. For *p* -CP, a decrease of toxicity was observed at 100 ppm when HA was coexisting. These toxicity influences were probably caused by increased bioavailability due to internalization of HA, electrostatic interaction by ionization of chlorophenols and adsorption of chlorophenols onto HA and chlorophenols in bulk. It was suggested that HA has two aspects of toxicity decrease effect and toxicity increase effect.

In addition, since a correlation was found between the adsorption equilibrium constant of chlorophenols to HA and the EC_{50}, it was suggested that adsorption mechanism between HA and chlorophenols and the adsorption of chlorophenols onto *Chlorella* cell wall surface is similar. The driving forces of adsorption between HA and chlorophenols are hydrophobic interactions and electrostatic interactions, and the adsorption behavior depends on the physical property of chlorophenols. In this experiment, when the number of Cl-(chloro-) groups, the growth inhibition rate greatly changed in case of both absence of HA

and coexistence of HA. This interesting phenomenon is predicted to occur naturally in the natural environment. Elucidation of the toxicity development mechanism of chemical substances in the presence of HA is thought to lead to toxicity expression prediction model and risk assessment.

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