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Binding of dicamba to soluble and bound extracellular polymeric substances (EPS) from aerobic activated sludge: A fluorescence quenching study

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ABSTRACT

Binding of dicamba to soluble EPS (SEPS) and bound EPS (BEPS) from aerobic activated sludge was investigated using fluorescence spectroscopy. Two protein-like fluorescence peaks (peak A with Ex/Em = 225 nm/342–344 nm and peak B with Ex/Em = 275/340–344 nm) were identified in SEPS and BEPS. Humic-like fluorescence peak C (Ex/Em = 270–275 nm/450–460 nm) was only found in BEPS. Fluorescence of the peaks A and B for SEPS and peak A for BEPS were markedly quenched by dicamba at all temperatures whereas fluorescence of peaks B and C for BEPS was quenched only at 298 K. A dynamic process dominated the fluorescence quenching of peak A of both SEPS and BEPS. Fluorescence quenching of peak B and C was governed a static process. The effective quenching constants (log K_a) were 4.725–5.293 for protein-like fluorophores of SEPS and 4.23–5.190 for protein-like fluorophores of BEPS, respectively. Log K_a for humic-like substances was 3.85. Generally, SEPS had greater binding capacity for dicamba than BEPS, and protein-like substances bound dicamba more strongly than humic-like substances. Binding of dicamba to SEPS and BEPS was spontaneous and exothermic. Electrostatic force and hydrophobic interaction forces play a crucial role in binding of dicamba to EPS.

1. Introduction

Activated sludge is the most widely used biological wastewater treatment process. Activated sludge can remove more than 50% of heavy metals in wastewater by adsorption and precipitation processes [1]. Extracellular polymeric substances (EPS), excreted by the microorganisms, play key role in removing pollutants from wastewater [2,3]. In the past several decades, quite a few studies reported the strong sorption capacity of EPS for heavy metals [4–7]. The strong sorption capacity of EPS for heavy metals is ascribed to their abundant functional groups such as carboxyl, hydroxyl and amine [5].

EPS can be divided into soluble EPS (SEPS) and bound EPS (BEPS) depending on their physical states in the activated sludge [8]. Both the soluble and bound EPS derived from activated sludge are mainly composed of proteins and polysaccharides with the soluble EPS having higher concentration of polysaccharides than the bound EPS [9]. SEPS can be shed into the surrounding environment as an amorphous slime and BEPS are loosely attached to the cell surface as peripheral capsules [10]. This means that the pollutants adsorbed by EPS may be released to water again. In this sense, performance of treatment of wastewater by activated sludge can be reduced more or less due to release of EPS to the water. Compared with BEPS, the pollutants bound to SEPS may be more easily released due to the high solubility of SEPS. Therefore, it is necessary to understand the differences between SEPS and BEPS for their complexation with pollutants and the stability of the complexes formed. A number of studies show that EPS are effective absorbents for removing heavy metals and organic pollutants such as dyes and pesticides [4–7], and few studies show that the biosorption capacity of SEPS for heavy metals is greater than that of BEPS [9]. Nevertheless, these studies mainly emphasized the biosorption rate and capacity of EPS for pollutants whereas the differences of biosorption of organic pollutants between SEPS and BEPS are still little known.

Dicamba (3,6-dichloro-2-methoxy benzoic acid), a post-emergent herbicide, is a chlorinated benzoic acid-derivate compound (Fig. 1). It is widely used in agriculture to control the growth of various unwanted vegetable species [11]. Because dicamba is highly soluble in water and extensively used in agriculture, it can easily enter the aqueous environments. It has been frequently detected in the surface water and ground water [12,13]. Dicamba is known to have toxic effects on aquatic plants and genotoxicity to animals [11]. It has received a great deal of attention due to its widespread contamination in aqueous environments and its potential ecological risk [12,13].

The fluorescence excitation emission matrix (EEM) spectroscopy is a simple, sensitive and rapid method for studying fluores-

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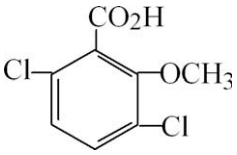

Fig. 1. The molecular structure of dicamba ($C_8H_6Cl_2O_3$).

cent organic compounds because this technique provides important information on fluorescence intensity and fluorescent functional groups for understanding the dynamics and chemical nature of organic compounds in aqueous media [14]. EEM spectroscopy has been successfully applied to investigate the DOM (dissolved organic matter) and its interaction with metals [15,16]. Recently, EEM spectroscopy has been employed to study the chemical properties of EPS [17] since protein and humic substances are also the key components that show fluorescence properties in EPS.

The aim of the present study was to investigate the complexation of the fluorescent compounds in SEPS and BEPS with dicamba and the stability of the complexes formed using EEM fluorescence spectroscopy. The binding modes of SEPS and BEPS with dicamba were also studied.

2. Materials and methods

2.1. Extraction of EPS

The aerobic activated sludge was collected from the aeration tanks in Hedong Municipal Sewage Treatment Plant, Urumqi, China. The activated sludge was centrifuged at 4300*g* for 10 min at 4 °C in order to remove the dissolved minerals and organic matter [9]. The residue was recovered and suspended in Milli-Q water and centrifuged at 4000*g* for 20 min at 4 °C. The supernatant was considered as SEPS sample [9]. The residue after extraction of SEPS was recovered and resuspended in 2% ethylenediamine tetraacetic acid disodium salt (EDTA) for 3 h at 4 °C. Then the suspension was centrifuged at 20,000*g* for 20 min at 4 °C. The supernatant was considered as BEPS sample. Both SEPS and BEPS samples were filtrated through 0.22 μm acetate cellulose membrane and purified with dialysis membrane (3500 Dalton) for 24 h at 4 °C for three times in order to remove the free ions, EDTA and components with low molecular weight from the EPS samples.

2.2. Preparation of dicamba solution

Dicamba (CAS No. 1918-00-9) was obtained from Accustandard (New Haven, CT, USA). Dicamba standard solution (0.01 M) was prepared by dissolving dicamba in milli-Q water.

2.3. The fluorescence quenching titration

The EEM fluorescence spectra of the EPS solution were recorded with a fluorescence spectrophotometer (F-7000, HITACHI, Japan). The EEM spectra were collected at 5 nm increments over an excitation range of 200–400 nm, with an emission range of 250–550 nm by every 2 nm. The excitation and emission slits were set to 5 and 10 nm of band-pass, respectively. The scan speed was 1200 nm min^{-1}. The Milli-Q water blank was subtracted from the sample's EEM spectra and EEM data was processed using the software SigmaPlot 10.0 (Systat, US).

10 ml of EPS solution (35 mg L^{-1}, pH 6.8) in a 25 ml beaker was titrated with incremental additions of 5 μL of 0.01 M dicamba at 283, 288, 293, and 298 K, respectively. After each addition of dicamba, the solution was fully mixed using a magnetic stirrer for 15 min and then its fluorescence spectrum was recorded. The equilibrium time was set as 15 min since fluorescence intensities at peaks varied little after 15 min reaction.

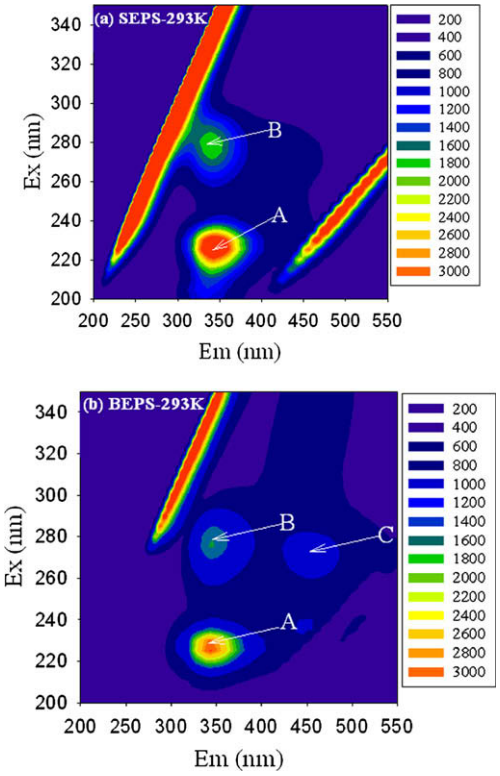

Fig. 2. The exemplified 3DEEM fluorescence spectra of SEPS (a) and BEPS (b) at 293 K.

3. Results and discussion

3.1. The EEM fluorescence spectra of EPS

Fig. 2 showed the exemplified EEM fluorescence spectra of SEPS and BEPS. Two fluorescence peaks (peak A with Ex/Em = 225 nm/342–344 nm and peak B with Ex/Em = 275/340–344 nm) were found in both SEPS and BEPS. Peaks A and B could be assigned to protein-like fluorescence [14,18]. The fluorescence of peaks A and B could be further identified as the aromatic amino acids and tryptophan [14,19,20]. The positions of peaks A and B for SEPS showed little difference from those for BEPS, suggesting that the fluorescence properties of protein-like substances did not altered after addition of EDTA (Fig. 2). Peak C (Ex/Em = 270–275 nm/450–460 nm), described as humic-like peak [21], was only identified in the fluorescence spectrum of BEPS. This suggested that the humic-like substances combined tightly with the cell wall and could be extracted with EDTA treatment. In comparison with the positions of fluorescence peak of humic-like substances (Ex/Em = 255/342 nm) [22], the position of peak C showed a red shift. Peaks A, B and C represent fluorophores A, B and C in EPS, respectively.

3.2. The fluorescence quenching of SEPS and BEPS by dicamba

The fluorescence intensities of peaks A and B for SEPS decreased markedly with increasing dicamba concentration at all experimental temperatures (Fig. 3a and b). This indicates that protein-like fluorophores in SEPS reacted strongly with dicamba. For BEPS, the fluorescence of peak A was clearly quenched by dicamba (Fig. 3c).

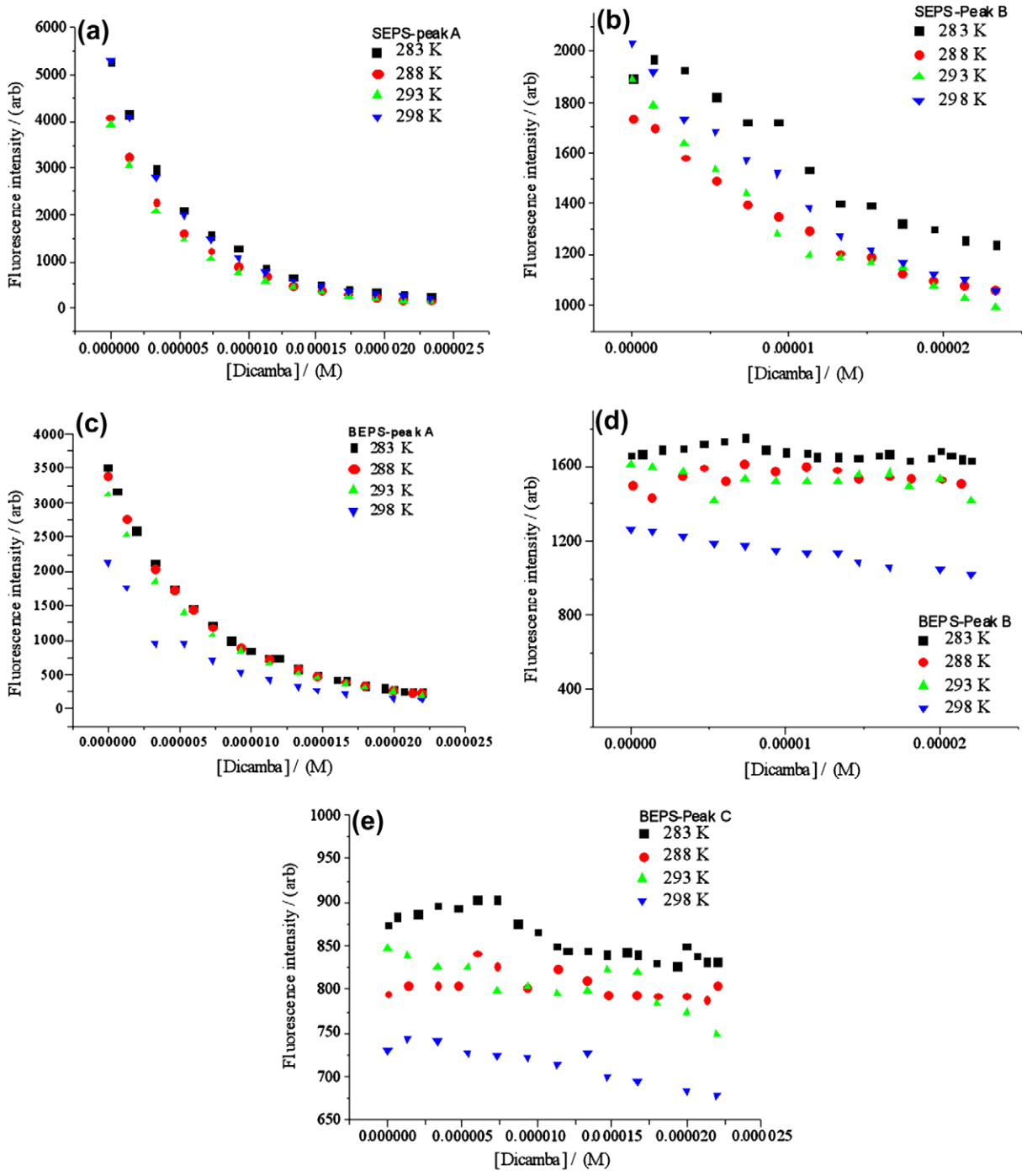

Fig. 3. The fluorescence intensity of peaks varied with increasing dicamba concentration at various temperatures. (a) peak A of SEPS, (b) peak B of SEPS, (c) peak A of BEPS, (d) peak B of BEPS and (e) peak C of BEPS.

However, fluorescence of peaks B and C for BEPS was only quenched at 298 K but not significantly quenched at other experimental temperatures (Fig. 3d and e). This implies that some of protein-like substances (represented by peak B) and humic-like substance reacted with dicamba at 298 K but not below this temperature.

3.3. Fluorescence quenching mechanisms

Generally, the fluorescence quenching process can be divided into static quenching process and dynamic quenching process. The dynamic quenching is attributed to the collision between fluorophore and quencher at excited state whilst the static quenching is assigned to the formation of a complex between fluorophore and quencher with the external forces [23,24]. The dynamic quenching constant increases with increasing temperature since the diffusion coefficients become larger at higher temperature. On the contrary, static quenching constant decreases with increasing temperature [24,25]. In order to understand the mechanisms involved in fluorescence quenching of the various fluorophores in SEPS and BEPS, the fluorescence titration data were fitted with Stern–Volmer Eq. (1).

$$F_0/F = 1 + k_q\tau_0[\text{dicamba}] = 1 + K_{sv}[\text{dicamba}] \quad (1)$$

where F_0 and F are fluorescence intensities of the fluorophores in absence and presence of dicamba, respectively; k_q is an energy transfer rate ($M^{-1}\,s^{-1}$), τ_0 refers as to lifetime of fluorescence (s), which taken as 10^{-8} s [26], K_{sv} is the Stern–Volmer constant, and [dicamba] is the concentration of dicamba.

Poor linear relationship between F_0/F and [dicamba] was observed for peak A of both SEPS and BEPS (Fig. 4). The nonlinearity of Stern–Volmer plot may be interpreted by two reasons [27]. One possible reason is that the dynamic quenching and static fluorescence quenching occur simultaneously. The other possible reason is that nonlinear binding isotherm involving a significant occupation of binding sites is present and the free quencher concentration decreases.

For peak B of both SEPS and BEPS and peak C of BEPS, the fluorescence titration data could satisfactorily fitted by the Stern–Volmer equation ($R^2 > 0.97$) (Fig. 4 and Table 1). This means that the quenching process of fluorophores B and C in EPS was governed by either dynamic process or static process singly. The values for K_q for peak B and peak C were 1 or 2 orders of magnitude greater than the maximum diffusion collision quenching rate constant ($2.0 \times 10^{10}\,M^{-1}\,s^{-1}$) for a variety of quenchers with biopolymers [28]. This indicates that the fluorescence quenching process of peaks B and C may be dominated by a static quenching process and some type of binding interaction occurs [26].

In order to understand more about the quenching mechanism, the fluorescence quenching data were further analyzed by the modified Stern–Volmer Eq. (2):

$$F_0/(F_0 - F) = 1/(fK_a[\text{dicamba}]) + 1/f \quad (2)$$

where F_0 and F are the fluorescence intensities in absence and presence of dicamba, respectively. f is the fraction of the initial fluorescence corresponding to the binding fluorophore, K_a is the effective quenching constant, and [dicamba] is the dicamba concentration.

In this modified equation, both the quenchable fluorophores and the quencher inaccessible fluorophores were considered. Following Lakowicz [26], the total fluorescence (F_0) fluorophores in the absence of quencher, F_0, equals to F_{0a} and F_{0b}.

$$F_0 = F_{0a} + F_{0b} \quad (3)$$

where F_{0a} is the fluorescence of the fluorophore moieties that can complex with quencher and F_{0b} is the fluorescence of the inaccessible fluorophore moieties.

In the presence of quencher, only the F_{0a} will change and the observed fluorescence intensity will be provided to form the modified Stern–Volmer equation to evaluate the complexation parameters, i.e. conditional stability constants and binding capacities.

$$f = F_{0a}/(F_{0a} + F_{0b}) \quad (4)$$

The parameters f_a and K_a can be obtained from Eq. (2). In the present study, the titration data were well represented by the modified Stern–Volmer equation ($R^2 > 0.997$). The quenching constants ($\log K_a$) for EPS by dicamba at various temperatures were listed in Table 2. The values of $\log K_a$ showed an increasing trend with increasing temperature for peak A of both SEPS and BEPS, suggesting the fluorescence quenching of both SEPS and BEPS by dicamba is dominated by a dynamic quenching process. For peak B of SEPS and peaks B and C of BEPS, the values of $\log K_a$ at temperatures below 298 K were not given or calculated because their values were negative values or no significant fluorescence quenching were observed at these temperatures. It was found that the values

Table 1
Stern–Volmer quenching constants for interaction of fluorophores in EPS with dicamba at 298 K.

	K_{sv} ($\times 10^4$ L mol^{-1})	k_q ($\times 10^{12}$ L mol^{-1} s^{-1})	R^2
Peak B (SEPS)	40.73	40.73	0.991
Peak B (BEPS)	1.09	1.09	0.977
Peak C (BEPS)	0.47	0.473	0.987

Table 2
The effective quenching constant $\log K_a$ for dicamba binding to SEPS and BEPS at different temperatures.

Peaks	T (K)	SEPS		BEPS	
		$\log K_a$	R^2	$\log K_a$	R^2
Peak A	283	5.207	0.992	5.033	0.999
	288	5.223	0.995	5.100	0.999
	293	5.290	0.994	5.167	0.999
	298	5.293	0.997	5.190	0.997
Peak B	283	[a]	0.906	[b]	[b]
	288	[a]	0.969	[b]	[b]
	293	4.568	0.997	[b]	[b]
	298	4.725	0.993	4.23	0.979
Peak C	298	[b]	[b]	3.85	0.936

[a] Negative values of $\log K_a$ were obtained from the modified Stern–Volmer equation.
[b] Since no significant fluorescence quenching at this temperature was observed, the value of $\log K_a$ was not calculated.

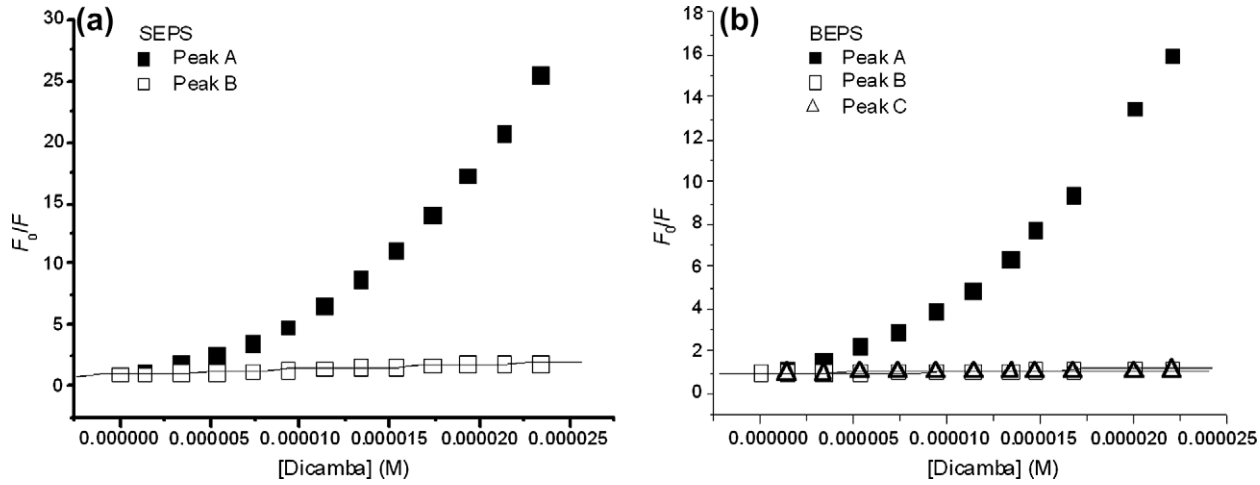

Fig. 4. Stern–Volmer plots for F/F_0 for SEPS (a) and BEPS (b) versus [dicamba] at 298 K.

Table 3
The binding constants log K_b and binding sites n for SEPS-dicamba system and BEPS-dicamba system at various temperatures.

Peaks	T (K)	SEPS			BEPS		
		n	log K_b	R^2	n	log k_b	R^2
Peak A	283	1.57	8.51	0.984	1.45	7.78	0.984
	288	1.57	8.54	0.984	1.46	7.84	0.984
	293	1.57	8.58	0.989	1.47	7.89	0.985
	298	1.57	8.60	0.984	1.52	8.20	0.987
Peak B	283	1.48	6.49	0.926	[a]	[a]	[a]
	288	0.96	4.15	0.957	[a]	[a]	[a]
	293	0.75	3.19	0.970	[a]	[a]	[a]
	298	0.70	3.14	0.985	1.19	4.71	0.974
Peak C	298	[a]	[a]	[a]	1.07	3.98	0.864

[a] Since no significant fluorescence quenching at this temperature was observed, the value of log K_b was not calculated.

of log K_a for peak A or peak B of SEPS were greater than those for BEPS, implying that the SEPS-dicamba complexes were more stable than BEPS-dicamba complexes. For the same type of EPS (SEPS or BEPS), log K_a followed the order of peak A > peak B > peak C (if present), indicating that the stability of compexes of dicamba-fluorophores in the same type of EPS followed the order of fluorophores A > fluorophores B > fluorophores C (if present). This also showed that interaction of dicamba with protein-like substances formed more stable complexes than with humic-like substances.

3.4. Binding constants and binding sites

When small molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant (K_b) and the numbers of binding sites (n) were calculated from the modified Hill Eq. (3)[29]:

$$\log[(F_0 - F)/F] = \log K_b + n \log[\text{dicamba}] \quad (5)$$

where F_0 and F are the fluorescence intensities of fluorophores in absence and presence of dicamba, respectively. F_0–F, the fraction of quenched fluorescence, indicating the fraction of ligand binding sites filled; K_b is the binding constant, n is the number of binding sites, and [dicamba] is the dicamba concentration.

The values of the number of binding sites (n) and the binding constants (log K_b) were listed in Table 3. For SEPS, the values of log K_b and n for peak A were much greater than those for peak B. For BEPS, the values of log K_b and n followed the order of peak A > peak B > peak C. This indicates that protein-like substances in SEPS and BEPS have greater binding capacity for dicamba than humic-like substances.

The binding constant, log K_b increased with increasing temperature for peak A of both types of EPS, indicating that binding capacity for diacamba of the protein-like fluorophores A in SEPS or BEPS becomes stronger with increasing temperature. The values of log K_b for peak A of SEPS were greater than those for peak A of BEPS at all temperatures. This suggests that fluorophores A in SEPS have stronger affinity for dicamba than those in BEPS. In the case of peak B of SEPS, the values of log K_b showed a decreasing trend with increasing temperature. This suggests that the protein-like fluorophores B in SEPS can bind more dicamba at lower temperatures. Humic-like fluorophores (represented by peak C) were found to have minimum log K_b. That is, humic-like fluorophores in EPS have weaker binding capacity for dicamba.

The binding sites numbers n for dicamba binding to fluorophores A in SEPS did not change with increasing temperature (n = 1.57). For peak A of BEPS, n increased with increasing temperature. This means that an increase in temperature resulted in more binding sites in BEPS exposed to dicamba. The values of n for peak A of both SEPS and BEPS were >1, suggesting that there were more than one class of binding sites in EPS for dicamba. In addition, values of n for peak A of SEPS were greater than those for BEPS. For peak B of SEPS, The values of n decreased with increasing temperature.

3.5. The binding mode of dicamba to EPS

The thermodynamic parameters for fluorescence quenching of EPS by dicamba, including the Gibbs free energy change (ΔG), the enthalpy change (ΔH) and the entropy change (ΔS), were calculated. These parameters were usually used to study the binding forces. The values of enthalpy change (ΔH) and the entropy change (ΔS) can be estimated from Van't Hoff Eq. (4) [30]:

$$\log K_b = -\Delta H/RT + \Delta S/R \quad (6)$$

where R is the universal gas constant (8.314 J^{-1} mol^{-1}), T is the absolute temperature (K), and K_b is the biding constant at the corresponding temperature. The ΔH and ΔS can be constants if the temperature changes within a narrow range [31]. ΔG was calculated from the following Eq. (5):

$$\Delta g = \Delta H - T\Delta S \quad (7)$$

In the present study, thermodynamic parameters for peaks B and C were not calculated because no valid log K_a values for these two peaks were available at temperatures below 298 K. ΔG, ΔH and ΔS for dicamba-flurophore-A system were calculated and summarized in Table 4. The negative values of ΔG suggest that binding of dicamba to both types of EPS was spontaneous. The positive ΔS values indicate that the binding of dicamba to SEPS and

Table 4
The thermodynamic parameters for SEPS-dicamba system and BEPS-dicamba system.

Peaks	T (K)	SEPS				BEPS			
		ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J $K^{-1}mol^{-1}$)	R^2	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J $K^{-1}mol^{-1}$)	R^2
Peak A	283	−46.0819	10.02	198.24	0.986	−41.881	42.02	296.47	0.806
	288	−47.0731				−43.3634			
	293	−48.0643				−44.8457			
	298	−49.0555				−46.3281			

BEPS is exothermic. $\Delta H > 0$ and $\Delta S > 0$ suggested that electrostatic force and hydrophobic interaction forces may play a major role in the binding between EPS (both SEPS and BEPS) and dicamba [26].

This study shows that both of the two types of EPS have strong binding capacity for dicamba, and SEPS generally have stronger binding capacity than BEPS. Similarly, Comte et al. [9] demonstrated that SEPS had greater biosorption capacity for heavy metals (Cu^{2+}, Pb^{2+} and Ni^{2+}) than BEPS. They consider that the ability of polysaccharides to bind metallic cations could be involved in the greater biosorption abilities of soluble EPS. They also pointed out that SEPS may serve as a protective barrier against heavy metals. Of course, in the present study, SEPS may play the same protective role in against toxicity of organic pollutants. On the other hand, from the viewpoint of wastewater treatment by activated sludge, the strong binding capacity of SEPS for organic pollutant may be not a good thing. SEPS are loosely attached to the cell surface and they may be easily released into water with the organic pollutants they bound. Therefore, the possibility of release of SEPS-organic pollutant complexes from the cell surface and their stability in the aqueous environments need further study.

4. Summary

(1) Two protein-like peaks were identified in the EEM fluorescence spectra of SEPS and BEPS, and one humic-like peak was found in the EEM spectra of BEPS.
(2) Fluorescence of the peaks A and B for SEPS and fluorescence of peak A for BEPS were markedly quenched by dicamba at all experimental temperatures whereas fluorescence of peaks B and C for BEPS was quenched only at 298 K. A dynamic process dominated the fluorescence quenching process of peak A of both SEPS and BEPS. For peak B and C, their fluorescence quenching was governed a static process.
(3) Generally, protein-like substances in SEPS formed more stable complexes with dicamba than those in BEPS. Protein-like substances have stronger binding capacity for dicamba than humic-like substances.
(4) Binding of dicamba to EPS was spontaneous and exothermic. Electrostatic force and hydrophobic interaction forces play a crucial role in binding of dicamba to EPS.

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