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Full Length Research Paper

Interaction of acetamiprid with extracellular polymeric substances (EPS) from activated sludge: A fluorescence study

Wenjuan Song[1], Guijin Mu[1], Daoyong Zhang[2] and Xiangliang Pan[1]*

[1]Laboratory of Environmental Pollution and Bioremediation, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, 818 Beijing Southern RD, Urumqi, China.
[2]State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, 46 Guanshui RD, Guiyang, China.

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Extracellular polymeric substances (EPS) are important components of activated sludge and it plays an important role in removing pollutants. The interaction between EPS and organic pollutants is still little known. In the present study, the interaction of soluble/bound EPS with acetamiprid, a neonicotinoid insecticide, was investigated using the three-dimensional excitation–emission matrix (EEM) fluorescence spectroscopy. The fluorescence spectra of EPS revealed that there were two classes of protein-like fluorophores in soluble/bound EPS and one class of fulvic acid-like fluorophore, in addition, in bound EPS. The quenching of protein-like fluorescence by acetamiprid indicated that static quenching (at peak B) and combined quenching (at peak A) occurred simultaneously. The interaction of acetamiprid with EPS *was* observed to have resulted in the formation of acetamiprid-EPS complexes. The binding constants of the soluble EPS for acetamiprid were greater than those of the bound EPS, indicating the soluble EPS had stronger binding capacity for acetamiprid than the bound EPS. This study confirmed that EPS (soluble/bound) play important roles in biosorption of organic pollutants by activated sludge and also indicated that they may serve as a protective barrier against toxic organic matter, for the microorganisms.

Key words: Extracellular polymeric substance (EPS), activated sludge, fluorescence quenching, binding constant, acetamiprid.

INTRODUCTION

The activated sludge process is the most commonly used technology in municipal sewage treatment. Activated sludges produce high amounts of extracellular polymeric substances (EPS) which are metabolic products of bacteria accumulated on the cell surface (Morgan et al., 1990). EPS are mainly composed of polysaccharides, proteins, humic substances, nucleic acids and lipids (Wingender et al., 1999). EPS contain a lot of functional groups such as carboxylic, phosphoric, amino, and hydroxylic groups which are useful to remove pollutants in sewage (Liu and Fang, 2002).

EPS can be divided into soluble EPS and bound EPS depending on their physical states, in the activated sludge. The soluble EPS can be shed into the surrounding environment as a less organized (amorphous) slime and the bound EPS are loosely attached to the cell surface as peripheral capsules (Nielsen and Jahn, 1997). 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 (Comte et al., 2006). The soluble and bound EPS can be separated using physical and chemical methods (Wingender et al. 1999); while the soluble EPS can be extracted by centri-

*Corresponding author. E-mail: xiangliangpan@163.com.Tel: 086 - 991-7885446. Fax: 086-991-7885446.

Abbreviations: EPS, Extracellular polymeric substances; **EEM,** excitation–emission matrix; **EDTA,** ethylenediamine tetraacetic acid.

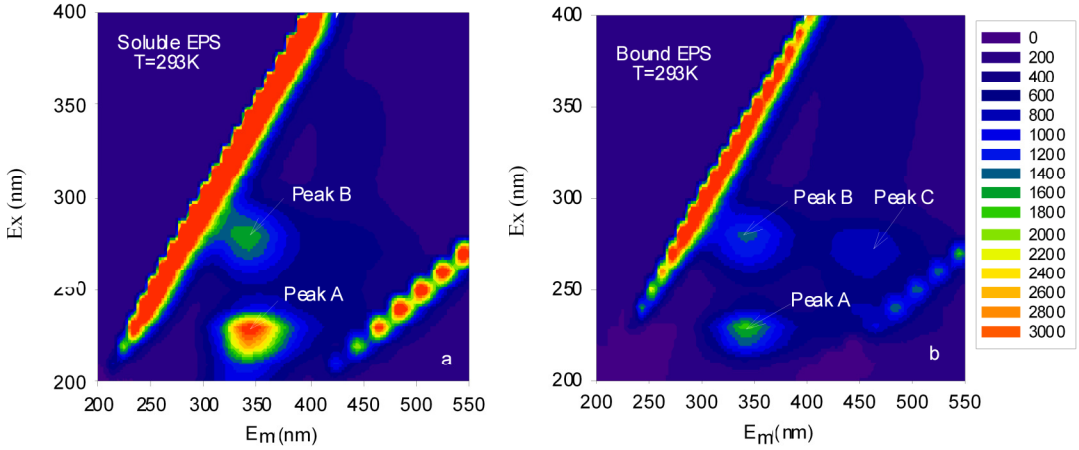

Figure 1. EEM fluorescence spectra of soluble EPS (a) and bound EPS (b).

fugation alone, the bound EPS require additional chemical treatment for their extraction.

Some studies have shown that EPS play a crucial role in biosorption and binding of heavy metals (Liu et al., 2001; Guibaud et al., 2006, 2009; Zhang et al. 2006) and the biosorption capacity of soluble EPS for heavy metals is greater than that of bound EPS (Comte et al., 2006). EPS were also demonstrated to be an effective absorbent for removing organic pollutants such as dyes and pesticides (Zümriye, 2005; Zhang et al., 2009). Nevertheless, these studies mainly emphasized the biosorption rate and capacity of EPS for organic pollutants, the biosorption mechanism involved and the difference of biosorption between soluble EPS and bound EPS are still not clear. The data of binding constants of EPS with organic pollutants are still not available.

Acetamiprid ((*E*)-*N*1-[(6-chloro-3-pyridyl) methyl]-*N*2-cyano-*N*1-methylacetamidine) is one of the new-effective-nicotinic pesticides with moderate toxicity (Figure 1). It has been widely used for the control of *Hemiptera* (mainly aphids), *Thyasnoptera* and *Lepidoptera* on various crops (Sanchez et al., 2003). The objectives of this study are (1) to comparatively investigate interaction of soluble EPS and bound EPS with acetamiprid, (2) to provide their binding constants and (3) to reveal the underlying mechanisms, using three-dimensional excitation-emission matrix (EEM) fluoresence spectroscopy.

MATERIALS AND METHODS

Chemicals

The acetamiprid with purity of 97.1% was purchased from United Pesticide and Industry Corporation (Shan Dong, China). The 0.005 M stock acetamprid solution was prepared with deionized water.

Extraction of EPS

The activated sludge sample was collected from the aerated basin of Hedong Municipal Sewage Treatment Plant in Urumqi, China.

Soluble EPS and bound EPS were subsequently extracted from the activated sludge according to Wingender's method (Wingender et al., 1999). The activated sludge was centrifuged at 5, 600 rpm for 10 min at 4°C. The activated sludge was recovered and re-suspended in ultra pure water. The separation of soluble EPS from activated sludge was carried out by centrifugation at 5, 500 rpm for 20 min at 4°C. The supernatant was considered as the raw soluble EPS. The residue was recovered and resuspended in 2% EDTA solution for 3 h at 4°C. Then, the suspension was centrifuged at 20, 000 rpm for 20 min at 4°C. The supernatant was regarded as the raw bound EPS.

The raw soluble and bound EPS samples were individually filtered through 0.22 µm acetate cellulose membranes. The raw EPS samples were then filtered through 0.22 µm acetate cellulose membrane at 25°C and purified with dialysis membrane (3500 Dalton) at 4°C for 24 h.

EEM fluorescence spectroscopy and quenching titration

All EEM spectra were measured by a fluorescence spectrophotometer (F-7000, Hitach, Japan) equipped with 1.0 cm quartz cell and a thermostat bath. EEM spectra were collected with subsequent scanning, emission spectra from 200 to 550 nm at 2 nm increments by varying the excitation wavelength from 200 to 400 nm at 5 nm increments. The width of the excitation/emission slit was set to 5.0 nm, and the scanning speed was set to 1200 nm/min. The fluorometer's response to a Milli-Q water blank solution was subtracted from the fluorescence spectra, recorded for samples containing EPS and acetamiprid under the same conditions. The 3DEEM data were processed using the software Sigma-plot 10.0 (Systat, US).

EPS solutions were titrated with incremental additions of 5 µL of 0.005 M acetamiprid. After each addition of acetamiprid, the solution was fully mixed using a magnetic stirrer for 15 min and the fluorescence spectra were recorded. All the experiments were done in triplicates and the mean values were used.

RESULTS AND DISCUSSION

EEM spectra of soluble EPS and bound EPS

The EEM spectra of soluble EPS and bound EPS were

Table 1. The fluorescence peak position in the EEM spectra of EPS from activated sludge in the…….Literature.

Origin of EPS	Component type			Reference
	Protein-like	Fulvic acid-like	Humic acid-like	
Activated sludge from Municipal sewage treatment plant	225/340-350, 280-285/340-350	-	330-340/420-430	Sheng and Yu, 2006
Activated sludge in sequencing bat reactor	280/350	340/400	390/450	Li et al., 2008
Activated sludge in Bench-scale sequencing bat reactor	280/340	320/400	-	Ni et al., 2009
Activated sludge from Municipal sewage treatment plant (soluble EPS)	225/342, 280/342	-	-	This study
Activated sludge from Municipal sewage treatment plant (bound EPS)	225/344, 280/344	275/454	-	This study

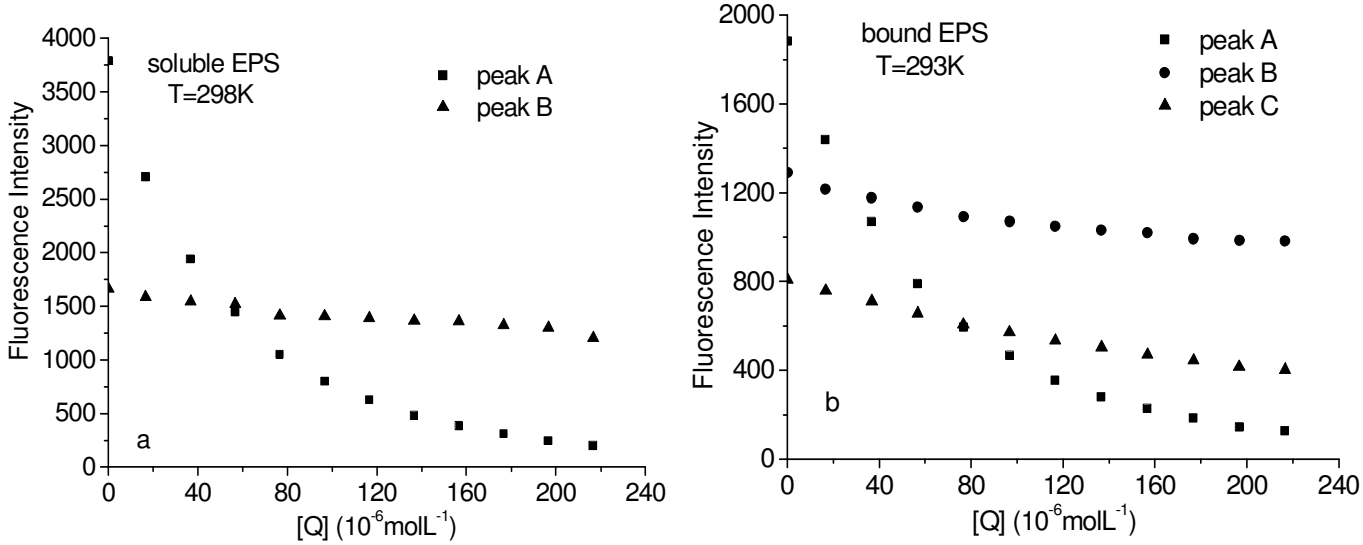

Figure 2. The fluorescence intensity of fluorescence peaks for soluble EPS (a) and bound EPS (b) in the presence of various concentrations of acetamiprid.

illustrated in Figure 1. Two peaks (peaks A and B), originating from protein-like peaks (Baker, 2001; Chen et al., 2003; Yamashita and Tanoue, 2003; Baker and Inverarity, 2004), were identified from the EEM spectra of both the soluble EPS and the bound EPS. Peak C in EEM spectra of the bound EPS can be indentified as fulvic acid like fluorophores (Sugiyama et al., 2005). Limited studies on the characteristics of EPS in activated sludge by fluorescence spectroscopy had suggested that there were several peaks in EEM of EPS from activated sludge, being assigned to protein-like substances, humic-like substances and fluvic-like substances (Sheng and Yu, 2006; Adav and Lee, 2008; Ni et al., 2009). In the present study, it was different from other studies about fluorescence peaks, of EPS from activated sludge (Table 1). In the present study, the position of protein-like peaks (peaks A and B) in this study was close to those of Sheng's study (Sheng and Yu, 2006), indicating that the position of fluorescence peaks are affected by the origin of activated sludge. In addition, the method used to extract EPS might also affect the position and type of fluorescence peak.

The fluorescence quenching

The values of fluorescence intensities and fluorescence peaks for EPS in the presence of various concentrations of acetamiprid are shown in Figure 2. The fluorescence intensities for both soluble EPS and bound EPS decreased with increasing acetamiprid concentration, indicating that acetamiprid could interact with EPS and

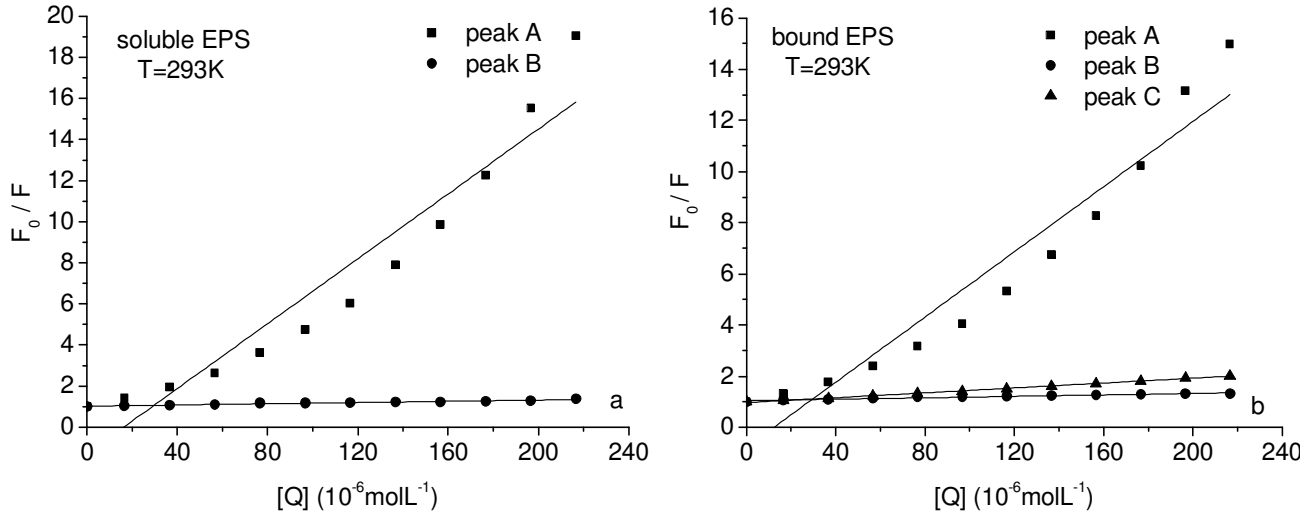

Figure 3. The Stern-Volmer plots for the fluorescence quenching of fluorescence peaks by acetamiprid for the soluble EPS (a) and the bound EPS (b).

Table 2. Stern-Volmer quenching constants K_{sv} and K_q of fluorescence peaks for EPS by acetamiprid at 293K.

Sample	Soluble EPS		Bound EPS		
	Peak A	Peak B	Peak A	Peak B	Peak C
Range of acetamiprid concentration (10^{-6} M)	0 - 100	0 - 220	0 - 100	0 - 220	0 - 220
K_{sv}($\times 10^3$ M^{-1})	38.135	1.469	31.450	1.402	4.780
K_q($\times 10^{11}$ $M^{-1}\cdot s^{-1}$)	38.135	1.469	31.450	1.402	4.780
R^*	0.986	0.973	0.988	0.974	0.998

* R is the correlation coefficient for the K_{sv} values.

quench its intrinsic fluorescence. The fluorescence intensity of peak B, for soluble EPS and that of peak B/peak C for bound EPS presented linear change with acetamiprid concentration, indicating the same quenching process of peaks B and C by acetamiprid. The fluorescence intensity of peak A for soluble/bound EPS decreased nonlinearly with acetamiprid concentration, suggesting that peak A might be quenched by acetamiprid in a different way from peak B/C.

The quenching process can be dynamic, resulting from collision between the fluorophore and quencher, or static, resulting from the complexation between the fluorophore and quencher. In order to understand the quenching process, the Stern-volmer equation was used to fit the quenching data (Lakowicz, 2006):

$$F_0 / F = 1 + K_q \tau_0 [Q] = 1 + K_{sv}[Q] \quad (1)$$

Where F_0 and F are the fluorescence intensities in the absence and presence of acetamiprid, respectively. K_q is the quenching rate constant of the EPS, K_{sv} is the dynamic quenching constant, τ_0 is the average lifetime of the EPS molecule without acetamiprid, which is taken as 10^{-8} s (Lakowicz, 2006) and [Q] is the concentration of the acetamiprid.

The linear plots also suggest that not more than a single quenching mechanism, either static or dynamic, occurrence. The nonlinear plots suggest that a combined quenching (static and dynamic) process occurred. The Stern-Volmer plots of the fluorescence quenching of EPS by acetamiprid were shown in Figure 3. The values of K_{sv}, K_q and R were summarized in Table 2. The Stern-Volmer plots of peak A for both the soluble/bound EPS exhibited an upward curve, deviating slightly from linearity toward the y-axis at higher concentrations of acetamiprid. This indicates that both dynamic and static quenching may be involved in the fluorescence quenching of peak A. In the linear range of the Stern-Volmer regression curve, the quenching rate constants for soluble EPS and bound EPS by acetamiprid at 293 K were 3.81×10^{12} $M^{-1}s^{-1}$ (R = 0.986) and 3.14×10^{12} $M^{-1}s^{-1}$ (R = 0.988), respectively. The values for K_q were two, orders of magnitude greater than the maximum diffusion collision quenching rate constant (2.0×10^{10} $M^{-1}s^{-1}$) for a variety of quenchers with biopolymers (Hu et al., 2005). This indicates that the fluorescence quenching process of peak A for soluble/

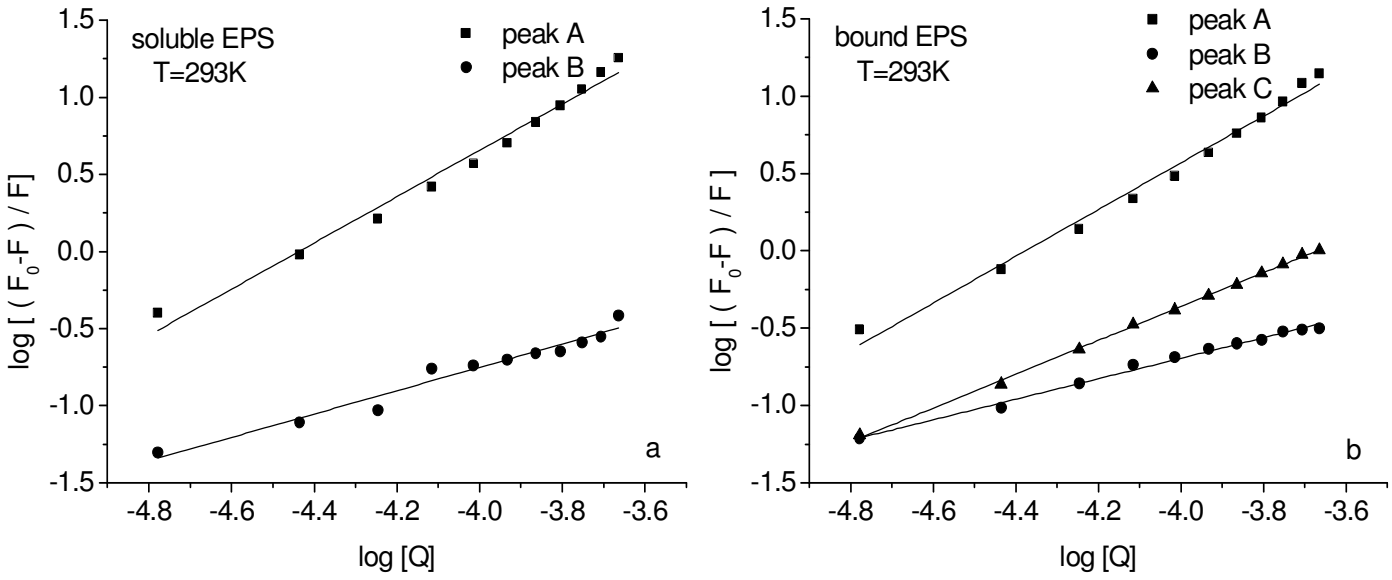

Figure 4. Plots of *log* [(*F*$_0$-*F*)/*F*$_0$] versus *log* [*Q*] for binding of acetamiprid with soluble EPS (a) and bound EPS (b).

bound EPS at low concentrations of acetamiprid may be mainly governed by a static quenching mec-hanism.

There was a good linear relationship (R = 0.9999) for the Stern-Volmer plots for peaks B and C (Figure 3) and the values of K_q were also far greater than the maximum diffusion collision quenching rate constant (Table 2). This suggests that the fluorescence quenching processes of peak B for soluble/bound EPS and peak C, for bound EPS by acetamiprid were mainly controlled by a static quenching process rather than a dynamic quenching process, as the values for K_q were much greater than the maximum diffusion collision quenching constant rate.

In summary, the probable quenching mechanism of acetamiprid-EPS interaction was predominated by complex formation.

The binding constant and binding sites

When small molecules bind independently to a set of equivalent sites on a macromolecule, fluorescence intensity data can be used to obtain the binding constants and the number of binding sites for the complex. The binding constant (K_b) and the number of binding site (n) can be determined by the following equation (Hill, 1985):

$$\log \frac{F_0 - F}{F_0} = \log K_b + n \log[Q] \quad (2)$$

Where, F_0 and F are the fluorescence intensities in the absence and presence of acetamiprid, respectively. K_b is the binding constant, n the number of binding sites, and [Q] the acetamiprid concentration. The binding constants (K_b) can reflect the interactive intensity between fluorophore and quencher. The greater the binding constant is, the greater the binding capacity of fluorophore is. The values of n express the binding sites provided by fluorophore to quencher molecule.

The plots of log [(F_0 - F)/F_0] as a function of log [Q] for soluble/bound EPS were shown in Figure 4. The correlation coefficients (R) for binding constants were larger than 0.98, indicating that the interaction of acetamiprid with EPS agrees well with the site-binding model depicted in Equation 2.

The binding constants and binding sites of acetamiprid-EPS system were listed in Table 3. The maximum binding constants for acetamiprid-soluble/bound EPS were 4.51 × 10^6 M^{-1} and 4.20 × 10^6 M^{-1}, respectively. The binding constants (K_b) of peak A were about one order of magnitude bigger than those of drug-protein and dye-protein systems (Table 3). In these studies, the binding constants of drug-BSA, dye-BSA and dye-HSA systems were usually smaller than 7 × 10^5 M^{-1}. For peaks B and C, K_b was several orders of magnitude smaller than those of peak A.

In addition, the same types of fluorophores in soluble EPS and bound EPS showed different binding capacity for acetamiprid. The binding constants of peak A for soluble/bound EPS were greater than those of peak B, though both peaks were protein-like peaks. The binding constants of the protein-like peaks (peaks A and B) for soluble EPS were greater than those for bound EPS, indicating that the soluble EPS may have a stronger binding ability to acetamiprid than the bound EPS. The values of n of peak A for both the soluble EPS and the bound EPS were greater than 1.0 (about 1.5), suggesting that more than one class of binding sites were present in

Table 3. The binding constants (K_b) and binding sites (n) for chemical-biological system in literature.

Fluorescence quenching			Binding Capacity			Reference
Fluorescent substance	Quencher	Temperature (K)	Binding constant (K_b)($Lmol^{-1}$)	Binding sites(n)	R	
BSA	*Trans*-resveratrol	293	1.95×10^6	1.230	0.999	Jiang et al., 2008
BSA	Luteolin	298	6.33×10^5	1.220	0.998	Yang et al., 2008
BSA	Azelnidipine	298	2.21×10^5	1.150	0.998	Wang et al., 2008
BSA	Sudan I	293	1.27×10^5	1.057	0.999	Zhang et al., 2008
BSA	Crystal Violet	291	2.92×10^4	0.989	0.999	Xu et al., 2009
Human serum albumin	Methyl blue	290	2.57×10^5	1.128	0.999	Song et al., 2009
Human serum albumin	C.I. Acid Red 2	298	2.55×10^5	1.190	0.998	Ding et al., 2009
Soluble EPS from activated sludge	Acetamiprid	293	4.51×10^6 (peak A)	1.499	0.992	This study
			1.89×10^2 (peak B)	0.757	0.980	
Bound EPS from activated sludge	Acetamiprid	293	4.20×10^6 (peak A)	1.513	0.994	This study
			0.91×10^2(peak B)	0.664	0.996	
			1.05×10^4 (peak C)	1.095	0.999	

fluorophores (represented by peak A) in EPS for acetamiprid. On the contrary, values of n were smaller than 1. For peak C of the bound EPS, the value of n was close to one. This means that there was exactly one class of binding sites in fulvic acid like fluorophores in EPS for acetamiprid.

Conclusion

Protein-like fluorophores and/or fluvic acid-like fluorophores were detected in the soluble EPS and bound EPS from aerobic sludge. Acetamiprid interacts with EPS and form complexes. The soluble EPS had stronger binding capacity for acetamiprid than the bound EPS. EPS of activated sludge, especially the soluble fraction, play an important role in the removal of organic pollutants.

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