Contrast of sludge toxicity variation during treatment of wastewater containing mixed chlorophenols and single chlorophenol

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Abstract
To address problems associated with the potential toxicity of sludge when it is applied in land utilization, this study investigated the variations by which activated sludge becomes toxic when fed with wastewater containing mixed chlorophenols, and compared variations in sludge toxicity due to inputs of mixed chlorophenols and single chlorophenol groups. In this study, 4-chlorophenol (4-CP), 2,4,6-trichlorophenol (2,4,6-TCP) and mixed chlorophenols which consist of both 4-CP and 2,4,6-TCP were studied in sequencing batch reactors (SBR). The results indicate that in 0-30 d, the toxicity of sludge fed with mixed chlorophenols is higher than that of sludge fed with single chlorophenol groups. During 50-100 d, the toxicity of sludge fed with mixed chlorophenols ranged between two single chlorophenol groups. EPS and protein in EPS had a significant relationship with sludge toxicity, but there was no significant relationship between polysaccharide in EPS and sludge toxicity.

Keywords: Sludge toxicity, Mixed chlorophenols, SBR, EPS, Wastewater treatment

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1. Introduction

The activated sludge process is widely used in wastewater treatment plants (WWTPs) at present, but it generates a large amount of excess sludge during the biological treatment process [1]. Excess sludge is generated during the aerobic biological treatment of municipal sewage and industrial wastewater [2]. Excess sludge is rich in nitrogen, phosphorus and other nutrients necessary for plant growth, and when used as fertilizer it improves soil properties, so it is usually discharged through landfill [3, 4]. To treat sludge in a cost-effective and safe manner and improve plant nutrients circulation in the nature environment, land utilization is predicted to be the dominant way for excess sludge treatment [5]. According to the EU, the total amount of excess sludge will be 13 million tons in 2020 and 44% of them will be recycled to the land [6]. In America, approximately 60% of sludge was used to improve soil properties or as fertilizer in 2002 [7]. During anaerobic digestion or composting, the organic toxicity in sludge will remain. In addition, the organic toxicity in sludge will affect anaerobic digestion or composting treatment, making the organic toxicity more difficult to remove [8]. However, as the organic composition of wastewater becomes more complex, it contains many types of organic pollutants that are difficult to degrade. Heavy metals such as Ni, Co, Cu, Zn, Cu and toxic organic compounds accumulate in excess sludge; thus, organic sludge toxicity has become a key factor limiting its use as fertilizer [9].

Chlorophenol (CP) is widely used in industrial production of pesticides, medicine, synthetic materials, wood preservatives, and oil refining [10, 11]. Extensive use of chlorophenol lets much chlorophenol spread into the environment, which will do harm to the skin, eyes and respiratory tracts of humans and animals [12]. 4-CP has been designated as a priority pollutant
by the United States Environmental Protection Agency because of its carcinogenic and 
mutagenic properties [13]. 2, 4, 6-trichlorophenol (2, 4, 6-TCP) has high toxicity because it 
contains three chloride groups attached to the phenol ring. Therefore, treatment of wastewater 
containing chlorophenol has recently received increased public attention. To remove 
chlorophenol from wastewater, physico-chemical methods are sometimes used [14, 15]. Physico-
chemical methods, such as adsorption and chemical oxidation, cause other toxic products to form 
and are also costly [16]. Biological treatment of wastewater containing chlorophenol is relatively 
inexpensive and effective [17, 18]. Studies show that chlorophenol can be effectively 
biodegraded using sequencing batch reactors (SBRs) [19, 20]. Considering that actual 
wastewater contains multiple chlorophenols, this study examines treatment of wastewater 
containing mixed chlorophenols and compares the effects of mixed chlorophenols and single 
chlorophenol groups on sludge toxicity variation.

EPS are commonly produced by microorganisms during biological wastewater treatment 
[21]. EPS accumulate through different mechanisms, such as bacterial secretion, cell lysis and 
hydrolysis, leakage of exocellular constituents, and adsorption of organic matter from 
surrounding wastewater [22]. EPS bind with cells through complex interactions, and protect cells 
from dewatering and destruction by toxic substances [23]. EPS in activated sludge are thought to 
join neighboring bacterial cells to each other and to inert particulate matter [24, 25]. Recent 
studies have demonstrated that the spatial distribution of EPS influences bioflocculation, sludge 
settling, and dewatering properties [26]. EPS influence surface adhesion, formation of matrix 
structure, long-term sludge stability, control of microbial physiology [27] and sludge 
dewaterability [28]. EPS are a major component of activated sludge matrix and a key factor in
inducing sludge flocculation during biological wastewater treatment [29]. Therefore, it is desirable to explore the interaction between the presence of EPS and the variation of sludge toxicity during biological treatment processes.

At present, the research on the biodegradation of chlorophenols mainly focuses on the removal efficiency of chlorophenols in water phase and sludge phase [30], the study of degradation kinetics of chlorophenols by microorganisms [31, 32], and the search for microorganisms that can degrade chlorophenols [33]. However, this research focuses on the toxicity of excess sludge in the process of biodegradation of chlorophenols, which is different from the studies relevant to chlorophenols before.

The objective of this study is: (1) to compare the variations of sludge toxicity between mixed chlorophenols group and single chlorophenol groups during biological treatment of wastewater. (2) to investigate the correlation between EPS, protein and polysaccharide content in EPS and sludge toxicity.

2. Materials and Methods

2.1. Activated Sludge and Synthetic Wastewater

Four 5 L reactors were operated as shown in Fig. 1. The SBRs were started up with conventional activated sludge taken from the sewage treatment plant at East China University of Science and Technology in Shanghai, China. The initial MLSS of sludge was around 2,500 mg/L. Synthetic wastewater was used in the experiment and the synthetic wastewater was prepared based on municipal wastewater. Methanol was used as a carbon source to control influent chemical oxygen demand (COD) at 300 ± 20 mg/L because influent COD of municipal wastewater is
around 300 mg/L [34, 35]. Urea and KH$_2$PO$_4$ were added to the reactors to meet the N, P demand of activated sludge. Ratio of initial C:N:P was kept at 100:5:1 to meet nutrient requirement of activated sludge. Trace elements such as Ca, Mg, Mn, Fe and Al were supplied, as Table S1 shows [36]. Operating temperature was kept at 20 ± 2.0°C. Adjust pH to 7.4 ± 0.2 by adding HCl and supply NaHCO$_3$ to the influent wastewater. Hydraulic retention time (HRT) and sludge retention time (SRT) were respectively 8 h and 20 d [37, 38]. Dissolved oxygen (DO) was controlled at 2.5 ± 0.5 mg/L during the operation. During SBR operation, an intermittent aeration mode was used (aeration for 2 h and stop for 2 h). When aeration stopped, activated sludge and wastewater were evenly mixed by stirring. Influent wastewater of three experimental groups contained 1) 10 mg/L 4-CP, 2) 10 mg/L 2,4,6-TCP and 3) a mixture of chlorophenols (5 mg/L 4-CP and 5 mg/L 2,4,6-TCP). A blank group was set aside for comparison. Effluent was taken every ten days and used for COD analysis. Sludge was harvested every ten days for CP analyses. Take three parallel samples at a time.

**Fig. 1.** SBR reactor diagrammatic sketch. (a) Reactor; (b) thermometer; (c) agitator; (d) dissolved oxygen meter; (e) gas flow meter; (f) aeration pump; (g) dissolved oxygen probe; (h) aeration header.
2.2. Analytical Methods

Before analysis, mixed liquor samples were centrifuged at 5000 r/min for 10 min to separate the aqueous phase from the sludge phase, and the supernatant fluid was filtered through 0.45 μm membranes. Closed reflux, titrimetric method/5220C was used to analyze COD according to the Standard Methods for the Examination of Water and Wastewater [39]. DO and pH values were determined using a portable DO meter (YSI Inc., United States) and a laboratory-grade FiveEasy™ pH meter (METTLER TOLEDO, Switzerland).

2.3. Sludge Toxicity

The luminescent bacteria test method was used to test the acute toxicity of the sludge. Sludge acute toxicity was characterized by using the freeze-dried marine bacteria Photobacterium phosphoreum (P. phosphoreum) according to the standard methods of China (GB/T 15441-1995, China). P. phosphoreum was purchased from the Institute of Soil Science, Chinese Academy of Sciences (Nanjing, China). These bacteria emit light at wavelengths of 50-490 nm, and there is a correlation between the intensity of these emissions and toxic substance content [40]. Before the experiment, a small amount of 3 % sterilized NaCl solution was used to recover the freeze-dried bacteria, and the recovered luminous bacteria were inoculated into 50 ml of sterilized culture medium. The composition of the culture medium component is shown in Table S2 [41]. pH was adjusted to 7.0 ± 0.5, and the sterilization time and temperature were 20 min and 121 ℃, respectively. Before sludge toxicity test, ultrasonic pretreatment method was used to break up the sludge flocs because it can break and crack sludge structures and it can also release cellular
components into the supernatant [42]. With ultrasonic treatment, underestimate of the sludge toxicity value tested by luminescent bacteria can be avoided [43].

Specifically, 30 mL mixed liquor was centrifuged for 10 min at a speed of 5,000 r/min. Supernatant was decanted and the sludge was suspended in phosphate buffer solution (PBS). An ultrasonic probe was placed 3 cm below the surface of the liquid. Samples were fixed in ice water and agitated ultrasonically (4 s of ultrasound followed by 4 s with no ultrasound) for 10 min at a power of 240 W. Finally, the mixed liquor was centrifuged for 10 min at 12,000 r/min and 4°C. The supernatant liquors were used to test sludge toxicity.

Use biological toxicity tester DXY-2 made by Nanjing Soil Research Institute to measure visible light intensity. Sludge toxicity was expressed in terms of inhibiting luminosity (%), as is shown in Eq. (1). To ensure accuracy, each sample was tested three times in parallel. Many studies have confirmed that sludge toxicity can obviously inhibit the luminescence of the luminescent bacteria [42] (Cao 2005), that is, sludge toxicity is positively correlated with inhibiting luminosity.

\[
\text{Inhibiting luminosity(\%)} = (1 - \frac{\text{luminosity of the sample tube}}{\text{luminosity of the blank tube}}) \times 100
\]  

(1)

2.4. Chlorophenol Content

4-CP and 2,4,6-TCP contents were analyzed in parallel by using a high performance liquid chromatograph (HPLC). The HPLC used a reversed-phase C-18 column (250 nm × 4.6 nm, 5 μm) as the stationary phase and a methanol-H\(_2\)O mixture (80:20, v/v; containing 1% acetic acid in H\(_2\)O) as the mobile phase. Flow rate was controlled at 1 mL/min, and the wavelength was 280 nm. The sample injection volume was 10 μl and the column temperature was kept at 40°C.
centrifuged 30 ml mixed liquor for 10 min at a speed of 5,000 r/min. Then decanted the supernatant and suspended the sludge in PBS. An ultrasonic probe was placed 3 cm below the surface of the liquid. Samples were fixed in ice water and agitated ultrasonically (4 s of ultrasound followed by 4 s with no ultrasound) for 10 min at a power of 240 W. Finally, the mixed liquor was centrifuged for 10 min at 12000 r/min and 4 °C. After filtering the mixed liquor through 0.45 μm membrane, the chlorophenol content in the sludge phase was determined. Dissolve chlorophenol standard samples in methanol to make 1.0 g/mL standard stock solution. Dilute the standard stock solution into several concentration gradients in accordance with test requirements. Inject 2 mL standard solution into the liquid phase tubules after filtration through 0.45 μm membrane filter. Test for chlorophenol content by HPLC. Test the peak area at the maximum absorption wavelength. Take the concentration of chlorophenol as the X axis and take the corresponding peak area as the Y axis to draw the standard curve. Three parallel samples were tested during the experiment. Detection limits of 4-CP and 2,4,6-TCP were approximately 0.01 and 0.03 mg/L. According to their peak area at the absorption wavelength, the content of chlorophenol was calculated by standard curve.

2.5. EPS Content

The ultrasound method was also used in the extraction of EPS. Centrifuged 30 mL mixed liquor for 10 min at a speed of 5,000 r/min. Decant the supernatant and suspend the sludge in PBS. Samples were fixed in ice water and agitated ultrasonically (4 s of ultrasound followed by 4 s with no ultrasound) for 10 min at a power of 240 W. Finally, the mixed liquor was centrifuged
for 10 min at 12,000 r/min and 4°C. EPS content was calculated as the sum of protein, polysaccharide and DNA contents [44].

Protein content was analyzed by using a Lowry protein assay kit made by Lida Biological Technology Company, Shanghai, following procedures given in the kit’s instructions [45]. Draw the standard curve of standard protein solution, determine the maximum absorption wavelength of protein, determine the sample absorbance, and calculate the protein content in sludge according to the standard curve. Test each sample three times in parallel.

Polysaccharide content was determined by using the anthranone-sulfuric acid method [46], which is simple and fast. The main principle underlying this method is that anthrone reacts with free hexose and aldopentose in polysaccharides. The solution turns blue-green after reaction. The resulting solution’s maximum absorbance occurs at a wavelength of 620 nm. This absorbance has a linear relationship with polysaccharide content. Draw the standard curve of polysaccharide content and absorbance. Pretreated the sludge by ultrasound. Test absorbance of supernatant and calculate polysaccharide content of samples according to the standard curve. Test each sample three times in parallel.

DNA content was tested using the diphenylamine colorimetric method using calf thymus DNA as a standard [47]. Draw the standard curve of standard DNA solution, determine the maximum absorption wavelength of DNA, determine the sample absorbance, and calculate the DNA content in sludge according to the standard curve. Test each sample three times in parallel.

3. Results and Discussion

3.1. Effluent COD Variation
To assess the degradation efficiency of different groups, effluent COD of 4-CP, 2,4,6-TCP and mixed chlorophenols was tested. The results are shown in Fig. 2. The initial influent COD of blank group, 4-CP group, 2,4,6-TCP and mixed chlorophenols group were 302 mg/L, 305 mg/L, 295 mg/L and 298 mg/L. Obtain the standard deviation of three data from each sampling, and draw the error bar. Error bars in the figure indicate standard deviations associated with the measurements displayed. Fig. 2-5 were all created in Origin 8.0.

Fig. 2. Variations of effluent COD in different groups.

Fig. 2 indicates that effluent COD of 4-CP, 2,4,6-TCP and mixed chlorophenol groups went up at first and then decreased before stabilizing at a low level. Effluent COD was high during 10-30 d, because during this period sludge activity was inhibited by chlorophenols, so the degradation efficiency was low [48]. Effluent COD of 4-CP, 2,4,6-TCP and mixed chlorophenol groups fluctuated within ranges of 100-160 mg/L, 120-180 mg/L and 170-220 mg/L, respectively, and the highest COD values appeared at 20 d. During 30-50 d, effluent COD decreased sharply. The effluent COD of 4-CP group decreased from 107 mg/L to 53 mg/L, 2,4,6-TCP group from 122
mg/L to 69 mg/L, and mixed chlorophenols group from 174 mg/L to 68 mg/L. This result occurred because, as the activated sludge became acclimated to chlorophenols, microorganisms which could degrade the target pollutants began to accumulate and sludge activity increased gradually [49]. After 50 d, effluent COD reached a stable level because the activated sludge had successfully acclimated to the chlorophenols and COD removal efficiency was high [20]. COD values of 4-CP, 2,4,6-TCP and mixed chlorophenol groups were about 48 mg/L, 65 mg/L, and 58 mg/L, respectively. The COD removal efficiency of 4-CP, 2,4,6-TCP and mixed chlorophenols groups are 84%, 78% and 81%. Zheng reported that COD removal efficiency decreased from 90% to 70% after adding 2 mg/L of 2,4,6-TCP in the SBR, and finally stabilized at 84% after several days [50]. Effluent COD of blank group was obviously lower than chlorophenols groups at every stage. Although the activated sludge can acclimate to chlorophenol, it cannot completely eliminate the toxicity of chlorophenol, chlorophenol still has certain inhibitory effect on activated sludge, so the COD of chlorophenol groups is much higher than that of blank group. At the beginning, the COD of blank group 52 mg/L, which was relatively high. After 40 d, COD of blank group reached 23 mg/L and maintained at a stable level. Based on this analysis, 0-30 d was regarded as the early stage of SBR operation, 30-50 d was regarded as the transition stage of SBR operation, and 50-100 d was regarded as the stable stage of SBR operation.

3.2. Sludge Toxicity Variation

The research focused on variation of sludge toxicity during SBR operation and differences in sludge toxicity among 4-CP, 2,4,6-TCP and mixed chlorophenol groups. The sludge toxicity variations are shown in Fig. 3.
As is shown in Fig. 3, during the whole process of SBR operation, sludge toxicity of blank group remained at a stable and low level, fluctuated in 14.2-15.3%. On day 0, the organic toxicity of 4-CP group, 2,4,6-TCP group and mixed chlorophenols group were 25.4%, 27.5% and 30.5%. Compared with the blank group, whose toxicity was 14.2% on day 0, the toxicity increased by 78.9%, 93.7% and 114%, respectively, which indicated that the chlorphenol organics could significantly increase the organic toxicity of sludge. Sludge toxicity induced by all 4-CP, 2,4,6-TCP and mixed chlorophenols increased at first and then decreased before attaining a relatively stable level. During 0-30 d, sludge toxicity first reached the peak and then decreased, and sludge toxicity was highest for the mixed chlorophenols and lowest for 4-CP, with 2,4,6-TCP having intermediate toxicity. Sludge toxicity of 4-CP, 2,4,6-TCP and the mixed chlorophenol groups fluctuated over the ranges 25.4-32.3%, 27.5-35.9% and 30.5-50.5%, respectively. The highest sludge toxicity appeared at 20 d, and the highest toxicities were 32.3%, 35.9% and 50.5%, respectively, and the differences among different groups were 18.2% and 14.6%. Zhao reported the sludge acute toxicity in acclimated SBR was significantly higher than the control SBR, and
gradually increased before 37 d, and after 48 d, the sludge acute toxicity in acclimated SBR was relatively stable [51], which is similar to this study. During the transition stage, the sludge toxicity associated with mixed chlorophenol groups decreased sharply from 43.5% to 25.3%. During the stable stage, the sludge toxicity associated with mixed chlorophenol groups was lower than that of 2,4,6-TCP, so sludge toxicity was greatest for 2,4,6-TCP and lowest for 4-CP, with the mixed chlorophenols having intermediate toxicity. During the stable stage, the sludge toxicity of 4-CP, 2,4,6-TCP and mixed chlorophenol groups remained stable at a low level, fluctuating over ranges of 16.8-18.5%, 24.2-24.3% and 20.5-25.4%, respectively. Average sludge toxicity values were 17.2%, 24.3% and 21.7%, respectively. Differences in toxicity among the three groups were 4.5% and 2.6%. Based on the above analysis, it was inferred that, during the stable stage, the sludge toxicity associated with 2,4,6-TCP was highest and 4-CP showed the lowest values, with results for mixed chlorophenols in the middle. Sludge toxicity differences among three groups are less than 10% during the stable stage.

3.3. Correlations among Chlorophenol Content, EPS Content and Sludge Toxicity

In the treatment of wastewater containing toxic organic substances, sludge toxicity can be caused by residues from toxic substances, intermediate products produced by microbial metabolism [52] and secretions produced by activated sludge under stress [53]. Since EPS is a major component of activated sludge matrix and a key factor in inducing sludge flocculation during biological wastewater treatment [29], the removal of chlorophenol and correlation between EPS and sludge toxicity were analyzed in mixed chlorophenols group and single chlorophenol groups.
3.3.1. Variations of chlorophenol content

To investigate the removal efficiency of chlorophenol absorbed in the sludge phase, the concentration of chlorophenol was measured. The results are shown in Fig. 4. The concentrations of chlorophenol absorbed in the sludge phase were expressed in mg/L. The detection limits associated with 4-CP and 2,4,6-TCP concentrations were 0.01 mg/L and 0.03 mg/L, respectively.

![Graph showing variations of chlorophenol content](image)

**Fig. 4** Concentrations of chlorophenol absorbed in sludge phases in different groups.

As is shown in Fig. 4, concentrations of 4-CP, 2,4,6-TCP and mixed chlorophenol groups went up at first, then decreased, before finally stabilizing at a low level. Content of chlorophenol absorbed in sludge increased during 0-10 d because sludge activity was inhibited [54]. Therefore, degradation efficiency was low, and chlorophenol contents were high. Maximum concentrations of 4-CP, 2,4,6-TCP and mixed chlorophenol in the sludge phases appeared at 10 d, when concentrations were 4.59 mg/L, 5.97 mg/L and 9.05 mg/L, respectively. As the activated sludge acclimated to different chlorophenols, bacteria which could degrade chlorophenols became more concentrated in the sludge [55], and sludge activity started to recover [56]. During the period
between 10 and 60 d, content of chlorophenol absorbed in the sludge phases decreased rapidly because the activated sludge was in an adaptation period, and microbial communities which could degrade chlorophenols began to grow. During 10-60 d, concentrations of 4-CP, 2,4,6-TCP and mixed chlorophenol fluctuated over ranges of 0.20-4.59 mg/L, 0.21-5.97 mg/L and 0.50-9.05 mg/L, respectively. 60 days after reactors began to operate, concentrations of target pollutant residue in the sludge phases reached at a stable low level. After 60 d, concentrations of 4-CP, 2,4,6-TCP and mixed chlorophenol were about 0.18 mg/L, 0.21 mg/L and 0.24 mg/L. This result occurred because the activated sludge had acclimated successfully and therefore degraded chlorophenols effectively.

3.3.2. Correlation between EPS content and sludge toxicity

During the early stage, chlorophenol concentration in sludge phase was high, which may lead to the high sludge toxicity. During the stable stage, target pollutant residue was low, but sludge toxicity still existed. To further examine the relationship between secretions produced by activated sludge and sludge toxicity, EPS content variation and its correlation with sludge toxicity were investigated. EPS content variation are shown in Fig. 5.
Fig. 5. Variations of EPS content in different groups.

As Fig. 5 shows, EPS content in the sludge of blank group remained at a stable and low level, fluctuated in 37-43 mg/L. During the early stage, EPS content increased sharply over the period 0-20 d. The EPS content of 4-CP group increased from 80 mg/L to 98 mg/L, 2,4,6-TCP group from 83 mg/L to 107 mg/L, and mixed chlorophenol group from 105 mg/L to 145 mg/L. During this period, the activated sludge was disturbed by chlorophenols, and its stress response was obvious. To protect its cells, the activated sludge secreted large amounts of EPS [57]. The EPS content in sludge fed with mixed chlorophenol groups was higher than that in single chlorophenol groups. The EPS content was highest for mixed chlorophenols, lowest for 4-CP, and intermediate for 2,4,6-TCP. The highest EPS contents in 4-CP, 2,4,6-TCP and mixed chlorophenol groups appeared at 20 d, when they reached 99.3 mg/L, 108.7 mg/L and 143.5 mg/L, respectively. Because the activated sludge fed with mixed chlorophenol groups was disturbed by both 4-CP and 2,4,6-TCP at the same time, the microorganisms fed with mixed chlorophenols showed a stronger stress response, and secreted more EPS, than those fed with single chlorophenol groups. In biological wastewater treatment processes, some pollutants
seriously inhibit microbial activity [58]. It was inferred that the presence of 2,4,6-TCP in the sludge phase seriously inhibits sludge activity. This inhibition was stronger than that associated with 4-CP, so activated sludge fed with 2,4,6-TCP secreted more EPS than activated sludge fed with 4-CP. The EPS content in the sludge phase decreased sharply between 20 and 50 d because the activated sludge began to adapt to the disturbance caused by the target pollutant. The EPS content of 4-CP group increased from 98 mg/L to 54 mg/L, 2,4,6-TCP group from 107 mg/L to 72 mg/L, and mixed chlorophenol group from 145 mg/L to 75 mg/L. Some protein was degraded by the activated sludge [59], and EPS content decreased rapidly. After 50 d, the EPS content of sludge phase fed with 4-CP, 2,4,6-TCP and mixed-chlorophenol groups maintained relatively stable levels, and remained within the ranges 48.4-54.6 mg/L, 72.6-73.3 mg/L and 61.5-76.2 mg/L, respectively. This result occurred because the activated sludge had acclimated successfully in the stable stage. Therefore, the microbiological community grew steadily and could degrade the target pollutants under high loads [60], and the EPS content remained at a stable level.

Correlation between EPS content and sludge toxicity was investigated. EPS secreted by microorganisms of activated sludge protects cells from toxic substances [61]. There may be a correlation between EPS content and sludge toxicity. According to the data in Fig. 5, linear regression was performed, setting EPS content as the independent variable and sludge toxicity as the dependent variable. The results are shown in Table 1.

Table 1. Correlations between EPS Content, Protein content, Polysaccharide Content and Sludge Toxicity

| Substance Type of SBR | R2 |
|-----------------------|----|
|                       |    |
Table 1 indicates that the EPS content was highly correlated with sludge toxicity. For
sludge fed with 4-CP, 2,4,6-TCP and mixed chlorophenols, correlation coefficients ($R^2$) were
0.967, 0.990 and 0.949, respectively. Activated sludge microorganisms secreted large amount of
EPS under the stress of different chlorophenols. Therefore, when sludge toxicity was high, EPS
content fluctuated at a high level.

Protein and polysaccharide are major constituents of EPS [62]. Variation of protein and
polysaccharide content were measured. The results are shown in Fig. S1-S4.

As is shown in Fig. S1-S4, protein and polysaccharide contents of blank group remained at a low
level, protein content fluctuated in 21-26 mg/L, and polysaccharide content fluctuated in 10-13
mg/L. Protein and polysaccharide contents in chlorophenols groups were high during 10-40 d.
The protein content of sludge fed with 4-CP, 2,4,6-TCP and mixed chlorophenol groups varied
within the ranges of 38.70-69.58 mg/L, 49.50-75.22 mg/L and 62.70-96.10 mg/L, respectively.
Highest values of the three groups were 69.58 mg/L, 75.22 mg/L and 96.10 mg/L, respectively
and appeared at 20 d. Mixed chlorophenols produced the highest protein content, 4-CP produced
the lowest protein content, and 2,4,6-TCP yielded values in between. The polysaccharide
contents of sludge fed with 4-CP, 2,4,6-TCP and mixed chlorophenol groups varied within the
ranges of 19.10-28.09 mg/L, 24.75-32.70 mg/L and 19.62-45.17 mg/L, respectively. Highest values of three groups were 28.09 mg/L, 32.70 mg/L and 45.17 mg/L, respectively and appeared at 20 d. As with protein content, sludge fed with mixed chlorophenols yielded the highest polysaccharide content, 4-CP produced the lowest polysaccharide content, and 2,4,6-TCP yielded intermediate values. As for mixed chlorophenols group, the exposure to both 4-CP and 2,4,6-TCP caused microbial cells to secrete more stress proteins and polysaccharides to protect cell structure and function. Therefore, the protein and polysaccharide contents of sludge fed with mixed chlorophenols were high. 2,4,6-TCP inhibited sludge activity, and this inhibition was stronger than that of 4-CP, so activated sludge in 2,4,6-TCP group secreted more protein and polysaccharide than that of 4-CP group.

Protein and polysaccharide contents maintained relatively steady levels during 50-100 d. The protein content of sludge fed with 4-CP, 2,4,6-TCP and mixed chlorophenol groups varied over the ranges 29.16-32.76 mg/L, 43.56-43.98 mg/L and 36.90-45.72 mg/L, respectively. The polysaccharide content of sludge fed with 4-CP, 2,4,6-TCP and mixed chlorophenol groups varied over the ranges 17.12-19.10 mg/L, 21.78-21.99 mg/L and 18.83-19.60 mg/L, respectively. 2,4,6-TCP group yielded the highest protein and polysaccharide content, 4-CP group gave the lowest, and mixed chlorophenols produced intermediate content. The activated sludge was successfully acclimated during 50-100 d, and microbiological communities which can degrade chlorophenols grew steadily. Therefore, protein and polysaccharide secretion declined and remained at low levels. Activated sludge microorganism in the mixed chlorophenols formed microbiological communities which can degrade both 4-CP and 2,4,6-TCP, and the adaptability
to environment was better than 2,4,6-TCP group, so protein and polysaccharide content were lower than 2,4,6-TCP group.

Linear regression was performed, setting protein and polysaccharide content as the independent variable, and sludge toxicity as the dependent variable. The results are shown in Table 1.

Table 1 indicates that the protein content and sludge toxicity were highly correlated. Correlation coefficients ($R^2$) associated with 4-CP, 2,4,6-TCP and mixed chlorophenol groups were 0.929, 0.938 and 0.963, respectively. The polysaccharide content and sludge toxicity were not well correlated. Correlation coefficients ($R^2$) associated with 4-CP, 2,4,6-TCP and mixed chlorophenol groups were 0.693, 0.504 and 0.691, respectively. Since protein and polysaccharide are secreted by activated sludge cells, this result indicates that protein secreted by microorganisms in activated sludge had significant correlation with sludge toxicity, but polysaccharide had no significant correlation with sludge toxicity. Guang found that in activated sludge, 97.5% - 98.3% protein is concentrated in the inner layer of EPS, while polysaccharide is more evenly distributed in the of EPS [63]. In addition, studies have shown that the outer layer of EPS in sludge mainly play a role to adsorb 4-CP and transfer to the inner layer [64]. It is speculated that protein may be more important than polysaccharide in maintaining sludge activity and metabolism of chlorophenols [65]. Therefore, there is a significant correlation between the protein in the inner layer of EPS and the toxicity of sludge, while the correlation between the polysaccharide uniformly distributed in EPS and sludge toxicity is not significant.

4. Conclusions
During 0-30 d, strong stress response was induced in the activated sludge because of inhibition and activated sludge secreted EPS to protect cells. The toxicity of sludge and the content of EPS increased to a high level and then decreased. The toxicity of sludge fed with mixed chlorophenols is higher than that of sludge fed with single chlorophenol groups. During 30-50 d, the activated sludge began to adapt to the interference of target pollutants, the toxicity of sludge and the content of EPS kept decreasing. During 50-100 d, the activated sludge has been successfully adapted to the target pollutants in the stable stage. Toxicity of sludge kept stable and toxicity of sludge fed with mixed chlorophenols ranged between two single chlorophenol groups.

In order to explore the contribution of EPS and its components to sludge toxicity, the correlation between the content of EPS and its components and sludge toxicity was studied in detail. The results showed that EPS and protein in EPS had a significant relationship with sludge toxicity, but there was no significant relationship between polysaccharide in EPS and sludge toxicity. Possible reason is that protein is more important than polysaccharide in maintaining sludge activity and metabolism of chlorophenols.

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Author Contributions

Y.W. (Master student) conducted all the experiments and wrote the manuscript. X.C. (Professor)
revised the manuscript. Q.L. (Master student), Y.Y. (Master student), X.W. (Master student), S.W. (Master student), Q.L. (Master student), X.S. (Master student) wrote and revised the manuscript.

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