Correlation between the uncoupling metabolism induced by 2,4,6-trichlorophenol and sludge toxicity in sequence batch reactors

Xiurong Chen, Quanling Lu, Shanshan Wang, Xiaoli Sun, Qiuyue Li, Xiao Wei, Yingying Yang and Yuan Wang

ABSTRACT

The correlation between sludge reduction induced by 2,4,6-trichlorophenol (2,4,6-TCP) as an uncoupler and sludge toxicity was investigated in sequence batch reactors over a 100-d operation period. The influent concentrations of 2,4,6-TCP tested were 10 mg/L, 30 mg/L, and 50 mg/L. Sludge reduction, chemical oxygen demand (COD) removal rate, and sludge toxicity were measured. The results showed that from 30 to 80 d, when the COD removal rate was at an acceptable level, the sludge reduction levels for the 10 mg/L, 30 mg/L, and 50 mg/L groups were 9.7%, 31.6%, and 41.5%, respectively, and the average sludge toxicity values were 24.2%, 38.0%, and 53.0%, respectively. Sludge reduction was positively correlated with sludge toxicity. The two-dimensional polyacrylamide gel electrophoresis/results showed that extracellular and intracellular proteins secreted by the activated sludge during uncoupling metabolism were positively correlated with sludge toxicity. Taking the COD removal rate, sludge reduction, and sludge toxicity into consideration, the optimal influent concentration of the uncoupler 2,4,6-TCP was 30 mg/L when the initial mixed liquid suspended solids of sludge was 2,500 mg/L.

Key words | 2,4,6-TCP, SBR, sludge reduction, sludge toxicity, uncoupling metabolism

HIGHLIGHTS

- Significant positive correlation between sludge toxicity and uncoupling metabolism was found.
- There is negative correlation between chemical oxygen demand removal and sludge toxicity.
- Microbes secretions produced under 2,4,6-TCP inhibition led to sludge toxicity.
- Effective uncoupling and high sludge toxicity both occurred with 2,4,6-TCP inhibition.
- The optimal 2,4,6-TCP concentration for uncoupling and toxicity of sludge was 30 mg/L.

INTRODUCTION

Excess sludge generated during the biological treatment process for wastewater leads to significant environmental risks and high disposal costs because it contains high levels of organics and heavy metals (Li et al. 2016). Therefore, it is necessary to reduce the production of excess sludge during the treatment process rather than post-treatment. An effective approach to solve this problem is to develop in situ treatment technologies that can limit excess sludge production from the source (Yang et al. 2012). Different in situ treatment processes for excess sludge have been applied, which are based on four mechanisms: lysis-cryptic growth, predation on bacteria, uncoupling metabolism, and maintenance metabolism (Pérez-Elvira et al. 2006). Among these methods, the uncoupling metabolism method has been proven to be a promising approach, and it is convenient, easy to perform, and highly efficient because it is simple to add an aeration tank to a wastewater treatment plant, which avoids modifying the conventional wastewater treatment process (Rho et al. 2006).
The concept behind metabolic uncoupling involves dissociating the energy coupling between catabolism and anabolism; thus, a portion of the energy extracted from substrates would be wasted, which leads to the production of less bacterial cell mass (Rho et al. 2007).

The effects on reducing excess sludge and influence of chemical uncouplers on the removal of pollutants have been investigated widely, and the feasibility of using chemical uncouplers to reduce excess sludge has drawn much attention. 3,3’,4’,5-Tetrachlorosalicylanilide (TCS), a component in the formulation of soaps, rinses, and shampoos, has been considered an environmentally benign metabolic uncoupler (Li et al. 2022a, 2022b) and might be effective if applied to limit excess sludge generation (Chen et al. 2002). Strand et al. (1999) compared the effects of 12 chemical uncouplers on cell yields in batch cultures and found that 2,4,5-trichlorophenol (2,4,5-TCP) was the most effective. Feng et al. (2015) found that the combination of TCS and 2,4,6-TCP effectively reduced the sludge yield without seriously influencing substrate removal. Zheng et al. (2008) proved that 2 mg/L 2,4,6-TCP could reduce sludge generation by about 47% without an obvious effect on chemical oxygen demand (COD) removal efficiency and sludge settling performance in a sequence batch reactor (SBR). Breitenstein et al. (2007) found that 2,4,6-TCP could accumulate anaerobic degrading bacteria through domesticated sludge, and aerobic treatment could achieve complete mineralization after anaerobic removing chlorine substituents. Chen et al. (2006) proved that 2,4,6-TCP had a good effect on the reduction of the sludge, without causing a significant increase in the nitrogen/phosphorus (N/P) content of the SBR effluent, and was basically feasible in cost operation. However, characteristics such as sludge toxicity during uncoupling metabolism still require further investigation.

Most chemical uncouplers are potentially harmful to the microorganisms in the biological wastewater treatment process. Qiao et al. (2012) reported that 2,4,6-TCP might inhibit the activity of the microbial community, leading to poor performance during the treatment process. Toxins that accumulate in activated sludge when treating refractory organics can pose a risk to human health through the food chain when excess sludge is recycled into the soil (Wang & Zhang 2010). Therefore, it is necessary to take sludge toxicity into consideration during land applications. It has been proven that the biodegradation of complicated pollutants, intermediate biodegradation products, and bacterial secretions all contribute to sludge toxicity (Barret et al. 2002). Some studies have shown that stress effects from toxic pollutants stimulate microorganisms to produce toxic soluble microbial products (Yu et al. 2006) and stress proteins (Bott & Love 2001). Zhao et al. (2016) demonstrated that sludge toxicity was closely related to microbial diversity during the biological treatment of wastewater containing 4-chlorophenol. Yang et al. (2014) found a positive correlation between sludge toxicity and N,N-dimethylformamide (DMF) concentration in sludge during the biological treatment of wastewater containing DMF in an SBR. Henriques & Love (2007) concluded that extracellular polymeric substances (EPSs) in activated sludge, which provide a protective barrier for the bacterial community in activated sludge and affect the cell yield rate, possibly interact with some toxic chemicals. Exploring the secretion (especially proteins) of activated sludge during the biological treatment of wastewater is necessary for describing the sludge toxicity formation process.

Based on the above discussion, a more comprehensive understanding of the correlation between excess sludge reduction and sludge toxicity induced by metabolic uncouplers in the biological wastewater treatment system is of great importance and deserves further investigation. The purposes of this research are as follows: (1) to determine the correlation between sludge reduction efficiency and sludge toxicity caused by 2,4,6-TCP as an uncoupler during a 100-d operation period; (2) to explore the relationship between protein and sludge toxicity during the biological treatment of 2,4,6-TCP; and (3) to select the optimal influent concentration of 2,4,6-TCP for both better sludge reduction efficiency and lower sludge toxicity.

**MATERIALS AND METHODS**

**Cultivation of activated sludge**

Four 5-L available capacity reactors were used, as shown in Figure 1. SBRs were started up with activated sludge from a wastewater treatment plant at East China University of Science and Technology in Shanghai, China. Synthetic wastewater was used in the experiment, and methanol was used as a carbon source. Influent COD was kept at 300 ± 20 mg/L. Urea and potassium dihydrogen phosphate (KH2PO4) were added to the SBR to provide the N and P requirements. The initial C:N:P was kept at 100:5:1. Trace elements such as Ca, Mg, Mn, Fe, and Al were added to the SBR. The operating temperature was kept at 20 ± 2 °C. Sodium bicarbonate and hydrochloric acid were added to the influent to maintain the desired alkalinity and pH (7.4 ± 0.2). The hydraulic retention time and sludge retention time were set at 8 h and 20 d, respectively. Dissolved oxygen (DO) was controlled at 2.5 ± 0.5 mg/L. An
intermittent aeration process was used, i.e. an aeration reaction of 2 h (stirring) and anoxic process of 2 h (no stirring) through electrically switchable grating. During the aeration process, sludge and water were mixed evenly with an agitator. The 2,4,6-TCP concentrations tested in the influent were 10 mg/L, 30 mg/L, and 50 mg/L.

**Chemicals and analytical methods**

Before analysis, mixed liquor samples were centrifuged at 5,000 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. COD and mixed liquid suspended solids (MLSS) were measured according to the Standard Methods (APHA et al. 2017). DO and pH values were measured with a portable DO meter (YSI, USA) and laboratory FiveEasy™ pH meter (METTLER TOLEDO, Switzerland).

**Sludge toxicity analysis**

The luminescent bacteria test method was used to test the acute toxicity of activated sludge. Sludge toxicity was characterized using the freeze-dried marine bacteria *Photobacterium phosphoreum* according to the Analytical Methods of Water and Wastewater in China, (National Environmental Protection Agency Book Edition Committee 2002). *P. phosphoreum* was purchased from the Institute of Soil Science, Chinese Academy of Sciences (Nanjing, China). Recovered luminescent bacteria can emit a specific visible light intensity at wavelengths of 50–490 nm. There is a correlation between light intensity and the toxic substance content (Li et al. 2012a, 2012b). Before the experiment, 3% sterilized NaCl was used to recover the freeze-dried powder, and the recovered luminescent bacteria were inoculated into 50 mL sterilized culture medium. The components of the culture medium are shown in Table 1. The pH, sterilization time, and temperature were kept at 7.0 ± 0.5, 20 min, and 121 °C, respectively. Sludge toxicity was tested when the luminescent bacteria were in the exponential growth phase. Before testing, the sludge structure had to be broken and cracked; otherwise, sludge toxicity would be underestimated, and so activated sludge was pretreated as follows.

Ultrasound treatment was used to break sludge flocs because it could crack the sludge structure so that components inside cells were released into the supernatant. Then, 30 mL sludge–water liquor was centrifuged for 10 min at 5,000 r/min. The supernatant was removed, and the sludge was suspended in 0.1 M phosphate buffer solution. A blank SBR containing no chlorophenol was established as the comparison group. An ultrasonic probe was placed 3 cm below the liquid surface. The samples were ultrasonicated for 10 min (ultrasound for 4 s and rest for 4 s) at 240 W. After ultrasonating, the liquid mixture was centrifuged for 10 min at 12,000 r/min and 4 °C. Then, samples were filtered through 0.45-μm membranes.

Visible light intensity was tested using a DXY-2 biological toxicity tester made by Nanjing Soil Research Institute. Sludge toxicity was determined by the inhibiting luminosity (%), as shown in Equation (1). In order to ensure accuracy, each sample was tested three times in parallel. Many scholars have confirmed that sludge toxicity can inhibit the luminescence of luminescent bacteria (Li et al. 2012a, 2012b), that is, sludge toxicity is positively correlated with inhibition of luminosity:

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

![Figure 1](image-url)  
**Figure 1** SBR reactor diagrammatic sketch. 1: reactor; 2: thermometer; 3: agitator; 4: dissolved oxygen meter; 5: gas flow meter; 6: aeration pump; 7: dissolved oxygen probe; 8: aeration header.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg L⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Tryptone</td>
<td>5</td>
</tr>
<tr>
<td>Yeast powder</td>
<td>5</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3</td>
</tr>
<tr>
<td>NaCl</td>
<td>30</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>5</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: Table 1 shows the culture medium components for luminescent bacteria.
Protein analysis

Protein extraction

At the end of the SBR cycle, samples of 5 mL activated sludge were shocked with an ultrasonic instrument at room temperature (running 6 s and resting 3 s) for 15 min and then centrifuged for 15 min at 13,000 r/min and 4 °C. The supernatants were collected and transferred into 1.5 mL tubes.

The extracted protein samples were transferred to sterile dialysis bags and placed in flowing distilled water for 48 h. The dialyzed protein samples were collected into centrifuge tubes and placed in a –20 °C refrigerator for 24 h. Finally, the floculent proteins were obtained from the frozen samples after drying for 48 h in a vacuum freeze dryer. For contrastive analysis, an activated sludge sample from the blank group was also treated according to the above approach.

 Isoelectric focusing (IEF)

A tube of aqueous buffer was removed from the refrigerator and dissolved at room temperature. Then, 0.01 g dithiothreitol, 2.5 μL Bio-Lyte 4–6, and 2.5 μL Bio-Lyte 5–7 were added to the tubes and mixed evenly. A 400 μL sample of water buffer was removed from each tube, 100 μL of a sample was added, and they were mixed evenly. Prefabricated immobilized pH gradient (IPG) gel strips (17 cm, pH 4–10) were taken out of a –20 °C refrigerator, samples were added along the edge of the focusing disk, and the protective layer on the prefabricated IPG gel strips was removed with tweezers. Noting the positive and negative poles of the IPG gel strips, they were placed on the sample solution in the focusing disk making sure that the positive pole of the IPG gel strip corresponded to the positive pole of the focusing disk. Each strip was covered with 2–3 mL mineral oil to avoid water evaporation during the hydration process of the strip. The lid was placed, and the isoelectric focusing process was started.

 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

The floculent proteins were dissolved in distilled water, and the protein concentration was measured using a microplate reader (Multiskan MK3, Thermo). To maintain uniform protein concentrations among the samples, different amounts of protein samples were added to 1.5 mL Eppendorf tube and diluted with distilled water to 40 μL, and 10 μL SDS-PAGE sample loading buffer (5x) (Beyotime) was added to 50 μL for storage until used. Different protein samples were separated on a 4% stacking gel and a 10% separating gel in a Mini-PROTEAN 3 Tetra Electrophoresis System (Bio-Rad). Five microliters of the markers was used, and 40 μL of each sample was injected into the sample well. The voltage of the electrophoresis apparatus (EPS-300, Shanghai Tanon) was increased to 100 V after the protein sample moved away from the stacking gel. Separation continued until the bromophenol blue band reached the bottom of the gel. The entire SDS–PAGE process took approximately 2.5 h. The gel was washed 1–2 times with distilled water, stained with Coomassie Brilliant Blue R-250 for 1 h, and then destained and photographed. The gel was kept in a 4 °C refrigerator in plastic wrap until further analysis.

Protein identification

The significant protein bands in the SDS–PAGE image were first excised and digested according to Katayama et al. (2010) and then identified using ABI 5800 matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF)/TOF plus MS. The excised bands were rinsed twice with ultrapure water and destained for 30 min at room temperature with 25 mmol/L ammonium bicarbonate (NH₄HCO₃) and 50% acetonitrile (ACN). After removing the destaining solution, the bands were sequentially dehydrated for 30 min using dehydration solutions 1 (50% ACN) and 2 (100% ACN) and then vacuum freeze-dried. The dried gels were then sequentially treated for 1 h in reduction solution 1 (10 mM dithiothreitol and 25 mM NH₄HCO₃) at 57 °C and 30 min in reduction solution 2 (50 mM iodoacetamide and 25 mM NH₄HCO₃) at room temperature. The bands were sequentially dehydrated a second time for 30 min in dehydration solutions 1 and 2 after adding imbibition solution (25 mM NH₄HCO₃) at room temperature and rehydrated for 30 min in a cover solution of 25 mM NH₄HCO₃ and 10% ACN containing 0.02 μg/μL trypsin. A double volume of cover solution was added again for digestion for 16 h at 37 °C. The supernatant was transferred to another tube, and the gels were extracted for 30 min with extraction buffer containing 5% trifluoroacetic acid (TFA) and 67% ACN at 37 °C and centrifuged for 5 min at 5,000 r/min. The gels were combined with the supernatants and then completely freeze-dried. The dried samples were resuspended in 5 μL of 0.1% TFA, followed by mixing at a 1:1 ratio with a substrate containing a saturated solution of a-cyano-4-hydroxytrans-cinnamic acid in 50% ACN and 0.1% TFA. Finally, 1 μL of the mixtures was spotted onto a stainless steel sample target plate, and peptide MS and
tandem mass spectrometry (MS/MS) were performed on an ABI 5800 MALDI-TOF/TOF plus MS instrument.

Data acquisition and analysis

Data were acquired in a positive MS reflector using a CalMix 5 standard to calibrate the instrument (ABI 5800 Calibration Mixture). MS and MS/MS data were integrated with GPS Explore V 3.6 software (Applied Biosystems, USA) using the default parameters. From the combined MS and MS/MS spectra, proteins were identified based on a 95% or higher confidence interval of their scores in the MASCOT V 2.3 search engine (Matrix Science Ltd, London, UK) using the following search parameters: the National Center for Biotechnology Information nr database; trypsin as the digestion enzyme; one missed cleavage site; fixed modification of carbamidomethyl (C); partial modifications of acetyl (protein N-term), deamidated (NQ), deoxidation (W), and oxidation (M); 100 ppm for precursor ion tolerance; and 0.3 Da for fragment ion tolerance.

Expression of the uncoupling effect

The MLSS of the blank group was controlled at 2,500 ± 100 mg/L. The sludge discharge of the blank group was 100 mL, whereas no sludge was discharged in the control groups. The MLSS of sludge in each group was measured. Sludge dry weight was calculated according to Equation (2), taking the sludge discharge of the blank group into account. The difference in sludge dry weight was calculated according to Equation (3). A curve of the difference in sludge dry weight was drawn, and the uncoupling efficiency was evaluated using the slope of the curve (rate of variation of the sludge dry weight difference):

\[ W = \text{MLSS} \times \text{V} \quad (2) \]

\[ \Delta W = W_E + W_B - W_C \quad (3) \]

In Equation (2), \( W \) represents sludge dry weight, mg; MLSS represents mixed liquid suspended solids concentration, mg/L; and \( \text{V} \) represents reactor volume, L:

\[ \Delta W = W_E + W_B - W_C \]

In Equation (3), \( \Delta W \) represents sludge dry weight difference, mg; \( W_E \) represents discharged sludge dry weight of the blank group, mg; \( W_B \) represents sludge dry weight of the blank group, mg; and \( W_C \) represents sludge dry weight of the control groups, mg.

RESULTS AND DISCUSSION

Uncoupling efficiency

Figure 2(a) and 2(b) show the MLSS of the control and blank groups and the sludge dry weight difference between the blank and control groups. As shown in Figure 2(a), the MLSS of the blank group fluctuated at a stable level around 2,500 mg/L, whereas the uncoupling efficiency of the control groups decreased over time. From 10 to 60 d, the uncoupling efficiency of the control groups was strong, and the MLSS of the control groups decreased rapidly. The MLSS values of the 10 mg/L group, 30 mg/L group, and 50 mg/L group decreased by 16.1%, 36.2%, and 52.7%, respectively. The uncoupling efficiency of the control groups was weaker from 80 to 100 days of operation, and the MLSS remained...
at a stable level. During 10–60 d, bacteria in the sludge were not induced to the degree that can fully degrade a high concentration of 2,4,6-TCP, so the uncoupling efficiency was strong. From 80 to 100 d, the biodegradation mechanism of bacteria in the sludge was better regulated, and the inhibition on the sludge caused by the high concentration of 2,4,6-TCP was relieved; thus, the uncoupling efficiency decreased. The uncoupling efficiencies of the three 2,4,6-TCP groups during the same period were as follows: 50 mg/L group > 30 mg/L group > 10 mg/L group. During 80–100 d, the uncoupling efficiency increased gradually with the increase in the 2,4,6-TCP concentration. The average MLSS of the 10 mg/L group, 30 mg/L group, and 50 mg/L group were 2,015 mg/L, 1,422 mg/L and 1,066 mg/L, respectively. The higher the concentration of the uncoupler 2,4,6-TCP in the influent, the stronger the inhibitory effect on the activated sludge and the higher the uncoupling efficiency.

As shown in Figure 2(b), from 10 to 60 d, the sludge dry weight difference between the blank and control groups gradually increased over time because of the strong uncoupling efficiency of 2,4,6-TCP. The sludge dry weight difference between the blank and control groups was significant as the influent 2,4,6-TCP concentration increased (p < 0.05), which is shown in Table 2. The uncoupling efficiency caused by 2,4,6-TCP decreased during 60–80 d, and the difference of dry weight of 2,4,6-TCP and blank group began to stabilize. During 80–100 d, the uncoupling efficiency was weak, and the dry weight difference of 2,4,6-TCP and blank group were 2,363 mg, 5,325 mg, and 7,108 mg, respectively. In conclusion, the results shown in Figure 2(b) are similar to those in Figure 2(a), showing that the uncoupling efficiency was strong during 10–60 d. The higher the uncoupler 2,4,6-TCP concentration in the influent, the stronger the uncoupling efficiency. This is due to the fact that a high concentration of uncoupler strongly inhibits the sludge activity (Song et al. 2010), leading to a high uncoupling efficiency.

Effect of the uncoupling mechanism on substrate degradation and sludge toxicity

COD removal rate in the aqueous phase

Figure 3 shows the effluent COD of the blank and control groups. The effluent COD of the blank group was significantly lower than that of 2,4,6-TCP groups. During the significant uncoupling stage, the effluent COD values of the control groups all first increased and then decreased. From 0 to 30 d, the effluent COD was at a high level, and the effluent COD values of the 10 mg/L, 30 mg/L, and 50 mg/L groups were in the range of 100–180 mg/L, 150–210 mg/L, and 170–250 mg/L, respectively. During 30–60 d, the effluent COD decreased fast and fluctuated in the range of 60–130 mg/L, 100–170 mg/L, and 160–210 mg/L, respectively. After 60 d, the effluent COD of 2,4,6-TCP maintained at steady state, and the values were 67 mg/L, 99 mg/L, and 164 mg/L, respectively. This is because in the early stage, the organic pollutants in the influent had a certain inhibitory effect on the sludge, and the bacteria in the sludge were impacted (Chen et al. 2015). As seen in Figure 3, the COD removal rate in the control groups was significant and that of the 50 mg/L group was relatively poor compared with those of the 10 mg/L and 30 mg/L groups. This is because a higher concentration of organic contaminants in the influent has a stronger inhibitory effect on activated sludge, and the microorganisms take longer to grow steadily (Monsalvo et al. 2009). Figure 4 shows the variation of 2,4,6-TCP content in aqueous and sludge phase. Consistent with the change trend of COD, the 2,4,6-TCP content in aqueous phase and sludge phase both increased first, then decreased and remained at a stable level. After 50 days of SBR operation, the 2,4,6-TCP content in aqueous phase and sludge phase in the 10 mg/L, 30 mg/L, and 50 mg/L groups was 0.28 mg/L and 0.36 mg/L; 0.39 mg/L and 0.21 mg/L; and

Table 2 | LSD-T-test of sludge dry weight between blank group and control group

<table>
<thead>
<tr>
<th>Control group</th>
<th>Mean</th>
<th>Distinctiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/L group</td>
<td>2,284 ± 116</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>30 mg/L group</td>
<td>5,137 ± 116</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>50 mg/L group</td>
<td>7,048 ± 116</td>
<td>&lt; 0.001</td>
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</table>

Figure 3 | Effluent COD of the blank and control groups.
0.29 mg/L and 0.31 mg/L, respectively, and the removal rate was over 97%. It was considered that in the initial stage of the SBR operation, the impact of 2,4,6-TCP inhibited the activity of sludge, the removal of 2,4,6-TCP was mainly based on adsorption with less degradation, and so the 2,4,6-TCP content showed an increasing trend. With the extension of the acclimatization time, the dominant bacteria degrading 2,4,6-TCP gradually enriched, allowing the sludge activity to recover. The removal effect of 2,4,6-TCP gradually increased, and the content of 2,4,6-TCP gradually decreased to a stable level (Xie et al. 1977). Analyzing the results of the COD removal rate and sludge reduction efficiency, the significant uncoupling stage was from 30 to 80 days during the whole SBR operation period.

**Sludge toxicity**

*Figure 5* shows the sludge toxicity of the blank and control groups during the whole SBR operation period. As shown in *Figure 5*, sludge toxicity in the blank group was always stable and low (14.2–15.3%), whereas in the control groups, sludge toxicity fluctuated greatly. From 0 to 30 d, sludge toxicity in the 10 mg/L, 30 mg/L, and 50 mg/L groups was in the range of 27.5–35.9%, 43.1–49.9%, and 52.0–65.0%, respectively. The peak value appeared on the 20th day. After 60 d, sludge toxicity tended to be stable, and sludge toxicity in the 10 mg/L, 30 mg/L, and 50 mg/L groups fluctuated in the range of 24.2–24.3%, 36.0–39.4%, and 51.8–55%, respectively, and the average values were 24.2%, 37.7%, and 53.2%, respectively. Compared with the sludge reduction efficiency and COD removal rate in the aqueous phase, the period when sludge toxicity was at a high level was consistent with that when sludge reduction and COD removal efficiency were high. From 0 to 30 d, a higher concentration of 2,4,6-TCP in the influent led to significant inhibition of the activated sludge. Sludge toxicity increased as the 2,4,6-TCP concentration increased. The difference in the peak sludge toxicity between the 50 mg/L and 30 mg/L groups was 13.1%, and that between the 30 mg/L and 10 mg/L groups was 14.0%. This is because the higher the 2,4,6-TCP concentration in the influent, the stronger the inhibitory effect on the activated sludge.

**Correlation between sludge toxicity and the uncoupling mechanism**

From the above analysis, under the condition of an acceptable COD removal rate, the period for a significant uncoupling effect was 30–80 d during the whole SBR operation period. The correlation between sludge toxicity and the uncoupling effect was investigated during 30–80 d. Taking the sludge discharge rate of the blank group of 100 mL per day into account, the slope K (variation rate of the sludge dry weight difference) for the 30–80 d section of the curve in *Figure 2(b)* was calculated, and K was used to characterize the uncoupling effect induced by 2,4,6-TCP. Sludge toxicity was set as the x value, K was set as the y value, and a linear regression analysis of the x and y values was performed. The correlations between the K and sludge toxicity of the control groups are shown in Table 3, which proves that K was positively correlated...
with the sludge toxicity in the 10 mg/L, 30 mg/L, and 50 mg/L groups, and the correlation coefficients \( R^2 \) were 0.920, 0.902, and 0.932, respectively.

From 30 to 80 d, sludge toxicity and \( K \) both decreased, and the uncoupling effect gradually weakened. Sludge toxicity induced by 2,4,6-TCP was highly correlated with the uncoupling effect. During the uncoupling process, the absorbed 2,4,6-TCP in the sludge phase had an inhibitory effect on the luminescent bacteria. In the period when uncoupling effect was strong, activated sludge secreted some toxic substances, increasing sludge toxicity. In addition, the change of sludge characteristic may also induce the increase of sludge toxicity.

### Relationship between polysaccharide, protein, and sludge toxicity

#### Variation of polysaccharide content

Studies have shown that when activated sludge degrades organic pollutants with certain biological toxicity, the content of protein and polysaccharide in the extracellular polymer increases significantly. Figure 6 showed the variation of polysaccharide content in sludge phase. The polysaccharide content in the control group is obviously higher than that in the blank group at the initial stage of SBR operation. The polysaccharide content in the blank group was always stable at around 21 mg/L, and the maximum polysaccharide content in the control group reached 32.7 mg/L on the 20th day. It was considered that the sludge was impacted by 2,4,6-TCP in the initial stage of acclimation of activated sludge, resulting in a strong stress response, and microorganisms produced a lot of secretions under stress, so the polysaccharide content was at a high level. After SBR was operated for 50 days, the polysaccharide content in the control group was not significantly different from that in the blank group. The polysaccharide had little influence on sludge toxicity.

**Table 3** | Correlation between \( K \) and sludge toxicity

<table>
<thead>
<tr>
<th>SBR type</th>
<th>Linear formula</th>
<th>( R^2 )</th>
</tr>
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<tbody>
<tr>
<td>10 mg/L</td>
<td>( y = 8.108x - 176.97 )</td>
<td>0.920</td>
</tr>
<tr>
<td>30 mg/L</td>
<td>( y = 19.377x - 707.18 )</td>
<td>0.902</td>
</tr>
<tr>
<td>50 mg/L</td>
<td>( y = 25.134x - 1,355.2 )</td>
<td>0.932</td>
</tr>
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</table>

#### Two-dimensional polyacrylamide gel electrophoresis of protein

Proteins (including stress enzymes, metabolic enzymes, and constituent proteins) have a complex structure, and these species are significantly affected by the operating conditions and pollutant components as one of the sludge secretions. Thus, proteins are closely related to the response mechanism of sludge under inhibition conditions. In this study, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to compare differences in highly expressed proteins between the blank and control groups, and the contribution of total proteins secreted by activated sludge to the toxicity was studied.

2D-PAGE images from the blank and control groups are shown in Figure 7. There were many differences among the highly expressed proteins between the blank and control groups. Some protein spots were obviously up-regulated, whereas some were obviously down-regulated. These up-regulated proteins are stress proteins secreted in response to the effect of 2,4,6-TCP. The down-regulated proteins are proteins involved in transcription and translation for cell growth. Sludge toxicity caused by 2,4,6-TCP leads to slower cell growth and metabolism, leading to the reduced expression of these proteins.

#### Relationship between protein and sludge toxicity

Some highly expressed proteins with significant differences between the blank and control groups were identified by MS, and the results are shown in Table 4. When bacteria in activated sludge were inhibited by 2,4,6-TCP, the composition of their cell surface changed. Some components of microbial secretions attached to the cell surface to decrease the permeability of cells (Ramos et al. 2003). Similar over-expressed proteins shown in Table 4 include a hypothetical protein (protein spots 6) and periplasmic binding protein.
(protein spots 4), and the over-expression of these proteins can protect microbes from the toxic effects of 2,4,6-TCP. In addition, the expression of adenosine triphosphate (ATP) synthase (protein spot 3) and nicotinamide adenine dinucleotide dehydrogenase (protein spot 5) also significantly increased, because microbes required more energy to synthesize intracellular ATP and resist the toxic effects of 2,4,6-TCP. When microorganisms are exposed to toxic organic contaminants, large amounts of oxygen free radicals are induced during redox processes, which may damage the plasma membrane system and DNA, leading to protein denaturation and lipid peroxidation. Under stress conditions, elongation factor Tu, which is responsible for peptide extension during microbial protein synthesis, becomes expressed (Len et al. 2004). In this study, we identified a variety of over-expressed induction proteins caused by 2,4,6-TCP such as elongation factor Tu (protein spot 2) and ribosomal protein (protein spots 1 and 7). Therefore, during the significant uncoupling effect period, when using 2,4,6-TCP as an uncoupler, the total protein secreted by bacteria increased, which led to the increase of sludge toxicity.

Optimum influent concentration of 2,4,6-TCP

2,4,6-TCP significantly affected sludge reduction efficiency and toxic inhibition on the flora in activated sludge, which lead to sludge toxicity. According to the effluent COD, MLSS at 30 d and 80 d, and sludge toxicity from 30 to 80 d, the COD removal rate, sludge reduction rate, and average sludge toxicity were calculated (Table 5), and the optimal influent concentration of 2,4,6-TCP was determined considering these parameters.

It can be seen in Table 5 that the COD removal rate decreased as the influent 2,4,6-TCP concentration increased, and the sludge reduction rate and average sludge toxicity increased as the influent 2,4,6-TCP concentration increased. Pearson correlation analysis (Table 6) showed that influent 2,4,6-TCP concentration was significantly correlated with COD removal rate, sludge reduction rate, and sludge toxicity ($p < 0.05$). A t-test showed that the difference in sludge reduction rate between 10 to 30 mg/L and 30 to 50 mg/L ($p = 0.0001$) was more significant than the difference in the sludge toxicity ($p = 0.01$). The difference in the COD removal rate between the 10 mg/L and 30 mg/L groups

Table 4 | identification of highly expressed differential proteins

<table>
<thead>
<tr>
<th>Number</th>
<th>Protein sequence number</th>
<th>Protein</th>
<th>Molecular weight</th>
<th>Score/matching polypeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gi</td>
<td>672855373</td>
<td>30S ribosomal protein S1, partial</td>
<td>58,270</td>
</tr>
<tr>
<td>2</td>
<td>gi</td>
<td>445953923</td>
<td>elongation factor Tu, partial</td>
<td>41,465</td>
</tr>
<tr>
<td>3</td>
<td>gi</td>
<td>519088784</td>
<td>F0F1 ATP synthase subunit alpha</td>
<td>55,146</td>
</tr>
<tr>
<td>4</td>
<td>gi</td>
<td>485769266</td>
<td>periplasmic oligopeptide-binding protein</td>
<td>60,681</td>
</tr>
<tr>
<td>5</td>
<td>gi</td>
<td>477071656</td>
<td>NADP-specific glutamate dehydrogenase</td>
<td>45,164</td>
</tr>
<tr>
<td>6</td>
<td>gi</td>
<td>446472797</td>
<td>hypothetical protein</td>
<td>38,647</td>
</tr>
<tr>
<td>7</td>
<td>gi</td>
<td>851933140</td>
<td>30S ribosomal protein S3, partial</td>
<td>18,688</td>
</tr>
</tbody>
</table>
was 10.7%, whereas the differences in the sludge reduction rate and average sludge toxicity were 21.9% and 13.8%, respectively, which were significant. When the influent 2,4,6-TCP concentration increased from 30 mg/L to 50 mg/L, the sludge reduction rate did not reach the same magnitude as that when the concentration was increased from 10 mg/L to 30 mg/L. The difference in the sludge reduction rate between the 30 mg/L and 50 mg/L groups was only 9.9%, whereas the sludge toxicity of the 50 mg/L group was significantly higher than that of the 30 mg/L group (15.0%) and the COD removal rate decreased by 22.0%, indicating a small decrease in the COD removal efficiency. Considering the effect of the 2,4,6-TCP influent concentration on the substrate removal rate, sludge reduction, and sludge toxicity, the optimum influent concentration of 2,4,6-TCP was 30 mg/L.

### CONCLUSION

During the stable operation of SBR, the sludge reduction in the 10, 30, and 50 mg/L groups can reach 9.7%, 31.6%, and 41.5%, respectively, and the average sludge toxicity is 24.2%, 37.7%, and 55.2%, respectively. Microbes in the activated sludge were inhibited by 2,4,6-TCP, and they produced secretions, especially intracellular and extracellular proteins, which were significantly correlated with sludge toxicity. When the influent 2,4,6-TCP concentration increased from 10 mg/L to 30 mg/L and from 30 mg/L to 50 mg/L, the COD removal rate decreased 10.7% and 22.0%, respectively, and sludge toxicity increased 13.8% and 15.0%, respectively. The optimal influent concentration of 2,4,6-TCP was 30 mg/L when the initial MLSS of activated sludge was 2,500 mg/L.

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### DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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