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Original Research

Toxicity Evaluation of Antibiotics in Piggery Wastewater by Luminescent Bacteria

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Abstract

Antibiotics, as special pollutants in piggery wastewater, have negative effects on wastewater treatment processes. However, it is difficult to obtain a clear understanding of the toxic potency of antibiotics using the common inhibitory experiments of wastewater because of their low concentrations. Therefore, this study for the first time reports research on examining the toxic strength of piggery wastewater containing antibiotics by acute toxicity experiments using luminescent bacteria. Also reported herein for the first time were the mixed toxic effects of different antibiotics on luminescent bacteria using orthogonal experimental designs. Results showed that piggery wastewater had high ecotoxicity equal to 24.6 $mg\cdot L^{-1}$ $HgCl_2$, and the toxicity units (TU) of chlortetracycline, amoxicillin, florfenicol, and sulfamethazine (SM_2) were 85.5, 64.72, 1.27, and 1.20, respectively, which fell under high or significant ecotoxicity according to the Toxicity Classification System (TCS). The IC_{10} values indicated that the antibiotics could impose single or mixed toxic effects on luminescent bacteria even in trace concentrations. Among these antibiotics, chlortetracycline was the most significant one needing more attention in the treatment of wastewater-containing antibiotics.

Keywords: piggery wastewater, luminescent bacteria, antibiotics, toxicity, trace concentration

Introduction

The treatment and disposal of piggery wastewater is a challenging task for its high concentration of chemical oxygen demand (COD), pathogenic bacteria, feed additives and inhibitors. Among the inhibitors, antibiotics commonly used for therapeutical purposes or as disinfectants in pig farms were found in piggery wastewater or water bodies [1-4] because of their low bioavailability [3, 5-7]. Christian et al. reported the concentration of amoxicillin, mezlocillin, flucloxacillin, and piperacillin in surface water to be 48 $ng\cdot L^{-1}$, and Campagnolo et al. found the maximum concentration of tetracycline antibiotics to be 540 $\mu g\cdot L^{-1}$. These antibiotics can have negative effects on the piggery wastewater treatment process. Some researchers have investigated the inhibitory effects of antibiotics on piggery wastewater treatment processes [3, 8-10]. For example, Lallai et al. studied the effects of amoxicillin trihydrate, oxytetracycline hydrochloride and thiamphenicol on anaerobic digestion of piggery wastewater using concentrations of 60 and 120 $mg\cdot l^{-1}$, 125 and 250 $mg\cdot l^{-1}$, and 80 and 160 $mg\cdot l^{-1}$, respectively. Sanz et al. investigated the inhibitory effects of antibiotics on wastewater treatment processes using a concentration range of 10 to 500 $mg\cdot l^{-1}$. However, all these studies, adopting the designated concentrations of antibiotics higher than commonly used in livestock, couldn't practically describe the toxic features of antibiotics in real concentrations in piggery wastewater. In the current study, the toxic-

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ity of antibiotics existing in wastewater at low concentrations was investigated by luminescent bacteria.

Luminescent bacteria can emit stable glaucous visible light with the maximum apex between 450 and 490 nm under normal physiological conditions, while the bioluminescence will be changed with the addition of toxic chemicals. The variations of bioluminescence are correlated with the concentration of the toxic chemicals [11-13]. Therefore, luminescent bacteria have been widely used in evaluating and monitoring toxicants in the environment for its specific photogenic characterization [14-18]. Belkin et al. used this technology for monitoring the environmental threat to bacteria. Gotzl et al. obtained similar results between the luminescent bacteria method and the standard toxicological test method during toxicity measurement.

The toxicity test method of luminescent bacteria was adopted in this study for:

1) quantitatively and qualitatively investigating the toxicity of piggery wastewater
2) determining the toxicity classification and toxic effects of four common antibiotics in trace concentrations by IC_{50} (50% inhibition concentration) and IC_{10} (10% inhibition concentration) values
3) finding the significant factor(s) and mixed effects of different antibiotics at trace levels

Materials and Methods

Chemicals

The mercury bichloride ($HgCl_2$) and sodium chloride (NaCl) used in this study were of analytical or technical grade (purity 99.5%), of which standard solution and stock solution (3% NaCl, v/v) were made separately. Chlortetracycline, amoxicillin, florfenicol, and sulfamethazine (SM_2) were all purchased from a special animal medicine company in China. Raw piggery wastewater was collected from a piggery wastewater treatment plant in Hangzhou, China, and its characteristics and antibiotics content were measured and listed in Table 1. Concentration levels of the antibiotics and dilution multiples of the raw piggery wastewater was prepared by 3% NaCl.

Microbial Agent

Freeze-dried powder of photobacterium phosphoreum (PP), packaged in ampules, was purchased from Nanjing Zhongkeyuan Kuake Sci. Tech. Co., Ltd, China. The PP was reactivated by injecting 1.0 ml of 3% NaCl into the ampule and thoroughly mixing. The bacteria suspension was transferred into a serum bottle prior to use. 10-μl of PP suspension was taken from the serum bottle into a 2-ml tube containing 2 ml of 3% NaCl solution and mixed completely by hand. The tube was then put into a biotoxicity analyzer for measuring the luminescent stability of PP: if the value displayed was above 600 mV, the PP could be used for this experiment, otherwise, new PP would be reactivated.

Table 1. Characteristics of raw piggery wastewater.

Parameters	Concentration
TCOD ($mg \cdot l^{-1}$)	8500±110
BOD ($mg \cdot l^{-1}$)	3700±120
NH_4-N ($mg \cdot l^{-1}$)	370±35
TP ($mg \cdot l^{-1}$)	55±4.2
Amoxicillin ($mg \cdot l^{-1}$)	0.45±0.15
Chlortetracycline ($mg \cdot l^{-1}$)	0.65±0.18
Florfenicol ($mg \cdot l^{-1}$)	0.20±0.12
Sulfamethazine (SM_2) ($mg \cdot l^{-1}$)	—
Zinc bacitracin ($mg \cdot l^{-1}$)	—
pH	6.7±0.3

Neither zinc bacitracin nor sulfamethazine was detected.

Experimental Design

Acute Toxicity Experiment of Piggery Wastewater

Acute toxicity experiments were conducted using standard method [19]. The relative and mean luminescence intensities were calculated based on equations (1) and (2):

$$\text{RLI (\%)} = \text{Luminescence of Standard solution or Samples (mV)} \,/\, \text{Luminescence of control (mV)} \qquad (1)$$

...where: RLI – Relative Luminescence Intensity,

$$\text{MRLI} = \Sigma\,(\text{RLIs}) / n \qquad (2)$$

...where: mean relative luminescence intensity – MRLI, "n" is the number of repeated tests, and in this experiment "n" was 3.

The concentration range of standard solution ($HgCl_2$) was prepared from 0.02 to 0.24 $mg \cdot l^{-1}$ and the dilution multiples of piggery wastewater were from 2 to 256 folds. Luminescence was measured using a DXY-2 biotoxicity analyzer (made from Nanjing Zhongkeyuan Kuake Sci. Tech. Co., Ltd, China). The reaction time was precisely controlled with 15 mins at ambient temperature below 15ºC.

Acute toxicity Experiment of Antibiotics

Four different antibiotics were selected in this section: chlortetracycline amoxicillin, florfenicol, and SM_2, separately belonging to tetracycline, β-lactam, chloramphenicol and sulfanilamide groups of antibiotics, which were commonly used as feed additives in livestock. The concentration gradients of antibiotics were listed in Table 2. Dose-response curves were fitted on the basis of the relationship between MRLI of PP and concentrations of antibiotics,

Table 2. Concentration gradients of antibiotics.

Concentration ($mg \cdot l^{-1}$)			
Chlortetracycline	Amoxicillin	Florfenicol	SM_2
0.4	5.0	2.0	5.0
0.8	20.0	4.0	20.0
1.0	40.0	8.0	40.0
2.0	80.0	16.0	80.0
4.0	160.0	32.0	160.0

The concentration gradients were confirmed from the pre-experiment results.

Table 3. Factors and levels in orthogonal experiment.

Levels ($mg \cdot l^{-1}$)	Factors		
	A	B	C
1	0.3	1.0	10.0
2	0.5	4.0	20.0
3	0.7	8.0	40.0

A, B, and C separately represented chlortetracycline, florfenicol and amoxicillin, and the selected concentration was from the acute toxicity of a single antibiotic.

from which IC_{10} and IC_{50} (10% inhibitory concentration and 50% inhibiting concentration) were calculated. Data analysis and curves fitting were performed using Microsoft Excel 2007.

Mixed Toxic Effects Experiment of Antibiotics

Chlortetracycline, amoxicillin, and florfenicol, separately representing different toxicity classifications obtained from acute toxicity experiment of antibiotics, were chosen for this experiment. The mixed toxic effects experiment was carried out by an L_{27} (3^{13}) orthogonal experimental design with three factors at three levels (Tables 3 and 4). There were in total 27 groups of runs according to the orthogonal experimental design and one test without any antibiotic was set as a control. The described testing protocol was performed in triplicate. The MRLI of all runs was measured using the method described in the chapter "Acute Toxicity Experiment of Piggery Wastewater."

Results and Discussion

Acute Toxicity of Piggery Wastewater

The relationships between MRLI and the concentration of standard solution ($HgCl_2$ solution), as well as dilution multiples of piggery wastewater, were established according to linear regressions (Fig. 1), from which two direct correlation equations, Eqs. (3) and (4), were separately obtained. The quantitative toxicity of piggery wastewater was estimated by comparing dilution folds with the concentrations of a standard solution based on IC_{50}. From equation (3), the IC_{50} of $HgCl_2$ was calculated to be 0.0985 $mg \cdot l^{-1}$, the toxicity unit (TU) of which was 1015 based on equation (5) and the toxicity classification is belonging to class 4, very high ecotoxicity, according to the toxicity classification system (TCS) [20]. From equation (4), the dilution multiple of piggery wastewater was 250 folds when the MRLI was half of the control, meaning that the concentration of total toxicants contained in the piggery wastewater was equal to 24.6 $mg \cdot l^{-1}$ of $HgCl_2$ (250×0.0985 $mg \cdot l^{-1}$). The raw piggery wastewater also belongs to Class 4, i.e. very high ecotoxicity according to the TCS system.

$$Y = -709.68x + 119.96 \qquad R^2 = 0.974 \qquad (3)$$

$$Y = 0.2081x - 1.7089 \qquad R^2 = 0.997 \qquad (4)$$

$$\text{Toxicity Unit (TU)} = 100/IC_{50} \qquad (5)$$

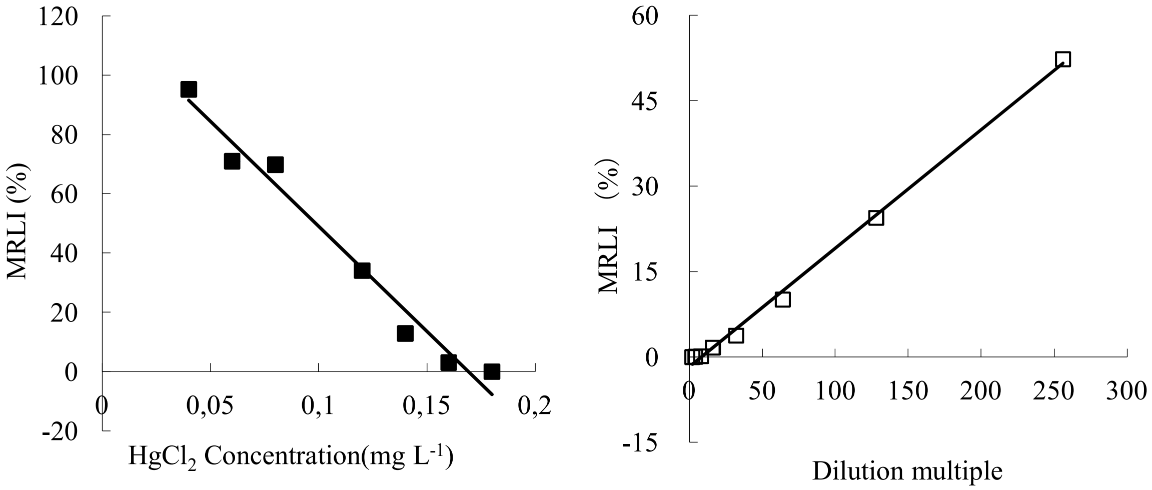

Fig. 1. Dependence of MRLI on $HgCl_2$ concentration and dilution multiple of piggery wastewater (MRLI, mean relative luminescence intensity).

Table 4. Orthogonal experiment of L_{27} (3^{13}).

Runs	A	B	$(A\times B)_1$	$(A\times B)_2$	C	$(A\times C)_1$	$(A\times C)_2$	$(B\times C)_1$	e_1	e_2	$(B\times C)_2$	e_3	e_4
1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	2	2	2	2	2	2	2	2	2
3	1	1	1	1	3	3	3	3	3	3	3	3	3
4	1	2	2	2	1	1	1	2	2	2	3	3	3
5	1	2	2	2	2	2	2	3	3	3	1	1	1
6	1	2	2	2	3	3	3	1	1	1	2	2	2
7	1	3	3	3	1	1	1	3	3	3	2	2	2
8	1	3	3	3	2	2	2	1	1	1	3	3	3
9	1	3	3	3	3	3	3	2	2	2	1	1	1
10	2	1	2	3	1	2	3	1	2	3	1	2	3
11	2	1	2	3	2	3	1	2	3	1	2	3	1
12	2	1	2	3	3	1	2	3	1	2	3	1	2
13	2	2	3	1	1	2	3	2	3	1	3	1	2
14	2	2	3	1	2	3	1	3	1	2	1	2	3
15	2	2	3	1	3	1	2	1	2	3	2	3	1
16	2	3	1	2	1	2	3	3	1	2	2	3	1
17	2	3	1	2	2	3	1	1	2	3	3	1	2
18	2	3	1	2	3	1	2	2	3	1	1	2	3
19	3	1	3	2	1	3	2	1	3	2	1	3	2
20	3	1	3	2	2	1	3	2	1	3	2	1	3
21	3	1	3	2	3	2	1	3	2	1	3	2	1
22	3	2	1	3	1	3	2	2	1	3	3	2	1
23	3	2	1	3	2	1	3	3	2	1	1	3	2
24	3	2	1	3	3	2	1	1	3	2	2	1	3
25	3	3	2	1	1	3	2	3	2	1	2	1	3
26	3	3	2	1	2	1	3	1	3	2	3	2	1
27	3	3	2	1	3	2	1	2	1	3	1	3	2

A, B, and C separately represented chlortetracycline, florfenicol, and amoxicillin. $(A\times B)_1$ and $(A\times B)_2$, $(A\times C)_1$ and $(A\times C)_2$, $(B\times C)_1$ and $(B\times xC)_2$ expressed the interaction between chlortetracycline and florfenicol, chlortetracycline and amoxicillin, florfenicol and amoxicillin. e_1, e_2, e_3, and e_4 stand for errors.

The results of this experiment explained the reason that methane production in anaerobic digestion of swine wastewater was lower than the expected values [21-24]. Pagilla et al. [24] obtained a methane production of 0.26-0.39 $L\cdot g^{-1}$ VS fed during a two-stage anaerobic process treating swine waste, but Chen [22] estimated the ultimate methane yields of anaerobic digestion from swine waste to be between 0.44 and 0.52 $L\cdot g^{-1}$ VS fed, regardless of the operating temperature. Our previous study reported that the methane production rate of anaerobic digestion of piggery wastewater was inhibited by 40% in comparison with that of sucrose synthetic wastewater. As is known, there are many kinds of antibiotics in piggery wastewater in low levels, which should deserve thorough investigation in terms of their individual or mixed toxic features.

Acute Toxicity of Antibiotics

As shown in Fig. 2, four kinds of antibiotics were all proved to be toxic to PP because MRLI decreased with the increasing concentration of antibiotics. IC_{10}, IC_{50}, and TU values could be calculated from the fitting equation based on the experimental data, as shown in Table 5, from which the IC_{50} of chlortetracycline appeared to be the lowest

Table 5. IC_{10}, IC_{50}, and TU values of antibiotics.

Antibiotics	IC_{10} (mg·l^{-1})	IC_{50} (mg·l^{-1})	R^2	TU	Toxicity classification
Chlortetracycline	0.30	1.17	0.9476	85.50	High ecotoxicity
Florfenicol	0.19	15.45	0.8560	64.72	
Amoxicillin	1.16	78.62	0.9326	1.27	Significant ecotoxicity
SM_2	2.81	83.00	0.8643	1.20	

IC_{10} and IC_{50} values were calculated from regression equation in Fig. 2. TU values were calculated from equation (5).

among the four antibiotics and that of SM_2 was the highest, indicating that chlortetracycline had the strongest toxicity to PP and SM_2 had the weakest. The toxicity classification of chlortetracycline amoxicillin, florfenicol, and SM_2 were separately belonging to Class 3 (high ecotoxicity) and Class 2 (significant ecotoxicity), which could fall under the category of significant ecotoxicity based on TCS. Also found herein was that the IC_{10} of the four antibiotics were very low, which indicated that the inhibitory effects could happen in trace concentrations. In the current experiment, it was found that the concentrations of chlortetracycline and florfenicol was 0.65±0.18 and 0.20±0.12 mg·l^{-1}, respectively, in piggery wastewater (Table 1), which exceeded the IC_{10} value and actually imposed a negative effect on water body and the water treatment plant. Varel and Hashimoto reported that the manure slurry from cattle fed on monensin completely inhibited methane production in mesophilic and thermophilic bioreactors, which required nearly 40 days for the microorganisms to recover and produce methane again [25]. Fischer et al. found that when lincomycin was added to swine diet for control of dysentery at a rate of 40 mg/kg of feed, a pilot scale digester failed twice and could not be revived [26]. All these findings suggest that the researchers and engineers must pay enough attention to the trace concentration of antibiotics existing in piggery wastewater.

Mixed Toxic Effects of Antibiotics

The mixed toxicity of chlortetracycline, florfenicol, and amoxicillin in different concentrations was investigated by an orthogonal experimental design. The MRLI inhibitory rate of each run was calculated from the percentage of mean D-value between samples and controls. The results were listed in Table 6, from which it could be seen that run 5 possessed the lowest inhibitory rate and run 27 bore the highest inhibitory rate. With the exception of run 1, where the

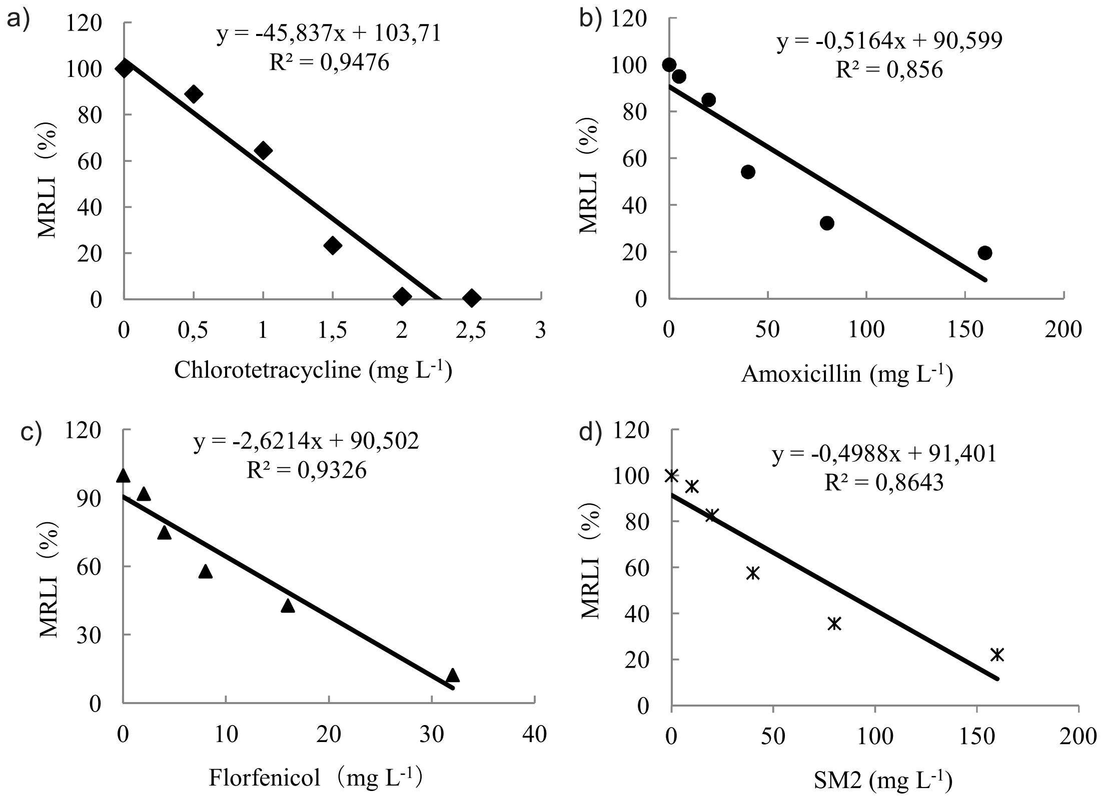

Fig. 2. Relationship between MRLI and concentration of antibiotics, and the linear fitting curve of the MRLI (a, b, c, and d were separately represented Chlorotetracycline, amoxicillin, florfenicol, and SM_2. MRLI was the mean relative luminescence intensity).

Table 6. MRLI inhibitory rate of compound antibiotic under different levels.

Run No.	Inhibitory rate (%)	Run No.	Inhibitory rate (%)
1	32.7	15	51.0
2	33.3	16	60.1
3	34.8	17	61.2
4	34.4	18	61.7
5	12.1	19	64.6
6	14.8	20	52.5
7	14.1	21	59.5
8	33.9	22	61.5
9	34.9	23	66.7
10	34.8	24	69.8
11	41.5	25	67.9
12	45.5	26	78.0
13	45.9	27	79.5
14	48.4	-	-

Table 7. Significance analysis of antibiotics.

	F-value	A	B	C	(A×B)	(A×C)	(B×C)
$F_{0.1}(2.8)$	3.11	-	3.52	0.43	1.30	0.1	0.6
$F_{0.05}(2.8)$	4.46	-	-	-	-	-	-
$F_{0.01}(2.8)$	8.65	25.7	-	-	-	-	-

A, B, and C were separately represented by chlortetracycline, florfenicol and amoxicillin. (A×B), (A×C), and (B×C) expressed the interaction between chlortetracycline and florfenicol, chlortetracycline and amoxicillin, and florfenicol and amoxicillin.

antibiotics were in first-level concentrations, the MRLI inhibitory rate of other runs were obviously lower than the simple addition of three antibiotics. This implied that the interactions of antibiotics were almost antagonistic. In other words, the toxic effects of three antibiotics were decreased when they existed simultaneously in the same media. So far, past studies on interactions of different groups of antibiotics have primarily centered on cases in the pharmaceutical field [27-29] and there is seldom information available in the environmental protection field [30]. Sun et al. studied the combined effects of four antibiotics on anaerobic digestion of piggery wastewater and reported that the relationships between florfenicol and doxycycline, florfenicol, and amoxicillin were antagonistic, which supported the results of ours. In the present investigation, the interactions of antibiotics were not observed to have a synergetic toxic effect regardless of low or high concentrations.

As discussed earlier, the MRLI inhibitory rate increased along with the concentration of mixed antibiotics; however, which antibiotic(s) was the most significant factor had yet to be determined, i.e. its change in concentration would significantly influence the inhibitory effects in the test. Therefore, a variance analysis method was employed to indentify the significant factor(s) through *F*-test, which is a statistically valid measurement of how well the factors describe the variation in the data about its mean. The calculated *F* values for the three antibiotics and the interaction of two antibiotics are shown in Table 7, from which it can be seen that chlortetracycline is the most significant factor followed by florfenicol, while amoxicillin and the interaction of two antibiotics were not significant. This result was no different than that with the results of acute toxicity experiment of a single antibiotic where chlortetracycline was found to have the lowest IC_{50} value.

The method adopted in the current study could be extensively used for finding principal contradiction among many inhibitors in wastewater and helping identify the valid measures to enhance the effectiveness of wastewater treatment processes according to the features of key inhibitors.

Conclusion

On the basis of the IC_{50} values obtained from this study, the toxicity of piggery wastewater was equal to 24.6 $mg \cdot l^{-1}$ of $HgCl_2$, belonging to the very high ecotoxicity classification, and antibiotics fell under the high or significant ecotoxicity category according to the Toxicity Classification System. The IC_{10} values of chlortetracycline, amoxicillin, florfenicol, and sulfamethazine were 0.3, 1.16, 0.19, and 2.81 $mg \cdot l^{-1}$, respectively, which suggested that antibiotics could cause an inhibitory effect even in trace concentrations. The mixed effects of antibiotics appeared to have no synergetic toxic effect. Chlortetracycline was found to be the most significant factor, followed by florfenicol and amoxicillin.

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