Optimization and mechanism of copepod zooplankton inactivation using ozone oxidation in drinking water treatment

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ABSTRACT

Bench-scale experiments were carried out to optimize ozone oxidation conditions for copepod inactivation by response surface methodology. Independent variables were ozone dosage (1, 3 and 5 mg/L), contact time (5, 15 and 25 min) and organic matter concentration (2, 5 and 8 mg/L). The results showed that all independent variables significantly influenced copepod inactivation rate. The observed inactivation rate was increased with the increasing ozone dosage and contact time. However, the trend of response gradually stabilized when the contact time was beyond 20 min. Copepod inactivation was more sensitive to high ozone dosage and short contact time under the same CT (dosage × contact time) value conditions. There was a negative effect of organic matter concentration on copepod inactivation. The polynomial response model for inactivation rate of copepod was established with $R^2 = 0.9933$ and adjusted-$R^2 = 0.9865$. The F-value of 165.93 implied the model significance and the p-value lower than 0.0001 indicated its good fit to use in the matrix. The 'lack of fit (LOF) F-value' of 0.0099 implied that the LOF was significant relative to pure error. The evaluation of the model showed it is able to make suitable predictions for the intended application.

Key words | copepod zooplankton, inactivation, oxidation, ozone, response surface optimization

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INTRODUCTION

In recent years, the ozone-granular activated carbon (O_3-GAC) technique has been used as a method of advanced treatment for drinking water purification in some waterworks. However, some reports have indicated that propagation of invertebrates can occur in GAC filters, thereby endangering the quality of the final product (Schreiber *et al.* 1997; Castaldelli *et al.* 2005; Weeks *et al.* 2007). In China, copepod densities gradually increase in the water source as the eutrophic status increases (Lin *et al.* 2004). The increasing number of copepods in the water source leads to the possibility of their presence in waterworks. Their motility means it is difficult to remove them by conventional drinking water treatment processes (Cui *et al.* 2005). Copepods have strong resistance to oxidation inactivation in the ozone contact tank. Therefore, the effluent from the contact tank, which enters the GAC filter, often contains copepods. Indeed, copepods such as Cyclopoida and Harpacticoida are commonly found in GAC filters. Excessive propagation of copepods occurs in GAC filters in some treatment plants in southern China (Li *et al.* 2007). The motility of copepods also enables them to easily penetrate the GAC filter and enter the clear water reservoir of waterworks, and they may even enter the municipal distribution network. They are visible to the naked eye, which could cause consumers to feel that the water is not sanitary. Furthermore, copepods may transmit pathogens, such as schistosomes and eelworms; therefore, they have the potential to threaten human health (Wolrnarans *et al.* 2005; Bichai *et al.* 2008). As a powerful oxidant, ozone not only oxidizes organic matter but inactivates organisms. It should be feasible to completely inactivate copepods by ozone inactivation prior to the GAC filtration. Previous studies on ozone oxidation focus on the disinfection efficiency of harmful microorganisms (Corona *et al.* 2002; Larson & Mariñas

doi: 10.2166/aqua.2012.026

2003; Sivaganesan *et al.* 2003). Information on factors influencing copepod inactivation by ozone is limited to a single study (Cui *et al.* 2004). Ozone oxidation is easily influenced by multiple external factors such as dosage, contact time and organic matter concentration. Copepod inactivation by ozone is also determined by the interaction of multiple factors. Therefore, experimental investigation of single factors is not sufficient to test the actual application in water treatment. Copepods are detected in the ozone contact tank effluent, which indicates that they may not be thoroughly removed due to the improper parameters of the ozone oxidation process. In addition, attempting to completely inactivate copepods by excessive ozone dosage is not appropriate because of production of more oxidation by-products and high cost. Therefore, the optimization of copepod inactivation of ozone will require optimal operating conditions and cost.

Classically, a method is optimized by investigating and altering one variable at a time, but this cannot solve the problem of dependence of multiple variables in obtaining optimal conditions (Marcos *et al.* 2008). Consequently, the response surface methodology (RSM) is designed to evaluate the relative significance of variables and determine optimal conditions for the desired response. There have been no previous reports of studies of RSM to optimize removal of unwanted organisms by oxidation. In this paper, experiments were conducted at bench-scale to optimize ozone oxidation conditions, including dosage, contact time and organic matter concentration, for the inactivation of copepods. The approach allowed for the simultaneous determination of the effects of important variables affecting the inactivation rate of copepods. In addition, the polynomial optimal model for copepod inactivation was established and the actual performance of model was investigated in the O_3-GAC process of waterworks.

MATERIALS AND METHODS

Ozone preparation and reactor

Ozone was generated from an ozonizer (Nippon Ozone Co., Ltd) and then sparged into cold highly purified water (Milli-Q system; Millipore Corp., USA), no longer than 1 h prior to use. It was stored under dark as well as refrigerated conditions prior to use. Ozone concentration in the stock solution ranged from 10 to 20 mg/L. Ozone from the stock solution was transferred to batch reactors using a syringe attached with Nalgene tubing so that ozone could be injected into the reactor bottom. Residual ozone in the batch reactors was measured by the indigo method. Three 2-L heat resistant glass beakers were used as reaction vessels. The beakers were immersed in a recirculating water bath to control temperature. All glassware was soaked in 2 mg/L or more ozone solution for 30 min and then dried at 110 °C for 5 h to satisfy ozone demand. The scheme of the pilot experiment is shown in Figure 1.

Culture of copepods

Mature copepods were initially collected from the raw water of a municipal waterworks in southern China (Jiangsu Province) and then artificially cultivated in the laboratory. Several species of copepod, mainly *Mesocyclops* and *Schmackeria*, were cultivated in an aerated glass container with a volume of 5 L, containing water sample collected from a GAC filter. GAC media (5 cm depth) was then added to the bottom of the container. The glass container was incubated at a constant temperature (25 °C) and photoperiod (16 h light/8 h dark), while the level of dissolved oxygen in the container was controlled at 7–10 mg/L.

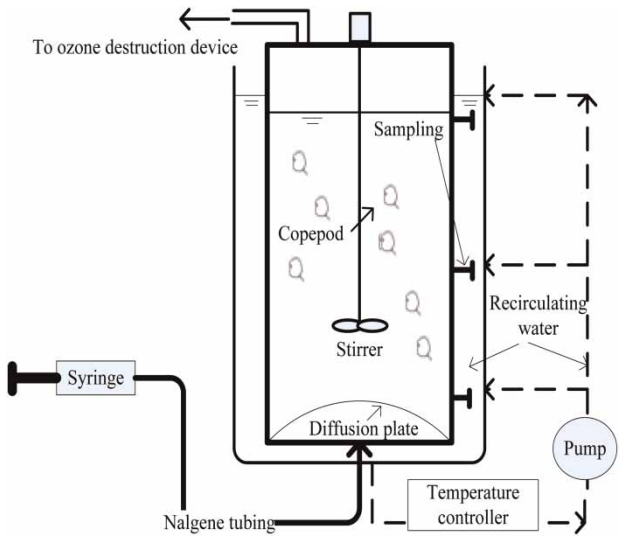


Figure 1 | Scheme of the pilot experiment.

Inactivation experiment

To investigate the influences of organic matter in the water on the inactivation rate, natural organic matter (NOM) in the water source served as the total organic carbon (TOC) source (International Humic Substances Society, Golden-Colorado, USA). Briefly, dried powdered NOM was suspended in highly purified water (Milli-Q system; Millipore Corp., USA) to prepare a stock solution of 100 mg/L as TOC. Aliquots of the stock solution were then added to the reactor water to give final concentrations of 2, 5 or 8 mg/L. The organic carbon concentrations were confirmed using a Sievers 800 TOC analyser (Ionics Sievers, Boulder, CO, USA). The experiments were conducted in 0.01 M buffer solutions prepared by adding reagent grade phosphates to obtain pH of 7.5. Distilled water was prepared as described in the Standard Methods (APHA 1998). For accuracy of analysis, each experiment was measured in triplicate and the average value was used for response surface analysis. In addition, the body structure changes of copepods were examined by scanning electron microscopy (JSM-5610LV, JEOL Ltd, Japan) during ozone inactivation. Leakage of inner protoplasm in copepods was detected by three-dimensional fluorescence spectra (LS55, Perkin-Elmer, USA).

The inactivation rate of copepod is calculated as follows in Equation (1).

$$\text{Inactivation rate} = \frac{C_0 - C_t}{C_0} \quad (1)$$

where C_0 is the initial copepod density at the start of the experiment and C_t is the residual copepod density at the end of the experiment.

Response surface design

The objective of the experimental design was to determine which factors influenced the sensitivity of copepod inactivation. The independent variables were designed as X_1, X_2 and X_3, which represented ozone dosage, contact time and organic matter concentration, respectively. In the response surface design, a minimum or low level (denoted as –1), a central or medium level (denoted as 0) and a high or maximum level (denoted as 1) are defined for each experimental factor (Table 1). The specific values for the levels for each experimental factor in Table 1 mainly depended on the actual conditions in some waterworks in China. Three replicates at the centre (0, 0, 0) of the design were performed to allow the estimation of the pure error.

Table 1 | Independent variables and levels for response surface design

		Variable levels		
Independent variables	**Coded factor**	**−1**	**0**	**+1**
Dosage (mg/L)	X_1	1	3	5
Contact time (min)	X_2	5	15	25
Organic matter/TOC (mg/L)	X_3	2	5	8

The polynomial equation of the response variable with respect to the discrete and continuous factors was regressed using the software package Design Expert 7.1.3 and Statistics Analysis System (SAS 8.2). A quadratic polynomial regression model was used to predict the response. The model proposed for response Y is shown in Equation (2):

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2 \quad (2)$$

where A_0 is a constant; A_1, A_2 and A_3 are linear coefficients; A_{12}, A_{13} and A_{23} are cross-product coefficients; and A_{11}, A_{22} and A_{33} are quadratic coefficients.

Experimental procedures

Solutions in distilled water were used as experimental water samples by adding organic precursors and phosphate buffers. The homogenous copepod population, having similar generation age and size, was transferred randomly to the reactor with a volume of 2 L, which was designed to contain 25 individuals. Therefore, the initial density of copepod was 12.5 ind./L. In each treatment group, there were three replicates and no oxidant was added to the control group. During the experiment, the temperature was $25 \pm 0.5\,^{\circ}C$ and pH value was 7.5 ± 0.1 in the reactor. At the desired time points, ozone was immediately neutralized with 1% sodium thiosulphate (filter sterilized) in each sample. Copepods in the reactor were then immediately collected using a

zooplankton net (pore size 200 mesh), which was then rinsed using distilled water to transfer the collected copepods to a 10-cm diameter glass vessel. After all 25 individuals were transferred from the reactor to the glass vessel, individual copepods were inspected with the aid of a magnifier and regarded as dead if they did not make at least one response when being stimulated with a pair of fine forceps. According to the number of residual living copepods, the residual density of copepods in the reactor was obtained. The inactivation rate was evaluated by the number of dead copepods being confirmed after disinfection. The experimental results were analysed by RSM and then the interaction effects of multi-factors were evaluated for copepod inactivation with ozone. In addition, the polynomial optimal model for copepod inactivation was established.

Subsequently, the application of the model was investigated in the actual O_3-GAC process of a water treatment plant receiving raw water from Taihu Lake, China, which is a eutrophic freshwater lake. The average density of copepods in raw water was about 3 ind./L. Ozone-GAC was used as an advanced treatment process following the conventional treatment. The sand filter effluent, which entered the ozone contact tank, had an average density of copepod about 1–2 ind./L due to poor removal of zooplankton in conventional treatment. The effluent of the ozone contact tank would enter the GAC filter together with copepods so as to cause the excess propagation of copepods in the GAC filter. Therefore it was essential to completely remove copepods by ozone inactivation in contact tank. The designed contact time in the ozone contact tank was 20 min. The influent TOC concentration varied from 3.5 to 3.8 mg/L in the ozone contact tank. The temperature was $23 \pm 0.5\ ^{\circ}C$ and pH value was 7.4 ± 0.1 during the experiment. Ozone dosage was designed in the range of 3.5–3.8 mg/L. A branch pipe (diameter 30 cm) had a valve set on the sampling point, located in the inlet and outlet of the connecting pipe between different treatment processes, to collect water samples. Water samples of 50 L were collected and then filtered with a plankton net (pore size 200 mesh). After each sampling process, the net was rinsed with distilled water in to a 10-cm diameter glass vessel. The rinsed sample was examined to calculate the variation in density of copepod. The influent and effluent of ozone contact tank were collected daily and the sampling process was performed for 1 month. The average inactivation rate was used to analyse experimental results.

RESULTS AND DISCUSSION

Model fitting

To express the influence of each parameter on the inactivation rate, the experimental results are shown in Table 2. The treatment groups in Table 2 were designed by the software package Design Expert 7.1.3. According to the level of independent variable in each treatment group, the copepod inactivation rate was investigated and the experimental results were used to the evaluation of model fitting. Analysis of variance (ANOVA) was used to evaluate the significance of the model equation and model terms are shown in Table 3. The results in Table 3 represented the accuracy

Table 2 | Inactivation rate of copepod with ozone under different experimental groups

Treatment group	X_1	X_2	X_3	Inactivation rate (%)
1	−1	1	1	0
2	−1	1	−1	24
3	0	0	0	55
4	1	−1	1	32
5	0	1	0	56
6	−1	−1	−1	15
7	0	−1	0	40
8	0	0	0	55
9	1	−1	−1	72
10	0	0	1	36
11	−1	0	0	12
12	1	1	−1	100
13	0	0	0	55
14	1	0	0	80
15	−1	−1	1	0
16	0	0	−1	72
17	0	0	0	52
18	1	1	1	60
19	0	0	0	54
20	0	0	0	55

Table 3 | Analysis of variance (ANOVA) table for fitting quadratic model

Regression	Degree of freedom	Sum of squares	*R*-square	*F*-value	Prob. > *F*
Model	9	13,158.09	0.9933	165.93	<0.0001
Linear	3	11,600	0.8757	438.85	0.0001
Cross-product	3	496.00	0.0374	18.76	0.0002
Quadratic	3	1,062.09	0.0802	40.18	<0.0001
Lack of fit	5	80.87	–	11.01	0.0099
Pure error	5	7.33	–	–	–
Total	19	13,246.20	–	–	–
$R^2 = 0.9933$		Adjusted-$R^2 = 0.9865$			

and general reliability in the polynomial model. The model had an *F*-value of 165.93, indicating that it was significant. The model *p*-value was lower than 0.0001, which showed that the model was fit for use in the matrix. The 'lack of fit (LOF) *F*-value' of 0.0099 implied that the LOF was significant relative to pure error. The adjusted-R^2 is adjusted for the number of terms in the model (Sereshti *et al.* 2009). The values of R^2 and adjusted-R^2 indicated that the polynomial model was adequate.

The regression equation consists of three main effects, three two-factor interaction effects and three curvature effects with the estimated coefficients listed in Table 4. As shown in Table 4, the *p*-values of variable X_1, X_2, and X_3 were lower than 0.0001 in regression analysis. In addition, there was also a lower value in cross-products of X_1 X_2, X_1 X_3 and quadratic of X_1. Using the data in Table 4, the second-order polynomial equation was used to express copepod inactivation rate (Y) as a function of the independent variables as follows (using coded factors):

$$Y = 54.53 + 29.2X_1 + 8X_2 - 15.6X_3 + 6X_1X_2 - 5X_1X_3 - X_2X_3 - 8.82X_1^2 - 6.82X_2^2 - 0.82X_3^2 \quad (3)$$

In the regression equation (Equation (3)), it can be concluded that the response has a complex relationship with the independent variables. All independent variables (ozone dosage, contact time and organic matter concentration), the cross-products of X_1 X_2 (dosage × contact time) and X_1 X_3 (dosage × organic matter concentration), and quadratic of dosage had significant influences on the response of the inactivation rate. The coefficients in Equation (3) denote the parameter of the model optimized iteratively to fit or model the data; a mathematical correlation model can be employed to predict and optimize the response Y with the range of variables in the experiment. A normal probability plot of the residual (shown in Figure 2) shows a nearly linear distribution, which means that errors are evenly distributed and which supports a least-square fit.

Table 4 | Results of regression analysis of a full second-order polynomial model

Variable	Degree of freedom	Sum of square	*F*-value	Prob. > *F*
X_1	1	8,526.40	967.71	<0.0001
X_2	1	640.00	72.64	<0.0001
X_3	1	2,433.60	276.20	<0.0001
X_1X_2	1	288.00	32.69	0.0002
X_1X_3	1	200	22.70	0.0008
X_2X_3	1	8	0.91	0.3631
X_1^2	1	213.84	24.27	0.0006
X_2^2	1	127.84	14.51	0.0034
X_3^2	1	1.84	0.21	0.6574

Effects of variables

To express the effects of any parameter on the response Y in a better way, three-dimensional response surface curves were created as a function of the interaction of any two of the variables by holding the other variable at its central level (0). The results are shown in Figures 3 to 5. The plots showed a similar relationship for the effects of dosage and contact time, whereas it was adverse in the effect of organic

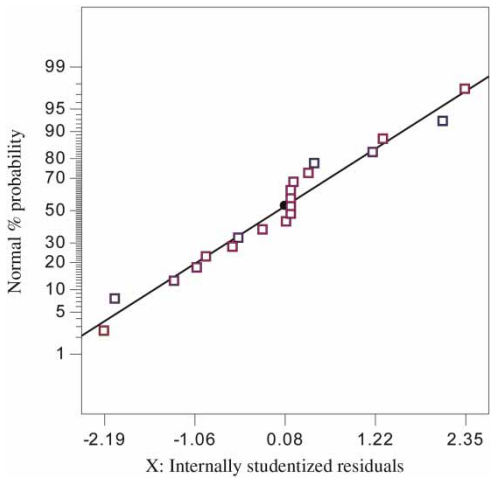
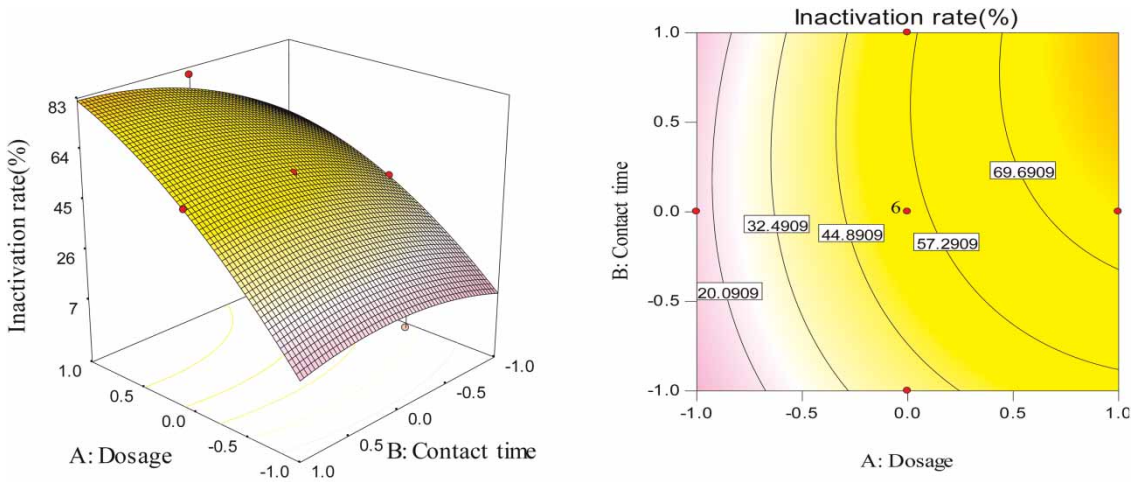


Figure 2 | Normal probability plot of residual for the response.

matter. As shown in Figure 3, the inactivation rate of copepods was remarkably increased with the increasing ozone dosage. For contact time (as shown in Figure 4), the observed response of inactivation rate was improved with the increase of contact time. However, when the contact time reached a certain duration, about 20 min (X_2 denoted as 0.5), the trend of inactivation response gradually stabilized. There were seldom changes in the copepod inactivation rate during ozone oxidation when the contact time beyond 20 min. As shown in Figure 5, there was an approximate linear and negative response relation between copepod inactivation rate and organic matter concentration, especially at lower ozone dosage.

The plot shape allows estimation of the significance of the mutual interactions between the independent variables. These plots are a visual representation of the relationship between the response and each experimental factor. From the plots, it can be concluded that the response is not convex indicating that the operating conditions are optimally well defined. The level of the convexity is not sufficient, suggesting that the response value is optimized based on the combined effect. The plot shape and response surface profile indicated that copepod inactivation was more sensitive to high ozone dosage and short contact time under the same CT (dosage × contact time) value conditions. For example, above 60% inactivation rate was attained by dosage of 5 mg/L (X_1 denoted as 1) and contact for 5 min (X_2 denoted as –1), whereas it was less than 20% by 1 mg/L (X_1 denoted as –1) and 25 min (X_2 denoted as 1). In contrast to common bacteria or viruses, copepods have a special surface structure consisting of several tissue layers, including bottom membrane, epithelium, calcific layer, etc. The body surface provides copepods strong protection against oxidation (Liu *et al.* 2007). So it is essential to thoroughly inactivate copepods by destroying surface structures, thus permitting oxidation of inner body protein structures. However, instability of ozone in water results in shorter available reaction times, so body structures may not be destroyed at



X_1=A: dosage; X_2=B: contact time; the holding factor C: organic matter (X_3 coded as 0, i.e.5 mg/L).

Figure 3 | Response surface and plot for copepod inactivation as a function of dosage and contact time (the values in the box mean the inactivation rate expressed by each contour).

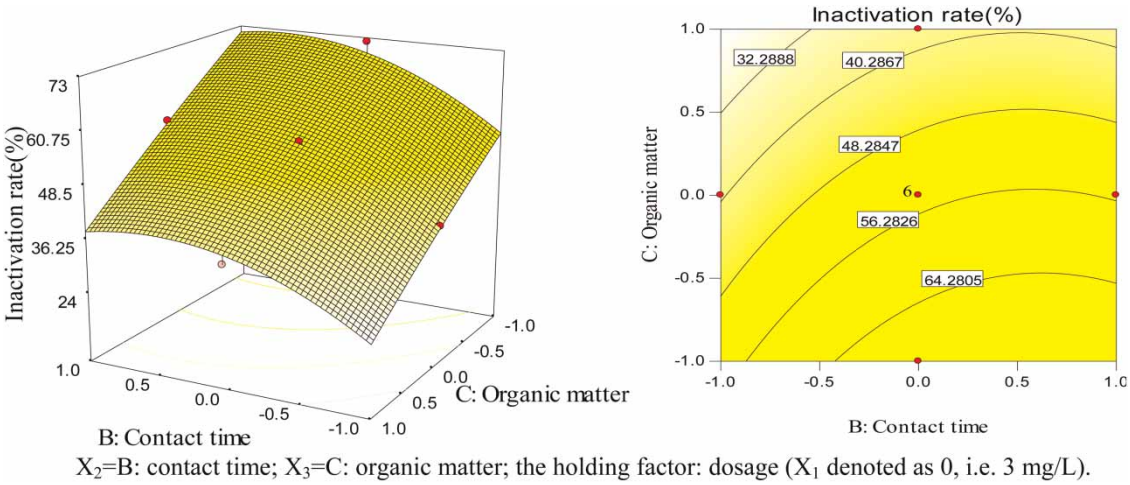


Figure 4 | Response surface and plot for copepod inactivation as a function of contact time and organic matter concentration (the values in the box mean the inactivation rate expressed by each contour).

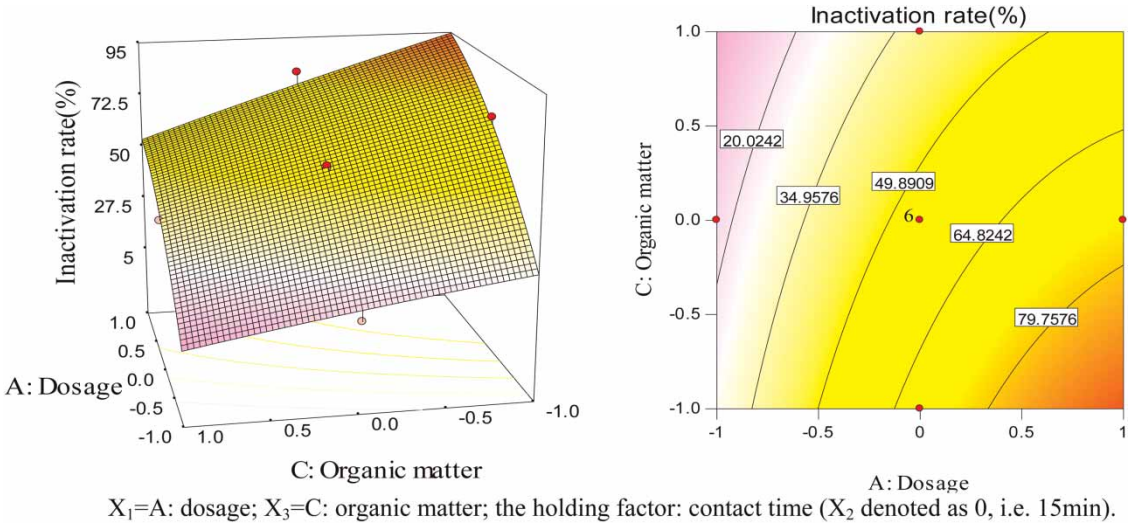


Figure 5 | Response surface and plot for copepod inactivation as a function of dosage and organic precursor content (the values in the box mean the inactivation rate expressed by each contour).

lower dosage. Experimental results indicate that greater dosage and, particularly, sufficient reaction time are essential to kill copepods. The copepod inactivation rate by ozone oxidation stabilizes after 20 min contact time, showing that the contact time has less influence on inactivation response than ozone dosage. Ozone's instability in water results in a shorter available reaction time for inactivating copepods, thus explaining why the inactivation rates of copepod were constant after 20 min for each ozone dosage experiment. As reductive substances, organic compounds may react with oxidant so as to decrease the available amount. Therefore, competition for ozone between oxidizing organic matter resulted in a decrease in the inactivation efficiency. The organic matter in water not only depletes the concentration of ozone, but also accelerates its decomposition, which results in a reduced exposure time for inactivation of the copepod. The negative influence of organic matter was mitigated by increasing dosage to give more residual ozone to inactivate copepods, as illustrated by varying trend of plot gradient and response surface profile in Figure 5.

In the above experiments, it can be deduced that the protective body surface is a major reason for the resistance of copepods to ozone inactivation. The copepod body structure was visualized with scanning electron microscopy and the leakage of inner protoplasm was detected by three-dimensional fluorescence spectra. The damaged body structure after inactivation is easily observed (Figure 6). Figure 7 indicates that there was an adsorption peak of fluorescence spectra in the water sample, in which copepods were inactivated by ozone, compared with the blank control. The peak mainly occurred at 220 nm/380 nm (Ex/Em) and 280 nm/360 nm (Ex/Em). The leakage of protoplasm from copepods may cause an increase of the dissolved organic carbon (DOC) following the destruction of the body surface. As can be seen in Figure 8, the concentration of DOC in distilled water solution was increased with increasing reaction time, particularly up to 20 min. The substances lost from the inner protoplasm including protein and aminoacids. The fluorescence spectra area of 220 nm/380 nm (Ex/Em) indicates presence of leucine and that of 280/360 nm (Ex/Em) indicates aminoacids such as lysine. Aminoacids are essential molecules in metazoa and their loss cause the death of copepods. As reductive organic matter, the leaking protoplasm will be oxidized by ozone to small fractions and hydrophilic organic substances, which causes an increase of DOC in water.

Figure 6 | Scan of copepod (*Mesocyclops*) body surface with electron microscope. (Left: before inactivation; Right: inactivation for 20 min.)

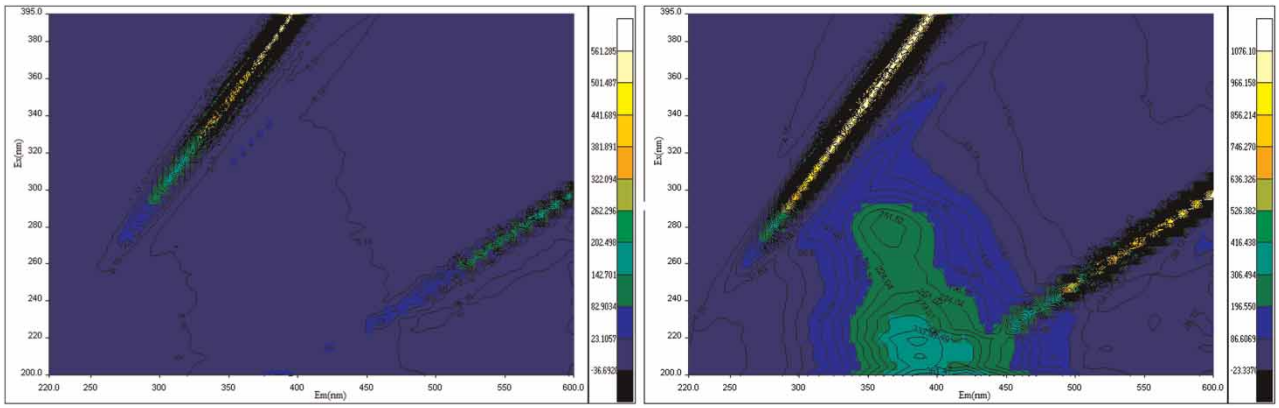


Figure 7 | Detection of three-dimensional fluorescence spectra before and after copepod inactivation with ozone. (Left: blank control; Right: experimental water sample for 20 min inactivation.)

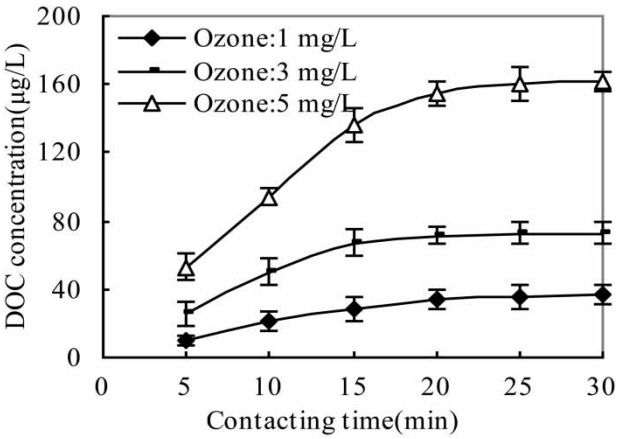


Figure 8 | Change of DOC concentration during ozonation ($n = 3$, $p < 0.05$).

Evaluation of the method performance

The performance of the model was evaluated in the O_3-GAC process in a water treatment plant (located in Jiangsu province). The designed contact time was 20 min (X_2 denoted as 0.5) and the influent TOC concentration varied from 3.5 to 3.8 mg/L (X_3 denoted from −0.5 to −0.40) in the ozone contact tank of waterworks. The temperature was $23 \pm 0.5\,^{\circ}C$ and pH value was 7.4 ± 0.1 during the experiment. The comparison of the copepod inactivation rate between actual detection and model prediction are shown in Table 5. As can be seen, the difference between detection and prediction was under 10%, which indicates the polynomial equation has a good fit to the prediction for the actual application. The difference was gradually reduced with the increasing ozone dosage, which shows that sufficient dosage will remedy the negative influence of adverse factors such as organic matter. In fact, the complete removal of copepods was achieved when ozone dosage was increased to 5.2 ± 0.1 mg/L. The performance of the model indicates that it is feasible to guide the operation in the actual waterworks.

CONCLUSIONS

The RSM was utilized to optimize ozone oxidation conditions for copepod inactivation. Independent variables (ozone dosage, contact time and organic matter concentration) were involved in response surface design. The inactivation rate was strongly influenced by ozone dosage and its variability gradually stabilized when the contact time extended to 20 min. Organic matter had a negative effect on copepod inactivation, which was improved by increasing ozone dosage. The leakage of inner aminoacids was essential to inactivate copepod by destroying body structure with ozone oxidation. The coefficient of determination (R^2) for the model was 0.9933. The high probability value ($p < 0.0001$) of the regression indicated that the model had a high significance level. The performance proved that the polynomial model had a good fit to the prediction for the intended application.

ACKNOWLEDGEMENTS

Financial support was received from the National Natural Science Foundation of China (Project 50808065) and the Funds sponsored by the Qing Lan Project.

Table 5 | Inactivation rate comparison between detection and prediction ($n = 40$, $p < 0.05$, mean ± SD)

Ozone dosage Actual (mg/L)	Code	Copepod inactivation response *E*: Experimental (%)	*P*: Predicted (%)	Difference % (*E* − *P*)/*E*
1	−1	19.7 ± 2.2	21.5	9.1
2	− 0.5	48.6 ± 1.4	44.5	8.0
3	0	60.5 ± 1.6	64.3	6.3
4	0.5	82.3 ± 2.1	77.4	5.9
5	1	95.2 ± 1.3	90.5	4.9

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First received 18 March 2011; accepted in revised form 29 June 2012