The process of activated sludge ozonation: effect of ozone on cells, flocs, macromolecules and nutrient release

Xianrong Meng, Dongfang Liu and Matthew Frigon

ABSTRACT

The purpose of this research is to investigate the activated sludge ozonation process. Results revealed that bacteria destruction and cell solubilization were not obvious when ozone dose was lower than 11 mgO_3/gMLSS (MLSS: mixed liquor suspended solids), while pores appeared on the sludge and bacterial disappeared from floc surface. In the range of 11–90 mgO_3/gMLSS, ozone had significant effect on cell permeabilization and disruption. Meanwhile, a large quantity of macromolecules and nutrients were released from bacteria cells. Additionally, efficiency of ozone utilization was low but specific solubilization related to cell lysis was high at this level. Greater than 90 mgO_3/gMLSS, the number of live cells and dead cells were both stable, and the quantity of material in bulk liquid increased slowly. The specific solubilization ratio decreased while the efficiency of ozone utilization began to increase. This indicated that ozone oxidized the macromolecules in the bulk liquid instead of bacteria cells at high ozone dose.

Key words | cell lysis, flow cytometry, sludge ozonation, sludge solubilization, sludge surface morphology

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INTRODUCTION

Production of large volumes of sludge that must be disposed of and low nitrogen and phosphorus removal efficiency are two issues that are a problem for many wastewater treatment plants (WWTPs) (Yang *et al.* 2011; Park *et al.* 2011; Xie *et al.* 2013). Development of new technologies for both sludge reduction and denitrification have not been able to completely resolve these two issues (Manterola *et al.* 2008; Kim *et al.* 2009; Tian *et al.* 2013). One family of techniques for sludge reduction that has received considerable attention for sludge reduction is in-situ methods (Guo *et al.* 2013). One in-situ method is solubilization and recirculation of returned activated sludge (RAS) within the wastewater treatment system. When the cells degrade, significant quantities of organic matter are released which can be recovered for use in anoxic/anaerobic reactors as an internal carbon source for nitrogen or phosphorus removal.

Ozone is a strong oxidizer and is mainly used in sludge treatment to degrade refractory organic matter, reduce sludge production and to disinfect (Beltran 2003; Dytczak *et al.* 2007; Chu *et al.* 2009b; Stalter *et al.* 2011; Lester *et al.* 2013; Bourgin *et al.* 2013). Sludge ozonation is widely used for sludge solubilization due to its strong oxidizing capacity and high efficiency. Most of the research regarding sludge ozonation have been focused on sludge reduction, soluble chemical oxygen demand (SCOD) solubilization, nitrogen removal, and phosphorus recovery (Zhang *et al.* 2009; Bougrier *et al.* 2006; Lou *et al.* 2011). Some studies have also investigated the effect of ozone on sludge bacteria population structure by using denaturing gradient gel electrophoresis (DGGE), or measured the indirect indicating parameters such as adenosine triphosphate (ATP) or protease (Yan *et al.* 2009; Chu *et al.* 2009a). In this study, flow cytometry (FCM) was used to examine the effect of ozone on bacteria cells during sludge ozonation. The use of FCM in conjunction with various fluorescent dyes provides a promising method for the detection and rapid enumeration of bacteria in activated sludge (Prorot *et al.* 2008; Foladori *et al.* 2010). Only a few papers have done an in depth investigation of the effect of ozonation on bacteria integrity, activity, permeability or death by using FCM (Ziglio *et al.* 2002). FCM allows the researcher to obtain a realistic view of bacteria physiological status and also a way to further evaluate the mechanisms that come into play during ozonation of activated sludge.

The purpose of the present study is to investigate the sludge ozonation process so that it can be applied more

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effectively during design and operation. Effective use of ozone as part of the activated sludge wastewater treatment process will allow this technology to be used more economically. The present study first analyzes how ozone oxidizes bacterial cells and flocs of activated sludge and macromolecules and nutrient release at varying ozone dose. The relationship between solubilization and bacteria cell lysis was analyzed. Additionally, the efficiency of ozone utilization and specific solubilization ratio were determined during the process of sludge ozonation to assist in analyzing the process of sludge ozonation.

MATERIALS AND METHODS

Activated sludge culture

Experiments were carried out with activated sludge collected from a municipal WWTP plant in Tianjin, China. The sludge was aerated and fed with synthetic wastewater for 3 days after collection from the plant's waste sludge storage tank. The characteristics of the synthetic wastewater were similar to those of municipal wastewater, containing carbon source, nitrogen, phosphorus and trace elements. The C/N/P ratio was 100/5/1. The cultured activated sludge was washed with deionized water three times before the ozonation test to remove residual organics in the supernatant.

Sludge ozonation

Ozone was produced by an ozone generator (XL401, Xinglu Water Limited Liability Company, Tianjin, China) supplied with pure oxygen. The oxygen inflow rate was maintained at 50 L/h. Concentration of ozone produced was measured by potassium iodide (KI) absorption and maintained at 53 ± 2 mg/L all the time. Ozone dose was defined as a ratio of mass (g) of transformed ozone during sludge ozonation to mass (g) of sludge mixed liquor suspended solids (MLSS) before ozonation. Two absorption bottles with KI solution were used to absorb the residual ozone and calculate the transformed ozone mass. Transformed ozone mass was calculated as the difference between applied ozone and residual ozone. For the batch ozonation experiments, the sludge concentration of MLSS was maintained at 6000 ± 100 mg/L; the ozone inflow time ranged from 0 to 45 minutes and the corresponding ozone dose range was 0–210 mgO_3/gMLSS. An aerator and a magnetic stirring apparatus were used to improve the efficiency of ozone diffusion and mass transfer between gas and liquid.

FCM analysis

The procedure for the use of FCM to count cells was as described by Foladori *et al.* (2010). Samples for FCM analysis were pretreated as follows.

Ultrasonic pretreatment for floc dispersion

A Scientz-JY99-IIDN Ultrasonifier (China) was used to disaggregate sludge flocs at 20–25 kHz. The apparatus was equipped with a horn stick and a temperature inductor. The horn stick was placed into the center of a 100 mL sample in a 250 mL glass beaker. Samples were treated by ultrasonics for 3 minutes, which was determined by ultrasonic testing completed prior to the ozonation experiments. No drastic damage to micro-organisms integrity occurred and the effectiveness of the FCM testing reached a maximum after 3 minutes of ultrasonic treatment.

Bacterial cell fluorescent staining and FCM analysis

The suspensions which received ultrasonic treatment were stained with SYBR-Green I and Propidium Iodide (PI) which was prepared by mixing SYBR-Green I (100 times dilution in 0.22 mm-filtered-DMSO) and PI (20 mmol/L in DMSO; Invitrogen) at a ratio of 1:50 (v/v). Fluorochromes (10 μL) were injected into 1 mL sample following the addition of 10 μL mL^{-1} EDTA (500 mmol/L at pH 8) and then kept standing for 25 minutes in the dark at room temperature. Bacterial cell count was performed with a Partec CyFlow Space flow cytometer (Partec GmbH, Münster, Germany); the wavelength was fixed at 488 nm. The instrumental gain settings were as follows: SSC = 320, FL1 = 360 and FL3 = 700. The sample was diluted with Millipore water before detection so that the measurement was kept at 500 cells/mL.

SEM analysis

The samples for scanning electron microscopy (SEM) analysis were collected after ozonation and prepared immediately as follows: samples were cleaned with normal saline and then fixed by 3% glutaraldehyde for more than 2 hours and rinsed with 0.1 M phosphate buffer three times. Next, the sample was dehydrated with ethanol. Ethanol concentrations used were 30, 50, 70, 80, 90 and 100%. Each concentration was used for 15 minutes. The samples were then desiccated by vacuum freeze drying for 48 hours. Finally, the samples were observed by SEM (QUANTA 200, USA).

Analytical methods

SCOD, total nitrogen (TN), and total phosphorus (TP) were measured in accordance with *Standard Methods for the Examination of Water and Wastewater* (1998). The method proposed by Lowry and modified by Frolund *et al*. was used to measure the concentration of protein (Frolund *et al*. 1995). Bovine serum albumin (BSA, Tianjin, China) was used as the protein standard. Carbohydrates content was obtained using the anthrone method. Glucose was used as the carbohydrate standard.

RESULTS AND DISCUSSION

Variation of bacteria during sludge ozonation evaluated by FCM

As shown in Figure 1, when the ozone dose was lower than 11 mgO_3/gMLSS, there was a little increase in the number of both viable and dead cells. This may be because ozone facilitated the process of sample pretreatment by ultrasonic treatment or the permeabilization of a few bacteria cells changed (Foladori *et al*. 2010). Isazadeh *et al*. (2014) also reported that bacteria were inactivated even at the lowest ozone dose. However, the alteration of cell numbers under low ozone dose was not significant, so the dose of 11 mgO_3/gMLSS was defined as the lowest effective dose for conspicuous cell damage.

When the ozone dose was 25–50 mgO_3/gMLSS, the number of viable cells declined and the number of dead cells increased sharply indicating that ozone was effective in killing cells by breaking the cytoderm and destroying the cell membrane. The number of viable cells remaining was steady at an ozone dose higher than 50 mgO_3/gMLSS. However, the quantity of measurable dead cells began to decrease at an ozone dosage of 50 mgO_3/gMLSS. The number of measurable dead cells continued to decline when the ozone dosage exceeded 90 mgO_3/gMLSS but the number of living cells remained steady. This phenomenon demonstrates that ozone initially damages the viable cells and destroys their cell membranes. When the quantity of dead bacteria cells increases past a certain concentration, the ozone begins to further degrade the dead cells but has little effect on the remaining living cells. The result is a declining number of measurable dead cells as a proportion of them have been oxidized into material which is not detected as cell material by FCM. When the ozone dose reached 140 mgO_3/gMLSS both viable cells and dead cells reached a steady state. At this level of ozone dose and above, ozone did not further degrade living or dead cells.

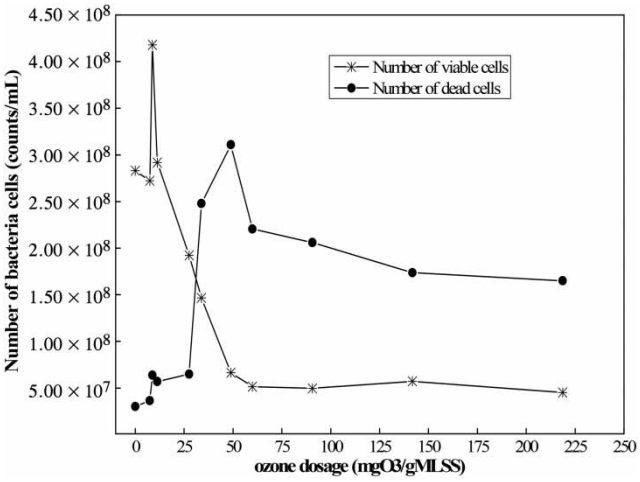

Figure 1 | Evolution of bacteria cells with the increase of ozone dose analyzed by FCM.

Effect of ozone on solubilization of macromolecules and nutrients during sludge ozonation

The effect of different ozone dose on sludge solubilization is presented in Figure 2. The release of all materials could be fitted well with the sigmoid function. The fit is presented in the graphs as the Adj.R-Square value calculated by the Origin Program.

The results demonstrate that the release of organic matter is a dynamic process. There was initially a small amount of organic and nutrient solubilization and then this rose sharply. After the sharp increase, further increase of organics and nutrients became slow and reached a plateau. When the ozone dose was lower than 11 mgO_3/gMLSS, a small amount of organics and nutrient were solubilized, which may have derived from the oxidation of particle organics or extracellular polymeric substance of sludge flocs. Little COD solubilization is from the inactivation of bacteria at low ozone dose (Isazadeh *et al*. 2014). When the ozone dose ranged from 11 to 90 mgO_3/gMLSS, soluble organic concentration increased rapidly. When the variation of viable and dead cell described previously is taken into consideration, the data presented in Figure 2 from the organic solubilization tests are a good fit. When the ozone dose ranged from 11 to 90 mgO_3/gMLSS, active bacteria cells were damaged and dead cells were destroyed. Correspondingly, the rate of material solubilized was high. During this period organic matter solubilized into the sludge mixed liquor which was mainly due to sludge cell lysis. When the ozone dose was higher than 140 mgO_3/gMLSS, few bacteria were disrupted and other organic material solubilization stopped increasing as well.

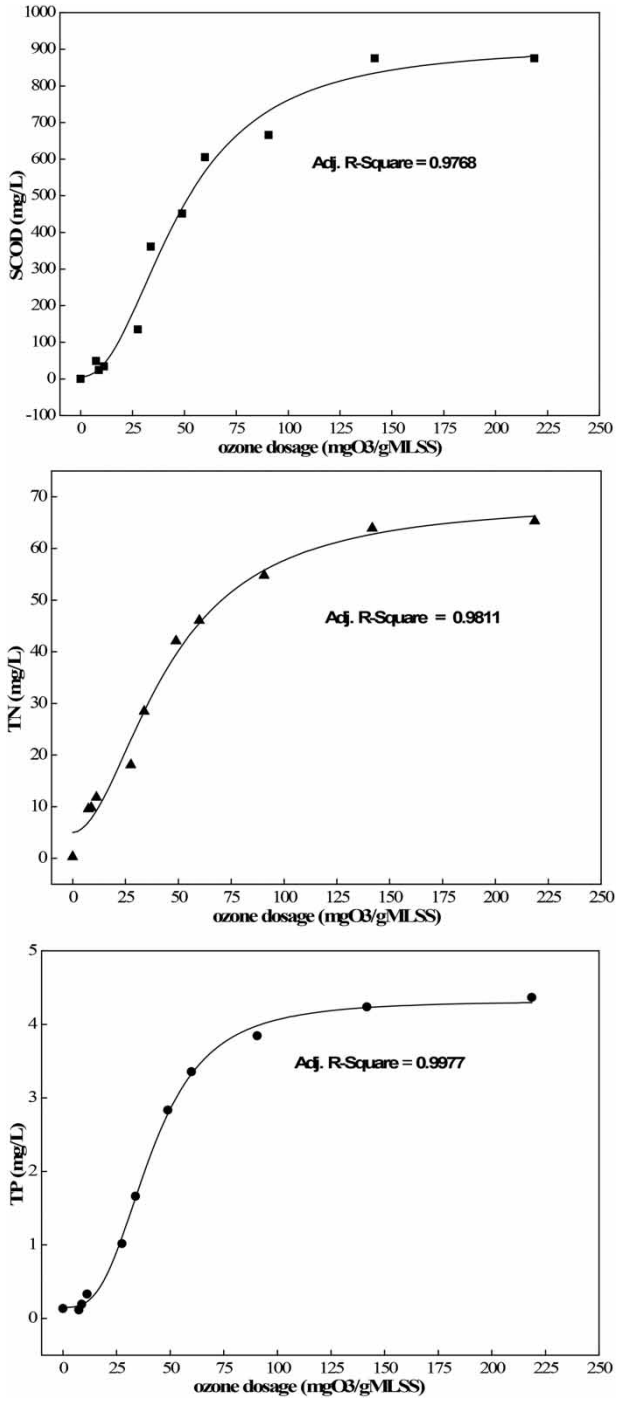

Figure 2 | Solubilization of SCOD, TN, TP, carbohydrate and protein during sludge ozonation.

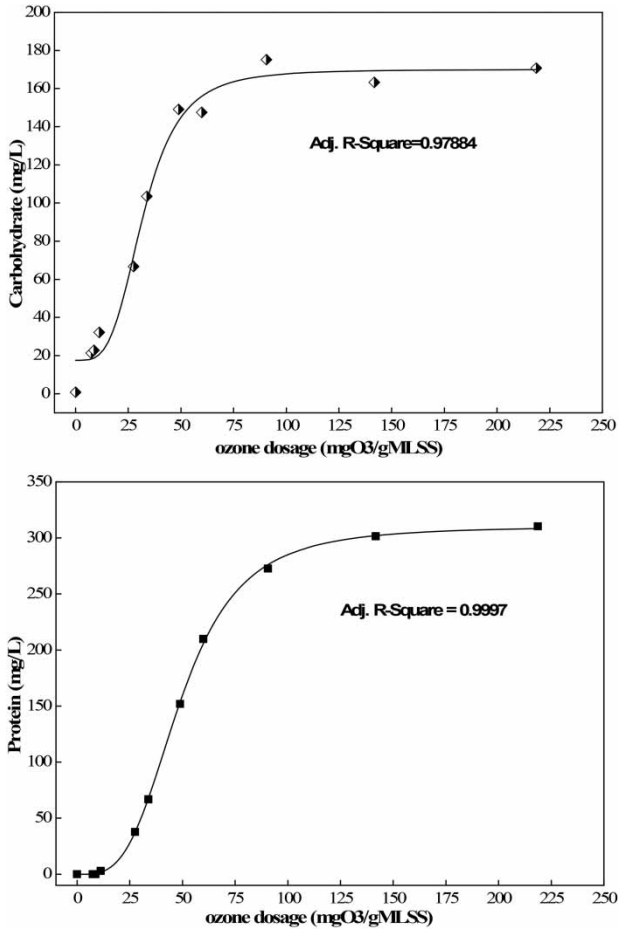

Figure 2 | (Continued).

Effect of ozone on sludge floc

Figure 3 shows the surface morphology of sludge at different ozone doses. In the right column (1000×), image 1 shows that the raw sludge surface is quite dense and smooth. Pie-shaped, sphere-shaped, and linear-shaped microbes can be seen on sludge surface. The ozonated sludge (images 2 and 3) has an irregular porous and rough surface structure like honeycomb with few microbes attached on the sludge surface. When the magnification is increased to 5000×, plentiful rod-shaped bacteria can be seen embedded in the raw sludge flocs (circled in image 4). At an ozone dose of 11 mgO_3/gMLSS, a few filamentous bacteria can be observed and some flocs are cut off. The dense floc has converted to a looser structure. Chaotic pore structure can be seen in image 6 which is similar to that in image 5. No micro-organisms were found to be attached on to the sludge surface or bound in flocs at an ozone dose of 30 mgO_3/gMLSS in image 6. The SEM images demonstrate that ozone primarily disaggregates sludge flocs resulting in cells released from the sludge at a low ozone dose.

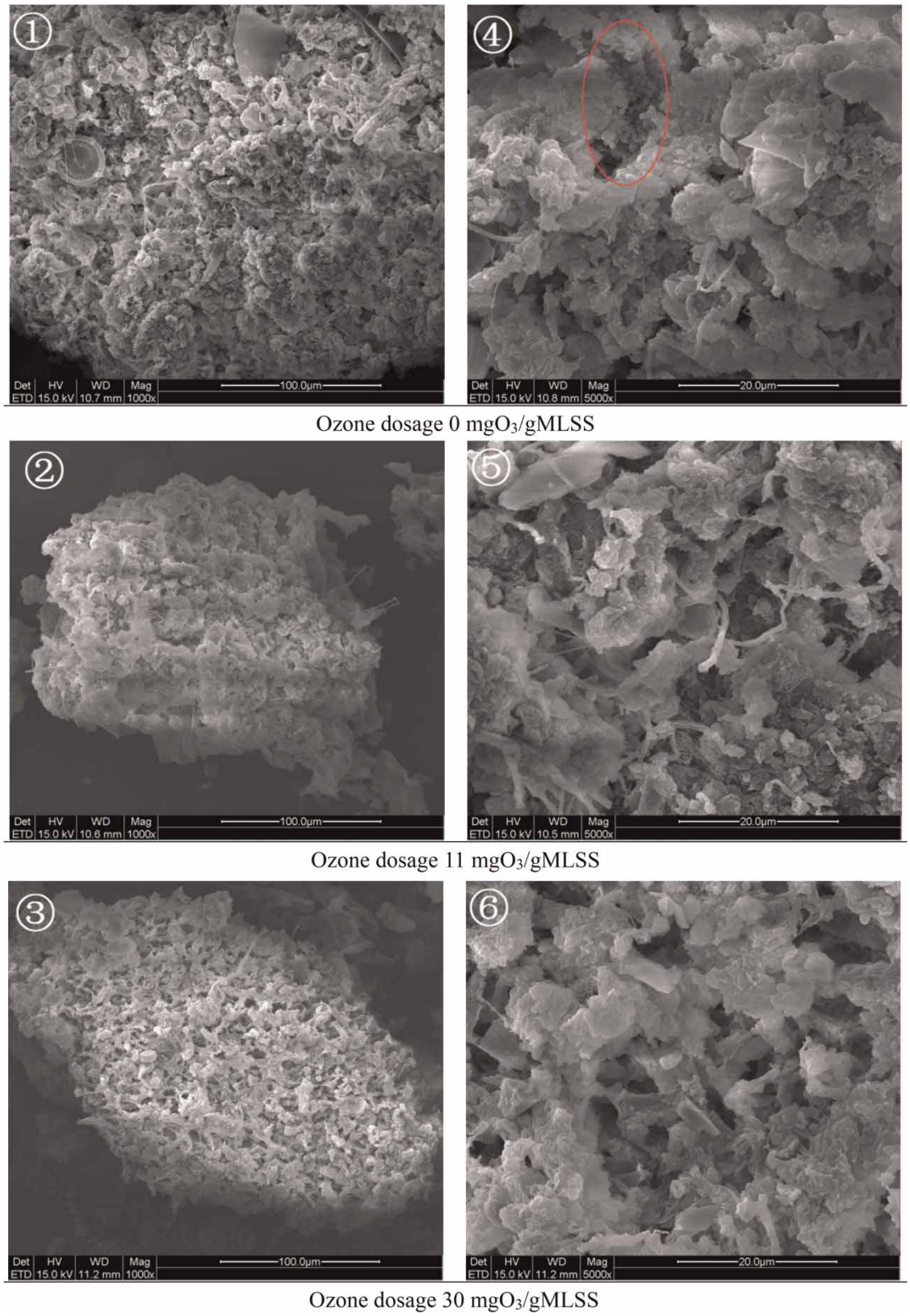

Figure 3 | SEM images of sludge at different ozone dose (right ×1000; left ×5000).

Efficiency of ozone utilization and specific solubilization ratio

During the sludge ozonation process, not all the ozone input into the ozonation reactor was used to disintegrate sludge and the efficiency of ozone utilization varied with increase of ozone input time. The efficiency of ozone utilization is defined here as the ratio of the mass of ozone consumed to the mass of ozone applied. Some ozone was consumed during oxidization and some was unable to be used rapidly enough and escaped from ozonation reactor. Figure 4(a) presents the ozone utilization efficiency vs. ozone input time data.

All the four curves in Figure 4(a) show efficiency of ozone utilization in different batch experiments (as described in the section 'Sludge ozonation'). Conditions for each experiment were identical. When the time of ozone inflow increased from 10 minutes to 28 minutes and the corresponding ozone dose was in the range 30–90 mg O_3/gMLSS, the efficiency of ozone utilization was low. When ozone was input continuously for more than 30 minutes and the corresponding ozone dose was over 90 mgO_3/gMLSS, the efficiency of ozone utilization began to rise. The efficiency of ozone utilization is related to the reaction between ozone and sludge. Figure 1 shows how the ozone reacted with the viable bacteria cells and destroyed the dead cells at 30–90 mg O_3/gMLSS. A significant mass of organic matter was released into the sludge mixed liquor when ozone dose was about 90 mgO_3/gMLSS (as shown in Figure 2). The reaction between ozone and cell is slower than that between ozone and macromolecules for the cell wall is a rigid structure. The increase of ozone utilization efficiency at 90 mg O_3/gMLSS indicated that ozone began to oxidize organics rather than bacteria cells. Degradation of the organics instead of bacteria cell destruction leads to lower release of carbon source from the activated sludge and loss of carbon source (small organics). Internal carbon source recycling is the goal of this research. The cost of ozone production and the loss of the carbon source caused by excess ozonation leads to the conclusion that ozone dose should not exceed 90 mgO_3/g MLSS.

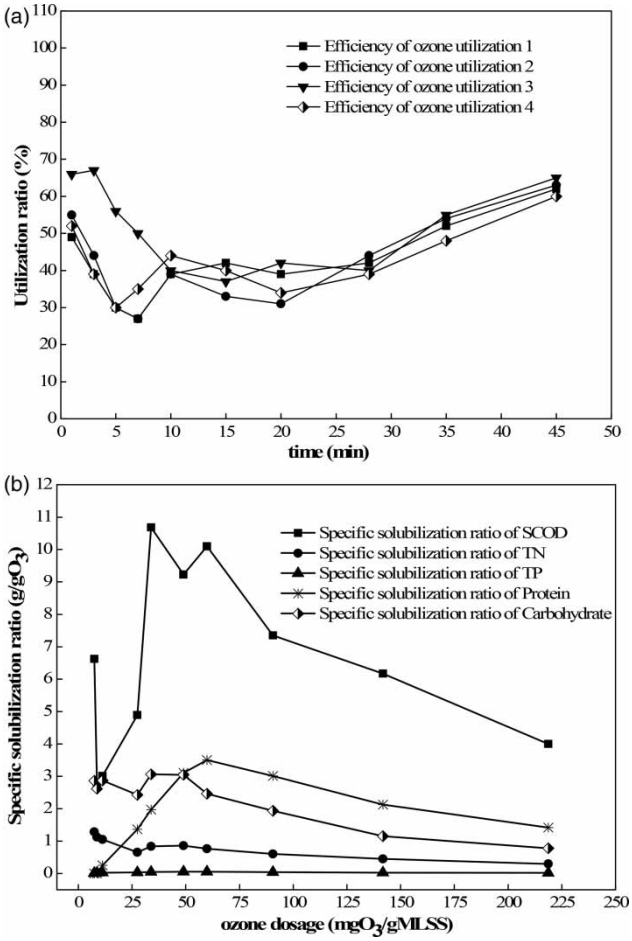

Figure 4 | (a) Efficiency of ozone utilization in four parallel experiments. (b) Specific solubilization ratio of SCOD, TN, TP, protein and carbohydrate.

The specific solubilization ratio is defined as the mass of SCOD, TN, TP, protein or carbohydrate released from microbial cells vs. the mass of ozone consumed (unit g/g). The specific solubilization ratio represents the effect of per unit ozone on sludge solubilization at different ozone doses. The specific solubilization ratio of SCOD, TN, TP, protein and carbohydrate increases for a time and then decreases. When the ozone dose was in the 30–90 mgO_3/gMLSS range, specific solubilization ratio for all five components was at its highest. When the ozone dose was higher than 90 mgO_3/gMLSS, the specific solubilization ratio of each parameter declined gradually. However, the efficiency of ozone utilization increased as shown in Figure 4(a). Considering the analysis in the above sections, high ozone dose did not lead to high specific solubilization ratio for two reasons. Firstly, the quantity of organic matter released from bacteria cells decreased and, secondly, the mineralization of some organics into carbon dioxide occurred at higher ozone dose.

CONCLUSIONS

A good understanding of the sludge ozonation process was achieved in this study. Low dose ozone has a significant effect on sludge structure but little effect on cell destruction and solubilization. At a dose of 11–90 mgO_3/gMLSS, variation of bacteria was consistent with organics and nutrient solubilization, which indicated that materials solubilized into the sludge mixed liquor was mainly due to sludge cell

lysis at this level. At a dose of greater than 90 mgO_3/gMLSS, cell concentration and material solubilization gradually stabilized. For carbon source production, an ozone dose of 90 mgO_3/g MLSS is effective. Various efficiencies of ozone utilization indicated that different matrices were oxidized at different ozone dosing ranges.

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