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Effects of inorganic salts on denitrifying granular sludge: The acute toxicity and working mechanisms

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HIGHLIGHTS

- The IC_{50} of NaCl, Na_2SO_4 and Na_3PO_4 on DGS were 11.46, 21.72 and 7.46 g/L.
- The LC_{50} of NaCl, Na_2SO_4 and Na_3PO_4 on DGS were 77.35, 100.58 and 67.92 g/L.
- The toxicity of low and high salinity had different working mechanisms.

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ABSTRACT

It is highly significant to investigate the toxicity of inorganic salts to denitrifying granular sludge (DGS) and its mechanism since the application of high-rate denitrification is seriously limited in the treatment of saline nitrogen-rich wastewaters. The batch experiments showed that the IC_{50} (half inhibition concentration) and LC_{50} (half lethal concentration) of NaCl, Na_2SO_4 and Na_3PO_4 on DGS were 11.46, 21.72, 7.46 g/L and 77.35, 100.58, 67.92 g/L respectively. Based on the analysis of specific denitrifying activity, the live cell percentage, the cell structure, and the DNA leakage, the toxicity of low salinity was ascribed to the inhibition of denitrifying activity and the toxicity of high salinity was ascribed to both the inhibition of denitrifying activity and the lethality of denitrifying cell.

1. Introduction

Denitrification is widely applied in the biological nitrogen removal from wastewaters, but limited in the treatment of saline nitrogen-rich wastewaters (Yu et al., 2012; Wen et al., 1999). The saline nitrogen-rich wastewaters contain high nitrogen pollutants and inorganic salts. Many inorganic salts exist in natural eco-environment at relatively low concentrations, while the industrial wastewaters have only several inorganic salts with much higher concentrations (Glass and Silverstein, 1999; Wan et al., 2014; Lefebvre and Moletta, 2006). Especially nowadays, some industrial factories have promoted their outputs to meet the market demands which results in vast high-salinity nitrogen-rich wastewaters. For example, it has been reported that the chlorate concentration in P-aminoazobenzenic salt production wastewater from a printing and dyeing plant was up to 100 g/L; the sulfate concentration in metal processing wastewater from a steel mill was up to 120 g/L; the phosphate concentration in imidazole aldehyde hydrolyzation wastewater from a pharmaceutical factory was up to 95 g/L (Hamoda and Al-Attar, 1995; Ramos et al., 2007; Chowdhury et al., 2010).

Every microbial species has its optimum growth salinity, and the microorganism would lose its activity beyond the tolerant limit (Shen et al., 2015). Halophiles enjoy the higher salinity than non-halophiles, and the separatrix of salinity (NaCl) for halophiles from non-halophiles was 17 g/L (Ollivier et al., 1994). Though marine microorganisms were considered as best inocula to start up the salinity-tolerant denitrifying reactor since halophiles are widely spread in seawaters (Babatsouli et al., 2015; Duan et al., 2015), it was not realistic to get the marine microorganisms in a large amount, thus highly active denitrifying sludge was often acclimatized to treat the saline nitrogen-rich wastewaters. The denitrifying sludges could display good performances at low salinities ($\leqslant$20 g/L) after acclimation, but not at high salinities (Miao et al., 2015; Yoshie et al., 2006). Researchers found that the denitrifying community changed greatly, sometimes even lost function, after exposure to high salinity. Yu et al. reported that *Marinobacter* dominated in denitrifying reactor with influent NaCl concentration of 110 g/L (Miao et al., 2015). Yoshie et al. found that *Halomonas*

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dominated in denitrifying reactor with NaCl concentration of 40–100 g/L (Yoshie et al., 2006). So far, little information is available about the toxicity of inorganic salts to denitrifying sludge and its mechanism.

In the practical application of high-rate denitrifying reactor, the large fluctuation of salinity might cause salinity shock, and lead to the collapse of denitrifying reactor (Duan et al., 2015; Liu et al., 2009). Therefore, it is important to investigate the toxicity of inorganic salts to denitrifying granular sludge (DGS) and understand the working mechanism. In this work, NaCl, Na_2SO_4 and Na_3PO_4 were chosen as representative salts of industrial wastewaters; their inhibition and lethality to DGS were evaluated by determining the specific denitrifying activity and the live cell percentage; and their mechanisms of toxicity were researched by observing cell structure and DNA leakage. All results obtained in this paper were used to guide the development of salinity-tolerant high-rate denitrifying reactor.

2. Methods

2.1. Denitrifying granular sludge

The denitrifying granular sludge (DGS) was taken from a lab-scale high-rate denitrifying reactor which was fed with sodium nitrate as electron accepter and methanol as electron donor. The nitrate loading rate of the reactor was 35.14 ± 0.38 kg N/(m^3 d) with hydraulic retention time of 7 h. The value of C/N in influent was 1:3.33, and a fixed recycling ratio of 2.0 was set to dilute the influent. The total solids (TS) and volatile suspended solid (VSS) of DGS were 136.68 g/L and 52.89 g/L, respectively (Li et al., 2013).

2.2. Synthetic wastewater

Based on the basal medium, series of salt concentrations were set to investigate the impacts of NaCl, Na_2SO_4 and Na_3PO_4 on DGS. According to the salt concentration in industrial wastewaters, the upper limit concentrations of NaCl, Na_2SO_4 and Na_3PO_4 were determined as 100, 120, 95 g/L respectively. Then, different concentrations of NaCl were set as 6, 14, 20, 40, 60, 80, 100 g/L. Different concentrations of Na_2SO_4 were set as 7, 14, 24, 48, 72, 96, 120 g/L. Different concentrations of Na_3PO_4 were set as 6, 13, 19, 38, 57, 76, 95 g/L.

The basal medium contained: $NaNO_3$ 0.61 g/L, CH_3COONa 0.64 g/L, KH_2PO_3 0.05 g/L, $CaCl_2$ 0.04 g/L, $MgSO_4{\cdot}7H_2O$ 0.01 g/L, and 1 ml/L of trace element solution.

The components of trace element solution were: EDTA 5 g/L, $MnCl_2{\cdot}4H_2O$ 5 g/L, $FeSO_4{\cdot}7H_2O$ 3 g/L, $CoCl_2{\cdot}6H_2O$ 0.05 g/L, $NiCl_2{\cdot}6H_2O$ 0.04 g/L, H_3BO_3 0.02 g/L, $(NH_4)_6Mo_7O_2{\cdot}4H_2O$ 0.02 g/L, $CuSO_4{\cdot}5H_2O$ 0.01 g/L and $ZnSO_4$ 0.003 g/L.

2.3. Determination of specific denitrifying activity

To test the inhibition of inorganic salts to the specific denitrifying activity, batch experiments were conducted in serum bottles with butyl rubber stoppers and aluminum caps (Wang et al., 2014). After washing three times by 0.9% NaCl solution, 5 g DGS (wet sludge) was put into 50 mL mineral medium with different salt concentrations. The initial concentrations of COD and NO_3^--N were set at 5000 mg/l and 100 mg/l, respectively. Experimental groups were added with different concentrations of NaCl or Na_2SO_4 or Na_3PO_4 respectively, while bottles without adding any inorganic salt were set as control. All tests were cultivated on a shaking table (120 rpm) at 30 °C for 5 h, conducted in duplicate. The specific denitrifying activity was calculated by Eq. (1).

$$r_s = \Delta c/(m \cdot t) \quad (1)$$

where r_s was the specific denitrifying activity (g N/(g VSS h)), Δc was the change of nitrate concentration (g N/L), m was the biomass concentration (g VSS/L) and t was the reaction time (h).

2.4. Determination of live cell percentage

After exposure to inorganic salts for 5 h, DGS was dispersed by ultrasonication to obtain cell suspension. Live/Dead® Baclight™ Bacterial Viability Kit (Molecular Probes, USA) was used to stain the nucleic acid so as to distinguish the live cells from the dead cells. The fluorescence microscope (Leica, Germany) was used to observe and photograph the cells. Image-Pro Plus 6.0 was used to count the number of live and dead cells (Wu and Xi, 2009).

2.5. Determination of DNA leakage

After exposure to inorganic salts for 5 h, the supernatant was sampled and filtrated through 0.22 μm membrane. Then, the absorbance of filtrate was determined at 260 nm which was the characteristic absorption peak of DNA.

2.6. Observation of cell structure

DGS sample was fixed with 2.5% buffering glutaraldehyde for 12 h at 4 °C, then post-fixed in 1% buffering osmium tetroxide for 1 h, stained with 1% uranyl acetate and dehydrated in a series of ethanol. After that, DGS sample was embedded by embedding medium for a whole night. Ultra-thin sections were prepared and stained with 1% uranyl acetate and sodium citrate. Microscopy was carried out with a Hitachi H-7650 (Tokyo, Japan) microscope (transmission electron microscope, TEM).

2.7. Analytical methods

All samples were measured immediately after sampling. The concentrations of nitrate and nitrite were determined according to the standard methods (APHA, 2005). The pH values were determined by a S20 K pH meter (Mettler Toledo, Switzerland).

3. Results and discussion

3.1. Determination of half inhibitory concentration

Batch experiments were conducted to test the effects of NaCl, Na_2SO_4 and Na_3PO_4 on the specific denitrifying activity of DGS. As shown in Fig. 1A–C, the trends of specific denitrifying activity versus salts concentrations could be easily divided into two sections. In the first section, the specific denitrifying activity decreased linearly; while in the second section, it leveled off along with the increase of salt concentration. The cut-off point of two sections was 20 g/L NaCl (24 g/L Na_2SO_4, 19 g/L Na_3PO_4). Hereafter, the salinity with 0–20 g/L NaCl (0–24 g/L Na_2SO_4, 0–19 g/L Na_3PO_4) was defined as the low salinity, while the salinity with 20–100 g/L NaCl (24–120 g/L Na_2SO_4, 19–95 g/L Na_3PO_4) was defined as the high salinity.

At the low salinity ($\leqslant$20 g/L for NaCl, 24 g/L for Na_2SO_4, 19 g/L for Na_3PO_4), the decrease of specific denitrifying activity was 20.47 g N/(g VSS h) for NaCl, 16.44 g N/(g VSS h) for Na_2SO_4, and 21.69 g N/(g VSS h) for Na_3PO_4, relatively decreased 73.73% for NaCl, 59.21% for Na_2SO_4, and 78.09% for Na_3PO_4 comparing with the control. At the high salinity (20–100 g/L for NaCl, 24–120 g/L for Na_2SO_4, 19–95 g/L for Na_3PO_4), the decrease of specific denitrifying activity was 27.69 g N/(g VSS h) for NaCl, 26.50 g N/(g VSS h) for Na_2SO_4, and 27.56 g N/(g VSS h) for Na_3PO_4, relatively decreased 99.72% for NaCl, 95.42% for Na_2SO_4, and 99.27% for