Effects of adding betaine on biological nitrogen and phosphorus removal from simulated pickled vegetables wastewater
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ABSTRACT
Laboratory-scale sequencing batch reactors (SBR) were used to examine the effects of adding dosage and ways of adding betaine on nitrogen and phosphorus removal from simulated pickled vegetables wastewater under two different concentrations of salt. The activated sludge was pre-acclimated in a salt environment prior to the experiment. Adding 0.5–2.0 mM betaine to the synthetic wastewater, all the levels were found to be effective at improving the ammonium nitrogen (NH$_4^+$-N) removal with increased salt concentrations from 8 to 16 g/L, in which 1.0 mM betaine was found to be the most effective. Rapid increase of salt concentration, however, showed to have a more pronounced negative effect on total phosphorus (TP) removal. Nevertheless, betaine-added enhanced TP removal was superior to that of NH$_4^+$-N in high salt content conditions compared with the absence of betaine. Both NH$_4^+$-N and TP removal rate were not significantly influenced by the ways of betaine-adding. Interestingly, the dynamic process on phosphate removal in a single cycle of SBR operation, was showed to have anomalous aerobic phosphorus desorption and anaerobic phosphorus absorption, the former could be caused by insufficiency of biodegradable organic matters and/or longer aeration time, and the latter may be attributed to the function of denitrifying phosphorus-accumulating bacteria in the sludge. As a result, a moderate betaine dosage can obtain a sufficient improvement effect for biological nitrogen and phosphorus removal even under high salt stress.

INTRODUCTION
High salinity wastewaters are generated by a number of industries (Lefebvre & Moletta 2006). These wastewaters are often difficult to treat using standard anaerobic treatment processes, since conventional biological treatment is strongly inhibited by salts, leading to decreased treatment efficiency or reactor failure (Welles et al. 2014; Wang et al. 2015; Cortes-Lorenzo et al. 2016; He et al. 2017). Saline effluents are conventionally treated by such physico-chemical means as electrokinetic (Jorfi et al. 2017), electrochemical (Sheng et al. 2015), coagulation (Mancini et al. 2016), reverse and forward osmosis (Chen et al. 2016; Zhang et al. 2016; Yan et al. 2017), and ultrafiltration (Lin et al. 2016). However, physicochemical techniques are energy-consuming and the costs are particularly high, so alternative systems for the treatment of saline wastewater are increasingly studied (Linarič et al. 2013; Castillo-Carvajal et al. 2014; Sharghi et al. 2014; Jiang et al. 2015; Wang et al. 2017), most of them involving anaerobic or aerobic biological treatment.

Most microorganisms involved in conventional wastewater treatment are non-halophilic, and can not normally tolerate high salt concentration without previous acclimatization (Linarič et al. 2013; Castillo-Carvajal et al. 2014). Therefore, halophilic and halotolerant microorganisms are of increasing interest in saline wastewater treatment (Bonfa et al. 2013; Sharghi et al. 2013; Zhang et al. 2014; Cui et al. 2016; Maharaja et al. 2017), due to their ability to degrade various environmental contaminants efficiently under high salt conditions (Castillo-Carvajal et al. 2014). The halophilic microorganisms have shown good removal efficiencies of pollutants such as hydrocarbons (Baltaci et al. 2017), PAHs (Pugazhendi et al. 2017), denitrification (Cui et al. 2016), phenol (Jiang et al. 2015, 2016), and aromatic...
compounds (Dalvi et al. 2014) from the saline wastewater.

When the halophilic bacterium Bacillus licheniformis strain YX2 was used to degrade nitrobenzene in a lysogeny broth medium, degradation of nitrobenzene (200 mg L⁻¹) exceeded 70% after 72 h at 7% NaCl (w/w) (Li et al. 2014). A halothermophilic bacterial consortium isolated from brine of a desalination plant was able degrade 86 ± 2.7% and 58 ± 4.1% of phenanthrene (200 ppm) at 20% and 50% NaCl concentration respectively; the bacterial consortium degraded the PAHs with 94 ± 3.8% chemical oxygen demand (COD) removal in petroleum refinery wastewater (Pugazhendi et al. 2017).

Moreover, the combination of anaerobic and aerobic processes can improve the performance of the overall treatment process and address biological nitrogen and phosphorus removal from saline wastewater. Adding halophilic bacteria and filamentous bacteria to a sequentialoxic-anoxic bioreactor for treatment of tannery wastewater resulted in a considerably higher organics removal at a high concentration of NaCl; the proteins, carbohydrates and lipids removal efficiencies reached 47.4%, 44.8% and 70.4%, respectively (Maharaja et al. 2017). Shi et al. (2014) showed that the combination of upflow anaerobic sludge blanket reactor (UASB) with a membrane bioreactor (MBR) or a sequencing batch reactor (SBR) enhanced the performance of the overall wastewater treatment systems; both the UASB + MBR and UASB + SBR systems achieved excellent COD removal efficiency, with respective removal of 94.7% and 91.8%. Recently, studies combining biological treatment processes for high salt-loading and high COD pickled vegetable wastewaters have been conducted (Chen et al. 2015; Kang et al. 2016). Combining an anaerobic sequencing batch biofilm reactor (ASBBR), SBR and UASB processing for high-salt mustard wastewater treatment, Chen et al. (2015) reported that removal rates of total nitrogen and COD reached 86.2% and 89.7%, respectively, under high salinity of 12.0 g NaCl/L. Kang et al. (2016) found that organic loading had a significant influence on effluent COD and phosphate in mustard wastewater treatment; an organic loading rate of 2.0 kg COD/m³/d for an integrated bioreactor should be considered.

In spite of the adverse effect of salt on microbial activity, adding compatible solutes to high salinity wastewater can improve adaptation of microorganisms and removal efficiency of pollutants (Li et al. 2015; Vyrides & Stuckey 2017). For instance, Liu et al. (2014) reported that the acclimation potential of anammox biomass under a salinity of 30 g/L increased significantly with glycine betaine addition; the recovery time in the reactor with glycine betaine addition (49 days), accompanied by a more stable stoichiometric ratio, was 2.65 times shorter than in the control reactor (130 days). The addition of 5 mM glycine betaine to a continuous submerged anaerobic membrane bioreactor (SAMBR) at 12 h hydraulic retention time can significantly enhance saline (55 g NaCl/L) synthetic sewage degradation and dissolved organic carbon removal from sewage (Vyrides & Stuckey 2017). He et al. (2012) found that adding 1.0 mM betaine to high salinity mustard tuber wastewater improved dehydrogenase activity of anaerobic microorganisms and the COD removal efficiency, compared to without addition of betaine. Moreover, with the addition of 1.0 mM glycine betaine in the activated sludge adapted to 15 g/L NaCl condition to handle the heparin sodium wastewater through an aerobic process, the removal rates of total organic carbon (TOC) and NH₄-N increased by 15.3% and 18.7%, respectively, with rapid shifts in salt concentrations from 15 to 25 g/L (Yang et al. 2015). However, there are very few studies in the literature on the use of compatible solutes as osmoprotectants for anaerobic biomass for biological nitrogen and phosphorus removal from saline.

The purpose of this study is to investigate the role of compatible solutes on anaerobic biomass when stressed with salinity. The activated sludge used by the study originated from the aeration pool of a sewage treatment station in a pickled vegetables plant (Meishan County, Sichuan), which was pre-acclimated in a salt environment for 30 days prior to the experiment in order to acquire satisfactory effluent treatment performance. The experimental wastewater was a high salt loading simulated pickled vegetables wastewater. A laboratory-scale SBR was carried out to examine the effects of adding dosage and ways of adding betaine on nutrient removal efficiency from the saline wastewater, and to investigate the change of nutrient removal rate at each stage in a single cycle of SBR operation.

MATERIALS AND METHODS

Experimental set-up

A schematic diagram of the experimental set-up is shown in Figure 1. A plexiglas jar with 5 L working volume was used as the SBR. The reactors were constructed of a plexiglas jar, sand blasting head, mixing device, aeration and timing socket. The SBR was controlled for aeration, agitation and dissolved oxygen (DO). Aeration was provided by using an air pump and a sand blasting head. DO and pH were continuously monitored by using the relevant detector (JPB-607,
Shanghai, China) and analyzer (PHS-P, Shanghai, China). The reason for choosing the SBR process is that SBR is a robust system, with a strong salt-tolerance ability, which has been successfully used in various wastewaters. Moreover, the SBR run mode is more favorable for the added betaine to be absorbed by microorganisms, which reduce betaine being quickly washed out of the system. Therefore, based on the SBR’s advantages, an SBR was employed to realize nitrogen and phosphorus processes in saline wastewater.

**Wastewater composition**

The experimental wastewater used in this study was simulating pickled vegetables wastewater. The wastewater quality is listed in Table 1.

**Experimental procedure**

The experiment was divided into two stages. The first stage was aimed at researching the effects of ways of adding betaine on nitrogen and phosphorus removal from the sewage, a batch reactors were used in this phase for parallel comparison, and the ways of adding betaine were in the influent, at the start of aeration, and the end of aeration, with a control without betaine; the concentration of the added betaine was 1.0 mM. The second stage employed five reactors to examine the effects of dosage level of the added betaine on the efficiency of removing nitrogen and phosphorus from the saline wastewater; the concentrations of added betaine were controlled at 0, 0.1, 0.5, 2.0 and 4.0 mM, respectively; the way of adding betaine in this phase was in the influent. For each condition studied, duplicate samples were used.

At the beginning of the experiments, each batch reactor was filled with seeding sludge taken from the aeration tank of the wastewater treatment station in a pickled vegetables plant in the city of Meishan, Sichuan, China. After seeding sludge were acclimatized at 8 g/L salt (Na⁺) for 30 days, the simulated wastewater was added into the reactors; the ratio of seeding sludge to water was controlled at 3.5/6.5, then batchwise with twice alternate anaerobic and aeration operations being applied in sequence for one cycle. The first betaine addition was at the second period, according to the ways of addition and dosages. Betaine added in the influent was dissolved in the water inflow according to the need to add dosage, while the two ways of adding betaine at the start of aeration and at the end of aeration were to extract 50–100 ml of the supernatant from the reactor to dissolve the betaine, then return the solutions to the system after the start of aeration and before the end of aeration, respectively. The second betaine addition was at the seventh cycle; at the same time, the salt concentration was increased from 8 to 16 g/L, to investigate the SBR performance in the case of salinity shock.

Four-step SBR operation, consisting of anaerobic/oxic/anoxic/oxic steps with HRTs of 2/7/1/0.5 h and a settling phase of 1 h, was used for all conditions. DO in each anaerobic phase was controlled under 0.5 mg/L, while in the aerobic phase it was controlled above 4 mg/L. 30 ml of samples were withdrawn from the reactor at the beginning and at the end of each cycle for analysis. At the end of each SBR operation, the organisms were sedimented for 0.5 h and 50% of supernatant wastewater was removed.

**Analytical methods**

Samples were withdrawn from the reactor at the beginning and at the end of each treatment period (anaerobic,
aeration) and were centrifuged at 5,000 rpm for 30 min to remove microorganisms from the liquid medium; the supernatant composition was immediately determined after sampling or it was stored at 4°C until analysis. Ammonium/nitrite nitrogen and phosphate-P contents were determined by using the national standard methods of China (SEPA 2002), of which ammonia nitrogen was measured by Nessler’s reagent colorimetric method, nitrite nitrogen was determined with ion chromatography (Dionex ICS3000, USA), and phosphate-P was analyzed by the ammonium molybdate spectrophotometric method. DO and pH measurements were done using the detector and analyzers associated with the SBR run, of which DO content was detected by DO meter (JPB-607, Shanghai, China), and pH value in the wastewater was monitored by pH meter (PHS-P, Shanghai, China). Samples were analyzed in triplicates, and average values were reported. Analysis of variance was used to compare the data, \( p \leq 0.05 \) was considered statistically significant.

RESULTS AND DISCUSSION

Effects of ways of adding betaine on nitrogen and phosphorus removal from the wastewater

Different ways of adding betaine were used to examine whether nitrogen and phosphorus removal from the simulating pickled vegetables wastewater could be affected by ways of adding betaine at two salt concentrations. Variations of nitrogen and phosphorus removal efficiencies (percent removal) with ways of addition and salt content are depicted in Figure 2. Ammonium nitrogen (\( \text{NH}_4^+ - \text{N} \)) removal from the wastewater do not seem to be obviously affected by the changed salt concentrations, in different ways of adding 1.0 mM betaine (Figure 2(a)). Adding betaine in the influent, at the start of aeration, and the end of aeration, average removal rates of \( \text{NH}_4^+ - \text{N} \) were 96.9%, 97.1% and 96.5%, respectively, compared with 96.4% without betaine addition, a significant difference between them is not observed (\( p = 0.390 \)). When the salt concentration was increased from 8.0 g/L to 16.0 g/L, removal of \( \text{NH}_4^+ - \text{N} \) was not inhibited by salinity, besides a sudden drop in the control at the beginning of the sudden salt increase, and these reactors with added betaine had a slight rise in \( \text{NH}_4^+ - \text{N} \) removal. This could indicate that slight salt loads do not have much effect on acclimated nitrification and denitrification microorganisms. Although there is also no difference among different ways of addition (\( p = 0.244 \)), biological nitrogen removal for betaine added groups is significantly higher than that without addition (\( p = 0.038 \)), possibly suggesting that betain added enhanced nitrification and denitrification performance in biological reactors.

Change in salt concentrations has a great influence on the removal of phosphorus (\( \text{PO}_4^{3-} - \text{P} \)) (Figure 2(b)). Increase in salt content from 8 to 16 g/L led to a reduction in total phosphorus (TP) removal, of which phosphorus in the control reactor decreased from 56.6% to 52.6% (\( p = 0.041 \)). For the three ways of adding betaine with two concentrations of salt, the removal dropped from 57.3% to 53.9% for influent (\( p = 0.085 \)), 59.1% to 54.4% for the start of aeration (\( p = 0.081 \)) and 56.7% to 52.1% for the end of aeration (\( p = 0.024 \)). Nevertheless, there are no dramatic differences between or among the control and treatments (\( p > 0.600 \)); a relatively higher removal was found in the reactors that had betaine added at inflow. These results possibly suggest that a rapid shift in salt concentrations produced adverse effects on nutrient removal rather than being positively affected by betaine addition, which is in accordance

![Figure 2](https://iwaponline.com/wst/article-pdf/77/10/2537/235009/wst077102537.pdf)
with the results of previously conducted studies (Sibel & Nusret 2009). Generally, a sharp increase in salt concentration leads to decreased sludge microbial activity; for instance, Sibel & Nusret (2009) showed that increase in salt concentrations from 0 to 50 g/L in the UASB reactors was responsible for significant inhibition of COD removal rate and efficiency.

**Effect of adding dosage on nitrogen and phosphorus removal**

Adding different betaine dosages to the simulated pickled wastewater are applied to investigate the effects of betaine on nitrogen and phosphorus removal by SBR at two concentrations of salt. After each group of SBR reactors operation stabilized, the removal rates of nitrogen and phosphorus, at four different betaine concentrations, are shown in Figure 3(a). It can be seen that the ammonium nitrogen removal efficiency can be significantly enhanced by adding different concentrations of betaine ($p = 0.007$). When 0.1, 0.5, 2.0 and 4.0 mM of betaine was added, average removal efficiency of $\text{NH}_4^+$-N was 97.5%, 98.4%, 97.8% and 96.8%, respectively, compared with 96.1% without adding betaine. This possibly demonstrates that dosing with betaine could improve the sludge microbial activity, such as nitrification and denitrification bacteria. Although betaine addition contributed to nitrogen removal, it is undeniable that the impact of salt on the removal rate is obvious, comparison of removal differences at four betaine dosages and two concentrations of salt illustrates this. In the case of low salinity (8 g/L), the removal of nitrogen under the conditions of four betaine addition concentrations, is not significantly different ($p = 0.240$), whereas these differences are considerably marked at high salinity (16 g/L) ($p = 0.032$). Thus, the findings could support the conclusions of Vyrides & Stuckey (2009), who showed that addition of betaine to anaerobic sludge increases the performance of anaerobic biomass under saline conditions. From the above results, adding 0.5–2.0 mM betaine was considerably effective in removing nitrogen from saline wastewater, which is in accordance with the results of He et al. (2012) who showed that the addition of 1.0 mM betaine can effectively enhance the performance of an anaerobic bioreactor.

The trend in TP removal at four betaine addition levels differs from nitrogen, the removal rates of TP varies with salinity as shown in Figure 3(b). Average TP removals for four addition concentrations of betaine with two salt contents are significantly different from the control ($p = 0.042$), whereas TP removal at different dosages has no obvious differences ($p = 0.065$). Adding 0.1, 0.5, 2.0 and 4.0 mM of betaine to the reactors with 8 g/L salinity, a considerably higher percent of TP removals was attained, 56.1%, 57.9%, 60.3% and 58.9%, respectively, when the saline concentration increased to 16 g/L, TP removal dropped to 52.4%, 53.5%, 57% and 56.6%, respectively. No significant difference in removal rate was found between the two salt content conditions for the same betaine addition dosage (all $p > 0.053$). These results could indicate that phosphorus removing micro-organisms (e.g. polyphosphate bacteria) are not sensitive to betaine, especially a higher salinity condition, also possibly in a low salt concentration (8 g/L) acclimated sludge does not have better tolerance to a sharp shift in salinity. The findings in this study are higher than those reported by Ugyur & Kargi (2004), who presented that with the addition of a halobacter strain to high salinity (5%) synthetic wastewater, $\text{PO}_4^{3-}$-P removal efficiencies reached 31%, whereas without the addition of halobacter removal efficiency was only 21%. This could suggest that adding betaine has a substantive improvement function for TP removal from saline wastewater.

**Figure 3** | Effects of betaine addition dosages on nitrogen (a) and phosphorus (b) removal from saline wastewater (Note: betaine was added at inflow of the second period).
The aforementioned results verify that betaine addition could effectively improve the performance of an anaerobic bioreactor, as antagonist for the anaerobic biomass to counteract salt toxicity (i.e. sodium). Higher or lower betaine dosages, however, would unbalance the internal and external osmotic pressure of microorganism cells, and therefore decreased activity, resulting in a sufficient improvement effect.

**Dynamic process in phosphate and nitrite removal in a single cycle**

After the SBR run attained stability, sampling in different time phases was used to investigate the trends in phosphate and nitrite removal in a single cycle. Changes in concentrations of nitrite and phosphate over time in a single cycle are shown in Figure 4. It can be seen from the figure that during the initial 2 hour anaerobic phase, the change in phosphate concentrations in both the control and the treatment are very similar, and an evident phenomenon of anaerobic phosphorus release can be observed during the first 30 min, in which phosphate content rose from 8.5 mg/L to 14 mg/L; from then, the phosphate gradually returned to its original level, whereas nitrite content in this stage dropped from 0.29 mg/L to 0.05 mg/L. When the SBR operation shifted to the first aeration stage, an aerobic phosphorus uptake emerged in the initial 180 min; hereafter, an abnormal aerobic phosphorus desorption appeared, though nitrite was always released at this stage. An anaerobic phosphorus absorption phenomenon occurred again at the second anaerobic stage, and then an aerobic phosphorus absorption happens again. When the single run cycle ended, the phosphate concentrations in both the control and the treatment were 4.17 mg/L and 3.65 mg/L, respectively; the nitrite contents were 6.17 mg/L and 5.98 mg/L, respectively.

It is generally believed that the polyphosphate bacteria are divided into denitrifying phosphorus-accumulating bacteria (DPB) and aerobic polyphosphate bacteria, in which DPB can use oxygen or nitrate as an electronic receptor, but aerobic polyphosphate bacteria can only use oxygen as an electronic receptor (Benammar et al. 2015). An anomalous anaerobic phosphorus absorption in the first anaerobic phase of this study may be due to DPB species predominating in the microbial community of the sludge, resulting in anaerobic phosphorus uptake. Similarly, an abnormal aerobic phosphorus desorption appeared at the aerobic stage, which could be caused by insufficiency of biodegradable organic matters and/or longer aeration time, since both factors could lead to polyphosphate bacteria growing slowly or decaying due to endogenous respiration, therefore releasing phosphorus. Moreover, accumulation of nitrite in the reactor probably inhibited the physiological activities of polyphosphate bacteria in the sludge (Maite et al. 2010), so biological phosphorus uptake declined.

**SUMMARY**

Betaine is effective as a compatible solute that can contribute to microorganisms adaptation to a high-permeability environment. Betaine improvement effects on biological nitrogen and phosphorus removal was investigated in a four-step SBR operation, and is conducive to biological nitrogen and phosphorus removal from simulated pickled vegetables wastewater; within the experimental salt concentration range, the rate of NH$_4^+$-N removal increased and TP removal decreased with increasing salt content. Salt inhibition effects were more pronounced for TP removal compared to NH$_4^+$-N removal. Nevertheless, betaine
addition enhanced TP removal was superior to that of \( \text{NH}_4^+ - \text{N} \) in high salt concentration conditions. As a result, a moderate betaine dosage can obtain a sufficient improvement effect for biological nitrogen and phosphorus removal even under high salt stress.

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