Integration of sodium hypochlorite pretreatment with co-immobilized microalgae/ bacteria treatment of meat processing wastewater

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Abstract: Wastewater with 0.2, 0.4, 0.8, 1.0 mg/L free chlorine was biologically treated using co-immobilized microalgae/bacteria. In contrast, non-pretreated wastewater was treated with beads (control) and blank beads (blank) under the same operating condition. Results showed that NaClO pretreatment removed 8-33% total nitrogen (TN), 31-45% true color and 0.7-2.5 log CFU/mL aerobic-bacteria. At the end of treatment, maximum algal biomass (2,027 dry weight mg/L) was achieved with 0.2 mg/L free chlorine. Bacterial growth in wastewater was decreased by NaClO pretreatment before reaching 7.2-7.7 log CFU/mL on the fifth day. Beads with microorganisms (control) removed 15% more chemical-oxygen-demand (COD), 16% more TN, and 13% more total phosphate (PO_4^{3-}) than blank. Pretreatment with 0.2 mg/L free chlorine increased TN removal from 75% to 80% while pollutant removal was substantially decreased with 0.4-1.0 mg/L free chlorine. Considering algal biomass growth and pollutant removal, 0.2 mg/L free chlorine pretreatment was recommended for microalgae/bacteria co-immobilized system.

Keywords: Wastewater; Microalgae; Activated sludge bacteria; Co-immobilization; Chlorine

1. Introduction

Microalgae, a category of fast-growing photosynthetic microorganisms in water, have demonstrated high efficiency for biological assimilation of nitrogen and phosphorus from various sources including highly polluted wastewater (Gonçalves et al., 2016). Wastewater treatment using microalgae is promising owing to the efficient uptake of CO_2 and the growth of algal biomass rich in compounds like lipid and protein (Maity et al., 2014). However, one significant pitfall of this technology is the recovery of suspended algal biomass attributed to the small size (3-30 μm) and negative charges of microalgae cell which prevents their auto-aggregation (Barros et al., 2015). To address this challenge, the technology of immobilization has been widely studied (de-Bashan and Bashan, 2010). Compared to physical centrifugation and chemical coagulation/flocculation, algal biomass harvesting through immobilization consumes less energy and the obtained biomass is less contaminated by metal hydroxides (Anthony et al., 2013). Among various immobilization approaches, co-immobilization of microalgae with symbiotic bacteria using alginate gel has been found efficient in wastewater treatment (de-Bashan and Bashan, 2010). In the co-immobilized algae/bacteria system, microalgae growth can benefit from the existence of symbiotic bacteria and also, organic pollutants removal by bacteria and nutrients assimilation by microalgae could possibly be achieved simultaneously (de-Bashan et al., 2002; Xie et al., 2018). Mujtaba et al. (2017) showed that the co-culture of immobilized microalgae and activated sludge removed 98–100% nitrogen, 92–100% phosphorus, and 94–96% COD from wastewater.

Nevertheless, most of the current studies were conducted using artificial or sterilized wastewater which has a different composition from real wastewater (Cheirsilp et al., 2017; Shen et al., 2017). Many factors need to be considered for the treatment of real wastewater. Firstly, most industrial wastewater is characterized by high turbidity and dark color which limits photosynthesis of microalgae. Also, alginate beads are susceptible to degradation by various pollutants in the case of real wastewater. For instance, under alkaline pH cross-linking ions of calcium in alginate gel will bind with chelating agents like phosphate or citrate to decay the mechanical strength of alginate beads (Ruiz-Marin et al., 2010). Apart from the complex chemical environment, the presence of alginate-degrading bacteria in wastewater is another crucial factor decreasing gel strength and stability of alginate beads (Cruz et al., 2013). Additionally, the existence of phytoplankton-lytic bacteria and protozoa in wastewater will compete with or predate microalgae and restrict biomass growth (Wang et al., 2013). Consequently, in most studies (see Table 1), industrial wastewater was usually pretreated with autoclave, membrane filtration and centrifugation or high dilution before being treated with microalgae which are not applicable considering the amount of wastewater produced by the food industry.

NaClO is a strong oxidizing agent and extensively used in municipal wastewater treatment plants to disinfect wastewater before being discharged to the environment. As a disinfectant, NaClO showed high efficiency inactivating a wide variety of aquatic microorganisms. Consequently, the lower bacteria load in the wastewater benefited microalgae growth and allowed higher alginate beads stability. Zhu et al. (2013) showed that performance (nutrients removal, biomass growth and lipid production) of *Chlorella zofingiensis* growing in NaClO-pretreated wastewater was similar to the autoclaved

88 samples. Besides, pretreatment of NaClO can generate free chlorine which can provide
89 lasting disinfection effect and control external contamination during microalgae cultivation.
90 Park et al. (2016) determined that chlorine dosage between 0.45 to 0.60 mg Cl/L with a
91 dosing interval of two hours could continually inhibit the predation of rotifer without
92 affecting microalgae growth in open pond system. Advanced oxidation with NaClO will
93 also bleach the dark color, improve light transmission and oxidize toxic pollutants like
94 detergents and phenols in wastewater, which in turn improves later microalgae-based
95 treatment. For example, pretreatment of olive mill wastewater using NaClO reduced 45%
96 toxic phenol and 75% turbidity making olive mill wastewater suitable for microalgae
97 growth (Markou et al., 2012). In NaClO pretreated dairy wastewater, algal biomass
98 increased from 0.861 to 1.339-1.870 g/L and was higher than the growth observed in UV
99 pretreated wastewater (Qin et al., 2014).

100 However, most of the present studies associated with pretreatment of NaClO are
101 conducted using a suspended microalgae system. Co-immobilization of microalgae and
102 symbiotic bacteria is a promising technology for biomass harvesting and pollutant removal.
103 Integration of pretreatment using NaClO with a co-immobilized microalgae/bacteria system
104 is expected to improve and simplify wastewater treatment while facilitating nutrients
105 recovering and algal biomass harvesting. Thus, in the present study primary treated meat
106 processing wastewater was sampled, and the effect of pretreatment with NaClO at different
107 free chlorine concentrations on quality of wastewater and pollutant removal as well as the
108 growth of microorganisms was evaluated.

109 **2. Materials and methods**

110 2.1. Meat processing wastewater

111 Wastewater was collected from a beef packaging plant located in the Midwest. Like
112 many food processing facilities, this plant used dissolved air flotation (DAF) as a primary
113 treatment. Wastewater was stored at 4 °C and large non-soluble particulate solids were
114 removed by sedimentation.

115 2.2. Beads preparation

116 Microalgae strains of *Scendesmus obliquus* UTEX B2630, *Chlorella vulgaris* UTEX
117 259, and *Chlorella sorokiniana* UTEX 1230 were obtained from Fatty Acid Transport,
118 Trafficking and Transcriptional Regulation Laboratory at the University of Nebraska-
119 Lincoln. They were inoculated and cultivated in modified Bold's Basal media (MBBM)
120 which was prepared according to the method described by Starr and Zeikus (1993).
121 Cultivation was conducted in a MAXQ4000 orbital shaking incubator (Thermal Scientific,
122 USA) at the rate of 125 rpm with 56 µmol photons/m^2/s continuous fluorescent light and 25
123 ± 2 °C for seven days. Light intensity was measured with LI-250 light meter (LICOR,
124 USA). Meanwhile, activated sludge bacteria were collected from secondary treatment of a
125 local municipal wastewater treatment plant and enriched in the laboratory. Cells of
126 microalgae and bacteria were harvested by centrifugation at 3,500 rpm for 10 min. Pellets
127 were re-suspended in deionized water to reach total suspended solids (TSS) concentration
128 of 32.63 g/L. Sodium alginate (2.5% w/v) was mixed with microalgae suspension and
129 activated sludge suspension at a volumetric ratio of 8:1:1 to achieve a final alginate mixture
130 of 2% (w/v). Uniform beads were formed by dropping alginate mixture into 1% (w/v)
131 $CaCl_2$ using a syringe pump (Harvard, USA). Blank beads were prepared following the
132 same method except that the microalgae and activated sludge portion were replaced by
133 deionized water. Gel beads with an average diameter of 3.4 mm were produced and

134 stabilized in 1% (w/v) $CaCl_2$ solution at 4 °C overnight. They were rinsed with deionized
135 water before being applied in wastewater treatment.

136 2.3. Pretreatment with NaClO

137 Wastewater pretreatment using NaClO was conducted with the method described by
138 Macauley et al. (2006) with modifications. Commercial bleach containing 8.25% (w/v)
139 NaClO was purchased from a local store and used as chlorinating agents. Sodium
140 hypochlorite has strong oxidant capacity and will oxidize organic compounds and
141 microorganisms in wastewater to be partially consumed. In a preliminary experiment, 550
142 mL wastewater was pretreated with 0.88, 1.76, 3.52, and 4.40 mL commercial bleach and
143 left at room temperature. After three hours of frequent mixing, the free chlorine
144 concentrations reached 0.2, 0.4, 0.8, 1.0 mg/L, respectively. All apparatus were sterilized to
145 avoid contamination. Free chlorine concentration was measured immediately at the end of
146 pretreatment. Meanwhile, concentrations of chemical oxygen demand (COD), total nitrogen
147 (TN), total phosphate (PO_4^{3-}), pH and color as well as aerobic-bacteria plate counts (APC)
148 were determined in wastewater before and after NaClO pretreatment.

149 2.4. Experimental setup

150 Three types of treatments were studied to determine the effect of pretreatment of
151 NaClO on co-immobilized microalgae/bacteria system. First, following the pretreatment of
152 NaClO beads were applied immediately to the wastewater with 0.2-1.0 mg/L free chlorine.
153 At the same time, control treatment (beads + non-pretreated wastewater) and blank (blank
154 beads + non-pretreated wastewater) were conducted by adding the same amount of beads
155 and blank beads to non-pretreated wastewater. All of these treatments were performed
156 following the same approach where beads were applied at the volumetric ratio of beads:

157 wastewater of 1:5. To increase the efficiency of light, gas, and nutrients diffusion among
158 beads, an annular reactor where beads being vertically laid against the glass wall in thin
159 layers was built instead of having beads stacking up at the bottom. All of the treatments
160 were conducted in a refrigerated incubator (Precision, USA) for seven days, under the
161 conditions as follows: 125 rpm, lightness: darkness = 16 h:8 h at 20 °C. The intensity of
162 fluorescent light was set to be 84 μmol photons/m^2/s, which was the highest value that the
163 incubator could provide. Pollutants removal of COD, TN and total PO_4^{3-} as well as the
164 growth of bacteria (using APC) in wastewater and microalgae immobilized in beads were
165 determined on the first, third, fifth and seventh day of the treatment.

166 2.5. Analytical methods

167 2.5.1. Wastewater characteristics

168 To determine pollutant concentration, 5 mL of wastewater was sampled and
169 centrifuged at 4000 rpm for 10 min. With the proper dilution of deionized water, the
170 concentration of soluble COD, TN, total PO_4^{3-} in wastewater were measured following
171 standard methods of dichromate digestion, molybdovanadate and persulfate digestion with
172 HACH analysis kits. TNT tubes were digested and read using DRB 200 digester (HACH,
173 USA) and DR 3900 spectrophotometer (HACH, USA). The percentage removal of COD,
174 TN, and total PO_4^{3-} were calculated with equation (1):

175 $$\text{The percentage removal of pollutant } (\%) = (a - b)/a \times 100 \quad (1)$$

176 Where a and b are the concentration of COD/TN/total PO_4^{3-} at the starting point and during
177 treatment, respectively.

178 The true color of wastewater was measured according to the platinum-cobalt standard
179 method (HACH, 2014) with a 3900 spectrophotometer (HACH, USA). To guarantee

readings were within the designed range, wastewater was diluted with deionized water. pH was measured with a digital pH meter (Fisher Scientific, USA). Due to the limitation of high turbidity and dark color in wastewater, a method based on colorimeter and titration could not be applied in determining the free chlorine concentration. Test strips (HF Scientific Inc, USA) which are widely used in industrial was adopted in this study (lowest detection limit = 0.05 mg/L). APC was measured following the technique of serial dilution with plate count agar (Thermo Scientific, USA) at 37 °C for 48 h.

2.5.2. Growth of immobilized microalgae

To determine the growth of immobilized microalgae, three beads were taken and the average weight of dry algal biomass in one bead was measured. Subsequently, it was multiplied with bead counts required to treat one liter of wastewater to estimate the weight of dry algal biomass grown in one liter of wastewater. The method described by Shen et al. (2017) was adopted with modifications to release algal cells from alginate beads and determine chlorophyll concentration which was correlated to the concentration of dry weight (DW) of algal biomass with the pre-developed standard curve. Three beads were dissolved in 5 mL of 4% (w/v) $NaHCO_3$. Microalgae cells were separated by centrifugation at 5000 rpm for 10 min and pellets were washed with 5 mL deionized water. Chlorophyll extraction was performed with 5 mL of dimethyl sulfide (DMSO). To accelerate cell rupture and increase chlorophyll extraction rate, a mixture of DMSO and algal cells were sonicated (Branson, USA) for 20 min, where an ice-water bath was used to avoid overheating. Finally, sonicated samples were left in darkness for two hours to complete chlorophyll extraction. Suspended cell debris was removed through centrifugation at 5000 rpm for 10 min at 10°C to obtain a clear supernatant for measuring chlorophyll

concentration at 663 nm (Abs 663 nm) using a UV/Vis UV-1800 spectrophotometer (Shimadzu, Japan). Algal biomass cultivated in one liter of wastewater was calculated following equation (2).

$$\text{Algal biomass(DWmg/L wastewater)} = 3.4306 \times Abs\ 663\ nm \times 2{,}700 \quad R^2 = 0.95 \qquad (2)$$

2.6. Statistical analysis

Two samples were prepared for each treatment, and all experiments were conducted twice. Measured parameters were reported as mean ± standard deviation. Pair-Wise comparison was conducted using SAS (9.4) to determine the significance of difference at the level of $P = 0.05$.

3. Results and discussion

3.1. Impact of NaClO pretreatment on wastewater quality

Concentrations of soluble COD, TN, total PO_4^{3-} in wastewater without (control) and with NaClO pretreatment are presented in Table 2. In contrast, to control, the concentration of COD in NaClO pretreated wastewater was higher but did not present a significant difference ($P > 0.05$). A similar phenomenon was also noticed by Plummer and Edzwald (2002) and it is attributed to the strong oxidation capability of chlorine which oxidizes part of large-non-soluble organic molecules such as fat into soluble ones (e.g., fatty acid) (Wang et al., 2017). The effect of NaClO pretreatment on total PO_4^{3-} concentration was insignificant ($P > 0.05$). On the contrary, 2-33% TN was removed from the wastewater after pretreatment and the concentration of TN was reduced from 154.6 mg/L to 103.1-151.4 mg/L. Also, the pH in wastewater decreased from 6.8 to 5.8. This is consistent with results obtained by Qin et al. (2014) who reported that total Kjeldahl nitrogen (TKN) was

225 reduced from 85.5 to 59.0 mg/L when available chlorine concentration in dairy wastewater
226 was increased from 10 to 90 mg/L. The pH reduction is explained by the hypochlorite acid
227 generated from the reaction of NaClO with water which can oxidize ammonia nitrogen into
228 nitrogen gas and produce hydrochloric acid ($2NH_3 + 3HClO \rightarrow N_2\uparrow + 3H_2O + 3HCl$)
229 (EPA, 2002; Estrela et al., 2002). As a strong and non-selective oxidant, chlorine can
230 oxidize a wide spectrum of organics and bacteria. NaClO pretreatment removed 31-45% of
231 true color and 0.7-2.5 log CFU/mL bacteria which improved light transmission and favor
232 photosynthetic growth of microalgae. However, unlike physical autoclave more than 1.9
233 log CFU/mL bacteria remained in the wastewater after NaClO pretreatment. Bacteria
234 survival suggests that a higher dose might be needed in the pretreatment to reduce the
235 bacteria load. However, the presence of chlorine-resistant bacteria strains was reported by
236 Macauley et al. (2006) who found that chlorine dose higher than 30 mg/L did not achieve
237 100% inactivation of bacteria in wastewater.

238 3.2. Effect of NaClO pretreatment on microorganism growth

239 3.2.1. Growth of immobilized microalgae in beads

240 Pretreatment of NaClO was reported to be able to benefit microalgae growth in
241 wastewater by improving light transmission and generating chloride ions as algal
242 micronutrients as well as reducing bacterial competition (Eyster, 1958). As shown in Fig.1,
243 following a one-day adaption period, immobilized microalgae growth in wastewater
244 pretreated with 0.2 and 0.4 mg/L free chlorine showed a consecutive increase from 822 to
245 2,026 and 1,547 DW mg /L wastewater at the end of treatment. Furthermore, microalgae
246 growth in wastewater pretreated with 0.2 mg/L free chlorine was significantly (P<0.05)

247 higher than control (1,714 DW mg/L wastewater) on day seven. However, despite the
248 benefit of reducing native bacteria, chlorine-based disinfection is non-selective and the
249 application of excess chlorine will increase salinity and be toxic to aquatic plants and
250 organisms (Lv et al., 2018). As indicated in this study, pretreatment with 0.8 and 1.0 mg/L
251 free chlorine extended the microalgae lag period. Therefore significant biomass increase
252 was not observed in these pretreatments. Mutanda et al. (2011) found that wastewater
253 pretreatment with 0.2 and 0.4 mg/L free chlorine supported higher algal biomass growth
254 while a dosage higher than 0.4 mg/L free chlorine was algicidal. Although they used a
255 different system to cultivate microalgae (suspended algae growth) similar results were
256 gained suggesting that chlorine oxidation is powerful and algal cells immobilized into
257 alginate gel beads cannot be exempt from the chemical disinfection. Therefore,
258 pretreatment with a free chlorine concentration of 0.2 mg/L is suitable to improve
259 wastewater quality while promoting the growth of microalgae. However, the increase in
260 algal biomass observed in this study (0.15 mg/bead) is significantly lower than the value
261 reported (0.45-0.62 mg/bead) obtained by Lam and Lee (2012). This is likely due to the
262 high concentration of immobilized activated sludge and microalgae cells which increased
263 the opacity of the beads and cause the self-shading effect to limit light penetration and
264 microalgae growth (Ruiz-Marin et al., 2010).

265 3.2.2. Growth of bacteria suspended in wastewater

266 Change of bacterial load in wastewater with and without (control and blank)
267 NaClO pretreatment was determined during the seven-day treatment. As shown in Fig.2,
268 after initiating the treatment, bacterial growth in non-pretreated (blank and control) and 0.2

mg/L free chlorine-pretreated wastewater entered into an exponential phase immediately while one-day lag phase was observed in wastewater pretreated with 0.4-1.0 mg/L free chlorine. This implies that free chlorine concentration higher than 0.2 mg/L was required to inhibit bacterial growth in wastewater. Also, a comparison of bacteria loads on the same day indicated that the adverse impact of free chlorine on bacterial growth in wastewater lasted for three days. On the third day, bacterial loads in 0.2, 0.4, 0.8, 1.0 mg/L free chlorine-pretreated wastewater were 6.9, 5.4, 4.7, and 4.4 log CFU/mL, respectively, which were lower than control (7.3 log CFU/mL) and blank (7.4 log CFU/mL). However, despite the initial inhibitive effect, bacteria in 0.4-1.0 mg/L free chlorine-pretreated wastewater subsequently followed exponential growth and reached 8.1-8.5 log CFU/mL at the end of treatment. This later recovery of bacterial loads in NaClO-pretreated wastewater was attributed to the instability of free chlorine in water which resulted in a decrease of free chlorine concentration in wastewater (Olivieri et al., 1986). Also, the presence of a large amount of digestible organic nutrients in wastewater provided favorable conditions for bacterial growth (Qiang et al., 2006). Therefore, NaClO pretreatment did not present a consistent inhibitive impact on the growth of bacteria suspended in wastewater despite the initial disinfection.

3.3. Impact of NaClO pretreatment on pollutants removal

3.3.1. Removal of COD

To determine the effect of indigenous bacteria and alginate gel on pollutant removal, the same amount of blank beads were applied in non-pretreated wastewater. As shown in Fig.3, blank treatment removed 67% COD in the end and immobilized system accounted for an additional 15% COD removal. This suggests that immobilized microorganisms

292 increased biodegradation of COD but their contribution was minor compared to the organic
293 mineralization by indigenous bacteria suspended in the wastewater. Consequently, the
294 effect of NaClO pretreatment on COD removal was found to be close to that on bacterial
295 growth discussed in section 3.2.2. In the first three days, the removal rate of COD was
296 reduced with the increase of free chlorine concentration. For instance, on the first day COD
297 removal from wastewater with free chlorine of 0.2 (48%) was significantly higher (P<0.05)
298 than 0.4 (33%), 0.8 (20%) and 1.0 (21%) mg/L which agrees with the low metabolic
299 activity of bacteria in lag phase. A similar phenomenon was also reported by Qin et al.
300 (2014), who found that increasing concentration of available chlorine from 10 to 50 mg/L
301 in dairy wastewater reduced COD removal from 65.8% to 51.5%. However, at the end of
302 treatment removal of COD in 0.8 (62%) and 1.0 (60%), mg/L free chlorine-pretreated
303 wastewater was significantly (P<0.05) lower than control (82%). This does not coordinate
304 with the observation of bacterial loads suspended in 0.8 (8.1 log CFU/mL) and 1.0 mg/L
305 (8.2 log CFU/mL) free chlorine-pretreated wastewater were higher than control (5.7 log
306 CFU/mL) at the end of treatment. Since the only difference between treatments of control
307 and chlorinated wastewater was the presence of free chlorine, thus the possible reason is
308 that immobilized microorganisms were inactivated by pretreatment of 0.8-1.0 mg/L free
309 chlorine which was proved by the poor growth of algal biomass discussed in 3.2.1.

310 3.3.2. Nutrients removal

311 Fig.4a demonstrated removal of TN from wastewater with and without (control and
312 blank) NaClO pretreatment as a function of the treatment period. In wastewater without
313 NaClO pretreatment, TN removal from the blank treatment kept a constant increase from

22% on day one until leveled off at 59%. It was found by the authors that alginate gel could remove 33.1 mg/L TN through physiochemical adsorption such as ionic interaction with positive charged ammonium ions. In contrast to blank, beads in control removed 48% TN on day one and peaked at 75% suggesting that immobilized microorganisms took up 16% more TN than blank. Since sludge bacteria are far less efficient in nutrients removal than microalgae and microalgal assimilation should be the dominating biological process for TN removal (Katam and Bhattacharyya, 2019).

Consequently, NaClO pretreatment generated a similar effect on the biological removal of TN to the growth of immobilized microalgae. It was noticed that increasing concentration of free chlorine applied in the process of wastewater pretreatment reduced the TN removal rate during the seven-day treatment. For example, on the first day, TN removal decreased from 47% to 26-29% when free chlorine concentration raised from 0.2 to 0.4-1.0 mg/L. Also, the removal rate of TN in wastewater pretreated with 0.8 (63%) and 1.0 (66%) mg/L free chlorine was close to those observed in the blank (59%) and significantly (P<0.05) lower than others. This implies that pretreatment with 0.8 and 1.0 mg/L free chlorine considerably lowered the activity of immobilized microorganisms, which correlates with the poor growth of immobilized microalgae. Nevertheless, pretreatment with 0.2 mg/L free chlorine slightly increased TN removal from 75% in control to 80% of TN at the end of treatment. Such effect is related to the higher concentration of algal biomass in 0.2 mg/L free chlorine-pretreated wastewater.

Similar to nitrogen, phosphorus can be removed in physicochemical and biological ways in the alginate gel co-immobilized microalgae-slduge system. In the previous study,

the authors noticed that alginate gel removed 30.6 mg/L more total PO_4^{3-} than pure wastewater. Alginate has been reported to physically adsorb phosphate ions and release calcium ions into wastewater to form precipitation with phosphate under alkaline pH (Angela et al., 2011). As a result, although there were no microorganisms immobilized in blank beads, this treatment still removed 23% total PO_4^{3-} on the first day and achieved a final removal of 79% from non-pretreated wastewater with the abiotic process (Fig.4b). The only difference between blank and control was the immobilization of microorganisms. Total PO_4^{3-} removal in control reached 63% and gradually increased to 92% at the end, which was significantly (P<0.05) higher than blank treatment. This means that immobilized microorganisms accelerated the process of phosphorus removal and removed 13% more total PO_4^{3}. However, pretreatment with NaClO did not enhance this biological process of removing phosphorus. Total PO_4^{3-} removal from wastewater pretreated with 0.2 and 0.4 mg/L free chlorine were close to control and increased steadily from 61% to 83% and 54% to 79% within the first three days before being stabilized at 86% and 81% in the end. While pretreatment with 0.8 and 1.0 mg/L free chlorine reduced removal of phosphorus and merely 22% total PO_4^{3-} was removed at the beginning of the treatment corresponding to the weak growth of microorganisms in wastewater. Sharp increases were observed later, and total PO_4^{3-} removal from 0.8 and 1.0 mg/L free chlorine increased to 79% and 74% on the fifth day. This trend is more related to the growth of bacteria in wastewater than immobilized microalgae. In terms of removal of phosphorus, intake by both microalgae and bacteria should be considered. In the activated sludge, some bacteria species can conduct luxury phosphorus uptake which refers to the storage of excess phosphorus in the form of polyphosphate under an aerobic environment and release them back to wastewater under

anaerobic condition (Wentzel, 1988). Pretreatments of 0.8 and 1.0 mg/L free chlorine were toxic to immobilized microalgae and phosphorus uptake by algal cells was limited. Thus, luxury phosphorus removal by activated sludge bacteria dominated.

Nitrogen and phosphorus are essential elements for microalgae growth. Although NaClO pretreatment improved wastewater environment, it did not significantly (P>0.05) increase nutrients removal, which suggests the tough resistance of this co-immobilized system to the harsh real industrial wastewater environment. After seven-day treatment, the concentration of TN and total PO_4^{3-} in control and 0.2 mg/L free chlorine-pretreated wastewater were reduced to 30-39.0 and 10.5-17.7 mg/L, respectively.

4. Conclusions

NaClO pretreatment improved wastewater environment, and the application of 0.2 mg/L free chlorine increased microalgae growth. The negative effect of free chlorine on bacterial growth was observed in the first three days but diminished later. Immobilized microorganisms functioned well and removed more pollutants from wastewater than blank gel beads. However, pretreatment with free chlorine > 0.2 mg/L decreased immobilized microorganisms' activity and pollutant removal. Finally, 72-82% COD, 75-80% TN and 86-92% total PO_4^{3-} were removed from control and 0.2 mg/L free chlorine pretreated wastewater. This study suggests that NaClO pretreatment was beneficial but not essential to improve the performance of the co-immobilized microalgae/bacteria system.

Acknowledgments

The authors would like to thank the support from the United States Geological Survey (USGS) and the Water for Food Daugherty Global Institute. The authors also would like to express their gratitude to the National Water Center at United Arab Emirates University (UAEU) for partly financing this project under [grant number G00003296].

Appendix A. Supplementary data

E-supplementary data for this work can be found in e-version of this paper online.

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404 **References**

405 1. Angela, M., Beatrice, B., Mathieu, S., 2011. Biologically induced phosphorus
406 precipitation in aerobic granular sludge process. Water Res 45(12), 3776-3786.
407 2. Anthony, R.J., Ellis, J.T., Sathish, A., Rahman, A., Miller, C.D., Sims, R.C., 2013. Effect
408 of coagulant/flocculants on bioproducts from microalgae. Bioresour Technol 149, 65-70.
409 3. Barros, A.I., Gonçalves, A.L., Simões, M., Pires, J.C., 2015. Harvesting techniques
410 applied to microalgae: a review. Renewable and Sustainable Energy Reviews 41, 1489-
411 1500.
412 4. Cheirsilp, B., Thawechai, T., Prasertsan, P., 2017. Immobilized oleaginous microalgae
413 for production of lipid and phytoremediation of secondary effluent from palm oil mill in
414 fluidized bed photobioreactor. Bioresour Technol 241, 787-794.
415 5. Cruz, I., Bashan, Y., Hernàndez-Carmona, G., De-Bashan, L.E., 2013. Biological
416 deterioration of alginate beads containing immobilized microalgae and bacteria during
417 tertiary wastewater treatment. Applied microbiology and biotechnology 97(22), 9847-9858.
418 6. de-Bashan, L.E., Bashan, Y., Moreno, M., Lebsky, V.K., Bustillos, J.J., 2002. Increased
419 pigment and lipid content, lipid variety, and cell and population size of the microalgae
420 *Chlorella spp.* when co-immobilized in alginate beads with the microalgae-growth-
421 promoting bacterium Azospirillum brasilense. Can J Microbiol 48(6), 514-521.

422 7. de-Bashan, L.E., Bashan, Y., 2010. Immobilized microalgae for removing pollutants:
423 review of practical aspects. Bioresour Technol 101(6), 1611-1627.
424 8. EPA, U., 2002. Development document for the proposed effluent limitations guidelines
425 and standards for the meat and poultry products industry point source category (40 CFR
426 432).
427 9. Estrela, C., Estrela, C.R., Barbin, E.L., Spanó, J.C.E., Marchesan, M.A., Pécora, J.D.,
428 2002. Mechanism of action of sodium hypochlorite. Brazilian dental journal 13(2), 113-
429 117.
430 10. Eyster, C., 1958. Chloride effect on the growth of *Chlorella pyrenoidosa*. Nature
431 181(4616), 1141.
432 11. Farooq, W., Lee, Y.-C., Ryu, B.-G., Kim, B.-H., Kim, H.-S., Choi, Y.-E., Yang, J.-W.,
433 2013. Two-stage cultivation of two *Chlorella sp.* strains by simultaneous treatment of
434 brewery wastewater and maximizing lipid productivity. Bioresource technology 132, 230-
435 238.
436 12. Gonçalves, A.L., Pires, J.C.M., Simões, M., 2016. Wastewater polishing by consortia of
437 *Chlorella vulgaris* and activated sludge native bacteria. Journal of Cleaner Production 133,
438 348-357.
439 13. Gupta, S., Pawar, S.B., 2018. An integrated approach for microalgae cultivation using
440 raw and anaerobic digested wastewaters from food processing industry. Bioresource
441 technology 269, 571-576.
442 14. HACH, 2014. Platinum-Cobalt Standard Method.
443 15. Hariz, H.B., Takriff, M.S., Yasin, N.H.M., Ba-Abbad, M.M., Hakimi, N.I.N.M., 2019.
444 Potential of the microalgae-based integrated wastewater treatment and CO2 fixation system

445 to treat Palm Oil Mill Effluent (POME) by indigenous microalgae: *Scenedesmus sp.* and
446 *Chlorella sp.* Journal of Water Process Engineering 32, 100907.
447 16. Hodaifa, G., Martínez, M.E., Sánchez, S., 2008. Use of industrial wastewater from
448 olive-oil extraction for biomass production of *Scenedesmus obliquus*. Bioresource
449 technology 99(5), 1111-1117.
450 17. Katam, K., Bhattacharyya, D., 2019. Simultaneous treatment of domestic wastewater
451 and bio-lipid synthesis using immobilized and suspended cultures of microalgae and
452 activated sludge. Journal of Industrial and Engineering Chemistry 69, 295-303.
453 18. Lam, M.K., Lee, K.T., 2012. Immobilization as a feasible method to simplify the
454 separation of microalgae from water for biodiesel production. Chemical Engineering
455 Journal 191, 263-268.
456 19. Lin, C.-Y., Nguyen, M.-L.T., Lay, C.-H., 2017. Starch-containing textile wastewater
457 treatment for biogas and microalgae biomass production. Journal of cleaner production 168,
458 331-337.
459 20. Lv, J., Liu, Y., Feng, J., Liu, Q., Nan, F., Xie, S., 2018. Nutrients removal from
460 undiluted cattle farm wastewater by the two-stage process of microalgae-based wastewater
461 treatment. Bioresour Technol 264, 311-318.
462 21. Macauley, J.J., Qiang, Z., Adams, C.D., Surampalli, R., Mormile, M.R., 2006.
463 Disinfection of swine wastewater using chlorine, ultraviolet light and ozone. Water Res
464 40(10), 2017-2026.
465 22. Maity, J.P., Bundschuh, J., Chen, C.-Y., Bhattacharya, P., 2014. Microalgae for third
466 generation biofuel production, mitigation of greenhouse gas emissions and wastewater
467 treatment: Present and future perspectives – A mini review. Energy 78, 104-113.

468 23. Markou, G., Chatzipavlidis, I., Georgakakis, D., 2012. Cultivation of *Arthrospira*
469 *(Spirulina) platensis* in olive-oil mill wastewater treated with sodium hypochlorite.
470 Bioresour Technol 112, 234-241.
471 24. Mujtaba, G., Rizwan, M., Lee, K., 2017. Removal of nutrients and COD from
472 wastewater using symbiotic co-culture of bacterium *Pseudomonas putida* and immobilized
473 microalga *Chlorella vulgaris*. Journal of Industrial and Engineering Chemistry 49, 145-151.
474 25. Mutanda, T., Karthikeyan, S., Bux, F., 2011. The utilization of post-chlorinated
475 municipal domestic wastewater for biomass and lipid production by *Chlorella spp.* under
476 batch conditions. Appl Biochem Biotechnol 164(7), 1126-1138.
477 26. Olivieri, V.P., Snead, M.C., Krusé, C.W., Kawata, K., 1986. Stability and effectiveness
478 of chlorine disinfectants in water distribution systems. Environmental Health Perspectives
479 69, 15-29.
480 27. Park, S., Van Ginkel, S.W., Pradeep, P., Igou, T., Yi, C., Snell, T., Chen, Y., 2016. The
481 Selective Use of Hypochlorite to Prevent Pond Crashes for Algae-Biofuel Production.
482 Water Environ Res 88(1), 70-78.
483 28. Plummer, J.D., Edzwald, J.K., 2002. Effects of chlorine and ozone on algal cell
484 properties and removal of algae by coagulation. Journal of Water Supply: Research and
485 Technology-AQUA 51(6), 307-318.
486 29. Qiang, Z., Macauley, J.J., Mormile, M.R., Surampalli, R., and Adams, C.D., 2006.
487 Treatment of antibiotics and antibiotic resistant bacteria in swine wastewater with free
488 chlorine. Journal of agricultural and food chemistry 54(21).

30. Qin, L., Shu, Q., Wang, Z., Shang, C., Zhu, S., Xu, J., Li, R., Zhu, L., Yuan, Z., 2014. Cultivation of *Chlorella vulgaris* in dairy wastewater pretreated by UV irradiation and sodium hypochlorite. Appl Biochem Biotechnol 172(2), 1121-1130.

31. Ruiz-Marin, A., Mendoza-Espinosa, L.G., Stephenson, T., 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. Bioresour Technol 101(1), 58-64.

32. Shen, Y., Gao, J., Li, L., 2017. Municipal wastewater treatment via co-immobilized microalgal-bacterial symbiosis: Microorganism growth and nutrients removal. Bioresour Technol 243, 905-913.

33. Starr, R.C., Zeikus, J.A., 1993. UTEX—The culture collection of algae at the University of Texas at Austin 1993 list of cultures 1. Journal of phycology 29, 1-106.

34. Su, H., Zhang, Y., Zhang, C., Zhou, X., Li, J., 2011. Cultivation of *Chlorella pyrenoidosa* in soybean processing wastewater. Bioresource Technology 102(21), 9884-9890.

35. Tan, X.-B., Zhao, X.-C., Yang, L.-B., Liao, J.-Y., Zhou, Y.-Y., 2018. Enhanced biomass and lipid production for cultivating *Chlorella pyrenoidosa* in anaerobically digested starch wastewater using various carbon sources and up-scaling culture outdoors. Biochemical Engineering Journal 135, 105-114.

36. Taşkan, E., 2016. Performance of mixed algae for treatment of slaughterhouse wastewater and microbial community analysis. Environmental Science and Pollution Research 23(20), 20474-20482.

37. Wang, H., Zhang, W., Chen, L., Wang, J., Liu, T., 2013. The contamination and control of biological pollutants in mass cultivation of microalgae. Bioresour Technol 128, 745-750.

512 38. Wang, W.L., Zhang, X., Wu, Q.Y., Du, Y., Hu, H.Y., 2017. Degradation of natural
513 organic matter by UV/chlorine oxidation: Molecular decomposition, formation of oxidation
514 byproducts and cytotoxicity. Water Res 124, 251-258.
515 39. Wang, Y., Guo, W., Yen, H.-W., Ho, S.-H., Lo, Y.-C., Cheng, C.-L., Ren, N., Chang,
516 J.-S., 2015. Cultivation of *Chlorella vulgaris* JSC-6 with swine wastewater for
517 simultaneous nutrient/COD removal and carbohydrate production. Bioresource technology
518 198, 619-625.
519 40. Wentzel, M.C., 1988. Biological excess phosphorus removal in activated sludge
520 systems. University of Cape Town.
521 41. Xie, B., Gong, W., Yu, H., Tang, X., Yan, Z., Luo, X., Gan, Z., Wang, T., Li, G., Liang,
522 H., 2018. Immobilized microalgae for anaerobic digestion effluent treatment in a
523 photobioreactor-ultrafiltration system: Algal harvest and membrane fouling control.
524 Bioresour Technol 268, 139-148.
525 42. Yang, L., Li, H., Wang, Q., 2019. A novel one-step method for oil-rich biomass
526 production and harvesting by co-cultivating microalgae with filamentous fungi in molasses
527 wastewater. Bioresource technology 275, 35-43.
528 43. Zhu, L., Qin, X., Xu, Z., Qin, L., Hiltunen, E., Takala, J., Wang, Z., Yuan, Z., 2013.
529 Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for biodiesel
530 production. Bioresour Technol 137, 318-325.

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537 **Figure captions**

538 Fig.1. Growth of microalgae in beads in wastewater with and without (control) NaClO

539 pretreated

540 Fig.2. Growth of bacteria suspended in wastewater with different free chlorine

541 concentration. Control: non-pretreated wastewater + beads, Blank: non-pretreated

542 wastewater + blank beads

543 Fig.3. Percentage removal of COD during seven-day treatment. Control: non-pretreated

544 wastewater + beads, Blank: non-pretreated wastewater + blank beads

545 Fig.4. Percentage removal of (a) TN and (b) total PO_4^{3-} from NaClO pretreated wastewater.

546 Control: non-pretreated wastewater + beads, Blank: non-pretreated wastewater + blank

547 beads

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558 **Tables and figures**

559 Color will not be used for any tables and figures in print

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581 Table 1. Pretreatment applied to wastewater before being treated with microalgae

Microalgae	Wastewater source	Pretreatment	Reference
C. vulgaris	Swine farm	Centrifugation + Membrane filtration + Dilution + Autoclave	Wang et al. (2015)
C. pyrenoidosa	Soybean processing	Centrifugation + Autoclave	Su et al. (2011)
S. obliquus	Olive-oil extraction	Membrane filtration + Dilution	Hodaifa et al. (2008)
Chlorella sp.	Brewery	Centrifugation + Autoclave	Farooq et al. (2013)
C. vulgaris	Molasses	Centrifugation + Autoclave	Yang et al. (2019)
C. sorokiniana, S. obliquus, S. abundans	Food processing	Centrifugation + Autoclave	Gupta and Pawar (2018)
Scenedesmus sp., Chlorella sp.	Palm oil mill	Centrifugation + Dilution	Hariz et al. (2019)
Scendesmus sp.	Textile desizing	Anaerobic digestion+ Dilution	Lin et al. (2017)
C. pyrenoidosa	Starch processing	Membrane filtration + Autoclave	Tan et al. (2018)
Mixed algae species	Slaughterhouse	Membrane filtration + Autoclave + Dilution	Taşkan (2016)

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590 Table 2. Wastewater characteristic before and after NaClO pretreatment

Free chlorine concentration (mg/L)	COD (mg/L)	Total PO_4^{3-} (mg/L)	TN (mg/L)	pH	True color (PtCo)	APC (log CFU/mL)
Control	1,868±2a	126.9±0.4a	154.6±8.3a	6.8±0.2a	1,562±101a	4.4±0.0a
0.2	1,937±57a	124.8±4.7a	151.4±7.8ab	6.5±0.3ab	1,295±106b	3.7±0.0a
0.4	1,943±91a	126.2±6.2a	142.2±8.7b	6.4±0.2ab	1,073±86c	3.4±0.4ab
0.8	1,967±83a	124.4±2.8a	120.1±6.2c	6.1±0.2ab	940±27cd	2.5±0.8bc
1.0	1,953±72a	127.7±3.6a	103.1±2.5d	5.8±0.3b	862±82d	1.9±0.4c

591 *Note*: values deviation with different letters indicate significant difference at level of
592 P=0.05. COD: chemical oxygen demand total PO_4^{3-}: total phosphate, TN: total nitrogen,
593 APC: aerobic plate counts

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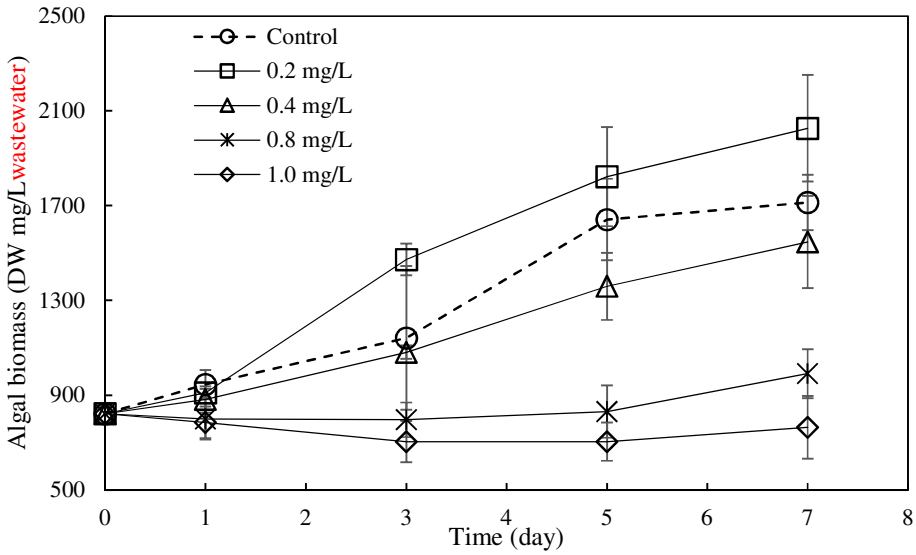
Control
0.2 mg/L
0.4 mg/L
0.8 mg/L
1.0 mg/L
Algal biomass (DW mg/Lwastewater)
Time (day)
2500
2100
1700
1300
900
500
0
1
2
3
4
5
6
7
8

Fig.1

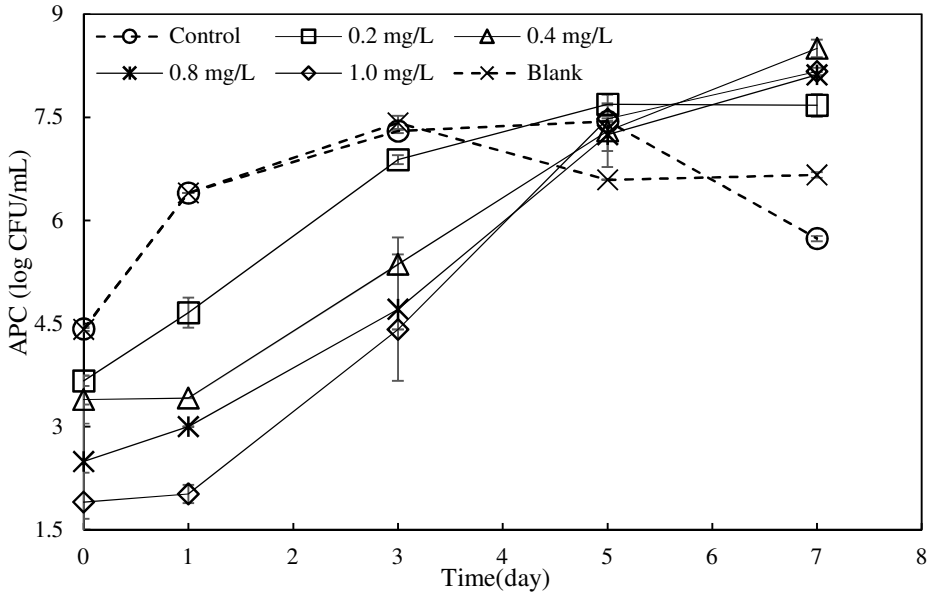
Control
0.2 mg/L
0.4 mg/L
0.8 mg/L
1.0 mg/L
Blank
APC (log CFU/mL)
Time(day)
9
7.5
6
4.5
3
1.5
0
1
2
3
4
5
6
7
8

Fig.2

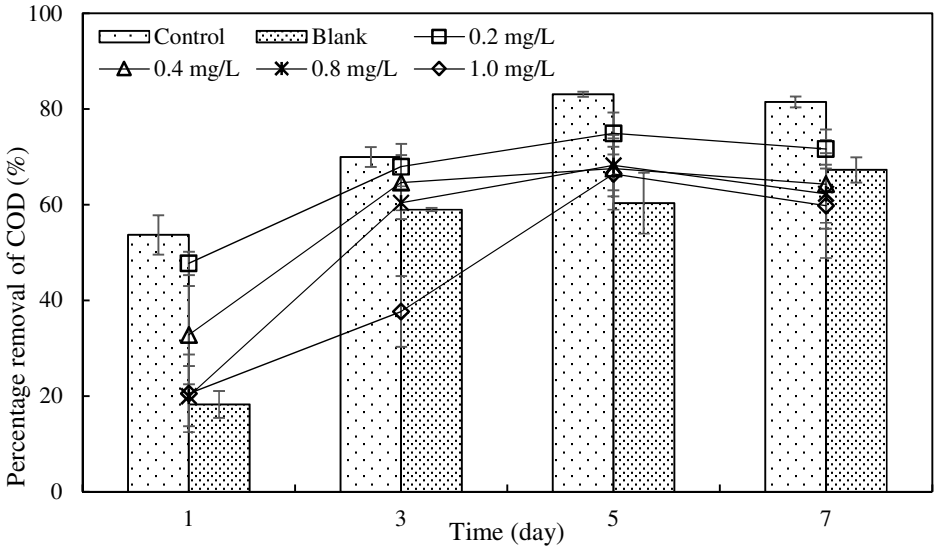
Control
Blank
0.2 mg/L
0.4 mg/L
0.8 mg/L
1.0 mg/L
Percentage removal of COD (%)
Time (day)
0
20
40
60
80
100
1
3
5
7

Fig.3

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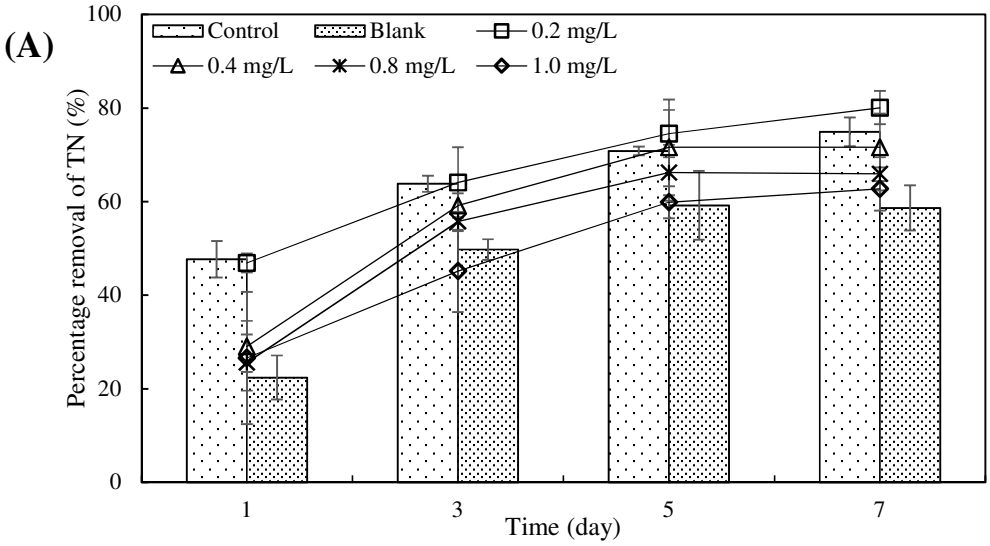

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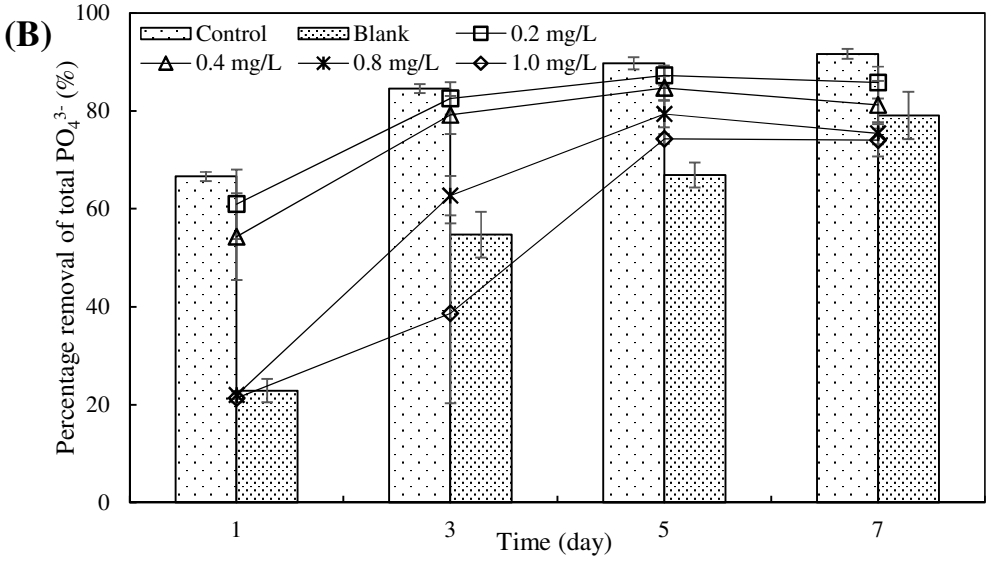

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647 Fig.4

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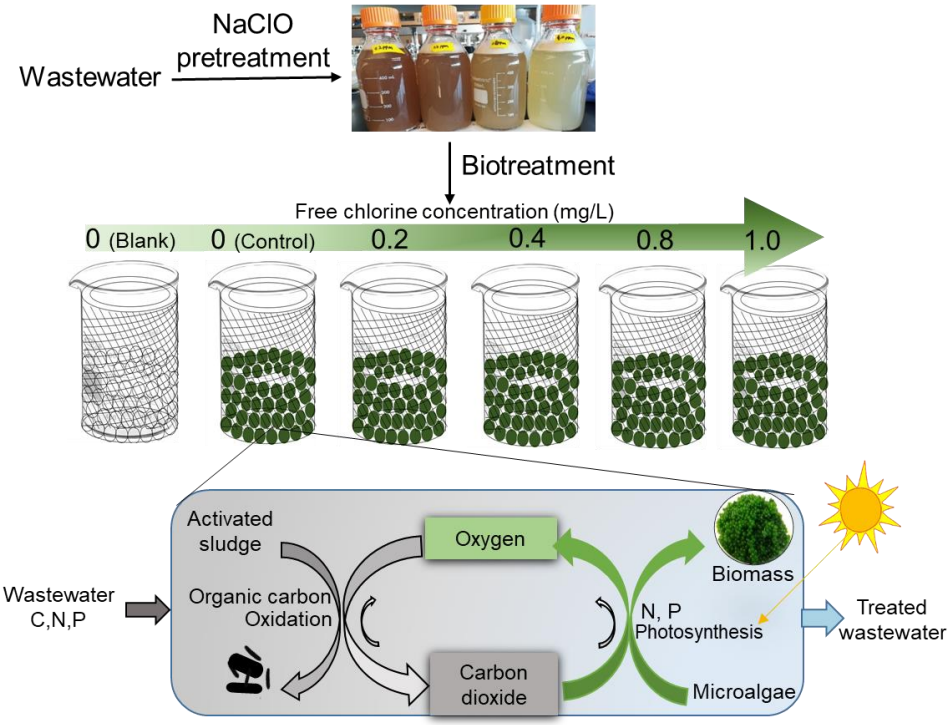
NaClO
pretreatment
Wastewater
Biotreatment
Free chlorine concentration (mg/L)
0 (Blank)
0 (Control)
0.2
0.4
0.8
1.0
Activated
sludge
Oxygen
Biomass
Wastewater
C,N,P
Organic carbon
Oxidation
N, P
Photosynthesis
Treated
wastewater
Carbon
dioxide
Microalgae