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1 **Impacts of antiseptic cetylpyridinium chloride on microbiome and its removal efficiency**

2 **in aerobic activated sludge**

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6

7 Luong N. Nguyen[a*] and Seungdae Oh[b**]

8 [a] Center for Technology in Water and Wastewater, School of Civil and Environmental

9 Engineering, University of Technology Sydney, NSW 2007, Australia

10 [b] Department of Civil Engineering, Kyung Hee University, 1732 Deogyeong-daero, Giheung-

11 gu, Yongin-si, Gyeonggi-do, 17104, Republic of Korea

12

13 Corresponding author

14 [*] Center for Technology in Water and Wastewater, School of Civil and Environmental

15 Engineering, University of Technology Sydney, NSW 2007, Australia

16 Phone: (+61) 468863865; E-mail: luongngoc.nguyen@uts.edu.au

17 [**] Department of Civil Engineering, Kyung Hee University, Yongin-si, Gyeonggi-do, Republic

18 of Korea. Phone: +82 (031) 201-3664. Fax: +82 (031) 202-8854. E-mail: soh@khu.ac.kr

19

20 **Abstract**

21 This study evaluated short- and long-term exposure of activated sludge (AS)
22 microbiome to cetylpyridinium chloride (CPC), a quaternary ammonium compound widely
23 used as biocidal additive or cationic surfactant. Toxicity assay in batch mode showed that CPC
24 (50 μg $L^{-1}$) inhibited cell growth. However, in a continuous reactor, CPC concentration in the
25 range of 50 to 500 μg $L^{-1}$ did not result in any observable impact on the biological activities of
26 the AS microbiome. Similarly, 16S rRNA gene-based community profiling revealed that CPC
27 had no observable impact on microbial diversity. At the phylogenetic structure, *Rhodobacter*
28 (15 ± 7% of the total) and *Asticcacaulis* (9 ± 3%) were the only two phyla with increasing
29 population in the 500 μg $L^{-1}$-exposed reactors. This was also supported by an observation of
30 no major change in the community structure. The reactors could remove >60% of CPC at initial
31 concentrations of 50–500 μg $L^{-1}$, primarily by adsorption and biodegradation. The enrichment
32 of *Rhodobacter* and *Asticcacaulis* genus might contribute to CPC biodegradation and emerge
33 as a potential microbial niche for the removal of CPC.

34 **Key words**: Cetylpyridinium chloride; Activated sludge microbiome; Microbial community
35 diversity; Community phylogenetic structure

36 **1. Introduction**

37 Quaternary ammonium compounds are commonly applied as biocidal additives or
38 cationic surfactants. They are extensively used in a number of personal care and domestic
39 products (e.g. shampoo, body wash and dishwashing detergents), pesticides, and industrial
40 applications (e.g. cleaning agents and lubricants) (Ko et al., 2007; Sundheim et al., 1998; Zhang
41 et al., 2015). They interact predominantly with phospholipid components of the cytoplasmic
42 membrane (e.g. bacteria) and the plasma membrane (e.g. yeast). Cetylpyridinium chloride
43 (CPC) is a frequently used quaternary ammonium compound. CPC is the active ingredient at
44 0.01 to 1% (w/w) of many personal care products such as antiperspirant deodorants, oral
45 hygiene products, and skin lotions (Costa et al., 2013) and surface-disinfecting agents in
46 poultry processing facilities (Zhang et al., 2015). CPC is also used as a detergent additive (up
47 to 5 mg $L^{-1}$) to improve the removal of phenols, reactive dyes, and other organic solutes by
48 micellar-enhanced ultrafiltration (Luo et al., 2010). Due to its extensive use, CPC has been
49 found at 52 μg $L^{-1}$ in river water and 47–88 μg $L^{-1}$ in wastewater (Shrivas and Wu 2007). CPC
50 causes acute toxicity to freshwater planarians (at 40 μg $L^{-1}$), rats (90 μg $L^{-1}$), and frog embryos
51 (531 μg $L^{-1}$) (Park et al., 2016), suggesting potential environmental health risks at the levels
52 found in the environment.

53 The impact of quaternary ammonium compounds on functionality and diversity of the
54 microbial community in activated sludge (AS) for wastewater treatment remains a topic for
55 further investigation. Several studies have shown that quaternary ammonium compounds may
56 disrupt the diversity and function of (AS) (Bessa et al., 2017; Delgado et al., 2010; Jiang et al.,
57 2017; Oh et al., 2014). Exposure of benzalkonium chloride reduced microbial community
58 diversity and resulted in the enrichment of resistant species (Oh et al., 2014). Furthermore,
59 quaternary ammonium compounds could inhibit both nitrifying and denitrifying bacteria in
60 biological nutrient removal (Carter 2008). While some effort has been made on understanding

CPC-mediated toxicity (Hrenovic et al., 2008; Imai et al., 2017), most studies focused on short-term toxicity effects (e.g. acute toxicity and lethality) using a single model organism. It was hypothesized that relatively low levels of CPC (i.e. sub-inhibitory concentrations), rather than pulsed inputs, are continuously emitted to wastewater treatment plants (WWTPs) through urban sewage disposal. In these WWTPs, complex microbial communities (e.g. AS) are exposed to CPC over long periods of time. The chronic toxicity effects of sub-inhibitory concentrations of CPC on AS communities, rather than pure cultures, have not been systematically investigated. These effects are highly relevant to the real-world ecotoxicological consequences of CPC in AS ecosystems.

WWTPs are an important barrier to limit the spread of CPC to the environment. Satisfactory removal of CPC (i.e. via the AS process) is highly desired to reduce the ecotoxicity of CPC. Thus far, there is a consensus in literature that adsorption and biodegradation are two main removal pathways of micropollutants. The contribution of these pathways to the overall removal depends on a number of factors such as physico-chemical properties, operational conditions and microbial community (Phan et al., 2016; Tadkaew et al., 2011). Since a substantial amount of CPC is released into WWTPs due to its extensive use in domestic and industrial applications, it is critical to address (1) whether CPC is removed effectively from the wastewater and (2) how CPC is removed in the AS process.

This study aimed to assess the effect of CPC and removal in AS. Laboratory bioreactors were inoculated with AS and fed with CPC-containing substrates. Both short- and long-term toxicity effects of CPC on the major functions of AS were investigated under different CPC levels. Bacterial community dynamics were also assessed using 16S rRNA gene sequencing and analysis.

84 **2. Materials and Methods**

85 2.1 Short-term toxicity assessment

86 AS taken from an aeration tank of a municipal WWTP (Jurong, Singapore) was
87 inoculated into three fed-batch reactors. The reactors were fed by the synthetic feed (Section
88 2.2) and maintained at pH 6.8 ± 0.2 under aerobic conditions (4.8 ± 0.8 mg $L^{-1}$ of dissolved
89 oxygen). After one month of acclimation to the laboratory conditions, the reactors showed
90 stable biomass (0.44 ± 0.16 g $L^{-1}$ of mixed liquor volatile suspended solids [MLVSS]) and
91 organic matter removal (91.6 ± 3.7% of soluble chemical oxygen demand [sCOD] removal).
92 Antimicrobial susceptibility testing for determining inhibitory concentrations was performed
93 to evaluate the short-term toxicity of CPC to AS (NCCLS 2003). Briefly, triplicate AS cultures
94 were taken from the reactors to use as inoculum and washed twice with saline phosphate buffer
95 (pH 7.4). The inoculum was added (100 μL) into pre-established 13-mL glass tubes containing
96 5 mL of synthetic feed supplemented with CPC at 0–8 mg $L^{-1}$. All the test tubes were incubated
97 in an orbital shaker at 150 rpm and 25 °C for 24 h. The relative growths of mixed cultures over
98 the range of CPC concentrations were measured after 24 h incubation using UV
99 spectrophotometer at wavelength of 620 nm. To determine the inhibition concentration, the
100 relative growth data were fitted using a four-parameter logistic model (Gadagkar and Call
101 2015).

102 2.2 Bioreactor operation

103 The AS from the three fed-batch reactors (Section 2.1) was mixed and used to develop
104 another set of six identical reactors. Three of these reactors (CPC_50) were exposed to 50 μg
105 $L^{-1}$ of CPC and another three (CPC_500) to 500 μg $L^{-1}$ of CPC. These two concentrations were
106 selected given that these are environmentally relevant levels (50 – 581 μg $L^{-1}$) in wastewater
107 (Clara et al., 2007; Shrivas and Wu 2007).

The synthetic feed (1 L) contained $C_6H_{12}O_6$ (1.83 g), $NH_4Cl$ (30 mg), $KH_2PO_4$ (340 mg), $K_2HPO_4$ (600 mg), $MgSO_4.7H_2O$ (270 mg), $FeSO_4$ (10 mg) and 10 mL of 100 x trace element solution (ZnSO4.7H2O 0.35 mg, $MnSO_4.H_2O$ 0.21 mg, $H_3BO_4$ 2.1 mg, $CoCl_2.2H_2O$ 1.4 mg, $CuCl_2.2H_20$ 0.07 mg, $NiSO_4.6H_2O$ 0.1 mg, $Na_2MoO_4.2H_2O$ 0.21 mg per liter) as described previously (Oh and Choi 2018). The ratio of COD, total nitrogen and total phosphorous (COD: TN: TP) in the synthetic feed was 80: 5: 1. The reactors (0.6 L working volume) were fed every 3.5 days by withdrawing one-third of a mixed liquor suspension and replacing with a freshly-prepared synthetic feed plus CPC at 50 and 500 µg $L^{-1}$, resulting in solid retention time of 10.5 days. The reactors were maintained at room temperature (22–23 °C) and under aerobic conditions ($4.8 \pm 0.8$ mg $L^{-1}$ of dissolved oxygen). CPC (analytical grade 98% purity) was purchased from Sigma Aldrich (Singapore) with the physicochemical properties as in Table S1. A CPC stock solution 0.1 g $L^{-1}$ was prepared and stored at 4 °C prior to use within one month. The reactors were operated for 1.5 months. The sCOD removal, and MLVSS levels were used were monitored at days 0, 3, 13, 27 and 42 to investigate the chronic impact of CPC on AS. The concentration of CPC in influent and effluent was also measured at days 0, 3, 13, 27 and 42. The AS microbial community samples were collected from day 0 and 42 (Control) and day 42 (CPC_50 and CPC_500) for characterisation of changes due to CPC exposure.

### 2.3 Analytical methods

sCOD was measured using a HACH colorimetric method after filtering the samples through a 0.22-µm filter. MLVSS were measured following the APHA Standard Method 2540 (Eaton. et al., 2005). pH was measured using an Orion 4-Star Plus pH/conductivity meter (Thermo Scientific, Waltham, MA). CPC concentrations were measured using a high-performance liquid chromatography system (Shimadzu Asia Pacific Pte. Ltd) equipped with a Shim-Pack GIST Phenyl column (5 µm, 4.6 x 250 mm) and a UV–vis detector. The mobile

133 phase solution consisted of 20 mM sodium dihydrogen phosphate monohydrate (pH 2.5) and
134 acetonitrile (99% purity) at a 40:60 (v/v) ratio. The injected sample volume and mobile phase
135 flow rate were 100 µL and 1.8 mL $min^{-1}$, respectively. CPC was detected at a wavelength of
136 254 nm and the detection limit was 10 µg $L^{-1}$.

137 To quantify CPC adsorption onto sludge, AS at the end of a feeding cycle from triplicate
138 reactors was collected and subjected to an ultrasonic solvent extraction method (Wijekoon et
139 al., 2013). In brief, the sludge was freeze-dried for 24 hours and ground to a fine powder using
140 a mortar and pestle. The powder was subsequently washed with 5 mL methanol in a 13-mL
141 tube. The resulting slurry was mixed well with a vortex mixer and ultrasonicated at 30 °C for
142 10 min, after which the resultant suspension was spun by centrifugation at 2851 x g for 10 min.
143 The supernatant was collected and the remaining solid mixture was subjected to another round
144 of extraction. The supernatants from all extraction steps were combined, filtered by 0.22 µm,
145 and subjected to HPLC analysis. Independent tests contained inactive (heat-killed biomass)
146 and abiotic settings, showing an extraction efficiency of 82 ± 5%. The mass of CPC adsorbed
147 onto the sludge in a given reactor was estimated using the following equation: adsorption = T
148 $\times C_{CPC} \times E$, where T (g), $C_{CPC}$ (µg $g^{-1}$), and E (%) denote the total sludge mass in the reactor,
149 the concentration of CPC extracted per one gram of sludge, and the extraction efficiency,
150 respectively.

151 2.4 DNA extraction and 16S rRNA gene sequencing

152 DNA was extracted from the mixed liquor samples using a MoBio PowerSoil ® DNA
153 isolation kit (MOBIO, Carlsbad, CA, USA) following the manufacturer's instructions. All
154 obtained DNA concentrations were > 0.5 µg µ$L^{-1}$ with absorbance ratios ($A_{260}/A_{280}$) > 1.8. PCR
155 amplification of the 16S rRNA gene was performed by Macrogen Inc. (Seoul, Republic of
156 Korea) using universal bacterial primers targeting the V3 to V4 region (341F-805R). The 16S
157 rRNA gene amplicon products were sequenced using the MiSeq™ platform at Macrogen Inc.

158 Raw paired-end (2×300 bp) 16S rRNA gene sequence data were analyzed according to
159 the MiSeq SOP pipeline (Kozich et al., 2013). In brief, raw sequences were preprocessed using
160 the following parameters: maxambig = 0, minimum length = 200, maximum length of
161 homopolymer = 8, and all other parameters at their default settings. The preprocessed
162 sequences were chimera-checked and classified using the commands chimera.vsearch and
163 classify.seqs, respectively. Chimeric sequences and sequences assigned to chloroplasts,
164 mitochondria, unknown, archaea, and eukaryotes were removed. The sequences were clustered
165 into representative OTUs based on a 97% nucleotide identity cutoff, using the commands
166 dist.seqs and cluster. The 16S rRNA gene sequencing generated 28,400 to 55,392 sequences
167 per sample after preprocessing using the MOTHUR pipeline (Kozich et al., 2013). The
168 rarefaction curves of the 12 datasets tended to approach the saturation plateau (> 99% of
169 Good's coverage) (Fig. S2), suggesting that the OTU diversity was almost saturated by the
170 sequencing depth used in this study. The sequences were rarefied to 28,000 sequences (the
171 lowest number of sequences per sample) to estimate alpha diversity indices (Chao, Ace,
172 Shannon, and Inverse Simpson) using the MOTHUR package. Statistical testing for differential
173 community characteristics was conducted using the Mann-Whitney U test. The 16S rRNA gene
174 sequences were deposited in GenBank with accession numbers. Control_0_1 (SRS2340183),
175 Control_0_2 (SRS2340176), Control_0_3 (SRS2340220), Control_42_1 (SRS2340175),
176 Control_42_2 (SRS2340198), Control_42_3 (SRS2340197), CPC_50_1 (SRS2340199),
177 CPC_50_2 (SRS2340202), CPC_50_3 (SRS2340203), CPC_500_1 (SRS2340200),
178 CPC_500_2 (SRS2340201), and CPC_500_3 (SRS2340195).

179 **3. Results and Discussion**

180 3.1 Short-term toxicity of CPC to activated sludge

181 Short-term toxicity assays showed that CPC was toxic to AS. The heterotrophic growth
182 of the AS was inhibited by 0.063–2 mg $L^{-1}$ of CPC and completely suppressed (99%) by 4 mg

183 $L^{-1}$ of CPC (Fig. 1). The relative growth data was fitted in a four-parameter logistic function
184 (Hill dose – response curve) (Gadagkar and Call 2015), and the model predicted that 20 mg $L^{-}$
185 $^{1}$ of CPC would completely inhibit cell growth. The model also predicted that environmentally
186 relevant concentrations of CPC (i.e. 50 and 500 µg $L^{-1}$) were sub-inhibitory levels (i.e. 20%
187 and 60% of cell growth inhibition at 24 hours, respectively).

188 [FIGURE 1]

189 This study provides conclusive evidence that CPC is toxic to the AS community in
190 short-term exposure. Thus far, CPC toxicity has only been demonstrated in a few pure cultures
191 (Table 1). A wide variation of CPC toxicity has been reported across different taxa, indicating
192 that toxicity levels may vary from one taxon to another. The toxicity threshold of CPC to
193 complex AS communities was quantitatively assessed in this study based on the effects on the
194 heterotrophic growths. Therefore, future investigations determining the effects and toxicity of
195 CPC on metabolic activities of other important nutrients (e.g., nitrogen and phosphorous) in
196 biological nitrogen removal and enhanced biological phosphorus removal processes will be
197 highly desired.

198 [TABLE 1]

199 3.2 Long-term exposure of activated sludge to CPC

200 Long-term exposure (1.5 months) of CPC (50–500 µg $L^{-1}$) to AS did not affect its
201 biological functions (e.g. organic matter removal and biomass yield). CPC_50 and CPC_500
202 reactors showed 89 ± 3% and 87 ± 3% of COD removal, respectively, which were marginally
203 lower than the COD removal in the control reactors (92 ± 4%) (Fig. S3) (i.e., no statistical
204 significance with $P > 0.05$ by Mann-Whitney U test). The range of COD removals observed in
205 both control and experimental reactors were similar to conventional AS process. Likewise,
206 MLVSS concentrations in the control, CPC_50 and CPC_500 were 0.75 ± 0.07, 0.76 ± 0.18

207 and 0.71 ± 0.16 mg $L^{-1}$, respectively (Fig. S3), which were comparable with each other ($P$ >
208 0.05 by Mann-Whitney U test). The MLVSS concentration was noticeably lower than that of
209 conventional AS, which could be attributed to the reactor operation without sludge recycling
210 in this study.

211 The difference between short-term toxicity assay and long-term exposure findings can
212 be attributed to the different incubation periods. Although a singular addition of CPC had short
213 term toxic effects on AS (Section 3.1), the continuous presence of the compound appeared to
214 have negligible impact on AS functionality in the reactors. One cycle duration of the fed-batch
215 reactors was 3.5 days, which was much longer than the cell growth period (24 hours) in the
216 acute toxicity testing. Therefore, toxicology assays in batch mode may not yield meaningful
217 results that can be applied for AS process. The impact of CPC and other micropollutants on the
218 AS process can only be ascertained through long-term evaluation of AS performance and
219 microbial community.

220 3.3 Effects of CPC on bacterial community diversity and structure

221 CPC addition at 50–500 μg $L^{-1}$ did not influence AS microbial diversity in reactors.
222 The diversity index measurements including species richness and evenness were compared
223 amongst control, CPC_50 and CPC_500 reactors. At 50 μg $L^{-1}$-exposed, the communities
224 showed a comparable species richness and evenness to the control communities (Table 2). At
225 higher CPC exposure, a slight increase in species evenness (Shannon and Inverse Simpson)
226 was observed. However, these differences in the community diversity amongst the three
227 community groups were not statistically significant ($P$ > 0.05).

228 [TABLE 2]

229 16S rRNA gene-based analysis also suggested that exposure to 50–500 μg $L^{-1}$ of CPC
230 did not significantly change community structure (Fig. 2). The OTU (a total of 796 OTUs)

231 relative abundance data were used to carry out nonmetric multidimensional scaling (NMDS)
232 analysis for community structural comparison. The CPC_50 (81.5 ± 3.0 by the Euclidean
233 distance similarity), and CPC_500 (78.4 ± 4.2) communities clustered closely within each
234 community group, confirming the high level of community structure similarity of the replicate
235 communities. The control communities showed only a moderate level of similarity (61.4 ± 14.5)
236 within the group. The pairwise similarity between communities was 41.5 ± 7.5 (control vs.
237 CPC_50) and 36.3 ± 6.0 (control vs. CPC_500). A permutational multivariate analysis of
238 variance (PERMANOVA) test (Anderson 2001) revealed that the community phylogenetic
239 structure did not differ significantly (Bonferroni-corrected $P > 0.05$) among the three
240 community groups.

241 [FIGURE 2]

242 The degree of perturbation posed by a micropollutant on AS microbial diversity and
243 structure can be influenced by compound concentrations (Jiang et al., 2017; Phan et al., 2016;
244 Zhang et al., 2016). A slight increase in species evenness was observed when AS community
245 was exposed to tetracycline or sulfamethoxazole at concentration of 5 μg $L^{-1}$. At higher
246 concentration 50 – 10,000 μg $L^{-1}$, the community diversity dropped significantly (Zhang et al.,
247 2016), indicating the effective dose at 50 μg $L^{-1}$ and above. Jiang et al. (2017) observed no
248 impact of diclofenac, ibuprofen and naproxen on AS communities at concentration of 5 μg $L^{-1}$
249 . It is indicated that each individual antimicrobial agent has its own effective dose level (i.e.
250 concentration for a significant biological response to be observed), below which no
251 deterministic effects occur. No significant effects of long-term CPC exposure on the phenotype
252 (biomass yield and organic matter removal) and overall community diversity of AS suggest
253 that the concentration (50–500 μg $L^{-1}$) of CPC exposure tested in this study is likely below the
254 threshold level.

255 3.4 Phylogenic structure of activated sludge after CPC exposure

256 CPC exposure affected the abundance of bacteria under the classes 257 *Gammaproteobacteria,* *Alphaproteobacteria,* and *Actinobacteria* (Fig. 3). While 258 *Gammaproteobacteria* was dramatically decreased in abundance from the control (32.9 ± 259 10.4%) to the CPC_50 (17.8 ± 12.2%) and the CPC_500 (6.3 ± 0.9%) communities, 260 *Alphaproteobacteria* was significantly enriched in the CPC_50 (30.1 ± 5.8%) and CPC_500 261 (31.2 ± 5.3%) communities compared to the control (17.7 ± 2.8%). The relative abundance of 262 *Actinobacteria* was 7.8 ± 2.3% in the CPC_500 communities, making this class significantly 263 more abundant than in the control (4.7 ± 1.3%). Although major variations were observed at 264 the class level, no significant change was observed at phylum levels. In fact, 16S rRNA gene 265 sequence analysis uncovered nine major phyla (> 1% of the total) of the AS communities (Fig. 266 S4) including *Proteobacteria* (64%), followed by *Bacteroidetes* (16%), *Verrucomicrobia* 267 (4.8%), *Candidatus Saccharibacteira* (2.5%), *Chloroflexi* (1.5%), *Phanctomycetes* (1.3%), 268 *Fimicutes* (1.2%), and *Acidobacteria* (1.0%). These major phyla exhibited similar levels of 269 abundance to those found in full-scale AS processes (Saunders et al., 2016; Zhang et al., 2012), 270 suggesting that acclimation of AS to laboratory condition could still preserve phylogenic 271 structure of microbial community.

272 [FIGURE 3]

273 Analysis of the major OTUs with >1% of the total abundance identified seven species 274 whose relative abundances significantly differed ($P < 0.05$) between the control and CPC- 275 exposed communities (Table 3). The two *Gammaproteobacteria* OTUs (OTU013 and OTU001) 276 were underrepresented in the CPC-exposed communities, consistent with the changes in the 277 class-level bacterial community composition (Fig. 3). OTU006, OTU014, and OTU009 278 (taxonomically affiliated with *Alphaproteobacteria*), OTU019 (*Actinobacteria*), and OTU007 279 (*Cytophagia*) were selectively enriched in the CPC-exposed communities. OTU006 and

280 OTU014 were two of the most abundant species (9.1 ± 4.1% and 15.2 ± 6.9%, respectively) in
281 the CPC_500 communities and were phylogenetically closely related (> 99% 16S rRNA gene
282 sequence similarity) to *Asticcacaulis excentricus* and *Rhodobacter maris* (Fig. 4).

283 [FIGURE 4]

284 The enrichment of the two species in response to CPC exposure might be attributable
285 to the biological removal (biotransformation/biodegradation) of CPC observed in this study
286 (Section 3.5). *Rhodobacter* are a genus of *Rhodobacteriaceae*, which have a diverse metabolic
287 range including aerobic/anaerobic respiration, photosynthesis and lithotrophy. *Rhodobacter*
288 spp. are capable of cleaning up soil and water environments contaminated with various organic
289 micropollutants (e.g. aromatic hydrocarbons and explosives) (Idi et al., 2015). *Rhodobacter*
290 spp. can generate an array of catalytic enzymes such as monooxygenase and dioxygenase
291 (Oberoi et al., 2015). These enzymes enable the N-dealkylation step in the degradation of
292 quaternary ammonium compounds. In addition, dioxygenase is a broad-substrate enzyme that
293 can catalyse the hydroxylation of aromatic compounds and N-dealkylation (Resnick et al.,
294 1996). The previously reported catabolic capabilities of *Rhodobacter* spp. and the selective
295 enrichment of *Rhodobacter* observed in this study upon CPC exposure imply the potential
296 participation of *Rhodobacter* in mediating CPC biotransformation (e.g. CPC dealkylation
297 and/or benzene ring hydroxylation). *Asticcacaulis* are a genus of *Caulobacteraceae*; two
298 species in this genus (*A. excentricus* and *A. biprosthecium*) were previously isolated from
299 freshwater environments (Liu et al., 2005). The *Asticcacaulis* genome (GenBank accession no.:
300 NC_014816.1) contains conserved genes encoding aldehyde dehydrogenase, alcohol
301 dehydrogenase, and malate dehydrogenase, which are involved in β-oxidation and the
302 tricarboxylic acid cycle of alkyl chain metabolism. A previous metatranscriptomic study of a
303 quaternary ammonium compound-degrading microbial community revealed significantly
304 higher expression of monooxygenase, dioxygenase, aldehyde dehydrogenase and alcohol

305 dehydrogenase (Oh et al., 2014). The results (e.g., putative genes) reported here would be a
306 useful basis for future investigations into the exact role of the *Rhodobacter* and *Asticcacaulis*
307 populations on CPC biodegradation. These results have important implications for biologically
308 engineered systems for treating CPC-bearing waste streams.

309 3.5 CPC removal by activated sludge

310 The removal efficiency of CPC from AS was impacted by influent CPC concentrations
311 (Fig. 5a). In the first feeding cycle, more than 95% of the fed CPC was removed from the
312 CPC_50 and CPC_500 reactors. The mean CPC removal by the CPC_50 reactors was slightly
313 decreased to 89 ± 6% at days 13–42, whereas the mean CPC removal by the CPC_500 reactors
314 decreased significantly from 96 ± 0.3% to 66 ± 15%. At steady state (day 13–42), the removal
315 efficiency was significantly different ($P < 0.05$) between CPC_50 and CPC_500.

316 The removal of CPC in AS reactors was mainly via adsorption and biodegradation. Due
317 to its low volatility (Henry's constant=1.05 x $10^{-8}$ atm $m^3$ $mole^{-1}$; Table S1), CPC is unlikely
318 to be eliminated via evaporation. Other abiotic pathways (i.e., hydrolysis and photolysis) were
319 examined in separate batch reactors under identical operational conditions. These batch
320 reactors showed no significant changes (within ± 2% difference) of CPC at 50 or 500 μg $L^{-1}$
321 for one week. These results are consistent with a consensus in the literature that adsorption and
322 biodegradation are the removal pathways of quaternary ammonium compounds (Tezel and
323 Pavlostathis 2015; Ying 2006; Zhang et al., 2015).

324 A substantial amount of CPC was adsorbed on sludge in AS reactors (Fig. 5b). CPC
325 concentrations on sludge were 41 ± 3.4 and 230 ± 15.6 μg $g^{-1}$ in the CPC_50 and CPC_500
326 reactors, respectively at the end of experiment. The octanol-water partition coefficient ($K_{ow}$) of
327 CPC is 1.7 (Table S1), suggesting a moderate level of hydrophobicity (Wells 2006). However,
328 the CPC molecule contains both hydrophobic and positively charged regions that could

329 facilitate its binding to negatively charged protein moieties of the cell membrane in AS. The
330 affinity of CPC to adsorb on sludge could explain for the instantly high removal of CPC at the
331 first cycle in this study. The results also implied that a considerable amount of CPC can be
332 released with wasted AS from WWTPs. Future investigations into the fate and effects of CPC
333 in post-treatment processes (e.g. anaerobic digestion and biosolid application) of activated
334 waste sludge are suggested.

335 [FIGURE 5a&b]

336 **4. Conclusions**

337 This study provides new insights on the effects of CPC on the phenotypes and bacterial
338 community structure of AS. Environmentally relevant concentrations of CPC (50–500 μg $L^{-1}$)
339 caused short term effects on AS heterotrophic growth. However, long term exposure to the
340 same concentrations had no impact on AS biological function (i.e. organic matter removal and
341 biomass yield). This was consistent with the lack of significant changes in the microbial
342 community diversity and structure between control and experimental reactors. In addition,
343 more than half of CPC was removed from CPC-containing waste streams through
344 biodegradation and adsorption. Two genera (*Rhodobacter* and *Asticcacaulis),* which were
345 enriched in the 500 μg $L^{-1}$-exposed reactors, potentially play important roles in CPC
346 biodegradation. A substantial portion of CPC was adsorbed on sludge, which may require
347 further treatment if beneficial re-use of biosolids is desired.

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