Cometabolic biotransformation and impacts of the anti-inflammatory drug diclofenac on activated sludge microbial communities

https://doi.org/10.1016/j.scitotenv.2018.12.094

Science of the Total Environment

Accepted Manuscript

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Highlights

- Activated sludge can remove 10–50% of DCF by biotransformation and adsorption
- Activated sludge biological function was resilient to DCF exposure at 50-5000 µg/L
- Microbial community was not altered by DCF exposure at 50-5000 µg/L
- *Nitratireductor Asticcacaulis* and *Pseudacidovorax* have potential to biotransform DCF

Abstract

This study evaluated the removal of diclofenac (DCF) in activated sludge and its long-term exposure effects on the function and structure of the microbial community. Activated sludge could remove less than 50% of 50 μg/L DCF. The removal decreased significantly to below 15% when DCF concentrations increased to 500 and 5000 μg/L. Quantitative assessment of the fate of DCF showed that its main removal routes were biodegradation (21%) and adsorption (7%), with other abiotic removals being insignificant (< 5%). The biodegradation occurred through cometabolic mechanisms. DCF exposure in the range of 50-5000 μg/L did not disrupt the major functions of the activated sludge ecosystem (e.g. biomass yield and heterotrophic activity) over two months of DCF exposure. Consistently, 16S rRNA gene-based community analysis revealed that the overall community diversity (e.g. species richness and diversity) and structure of activated sludge underwent no significant alterations. The analysis did uncover a significant increase in several genera, *Nitratireductor*, *Asticcacaulis*, and *Pseudacidovorax*, which gained competitive advantages under DCF exposure. The enrichment of *Nitratireductor*, *Asticcacaulis*, and *Pseudacidovorax* genus might contribute to DCF biodegradation and emerge as a potential microbial niche for the removal of DCF.

Key words: Diclofenac; Activated sludge; Adsorption; Biotransformation; Cometabolism; Microbial community

1. Introduction

DCF can be easily found over-the-counter medicine with a variety of trade names and has been extensively used as medicine for both humans and domestic livestock. About 1400 tons of DCF are consumed globally each year, giving DCF a market share comparable to that of other common nonsteroidal anti-inflammatory drugs (i.e. ibuprofen, mefenamic acid, and naproxen) (McGettigan & Henry, 2013). Therefore, DCF is one of the most commonly detected pharmaceutically active compounds in soil and aquatic environments. The occurrence of DCF was at up to 1 μg/L in (surface waters) (Vulliet et al., 2011), up to 10 μg/L (ground waters) (Vieno & Sillanpää, 2014), and up to 95 μg/L (urban wastewaters) (Luo et al., 2014; Muter et al., 2017). Even at very low concentrations, DCF causes toxicity to aquatic organisms such as rainbow trout (at 5–50 μg/L) (Hoeger et al., 2005) and hydra (0.1 μg/L) (Carlsson et al., 2006); thus DCF carries significant potential health risks at the level currently found in the environment. Accordingly, DCF is a highly prioritized emerging contaminant that needs to be regulated/monitored in natural water environments (e.g. drinking water sources) (de Voogt et al., 2009; Gerbersdorf et al., 2015).

WWTPs are an important barrier to limit the spread of DCF to the environment. However, DCF is one of the most poorly removed pharmaceuticals in conventional WWTPs (Gerbersdorf et al., 2015; Luo et al., 2014). Furthermore, the overall removal of DCF varies significantly (5–81%) across various full-scale WWTPs (Luo et al., 2014; Tran et al., 2018), suggesting that DCF removal is not only unsatisfactory but also unpredictable. Accordingly, to develop ways to control effectively DCF in WWTPs, it is highly desirable to determine quantitatively how DCF is removed, along with the underlying mechanisms that control its fate. Recent studies have shown that although DCF is considered to be not particularly biodegradable, microbial degradation of DCF using bacterial and fungal pure cultures is possible (Aissaoui et al., 2017; Bessa et al., 2017; Nguyen et al., 2013). *Enterobacter* from

activated sludge (AS) can degrade DCF (> 50%) as a sole carbon and energy source, and degradation improves (> 80%) with an additional carbon source (Aissaoui et al., 2017). *Brevibacterium* isolated from AS could remove > 30% of DCF at 10 mg/L for 30 days and increased removal up to 90% when acetate was used as a supplementary carbon source (Bessa et al., 2017). White-rot fungi such as *Trametes* (Nguyen et al., 2013) and *Ascomycota* (Gonda et al., 2016) are known to degrade up to 60% and 10% of DCF, respectively. Although the exact degradation pathways of DCF remain unclear, hydroxylation is involved in its biotransformation and detoxification, which leads to the formation of various metabolic byproducts, including 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one (Aissaoui et al., 2017). Those isolate-based studies have advanced understanding of DCF biodegradation by identifying strains, degradation kinetics, and metabolic byproducts. However, the microorganisms that inhabit full-scale environmental biochemical processes such as AS represent highly complex communities, not isolated individual. Therefore, whether the previously reported isolate organisms are relevant in complex AS microbial communities remains to be clearly elucidated. Further, if they are not relevant, what microbial taxa in those communities control the fate of DCF?

The impact of DCF on AS microbial community and its functionality remains a topic for further investigation. Recently, the development of next-generation sequencing technologies has paved the way for in-depth investigation of the microbial community from different environmental matrixes. The 16S rRNA gene has been widely used as the marker gene for the microbial community in biological wastewater treatment process such as AS, biological nutrient removal and anaerobic digester (Kang et al., 2018; Nguyen et al., 2019; Vasiliadou et al., 2018; Zhang et al., 2016). Several studies have initially indicated the impacts of micropollutants exposure to the AS microbial community and functionality (Jiménez-Silva et al., 2018; Liao et al., 2017; Vasiliadou et al., 2018). Schmidt et al. (2012) observed a

96 complete inhibition on nitrification at 7.2 mg/L of ciprofloxacin, gentamicin, sulfamethoxazole
97 and trimethoprim. Collado et al. (2013) observed a decrease in microbial diversity of AS
98 community at 50 µg/L sulfamethoxazole exposure in two months. However, biological nutrient
99 removal (COD and nitrogen) was unaffected at this concentration. Therefore, the compound
100 and its concentrations could have a specific level of impacts on AS community.

101 This study examines the removal mechanisms of DCF in AS process and its impacts on
102 the microbial community at a range of concentrations representing environmentally relevant
103 and catastrophic levels. Laboratory bioreactors were inoculated from a local AS process and
104 fed with DCF-containing substrates over two months. While the bioreactors exhibited stable
105 DCF removal performance, biochemical assays used in this study determined the detailed
106 removal routes. The high throughput Illumina MiSeq platform was utilized to elucidate the
107 response of the microbial community to DCF exposure. Diversity and structure of the microbial
108 community were characterized. Finally, impacts of DCF on AS functionality were evaluated.

109 **2. Materials and Methods**

110 2.1 Laboratory scale bioreactors

111 AS taken from an aeration tank of a municipal WWTP (Jurong, Singapore) which was
112 acclimated to laboratory conditions for one month in the fed-batch bioreactor. The acclimated
113 AS showed stable chemical oxygen demand removal (i.e. 91.6 ± 3.7 %) was then used for other
114 bioreactors. Twelve identical fed-batch bioreactors (0.6 L active volume) were operated over
115 two months. All reactors were fed every 3.5 days by withdrawing 0.2 L of the mixed liquor
116 suspension and replacing it with 0.2 L of synthetic feed (i.e. 10.5 days of hydraulic and solid
117 retention time). The reactors were aerated at a dissolved oxygen concentration of 4.8 ± 0.8
118 mg/L and kept at laboratory room temperature (i.e. 22–23 °C). The synthetic feed contained
119 per liter: glucose (1.83 g), NH_4Cl (30 mg), KH_2PO_4 (340 mg), K_2HPO_4 (600 mg), $MgSO_4$ (270

120 mg), $FeSO_4$ (10 mg), and 10 mL of 100 x trace element solution (ZnSO4.7H2O 0.35 mg,
121 $MnSO_4.H_2O$ 0.21 mg, H_3BO_4 2.1 mg, $CoCl_2.2H_2O$ 1.4 mg, $CuCl_2.2H_20$ 0.07 mg, $NiSO_4.6H_2O$
122 0.1 mg, $Na_2MoO_4.2H_2O$ 0.21 mg per liter) as described previously (Nguyen & Oh, 2019). The
123 synthetic feed has a ratio of COD, total nitrogen and total phosphorous (COD: TN: TP) of 80:
124 5: 1.

125 A stock solution of DCF (Sigma Aldrich Singapore) was prepared at a concentration of
126 1 g/L and stored at 4 °C prior to use. Each set of three reactors were exposed to 0 (i.e. control),
127 50 (DCF_50), 500 (DCF_500), 5000 µg/L (DCF_5000) of DCF. The concentration range
128 tested in this study included 50 µg/L, which is comparable to the concentration found in urban
129 wastewaters (0.01–95 µg/L) (Luo et al., 2014). The higher concentration range (500–5000
130 µg/L) in this study was thus higher than that found in urban wastewaters by a factor of 10–100.
131 Accordingly, the levels tested in this study are relevant for hospital/pharmaceutical wastewater
132 or exceptional maxima (accidental spills or highest peaks among temporal variations) in urban
133 municipal wastewaters.

134 2.2 Analytical methods

135 Volatile suspended solids (VSS) and chemical oxygen demand (COD) were measured
136 using standard methods. pH was determined with an Orion 4-Star Plus pH/conductivity meter
137 (Thermo Scientific, Waltham, MA). Samples were collected from influent and effluent, filtered
138 by a 0.22 µm pore-size filter for the assessment of DCF removal. A high-performance liquid
139 chromatography (HPLC) system (Shimadzu Asia Pacific Pte. Ltd) equipped with a Shim-Pack
140 GIST Phenyl, 5 µm, 4.6 x 250 mm column and a UV–vis detector was used to measure the
141 DCF concentration. The system was run on isocratic mode with a mobile phase containing
142 40:60% (v/v) of 20 mM sodium dihydrogen phosphate monohydrate and acetonitrile (pH 2.5),
143 which was delivered at 1.8 mL/min through the column. The detection wavelength used for the

144 DCF measurement was 220 nm. The sample volume injected to the HPLC was 100 μL and the
145 detection limit was 10 μg/L.

146 2.3 Evaluation of DCF fate in activated sludge

147 DCF removal in a fed-batch bioreactor was calculated using the following equation:
148 removal (%) = $(C_{inf} - C_{eff}) \times 100 \div C_{inf}$, where C_{inf} and C_{eff} denote the concentration of DCF
149 in the reactor influent and effluent, respectively. To determine the detailed routes of DCF
150 removal in AS (hydrolysis, volatilization, photolysis, adsorption, or biodegradation), six sets
151 of triplicate batch experiments (I through VI) were established (Table S1). The experiment
152 regarding inoculum (active or inactivated sludge), synthetic feed, DCF, aeration, and light
153 availability are described in Table S1. The biomass was collected from the mixed liquor
154 suspension of the DCF_5000 reactors at day 70. The biomass was washed two times with
155 phosphate saline buffer (pH 7.4). 50 μL of the DCF stock solution (1 g/L) was added to 50 mL
156 of the synthetic feed medium in 400 mL-Erlenmeyer flasks, resulting in 1 mg/L of initial DCF
157 concentration. The initial concentration of DCF was selected such that the concentration
158 loading exceeded the environmentally relevant concentration, thus allowing the direct
159 biotransformation of DCF to be conclusively observed. The biomass concentration inoculated
160 into each flask was 0.8 g VSS/L. The same amount of sludge autoclaved at 121 ℃ for 15 min
161 was used for experiment III. The DCF level and optical density from the batch experiments
162 were followed over 5 days.

163 2.4 16S rRNA gene sequencing and analysis

164 The total genomic DNA from a mixed liquor sample from a reactor was extracted using
165 a MoBio PowerSoil® DNA isolation kit (MOBIO, Carlsbad, CA, USA) following the
166 manufacturer's instructions. All DNA obtained in this study showed > 0.5 μg DNA/μL and >
167 1.8 absorbance ratios (A260/A280). 16S rRNA genes were PCR-amplified by Macrogen Inc.
168 (Seoul, Republic of Korea) using universal bacterial primers targeting the V3–V4 region

(341F–805R). The 16S rRNA gene sequences were determined using the Miseq^{TM} platform at Macrogen Inc. Paired-end (2 × 300 bp) 16S rRNA gene sequences were analyzed using the MiSeq SOP pipeline (Kozich et al., 2013). In brief, raw sequences were preprocessed with the following parameters, no ambiguous sequence, > 200 bp in length, and < 8 bp homopolymer, with other parameters at their defaults. The preprocessed sequences were chimera-checked using chimera.vsearch and then taxonomically classified with classify.seqs. Chimera sequences and those assigned to chloroplasts, mitochondria, archaea, eukaryotes, and unknown were excluded from further analyses. The remaining sequences were clustered into operational taxonomic units (OTUs) using a 97% nucleotide identity cutoff with the dist.seqs and cluster commands. The sequences were rarefied to the lowest number of sequences per sample to calculate alpha diversity indices across different datasets. The OTU level bacterial community composition data were used for beta diversity analysis. Rarefaction curves of the 12 datasets tended to approach the saturation plateau (> 99% of Good's coverage), indicating that the sequencing depth was adequate to capture most of the diversity in the AS communities (Fig. S1). The 16S rRNA gene sequence datasets used in this study were deposited in GenBank under the following accession numbers: DCF_50_1 (SRS2340272), DCF_50_2 (SRS2340268), DCF_50_3 (SRS2340266), DCF_500_1 (SRS2340271), DCF_500_2 (SRS2340267), DCF_500_3 (SRS2340264), DCF_5000_1 (SRS2340254), DCF_5000_2 (SRS2340273), DCF_5000_3 (SRS2340269), Control_0_1 (SRS2340183), Control_0_2 (SRS2340176), Control_0_3 (SRS2340220), Control_42_1 (SRS2340175), Control_42_2 (SRS2340198), and Control_42_3 (SRS2340197).

The Mann-Whitney U test was carried out to evaluate differential features. The *P* value threshold for statistical significance was set at $P < 0.05$.

3. Results and Discussion

3.1 DCF removal by activated sludge

DCF was not effectively removed by AS process (Fig. 1). After the introduction of DCF into the feed, the removal of DCF was below 50% in three tested DCF concentrations. The DCF_50 reactor exhibited 45 ± 2% of DCF removal at days 13–70, comparable to that (43 ± 2%) in the first feeding cycle. The removals in the reactors exposed to higher DCF concentrations (DCF_500 and DCF_5000) decreased to 22 ± 5% and 12 ± 2.0%, respectively, at days 13–70. Those overall results suggest that the AS could remove less than half of 50–5000 μg/L of DCF after one full retention time. The ordinary least squares analysis indicated a significant negative relation (Pearson's r = -0.92 with $P < 0.05$) between the DCF feeding concentration and the resulting DCF removal rate (Fig. 1b). The results further ascertain that DCF removal is dependent on initial concentration.

[FIGURE 1]

The low DCF removal (12–43%) at the wide range of DCF concentrations (50–5000 μg/L) is in good agreement with the poor removal characteristics of DCF previously reported from WWTPs (Luo et al., 2014). Furthermore, it is noteworthy that the fate of DCF was significantly affected by the amount of DCF in the reactor influent. The findings (decreased DCF removal with an increase in DCF concentration) suggest that the input DCF level is an important factor affecting the fate of DCF, in addition to other previously documented factors (e.g., biomass concentration and retention time). These results strongly suggest that the input DCF concentration is an important criterion to consider when designing/operating AS–associated biological processes to treat DCF-containing wastewaters.

214 3.2 Removal routes for DCF in activated sludge

215 The removal of DCF by hydrolysis, volatilization, photolysis, adsorption, and
216 biodegradation was 2.3 ± 1.4%, 2.5 ± 1.4%, 3.2 ± 2.1%, 6.5 ± 1.5%, and 21.3 ± 7.3%,
217 respectively (Fig. 2). These results suggest that DCF removal occurred primarily (a total of
218 28%) via biodegradation and adsorption, with other abiotic means (hydrolysis, volatilization,
219 and photolysis) being relatively less significant (a total of 8%). The adsorption of a compound
220 on sludge primarily depends on lipophilicity and environmental conditions (e.g. pH,
221 temperature, and sludge properties) (Tadkaew et al., 2011). The degree of adsorption on sludge
222 can be estimated by the adsorption-desorption distribution ratio (K_d), i.e. the ratio of the
223 compound concentration at equilibrium in the solid-phase and the liquid phase. The $\log K_d$
224 value of DCF in sludge varies from 1.3 to 2.7 across different sludges (e.g. primary, secondary,
225 MBR, and anaerobically digested) (Vieno & Sillanpää, 2014). Because > 2.5 $\log K_d$ is often
226 associated with efficient adsorption, DCF is thought to have low adsorptive potential to sludge.
227 The DCF removal via adsorption observed in this study was 6.5 ± 1.5%, which is comparable
228 to previous measurements in primary sludge (5–15%) (Ternes et al., 2004). Together with
229 adsorption, the biological route (21.3 ± 7.3%) accounted for the highest fraction of total DCF
230 removal. The biological degradation constant (K_{biol}, L/g VSS·d) of a pollutant is often used to
231 infer pollutant biodegradability. K_{biol} values are sorted into four classes (Joss et al., 2006): <
232 0.5 (hard biodegradability), 0.5–1 (moderate), 1–5 (high), and > 5 (very high). The K_{biol}
233 constant of DCF was estimated using our experimental data based on the DCF that was
234 biologically removed. The K_{biol} constant was 0.14 ± 0.2 (L/g VSS·d) during the first day of the
235 experiment, when the maximum biodegradation occurred. Our and previous findings
236 (Fernandez-Fontaina et al., 2013; Joss et al., 2006) on the K_{biol} constant collectively support the
237 low biodegradation potential of DCF in AS. The synergistic effect from adsorption and
238 biodegradation of sludge on DCF removal has not been indicated in the literature. Previous

239 studies reported the addition of adsorbents such as activated carbon in sludge facilitates the
240 removal of DCF from the liquid phase (Nguyen et al., 2014; Semblante et al., 2015). However,
241 the conceptual expectation of adsorption enhanced biodegradation is often not accomplished,
242 which require frequent addition of adsorbents (Nguyen et al., 2014). It would be expected that
243 there is no complementary of adsorption and biodegradation on observed DCF removal in this
244 study.

245 The biodegradation of DCF in AS can be due to the co-metabolism (Fig. 2a). The
246 removal of DCF was 17.3 ± 1.4% when the feed containing DCF as sole carbon and energy
247 source, which was comparable with the removal due to adsorption. Whereas, the removal of
248 DCF was significantly higher ($P < 0.05$) when the feed containing DCF and glucose.
249 Consistently, the optical density, which indicates microbial growth, was ca. 0.69 – 0.88,
250 suggesting no microbial growth with DCF only in the culture medium. In wastewater, DCF
251 occurs at very low levels (generally up to at μg/L) compared to other organic matter (generally
252 up to mg/L). Accordingly, at the level typical in wastewater, DCF might not act as a primary
253 carbon and energy source for microbial growth. Instead, cometabolic degradation of DCF may
254 be the predominant biological removal route. Cometabolism is the transformation of a non-
255 growth substrate in the presence of a growth substrate. The term 'non-growth substrate'
256 describes compounds that are unable to support cell growth as sole carbon source (Tobajas et
257 al., 2012). A nitrifying microbial community could significantly increase DCF removal by
258 adding an external carbon source (acetate) (Tran et al., 2009). Although several studies
259 investigated the biodegradation of DCF in the WWTPs without considering direct and
260 cometabolic processes, the contribution of cometabolism for the DCF removal (non-detectable
261 direct metabolism) in the AS systems need to be further examined for understanding the
262 involvement of enzymatic biotransformation and by-products. Currently, this study provided an

263 investigation on the microbial community control over the cometabolic processes of DCF
264 removal.

265 [FIGURE 2]

266 3.3 Dissecting activated sludge communities metabolizing DCF

267 DCF exposure decreased ($P < 0.05$) the abundance of *Gammaproteobacteria*,
268 *Deltaproteobacteria*, and *Actinobacteria*, but dramatically increased the abundance of
269 *Alphaproteobacteria, Cytophagia*, and *Sphingobacteriia*. Therefore, we conducted a further,
270 detailed investigation at the finer level of the taxa that are differentially enriched upon DCF
271 exposure.

272 OTU clustering generated 796 OTUs, of which ten were selectively enriched (with
273 statistical significance) under DCF-exposure (Fig. 3). Four OTUs (OTU015, OTU020,
274 OTU023, and OTU026) increased significantly at 5000 µg/L of DCF compared to the Control,
275 and three OTUs (OTU025, OTU002, and OTU008) increased significantly at both 5000 µg/L
276 and 500 µg/L of DCF. Of particular note were OTU006, OTU009, and OTU012, which were
277 overrepresented even at a low DCF level (50 µg/L). OTU009 increased from 1.2 ± 0.7%
278 (Control) to 2.6 ± 0.8% (DCF_50), 3.2 ± 0.3% (DCF_500), and 4.0 ± 0.6% (DCF_5000).
279 OTU012 was selectively enriched from 1.7 ± 0.6% (Control) to 5.1 ± 1.4% (DCF_50), 4.9 ±
280 0.4% (DCF_500), and 4.3 ± 0.6% (DCF_5000). OTU006 increased by more than 2-, 2.5- and
281 4.5-fold in DCF_50, DCF_500 and DCF_5000, respectively. Those three organisms accounted
282 for a substantial fraction (> 68%) of the communities in the reactors exposed to 5,000 µg/L of
283 DCF. Phylogenetic analysis of the ten selectively enriched OTU sequences revealed that
284 OTU009, OTU012, and OTU006 were closely related (99% 16S rRNA gene sequence
285 similarity) to *Nitratireductor, Pseudacidovorax,* and *Asticcacaulis*, respectively (Fig. 4).

286 *Nitratireductor* are aerobic gram-negative bacteria capable of oxidizing nitrate to nitrite
287 in anoxic conditions (Manickam et al., 2012). *Pseudoxanthomonas* are metabolically versatile
288 and have nitrogen-fixing ability (Wang et al., 2013). *Nitratireductor* and *Pseudoxanthomonas*
289 are frequently detected in contaminated sites and are associated with detoxification of organic
290 pollutants (e.g. pesticides and xenobiotics) (Manickam et al., 2012). Although *Asticcacaulis*
291 are distributed across natural freshwater and soil environments, little is known about their
292 physiological characteristics and biotic/abiotic interactions in their ecological niches. Previous
293 studies have identified direct and cometabolic degradation of DCF by pure cultures of
294 *Enterobacter* and *Brevibacterium*, but our results reveal that those organisms were very rare (<
295 0.7%) in the DCF-exposed communities and were not enriched under DCF exposure. Thus,
296 isolate organisms might have low biotechnological application potential in wastewater
297 treatment systems for DCF, despite their experimentally verified metabolic capability for DCF.
298 Instead, the 16S rRNA gene-based community profiling revealed that *Nitratireductor,*
299 *Pseudoxanthomonas,* and *Asticcacaulis* gained competitive advantages (e.g. cometabolic
300 capability for DCF) under DCF exposure, enabling them to outcompete other populations in
301 the AS communities. Isolation of these species from AS after long-term exposure could provide
302 some bacterial niches that can be used as inoculum source in bioaugmentation technique. For
303 instance, Terzic et al. (2018) observed an increase from none to 99% removal of antibiotic
304 macrolide after two months of exposure. Likewise, Nguyen et al. (2018) retrieved a
305 *Bradyrhizobium sp*. from AS via an enrichment and isolation process, which showed the ability
306 to cometabolite antibiotic ciprofloxacin. Therefore, future experiment on the isolated
307 *Nitratireductor, Pseudoxanthomonas,* and *Asticcacaulis* could provide new insights into
308 devising biological means for treatment of DCF-bearing waste streams.

309 [FIGURE 4]

310 3.4 Long-term effects on activated sludge function

311 The results of this study suggest that 50–5,000 μg/L of DCF exposure does not
312 significantly alter the species richness, diversity, and composition of AS communities (Fig. 5).
313 A principal coordinate analysis with the Euclidean distance metric (for bacterial community
314 composition at the OTU level) indicated no shifts in community phylogenetic structure (Fig.
315 5a). The DCF_50 (83.2 ± 1.5 by the Euclidean distance similarity), DCF_500 (79.5 ± 8.2), and
316 DCF_5000 (77.3 ± 7.6) communities clustered closely, suggesting that the community
317 structure among the three replicate communities was similar. We noticed that the Control
318 communities showed more profound variation (61.4 ± 14.5) among them compared with the
319 other three groups. The pairwise distance was 30.9 ± 5.4 (Control vs DCF_50), 29.1 ± 3.6
320 (Control vs DCF_500), and 32.3 ± 4.3 (Control vs DCF_5000). Although inter-community
321 distances were lower than intra-community distances, a PERMANOVA test revealed no
322 significant difference (Bonferroni-corrected $P > 0.05$) in community phylogenetic structure
323 among the four community groups. We also estimated alpha diversity indices using 33,000
324 sequences per sample (rarefied to the lowest number per sample). The species richness and
325 diversity indices did not show significant differences between the Control and DCF-exposed
326 communities (Figs. 5b and 5c).

327 [FIGURE 5]

328 DCF at concentration of 50-5000 mg/L had no impacts on heterotrophic and microbial
329 growth in AS. VSS values were 0.75 ± 0.06, 0.78 ± 0.12, 0.73 ± 0.07, and 0.74 ± 0.14 g/L in
330 the Control, DCF_50, DCF_500, and DCF_5000 reactors, respectively. The soluble COD
331 removal rates in the DCF-exposed reactors (93 ± 2.5%, 91 ± 3.4%, and 92 ± 2.5% for DCF_50,
332 DCF_500, and DCF_5000, respectively) were relatively constant over two months and
333 comparable to those (91.6 ± 3.7%) of the Control reactors. Statistical testing using the Mann-

Whitney U test revealed no significant differences ($P > 0.05$) between the Control and DCF exposure reactors.

Previous studies documented acute toxicity values for several isolates by determining their minimum inhibition concentrations (MICs) against DCF at grams per liter levels: *Enterobacter cloacae* (1.6 g/L), *Pseudomonas aeruginosa* (1.6 g/L), and *Acinetobacter baumannii* (0.8 g/L) (Laudy et al., 2016). These levels are significantly higher than both the dose level tested in this study and the environmentally relevant level in wastewaters. In addition, our antimicrobial susceptibility testing of the Control communities against DCF revealed > 1 g/L of MIC. DCF is a pharmaceutically active compound, which is indeed intended to be biologically active. However, unlike antimicrobial pharmaceuticals, DCF is designed to reduce inflammation in humans and animals, rather than act as a bactericidal or bacteriostatic drug. Taken together, the present data (16S rRNA gene-based and experimental results given in Fig. 5) and previously reported results suggest that DCF exposure 100 times greater than environmentally relevant in urban wastewaters (i.e. potential environmental maxima representing accidental spills or the highest peaks among temporal variations) might not cause acute or chronic toxicity to major ecosystem functions (e.g. microbial growth and heterotrophic activities) and the overall biodiversity of AS communities. These results have important implications for designing and operating environmental biochemical processes treating DCF-bearing waste streams.

4. Conclusions

This study showed that DCF was poorly removed by AS (< 50%). Our quantitative analyses revealed that biodegradation and adsorption were the major two removal pathways in AS, and biodegradation occurred via cometabolic degradation rather than direct metabolism. Long-term exposure to DCF at 50–5000 μg/L did not cause disturbances in the major functions of AS ecosystems, which is consistent with our 16S rRNA gene-based results. Several bacterial

taxa (*Nitratireductor*, *Asticcacaulis*, and *Pseudoxanthomonas*) increased significantly with exposure to DCF, suggesting the need for further experimental investigations of their functional capacity in the cometabolism of DCF.

Acknowledgements

This work was supported by the "Leaders in INdustry-university Cooperation +" Project, supported by the Ministry of Education and National Research Foundation of Korea.

Conflicts of interest

There are no conflicts of interest to declare.

List of Figures:

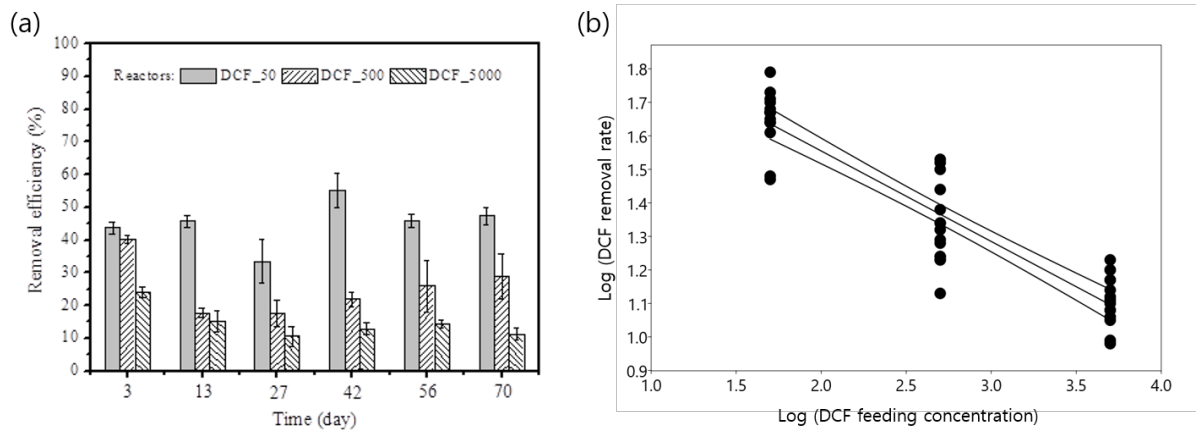
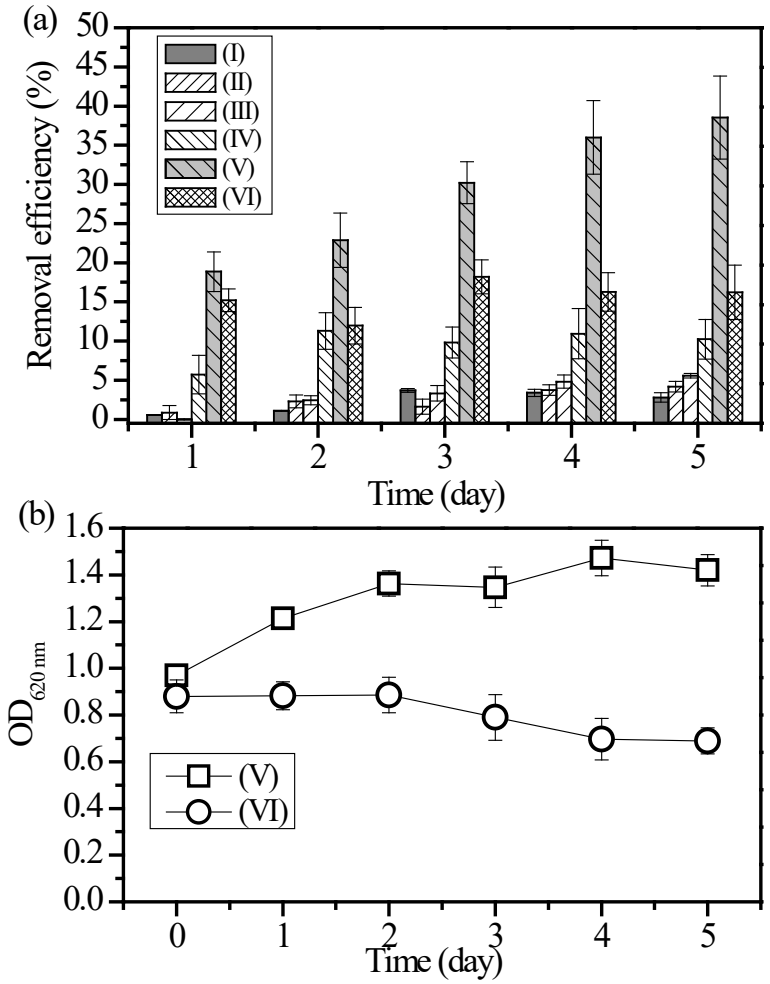
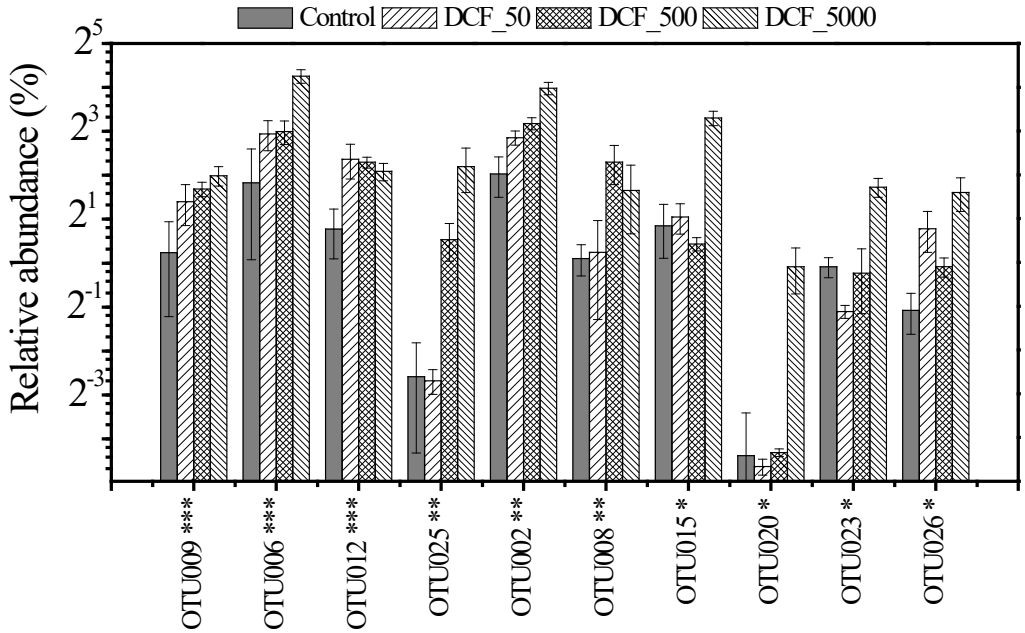


Figure 1: Time course removal of DCF in fed-batch reactors (a) and correlation between the DCF feeding concentration and DCF removal rate (b). The ordinary least squares (OLS) regression analysis shows a significant negative correlation (Pearson correlation = -0.92 with $P < 0.05$) between the DCF removal rate and DCF feeding concentration. The center and outer lines represent the OLS slope and 95% confidence bands, respectively.



374

375 **Figure 2**: DCF concentrations in batch tests under six different conditions. Time course
376 concentration of DCF (a) and optical density (OD_{620nm}) (b). Error bars present the standard
377 deviation of triplicate samples. Each experiment (I through VI) is described in detail in Table
378 S1.



379

380 **Figure 3**: Relative abundance of ten major OTUs (> 1% of the total). Asterisks indicate
381 differential relative abundance with statistical significance ($P < 0.05$ by Mann-Whitney U test):
382 *** (Control vs DCF_50, DCF_500, DCF_5000), ** (Control vs DCF_500 and DCF_5000),
383 and * (Control vs DCF_5000).

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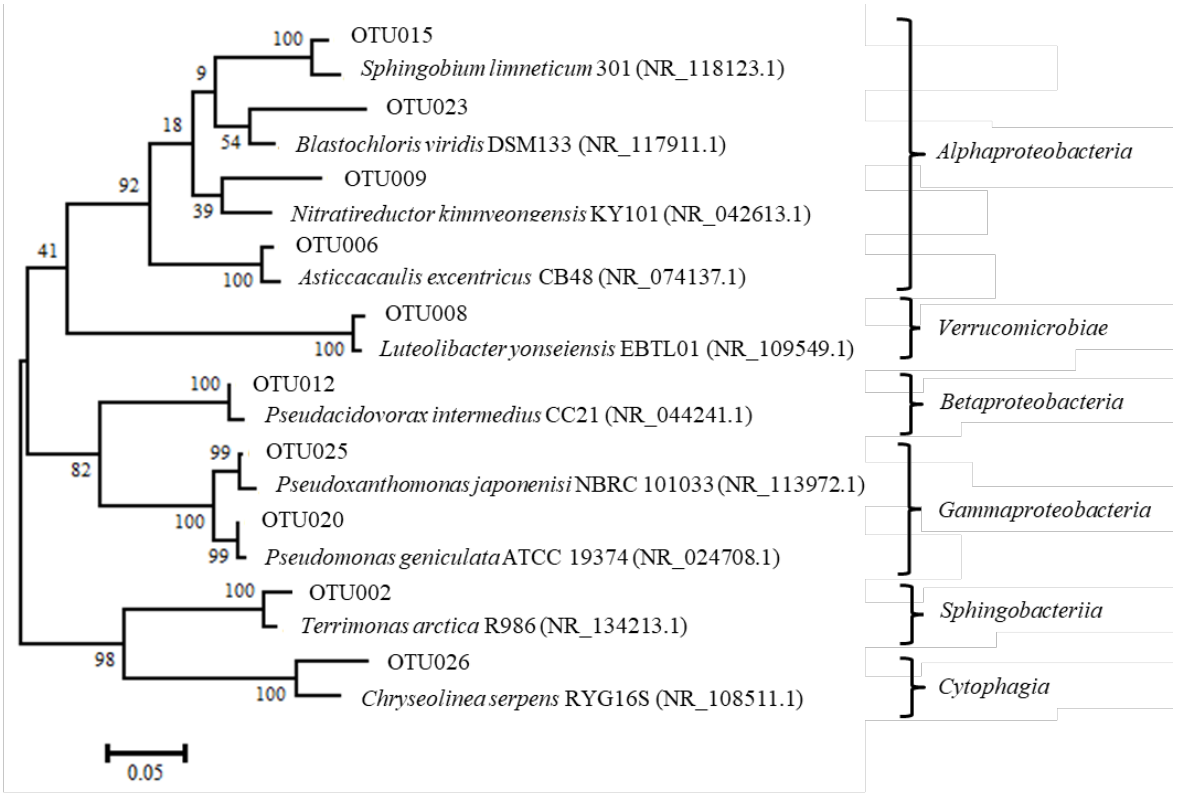

385

386 **Figure 4**: Phylogenetic tree of the ten selectively enhanced OTUs. The OTUs shown here are
387 the same as those listed in Fig. 3. The tree was constructed using MEGA7.0 (Kumar et al.,
388 2016) with the maximum likelihood method and the Tamura-Nei model. The closest relative
389 (> 99% nucleotide identity) of each OTU was obtained from the 16S ribosomal RNA sequence
390 database (GenBank) and is included to deduce the phylogenetic affiliation of each OTU. The
391 bootstrap support with 100 replicates is shown on the tree nodes. The accession number of the
392 reference strain is shown in parentheses. The taxonomic affiliation of each OTU at the class
393 level is listed on the right side.

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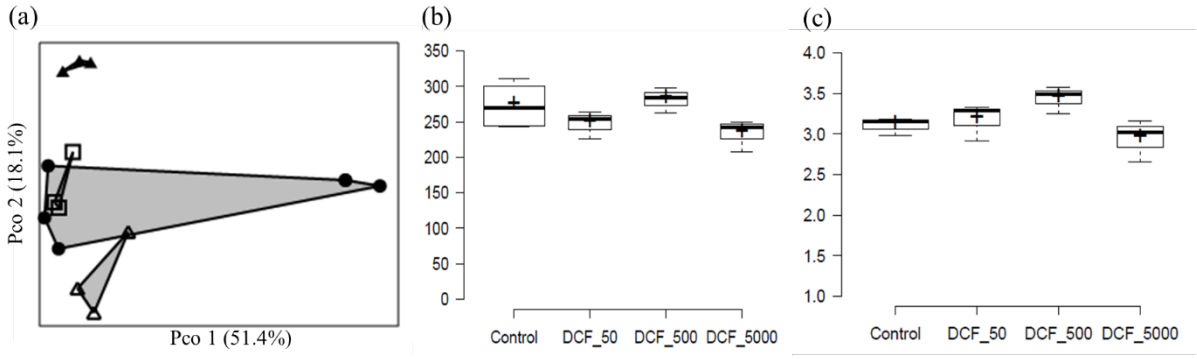

Figure 5: Shifts in community phylogenetic structure and diversity. Principal coordinate analysis of community structure using the Euclidean distance metric (a). Solid circles, open squares, open triangles, and solid triangles represent the Control, DCF_50, DCF_500, and DCF_5000 communities, respectively. Alpha diversity indices of the control and DCF-exposed communities: Chao1 (b) and Shannon (c). The whiskers of the box represent the minimum and maximum values. The bottom and top of the box are the first and third quartiles, respectively, and the line inside the box denotes the median.

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