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Safe reuse of wastewater: effect of disinfection methods on microbial community --Manuscript Draft--

Manuscript Number:	JCLEPRO-D-23-08618R3
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Keywords:	Wastewater reuse; Performic acid; Disinfection efficiency; Disinfectant sensitive biomarker; Microbial community; Microbial function
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Abstract:	<p>Performic acid (PFA), a new generation of disinfectant, has shown outstanding performance for wastewater treatment. However, the effect of PFA on wastewater microbial community changes as well as the differences in comparison to the commonly disinfectants used are poorly documented. We provide an overview of the PFA effect and that of chlorine and UV, two commonly used disinfectants, on microbial community diversity and structure. The study was performed using wastewaters from three municipal wastewater treatment plants (WWTPs) processed by activated sludge, lagoon and reed beds, and biofilter. We focused on disinfection of pathogen microorganisms, identifying sensitive and resistant bacteria to disinfectants that can serve as biomarkers for evaluating the disinfectant performances. As well, we investigated the modifications of microbial functions after treatment by functional predictive analysis. The more efficient <i>E. coli</i> inactivation was obtained with initial concentration of 4 mg/L of PFA with 15 min disinfectant contact time that achieved 95.71-99.99% inactivation efficiency. Although the origin of wastewater was the main factor determining the microbial community diversity and composition, the microbial communities were specific to the disinfectant used. Linear discriminant analysis Effect Size (LEfSe) identified 18 and 10 biomarkers, including pathogenic microorganisms affiliated to <i>Chromobacterium</i>, <i>Flavobacterium</i>, <i>Aeromonas</i>, <i>Enterobacteriaceae</i>, <i>Escherichia_Shigella</i> and <i>Fusobacteriaceae</i>, which were sensitive to PFA, while being resistant to chlorine and/or UV. Consistently, the predicted bacterial functional patterns diverged according to the wastewater origin and the disinfectant used. Pathogen microorganisms were more sensitive to PFA than the other treatments, demonstrating its potential for implementing a safe reuse of wastewater.</p>
Suggested Reviewers:	Maren Ortiz-Zarragoitia maren.ortiz@ehu.eus Jean Baptiste Ramond jbramond@bio.puc.cl Tibor Benedek benedek.tibor@fh.szie.hu Jianli Li ljl2489@163.com



July the 26th, 2023

Dear Editor,

Please find as attached file, a revised version of the manuscript JCLEPRO-D-23-08618R3. As you will see, we have made revisions to the manuscript in line with the reviewers' comments and criticisms.

The modifications have been highlighted in blue. The responses to reviewer's comments are in the rebuttal letter.

We hope that this new version will be now acceptable for publication.

If there are any points we haven't addressed, please contact us and we will be happy to clarify them.

On behalf of authors,

Sincerely,

Pr. Robert Duran



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Rebuttal letter

Reviewer #4:

The article has been strongly improved. Still, it is not easy to be read and, in my opinion, some informations (i.e.the characterization of the effluents rom the three WWTPs) should be included in the text and the English should be revised. However, it contains interesting data and now I thing it could be published after minor revisions.

[Response]: Thank you for the comments that are useful to further improve the MS.

The information of the characterization of WWTP, which was presented in supplementary material Table S1, it is now described in the text as follows:

"Indeed, the WWTPs' effluents showed different physical-chemical characteristics (Table S1). The conductivity was lower in WWTP1 effluent (718 $\mu\text{S}/\text{cm}$) than in WWTP2 (1000 $\mu\text{S}/\text{cm}$) and WWTP3 (1254 $\mu\text{S}/\text{cm}$) effluents (Table S1). Consistently, WWTP1 effluent exhibited the lower contents of COD (36 mg/L), TSS (2 mg/L), TOC (18 mg/L), and TN (3.5 mg/L), while WWTP2 effluent showed the higher COD (80 mg/L) and NT (60.6 mg/L) contents (Table S1)."

Also the information of statistics comparing the WWTP and treatments presented in supplementary material tables has been included in the text.

The modifications of text for English revision are also indicated.

Safe reuse of wastewater: effect of disinfection methods on microbial community

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Highlights

- PFA was **an** efficient disinfectant comparing to chlorine and UV
- Microbial communities from treated water were specific to the disinfectant used
- Biomarkers sensitive to PFA and resistant to chlorine and/or UV were identified
- Bacterial predicted functions diverge according to WWTP and disinfectants

1 Safe reuse of wastewater: effect of disinfection methods on microbial community

2
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7 Biological and Medical Engineering, Guangdong Academy of Sciences, Guangzhou, China

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19 **Abstract**

20 Performic acid (PFA), a new generation of disinfectant, has shown outstanding
21 performance for wastewater treatment. However, the effect of PFA on wastewater
22 microbial community changes as well as the differences in comparison to the
23 commonly disinfectants used are poorly documented. We provide an overview of the
24 PFA effect and that of chlorine and UV, two commonly used disinfectants, on
25 microbial community diversity and structure. The study was performed using
26 wastewaters from three municipal wastewater treatment plants (WWTPs) processed
27 by activated sludge, lagoon and reed beds, and biofilter. We focused on disinfection
28 of pathogen microorganisms, identifying sensitive and resistant bacteria to
29 disinfectants that can serve as biomarkers for evaluating the disinfectant performances.
30 As well, we investigated the modifications of microbial functions after treatment by
31 functional predictive analysis. The more efficient *E. coli* inactivation was obtained
32 with initial concentration of 4 mg/L of PFA with 15 min disinfectant contact time that
33 achieved 95.71-99.99% inactivation efficiency. Although the origin of wastewater
34 was the main factor determining the microbial community diversity and composition,
35 the microbial communities were specific to the disinfectant used. Linear discriminant
36 analysis Effect Size (LEfSe) identified 18 and 10 biomarkers, including pathogenic
37 microorganisms affiliated to *Chromobacterium*, *Flavobacterium*, *Aeromonas*,
38 Enterobacteriaceae, *Escherichia_Shigella* and Fusobacteriaceae, which were sensitive
39 to PFA, while being resistant to chlorine and/or UV. Consistently, the predicted
40 bacterial functional patterns diverged according to the wastewater origin and the
41 disinfectant used. Pathogen microorganisms were more sensitive to PFA than the
42 other treatments, demonstrating its potential for implementing a safe reuse of
43 wastewater.

44
45 **Keywords:** Wastewater reuse; Performic acid; Disinfection efficiency; Disinfectant
46 sensitive biomarker; Microbial community; Microbial function

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47 1. Introduction

48 Water scarcity is becoming a global concern in recent decades. The
49 overexploitation of water is one of the major threats to the EU freshwater
50 environment, especially for agricultural irrigation (EC, 2018). More than 40,000
51 million m³ wastewater is treated each year in the EU, only less than 3% being reused
52 (EC, 2022). Thus, there is place to improve water reuse, which represents a potential
53 on easing water supply pressure, also contributing to a sustainable agriculture.

54 Domestic wastewater owns microorganism associated to human diseases
55 (Diamond et al., 2022; McCall et al., 2020). It is thus critical to establish efficient and
56 economical disinfection frameworks in wastewater treatment in order to supply safe
57 water. Tertiary treatment aims to remove stubborn pollutants (organics, turbidity,
58 nitrogen, phosphorus, metals, and pathogens), including also an additional
59 disinfection step (Pei et al., 2019). The tertiary treatment provides additional
60 assurance of effluent quality, protecting thus wildlife in the discharged water system.
61 It also ensures the safety of wastewater reuse for various purposes, such as irrigation
62 and industrial applications (Gerba and Pepper, 2019).

63 Several disinfection approaches are commonly implemented in tertiary treatment
64 for the inactivation of pathogen microorganisms, such as chlorination, ultraviolet (UV)
65 irradiation, ozonation and filtration (USEPA, 2004). Chlorine and its various forms
66 are powerful oxidants for the inactivation of pathogens (Furst et al., 2018), but it
67 forms toxic by-products that might impact the receiving environment threatening the
68 human health (Bull et al., 1995; Nieuwenhuijsen et al., 2000; Victoria, 2002). The UV
69 irradiation has been considered as an alternative to chlorine for wastewater treatment
70 due to its short contact time, and it has the advantage that toxic by-products are not
71 produced (Zyara et al., 2016). However, UV irradiation has the disadvantage to be
72 expensive because of high-energy demand, high costs of the equipment and its
73 management. The investment cost is estimated to be 10 times more expensive than
74 that of chlorine and PFA treatments (Ragazzo et al., 2013). Also, the practical
75 application of UV irradiation carries many limitations because it is dependent on

1 76 water quality parameters, such as turbidity, colour and suspended solid (Oguma et al.,
2 77 2001; Zimmer and Slawson, 2002).

3
4 78 Performic acid (PFA), a high oxidative peracid, has been considered as an ideal
5
6 79 alternative for industrially wastewater disinfection due to its outstanding
7
8 80 characteristics (Gehr et al., 2009; Ragazzo et al., 2013). PFA is a wide-spectrum
9
10 81 disinfectant with deodorizing ability and low corrosive effect, exhibiting toxicity to
11
12 82 viruses, bacteria and bacterial spores, mycobacteria as well as microscopic fungi, but
13
14 83 not to higher forms of life (Gehr et al., 2009; Luukkonen and Pehkonen, 2017). The
15
16 84 PFA treatment cost has been estimated at 0.0114 €/m³ (Luukkonen and Pehkonen,
17
18 85 2017), while that of chlorination at 0.004 €/m³ (Zagklis and Bampos, 2022). Although
19
20 86 the operation cost of PFA treatment is estimated to be 2.5 times more expensive than
21
22 87 that of chlorine (Luukkonen et al., 2015). It has the advantage to produce by-products
23
24 88 (hydrogen peroxide and formic acid) that are nontoxic to aquatic fauna at the
25
26 89 disinfection doses (Gehr et al., 2009; Ragazzo et al., 2013). Thus, its reasonable cost
27
28 90 and splendid performance at ambient temperature demonstrate that PFA has potential
29
30 91 applicability for large wastewater treatment plants (WWTPs) (Luukkonen et al.,
31
32 92 2015).

33
34
35 93 Several studies have reported on the effect of the commonly used disinfectants on
36
37 94 microorganisms. Most studies have considered the decrease of bacterial cell counts
38
39 95 and species richness, the inactivation of viruses, and the removal of microorganisms
40
41 96 carrying antibiotic resistance genes (ARGs) (Kantor et al., 2019; Kulkarni et al., 2018;
42
43 97 Ng et al., 2019; Stamps et al., 2018; Zaouri et al., 2020). Nevertheless, the effect of
44
45 98 disinfectant treatments on the microbial community structure and functions is scarce,
46
47 99 especially for the PFA treatment (Kantor et al., 2019; Numberger et al., 2019; Stamps
48
49 100 et al., 2018). The release of potential pathogens and the modification of microbial
50
51 101 community in the receiving environment have been reported during reclaimed water
52
53 102 discharging process (Numberger et al., 2019). Thus, it is important to consider the
54
55 103 effect of wastewater treatments on the microbial community. Precisely, the objective
56
57 104 of our study was to examine the effect of PFA treatment on the microbial diversity,
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59 105 microbial structure and function. The disinfection efficiency of PFA treatment against

106 pathogens was also evaluated and compared to that of other conventional treatments.
107 We hypothesize that the microbial communities will be modified differently
108 according to the treatment because they involve different action mechanisms.

109 In order to examine the possibility to safely reuse treated wastewater for irrigation,
110 industrial applications, groundwater recharge and other agricultural activities, this
111 study aimed to compare PFA, UV and chlorine treatments regarding their effect on 1)
112 the microbial community diversity and structure, and 2) the disinfection efficiency for
113 the inactivation of pathogens. For this purpose different WWTPs were treated in order
114 to identify sensitive and resistant bacteria to each disinfectant, representing potential
115 microbial biomarkers according to treatment. The bacterial functions were predicted
116 and correlated with the wastewaters resulting from the treatments.

117 **2. Materials and methods**

118 **2.1 Sampling sites and physical-chemical characterization of wastewaters**

119 Three domestic WWTPs with different treatment processes, activated sludge,
120 lagoon and reed bed and biofilter were selected. Brief information of the sampling
121 WWTPs is listed in the [Table S1](#).

122 The main physical-chemical parameters were determined on the wastewater
123 samples. Conductivity was measured by 3510 WTW multiparameter-probe. Chemical
124 oxygen demand (COD) was determined using colorimetric method by Hach reactants
125 and Hach DR3900 spectrophotometer. Total suspended solid (TSS) was determined
126 after filtration through 0.45 μm on 47 mm glass fiber filter and drying at 105 $^{\circ}\text{C}$ until
127 constant mass. Total organic carbon (TOC) and total nitrogen (TN) were determined
128 by a Shimadzu TOC VCN analyzer with a TN unit (chemiluminescence measurement
129 of NO_x) according to (Behera et al., 2017). TOC was obtained by the difference of
130 total carbon and inorganic carbon analyses.

131 2.2 Sample collection and disinfection

132 The effluent wastewater samples were collected from each WWTP with sterile
133 Scott bottles. All the samples were collected in the morning and transported to the
134 laboratory at 4 °C. The disinfections were generated by three disinfectants (PFA,
135 chlorine and UV) just right after the water samples arrived to the laboratory. Each
136 disinfection treatment was performed in true biological triplicates. Thus, a total of 45
137 samples (3 WWTP x triplicate x 5 treatments (PFA_L, PFA_H, Chlorine, UV and
138 untreated wastewater as control)) were analysed. All samples were maintained at 4 °C
139 prior further processing.

140 The optimal disinfection concentration of all the disinfectants used was
141 determined in a pre-experiment using *E. coli* and total coliforms as indicators. The
142 initial doses range were determined based on official wastewater treatment manual
143 and previous studies, also adjusted according to the wastewater treatment plants
144 conditions (Anastasi et al., 2013; EPA, 2011; Gehr et al., 2009; Ragazzo et al., 2013;
145 USEPA, 2003). More specifically, previous reports indicated that UV was effective at
146 10 mJ/cm² although a higher dose (30-40 mJ/cm²) would be required for complete
147 disinfection (Andreadakis et al., 1999), while hypochlorite was found effective at 2
148 mg/L (Collivignarelli et al., 2017) and PFA in 1-4 mg/L range (Chhetri et al., 2014).

149 2.2.1 PFA preparation and disinfection

150 For preparing PFA, 10 mL of formic acid was mixed with 1 mL sulfuric acid
151 (98%, Aldrich) as a catalyst on ice and wait about 10 min to cool. 14.2 mL of
152 hydrogen peroxide (H₂O₂, 50% wt, Aldrich) was then added slowly into the mixture.
153 Then, the mixed solution was maintained for 60 min to get ready to use. The whole
154 process was performed in ice bath and mixed with a magnetic stirrer. 100 mg of the
155 mixture was then mixed with 0.1M H₂SO₄ solution and titrated to determine the
156 concentrations of H₂O₂ and PFA. The H₂O₂ titration was conducted with potassium
157 permanganate solution (KMnO₄, 0.02 mol/L, Aldrich). An excess of potassium iodide
158 was then added and oxidized by PFA. Iodine was then titrated with sodium thiosulfate

159 (Na₂SO₃, 0.02 mol/L, Aldrich). The PFA solution was kept at -20 °C and titrated each
160 time right before use.

161 Because PFA is rapidly decomposed (Karpova et al., 2013), two doses of PFA, a
162 lower initial concentration at 1 mg/L with 15 min disinfectant contact time (PFA_L)
163 corresponding to a dose know to inactive most microorganisms (Karpova et al., 2013)
164 and a higher initial concentration at 4 mg/L with 15 min disinfectant contact time
165 (PFA_H) corresponding to the optimal dose for disinfection (Karpova et al., 2013;
166 Chhetri et al., 2014), were used in the disinfection. Na₂SO₃ solution (0.1 mol/L) was
167 used for quenching the reaction.

168 **2.2.2 Chlorination**

169 A solution of 100 mg/L of total chlorine was prepared from pure calcium
170 hypochlorite (Aldrich) and stored at 4 °C. The prepared solution was titrated by
171 iodometric titration in acetic acid medium before use. The chlorination was performed
172 with the initial concentration at 2 mg/L for 30 min (Chl). Na₂SO₃ solution was used
173 for quenching the reaction.

174 **2.2.3 UV disinfection**

175 The laboratory scale UV disinfection was performed using low-pressure mercury
176 vapour lamp to perform the disinfection at the dose of 10 mJ/cm². The irradiance rate
177 was measured by UV radiometer (Avantes Avaspec 2048L equipped with a 600 µm
178 core optical fiber and a corrector cosinus (4.9 mm diameter)). The solutions were
179 irradiated by a home-made device consisting of four Phillips TL UV 8W 30 cm
180 fluorescent lamps. The device was placed on top of the solutions and the height was
181 adjusted until obtaining the desired irradiance. The lamp was preheated for more than
182 15 min before disinfection. 250 mL of wastewater was treated each time in the
183 specific glass beaker. The exposure time was calculated by the desired dose,
184 irradiance rate and transmittance value of the wastewater sample.

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185 2.3 Microbial analysis

186 The *Escherichia coli* (*E. coli*) counts were determined by a standard
187 beta-glucuronidase positive method (APHA, 2005).

188 The water samples were filtered by sterile disposable filtration units (MicroFunnel,
189 Pall Corporation, USA) with 0.2 µm Supor membrane to concentrate the microbial
190 biomass. The iQ-Check Free DNA Removal Solution (FDRS, Bio-Rad Laboratories,
191 USA), which degrades free DNA by enzymatic treatment, was used to ensure that the
192 samples are devoid of free DNA prior to DNA extraction. For the free DNA
193 elimination, we followed standard protocol. In short, the membranes were folded into
194 cones and transferred into Eppendorf tubes containing 40 µl FDRS and 460 µl
195 distilled Milli-Q water, followed by 30 min incubation at 37 °C. To inactivate the
196 FDRS and for DNA extraction, 1 mL lysis solution (R1) from Aquadien™ Bacterial
197 DNA Extraction and Purification Kit (Bio-Rad Laboratories, USA) were then added
198 and incubated for 15 min at 95 °C with 1,300 rpm in a shaking heating block
199 (Eppendorf ThermoMixer C, Eppendorf, Germany). We followed the standard
200 protocol for the Aquadien™ Bacterial DNA Extraction and Purification Kit for the
201 following steps.

202 The V3-V4 region of 16S rRNA gene was amplified using the primer pair 341F
203 (5' - CCTAYGGGRBGCASCAG - 3') and 806R (5' -
204 GGACTACNNGGGTATCTAAT - 3') (Gao et al., 2020) connecting with barcodes.
205 PCR amplification, library preparation and sequencing were conducted by Novogene
206 Europe (Cambridge, UK) using their proprietary methods. To ensure an accurate
207 recognition of the samples, the index pairs were specific for each sample. Quality
208 control was performed at each step of the procedure. The PCR products were purified
209 using AMPureXP magnetic beads, pooled in equimolar and the libraries generated
210 with NEBNext® Ultra™ DNA Library Prep Kit for Illumina and quantified via
211 Qubit and Q-PCR. Paired-end 250 bp sequencing was then performed on Illumina
212 NovaSeq 6000 sequencer (Illumina, San Diego, CA, USA).

213 2.4 Bioinformatics analysis and visualization

214 Paired-end reads were assigned to samples based on the sample-specific barcode
215 and truncated by cutting off the barcode and primer sequences, and were merged
216 using FLASH (V1.2.7) (Magoc and Salzberg, 2011). Qiime2 (v 2019.4) was used for
217 the data processing and analysis. The sequence quality control and amplicon
218 sequencing variants (ASVs) assignment were performed using DADA2 plugin.
219 Taxonomy assignment was completed using the q2-feature-classifier plugin against
220 SILVA database (v132) with a clustering threshold of 97% similarity. The ASVs
221 assigned to Eukaryota, Chloroplast, Mitochondria and unassigned ASVs were
222 removed and the filtered dataset was then rarefied at the depth of 71673 using
223 *alpha-rarefaction.py* script. The rarefied data were then used for the following
224 analyses without threshold. The complete dataset was deposited in the Sequence Read
225 Archive (SRA) of the National Center for Biotechnology Information (NCBI) and is
226 available under the Bioproject ID PRJNA907723.

227 For the alpha-diversity, the Shannon, Simpson, observed features, Chao1, ace
228 indices were analyzed using *alpha_diversity.py* in Qiime2. Statistical analysis
229 (ANOVA, Tukey HSD) and visualization were performed in GraphPad Prism.
230 Beta-diversity was calculated in Qiime2 and R based on weighted Unifrac and
231 Bray-Curtis dissimilarity. Permutational multivariate analysis of variance
232 (PERMANOVA) was performed with 999 times permutations using the
233 *beta_significance.py* script in Qiime2 and the “adonis” function in the vegan package
234 in R. Principal coordinate analyses (PCoA) were performed and visualized by
235 EMPERor (Vazquez-Baeza et al., 2013) and the Ape and ggplot2 packages in R. The
236 significances of physical-chemical parameters associated with microbial community
237 were identified by “envfit” function in the “vegan” package in R.

238 Linear discriminant analysis Effect Size (LEfSe, v 1.0) (Segata et al., 2011) was
239 performed to identify the biomarkers between the groups, which emphasizes both
240 statistical significance and biological relevance. LEfSe analysis was performed using
241 the ASV table, at the ASV level spanning all the taxonomic levels. The

1 242 non-parametric factorial Kruskal-Wallis sum-rank test was performed first to detect
2 243 features with significant differential abundance between the classes (groups). The
3
4 244 pairwise Wilcoxon test was then applied to check if the subclasses agree with the
5
6 245 class level trend, which was performed only among subclasses in different classes
7
8 246 with the same name. The resulting subset of vectors was then used to build a Linear
9
10 247 Discriminant Analysis model. The alpha-value for both Kruskal-Wallis and Wilcoxon
11
12 248 tests were at 0.05 and the logarithmic LDA threshold score for discriminative features
13
14 249 was 2.
15

16 250 The PICRUST2 was used for the prediction of microbial community functions
17
18 251 following the following 4 steps: (1) sequence placement, (2) hidden-state prediction
19
20 252 of genomes, (3) metagenome prediction, (4) pathway-level predictions. The
21
22 253 pathway-level predictions were performed using a customized mapping file based on
23
24 254 the KEGG database (KEGG Brite orthologs to pathways, updated on 22 Nov. 2020,
25
26 255 [Table S2](#)). The descriptions were then added to the Enzyme Classification (EC)
27
28 256 numbers, KEGG orthologs (K) with default mapping table, and a customized mapping
29
30 257 table including descriptions of higher hierarchical levels was used for the KEGG
31
32 258 pathways (ko, [Table S3](#)). STAMP v 2.1.3 was used for visualizing PICRUST2 output
33
34 259 data. ANOVA and Kruskal-Wallis H-test were used for the multiple groups' statistical
35
36 260 analysis. Tukey-Kramer and Welch's at 0.95 were performed as Post-hoc tests.
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40 261 **3. Results and discussion**

41 262 **3.1 Disinfection treatment efficiency**

42
43 263 The disinfection efficiency of PFA, UV and chlorine was determined for the three
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45 264 different wastewater treatment plants by determining the reduction of the *E. coli*
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47 265 content, which represents a key parameter for characterizing reclaimed water quality
48
49 266 in wastewater reuse (EC, 2018; EPA, 2004). The initial *E. coli* cell concentration
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51 267 (before treatment BT, [Fig. 1](#)) was 2.8×10^4 , 2.5×10^4 and 5.8×10^3 CFU/100 mL for
52
53 268 WWTP1, 2, and 3, respectively. In WWTP1 and WWTP2, the *E. coli* cell
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55 269 concentration significantly decreased in all the treated wastewaters ($p < 0.001$, [Fig. 1](#)).
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1 270 Indeed, the WWTPs' effluents showed different physical-chemical characteristics
2 271 (Table S1). The conductivity was lower in WWTP1 effluent (718 $\mu\text{S}/\text{cm}$) than in
3 272 WWTP2 (1000 $\mu\text{S}/\text{cm}$) and WWTP3 (1254 $\mu\text{S}/\text{cm}$) effluents (Table S1). Consistently,
4 273 WWTP1 effluent exhibited the lower contents of COD (36 mg/L), TSS (2 mg/L),
5 274 TOC (18 mg/L), and TN (3.5 mg/L), while WWTP2 effluent showed the higher COD
6 275 (80 mg/L) and NT (60.6 mg/L) contents (Table S1).

7 276 Noteworthy, the PFA treatment was more efficient with WWTP1, which exhibited
8 277 the lowest TSS content (Table S1, Fig. 1). This result is consistent with previous
9 278 studies showing that the disinfection efficiency of UV and peracetic acid increased
10 279 with low TSS (Dominguez Henao et al., 2018; Walters et al., 2014).

11 280 The Chl and both low and high doses of PFA (PFA_L and PFA_H) treatments
12 281 showed similar *E. coli* content reduction for WWTP1 and WWTP2 ($p > 0.05$),
13 282 although the *E. coli* concentration (277 CFU/100 mL) was higher for the PFA_L
14 283 treatment of WWTP2. All these three treatments were significantly more efficient in
15 284 decreasing the *E. coli* concentration than the UV treatment ($p < 0.001$). The Chl and
16 285 PFA_H treatments had the best efficiency, reducing the *E. coli* concentration below
17 286 40 CFU/100 mL. A reduction of *E. coli* counts by 3-4 \log_{10} magnitude was observed
18 287 with the PFA_H treatment for WWTP1 and WWTP2, while for WWTP3 the
19 288 reduction of *E. coli* counts was 1 \log_{10} magnitude (Fig. 1). The treatment of WWTP3
20 289 showed different trends, except for the PFA_H treatment that was also the most
21 290 efficient for reducing the *E. coli* content. These observations suggested that the
22 291 disinfection efficiency, evaluated by the decrease of *E. coli* concentration, was
23 292 dependent on the water physical-chemical parameters. The treatment with the high
24 293 dose of PFA (PFA_H) provided better performance than chlorine and UV,
25 294 irrespective of the wastewater origin, observation that was in agreement with a
26 295 previous report (Ragazzo et al., 2020).

27 296 The UV and PFA treatments were less efficient in decreasing the *E. coli*
28 297 concentration for the WWTP3 wastewater than for WWTP1 and WWTP2, even if the
29 298 TSS of WWTP2 and WWTP3 were at a similar level (16 and 18 mg/L, respectively).
30 299 It is likely that the high TSS content of WWTP3 might relate high *E. coli*

1 300 concentration since WWTP3 exhibited also the highest conductivity (1254 $\mu\text{S}/\text{cm}$,
2 301 Table S1), which has been shown to be related to the *E. coli* concentration (Foschi et
3 al., 2021). The inactivation efficiency of *E. coli* has been shown to be dependent on
4 302 the WWTP treatment processes (Biswal et al., 2014). Although UV disinfection
5 303 significantly decreased *E. coli* concentration, it was not efficient as Chl and PFA in
6 304 WWTP1 and WWTP2. Among the disinfection treatments, only the PFA_H was
7 305 effective for reducing the *E. coli* concentration. Such observation was in agreement
8 306 with previous studies showing that PFA was more efficient in reducing *E. coli*
9 307 concentration than the disinfectants commonly used for municipal wastewater
10 308 treatment (Maffettone et al., 2020; Wang et al., 2023).
11 309

12 310 **3.2 Effect of disinfection treatment on microbial community**

13 311 A total of 5,724,372 raw reads was obtained and assigned to 17,151 amplicon
14 312 sequence variants (ASVs). The sequences were then rarefied at the minimum 71,673
15 313 reads per sample. The final dataset, including 3,153,612 reads dispatched within
16 314 14,838 ASVs, was used for the microbial community analysis.

17 315 **3.2.1 Impact of disinfection treatments on microbial community diversity**

18 316 The diversity indices (Shannon, Simpson, observed richness, Chao1 and ace) were
19 317 significantly different (two-way ANOVA, $p < 0.001$) according to the WWTP origin
20 318 and the treatment, WWTP origin explaining most of the variation (62.8 %, $p < 0.001$;
21 319 Table S4). The microbial community of the wastewater from WWTP2 exhibited
22 320 significantly higher diversity indices than those from WWTP1 and WWTP3 (two-way
23 321 ANOVA, $p < 0.001$; Fig. S1). The microbial community of wastewater from WWTP3
24 322 had significantly higher Shannon index than that from WWTP1 (two-way ANOVA, p
25 323 < 0.001 ; Fig. S1).

26 324 Noteworthy, the treatments for a given WWTP decreased the microbial diversity
27 325 (Shannon index and the observed richness, two-way ANOVA, $p < 0.05$; Fig. S2),
28 326 except for WWTP2 (Fig. S2). Interestingly, the WWTP2, lagoon and reed beds
29 327 process, showed the higher COD, TOC and TN contents (Table S1). It has been

1 328 shown that the efficiency of a treatment in decreasing the microbial diversity is
2 329 dependent on the wastewater physical-chemical characteristics such as COD, TOC
3 330 and TN (Tian and Wang, 2020; Wu et al., 2022), the treatment processes (Sauter et al.,
4 331 2023), and even on the different type of reed beds systems (Kowal et al., 2021). Thus,
5 332 it is likely that the high content of organic matter (COD, TOC and TN) might explain
6 333 the fact that the microbial diversity was not decreased by the treatments in WWTP2.

7 334 The WWTP3 showed higher diversity (Shannon and observed richness) indices
8 335 before treatment (BT) than the two other WWTPs (two-way ANOVA, $p < 0.05$; Fig.
9 336 S2), which may explain the lower *E. coli* inactivation observed with WWTP3
10 337 wastewater (Fig 1) despite that it showed the lowest *E. coli* concentration. Such
11 338 observation was consistent with a previous report showing that the *E. coli* inactivation
12 339 is dependent on the microbial diversity (Qian et al., 2022).

13 340 For the WWTP1, the Shannon index was significantly lower (two-way ANOVA,
14 341 $p < 0.001$; Fig. S2) for PFA_H than for before treatment (BT), and for the three other
15 342 treatments. Similarly, the observed richness for PFA_H was significantly lower than
16 343 for Chl (two-way ANOVA, $p < 0.05$; Fig. S2). The WWTP3 treated by PFA_H and
17 344 Chl showed significantly lower Shannon index and observed richness than BT
18 345 (two-way ANOVA, $p < 0.05$; Fig. S2).

19 346 **3.2.2 Impact of disinfection on microbial community structure**

20 347 The principal coordinate analysis (PCoA), based on either the weighted UniFrac
21 348 or Bray-Curtis dissimilarity matrices (Fig. 2), further confirmed that the microbial
22 349 communities of the wastewaters were different according to the WWTPs, revealing
23 350 also the treatments effects. The PCoA clustered the microbial communities according
24 351 to their origin (i.e. WWTP), separating the different WWTPs along the axis 1, which
25 352 explained 66.7% of the variation (Fig. 2a). This observation was supported by the
26 353 permutational multivariate ANOVA (PERMANOVA) indicating that the WWTP
27 354 origin was the main factor explaining the differences between the microbial
28 355 communities (pseudo-F = 79.68, $p = 0.001$). Within a WWTP cluster, the microbial
29 356 communities clustered by treatment (Fig. 2a). Thus, in order to reveal the effect of the

1 357 disinfection treatments on the microbial community, PCoA were performed for each
2 358 WWTP (Fig. 2b, 2c, 2d). For WWTP2 and WWTP3, wastewater samples from the
3
4 359 same treatment tightly clustered together (Fig. 2c, 2d). Envfit (Fits an Environmental
5
6 360 Vector or Factor onto an Ordination) analysis also revealed that the composition of
7
8 361 wastewater microbial communities significantly varied by treatment in WWTP2 ($r^2 =$
9
10 362 $0.94, p < 0.001$) and WWTP3 ($r^2 = 0.89, p < 0.001$), but not in WWTP1.

11
12
13 363 Before the treatments, COD, TOC and TN were identified as the main factors
14
15 364 driving the microbial communities of WWTP2 (Envfit; COD: $r^2 = 0.9712, p = 0.004$;
16
17 365 TOC: $r^2 = 0.9688, p = 0.004$; NT: $r^2 = 0.9682, p = 0.009$), while the conductivity
18
19 366 (Envfit; $r^2 = 0.9930, p = 0.001$) and TSS (Envfit; $r^2 = 0.9844, p = 0.001$) were the
20
21 367 main factors driving the microbial communities from WWTP3. After the disinfection
22
23 368 treatments, the same factors were found to drive the microbial communities from
24
25 369 WWTP2 and WWTP3 irrespective of the treatment: COD (Chl: $r^2 = 0.9568, p = 0.004$;
26
27 370 UV: $r^2 = 0.9955, p = 0.004$; PFA_L: $r^2 = 0.9664, p = 0.003$; PFA_H: $r^2 = 0.9251, p =$
28
29 371 0.003), TOC (Chl: $r^2 = 0.9522, p = 0.004$; UV: $r^2 = 0.9946, p = 0.004$; PFA_L: $r^2 =$
30
31 372 $0.9601, p = 0.003$; PFA_H: $r^2 = 0.9139, p = 0.004$) and NT (Chl: $r^2 = 0.9503, p =$
32
33 373 0.020 ; UV: $r^2 = 0.9927, p = 0.004$; PFA_L: $r^2 = 0.9547, p = 0.009$; PFA_H: $r^2 =$
34
35 374 $0.9051, p = 0.026$) for WWTP2; conductivity (Chl: $r^2 = 0.9936, p = 0.001$; UV: $r^2 =$
36
37 375 $0.9907, p = 0.008$; PFA_L: $r^2 = 0.9968, p = 0.001$; PFA_H: $r^2 = 0.9866, p = 0.002$)
38
39 376 and TSS (Chl: $r^2 = 0.9798, p = 0.006$; UV: $r^2 = 0.9943, p = 0.009$; PFA_L: $r^2 = 0.9883,$
40
41 377 $p = 0.006$; PFA_H: $r^2 = 0.9678, p = 0.006$) for WWTP3.

42
43 378 These results suggested that the origin (i.e. WWTP) was the main factor
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45 379 determining the composition of the microbial community. The different disinfection
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47 380 treatments resulted in specific microbial communities, which effects were different
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49 381 according to the wastewater origin (WWTP).

50 51 52 53 382 **3.2.3 Microbial composition differences between WWTPs: identification of** 54 55 383 **specific biomarkers**

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57
58 384 The distinction of the microbial communities according to their origin (i.e.
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60 385 WWTP) was confirmed by the presence of different dominant phyla (Fig. 3).

1 386 Proteobacteria (60.5%) was the most dominant bacterial phylum in all three WWTPs,
2 387 consistent with previous studies in both domestic and industrial WWTPs (Sharma et
3 al., 2021; Tang et al., 2020a). The relative abundance of the Proteobacteria
4 388 significantly decreased from WWTP2 (70.7%) to WWTP3 (61.9%), and to WWTP1
5 389 (48.9%) (ANOVA, $p < 0.001$).
6 390

7 391 The second dominant phylum in the three WWTPs was Bacteroidetes (11.4%),
8 392 followed then by Actinobacteria (8.1%), Nitrospirae (4.3%) and Nanoarchaeaeota
9 393 (3.7%). The relative abundance of the Actinobacteria significantly decreased from
10 394 WWTP1 (15.1%) to WWTP2 (7.1%), and to WWTP3 (2.3%) (ANOVA, $p < 0.01$).
11 395 Nitrospirae was significantly more abundant in WWTP3 (11.9%) than in WWTP1
12 396 (0.6%) and WWTP2 (0.6%) (ANOVA, $p < 0.001$). Nitrospirae includes nitrifying
13 397 taxa, nitrite-oxidizing bacteria (NOB) and comammox bacteria (Koch et al., 2019).
14 398 Nitrospira, the largest class, have been found to be the dominant nitrifiers in
15 399 wastewater treatment plants (Forrez et al., 2011). Nitrospira, an aerobic
16 400 chemolithoautotrophic nitrite-oxidizing bacterium, plays important roles in
17 401 nitrification and oxidizes nitrite to nitrate (Daims et al., 2015; Daims and Wagner,
18 402 2018). It has been identified as keystone taxon, an ideal partner for establishing
19 403 microbial relationships under various conditions, in aquatic environment (Veloso et
20 404 al., 2023).

21 405 The linear discriminant effect size (LEfSe) analysis revealed microbial taxa
22 406 specifically more abundant according to the origin of the WWTP. Such taxa found
23 407 significantly more abundant to a specific condition by LEfSe analysis has been
24 408 proposed to serve as biomarkers (Segata et al., 2011). The LEfSe comparing the
25 409 microbial communities before treatment (BT) revealed biomarkers for each WWTP
26 410 (Fig. 4). For WWTP1, Actinobacteria, Elusimicrobia, Epsilonbacteraeota,
27 411 Latescibacteria, Omnitrophicaeota and Spirochaetes phyla, and Parcubacteria and
28 412 Campylobacteria class were identified as biomarkers (Fig. 4). Actinobacteria, known
29 413 for carrying antibiotic resistant genes (ARGs) (Tang et al., 2020b), have been reported
30 414 dominant in activated sludge processed municipal wastewaters (Kang et al., 2020; Xu
31 415 et al., 2017). Bacteria affiliated to Parcubacteria have been reported to be involved in

1 416 sulphamethoxazole antibiotic degradation (Song et al., 2019). *Campylobacter* species
2 417 are known as bacterial indicators for faecal contamination by animals (Ogden et al.,
3 418 2009).

4 419 For WWTP2, the LEfSe analysis revealed Proteobacteria, Cyanobacteria phyla
5 420 and Oxyphotobacteria class as biomarkers (Fig. 4). Cyanobacteria are considered to
6 421 be well adapted to high organic and low oxygen contents (Saqrane et al., 2020).
7 422 WWTP2 process is based on lagoon system and reed beds, providing probably
8 423 appropriate growth condition for Cyanobacteria. Cyanobacteria are known to release
9 424 cyanotoxins (Saqrane et al., 2020). Nevertheless, after disinfection treatments, the
10 425 relative abundance of Cyanobacteria in WWTP2 decreased to the same level of that
11 426 found in WWTP1 and WWTP3 (Fig. S3).

12 427 For WWTP3, the biofiltration based process, the LEfSe analysis revealed
13 428 *Armatimonadetes*, *Verrucomicrobia*, *Acidobacteria*, *Nitrospirae* and
14 429 *Gemmatimonadetes* phyla, and the *Acidobacteria*, *Nitrospira* and *Planctomycetacia*
15 430 classes as biomarkers (Fig. 4). Such observation was consistent with previous studies
16 431 showing that these taxa were found dominant in three different types of biofilters
17 432 (Gulay et al., 2019; Lautenschlager et al., 2014; Li et al., 2020). *Nitrospirae* and
18 433 *Verrucomicrobia* have been demonstrated to be involved in the transformation of
19 434 organic nitrogen (Tang et al., 2021).

20 435 After the disinfection treatments, most of the detected biomarkers differed from
21 436 those of the non-treated WWTPs (Fig. S3). However, the *Actinobacteria*,
22 437 *Alphaproteobacteria* and *Acidobacteria* classes were still identified as biomarkers for
23 438 WWTP1, WWTP2 and WWTP3 respectively, irrespective of the disinfection
24 439 treatment (Fig. 4, Fig. S3). Such result further confirmed that the origin of
25 440 wastewaters is an important factor shaping the microbial community (habitat filtering).
26 441 Thus, it is likely that the WWTP specific biomarkers are reflecting not only the
27 442 differences on wastewater composition, but also the processing methods involved in
28 443 the wastewater treatment systems (activated sludge, lagoon and reed beds, and
29 444 biofilter, for WWTP1, WWTP2 and WWTP3 respectively).

3.3 Identification of biomarkers sensitive and resistant to the disinfection treatments

The LEfSe analysis comparing the bacterial communities before (BT) and after each treatment revealed biomarkers, taxa significantly more abundant in a specific condition. Such biomarkers reflect thus the disinfection efficiency of each treatment towards microbial taxa (Fig. 5). Two groups of biomarkers were detected: i) treatment-sensitive biomarkers corresponding to taxa significantly found more abundant in BT (i.e. depleted in the wastewater treated by a disinfectant), and ii) treatment-resistant biomarkers corresponding to taxa significantly found more abundant in treated water.

The numbers of treatment-sensitive biomarkers decreased as follow PFA_H, PFA_L, UV, and chlorine. For PFA_H, 39 treatment-sensitive biomarkers were detected including ASVs related to Flavobacteriaceae, Chromobacteriaceae, *Chromobacterium* and *Escherichia_Shigella* (Fig. 5), members of which have been reported as dominant taxa in sewage and WWTP (Ahmed et al., 2017; Leroy-Freitas et al., 2022; Yousra et al., 2017). For the PFA_L, 11 treatment-sensitive biomarkers including the previous discussed taxa Chromobacteriaceae, *Chromobacterium* and *Escherichia_Shigella* were revealed. For chlorine and UV, only 2 and 4 treatment-sensitive biomarkers were detected, respectively. Additionally, 11, 3, 3 and 1 treatment-resistant biomarkers were detected for the UV, chlorine, PFA_H and PFA_L, respectively (Fig. 5). Paracaedibacteraceae, identified as treatment-resistant biomarker for both Chl and UV, has been reported to be significantly enriched after hydrogen peroxide treatment (Piel et al., 2021). *Acetoanaerobium* and *Acetobacterium* were identified as treatment-resistant biomarker of Chl and UV, respectively. *Ottowia*, identified as UV resistant biomarker, was isolated from different types of wastewater treated by activated sludge (Spring et al., 2004). Obscuribacterales, identified as treatment-resistant biomarkers for both PFA_L and PFA_H, have been found in activated sludge of aerobic reactor, aquifer, soil and animal (McGorum et al., 2015; Salmaso et al., 2018; Soo et al., 2014). The genome analyses of bacteria belonging to

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474 Obscuribacterales revealed their ability to perform both aerobic and anaerobic
475 respiration, store polyphosphate, utilize nitrate, as well as their potential to use
476 carbohydrates and/or fatty acids, but their harmful potential has not been reported so
477 far (Dueholm et al., 2022; Salmaso et al., 2018).

478 Such results, showing more sensitive biomarkers and less resistant biomarkers for
479 PFA than for the other treatments, demonstrated a more efficient disinfection of
480 wastewater by the PFA treatment than the treatments involving UV and chlorine
481 disinfectants.

482 In order to better understand the differences in effectiveness of disinfectants, we
483 further performed LefSe analysis comparing all pairs of two treatments (Fig. 6).
484 Twelve taxa were sensitive to PFA_L and resistant to chlorine, including
485 microorganisms affiliated to i) Aeromonadales, Aeromonadaceae and *Aeromonas* that
486 have been reported as typical taxa in WWTP (Gao et al., 2023; VandeWalle et al.,
487 2012), and ii) Enterobacteriales and Enterobacteriaceae (Fig. 6, Fig. S4a), members of
488 which represent faecal indicators (Kausar et al., 2019). Also, 10 taxa were sensitive to
489 PFA_L and resistant to UV corresponding, in addition to the above-mentioned taxa, to
490 *Chromobacterium* and Fusobacteriaceae (Fig. 6, Fig. S4b), also known as faecal
491 indicators (Hutinel et al., 2022). Thus, PFA exhibits superior disinfection efficacy over
492 chlorine and UV because taxa related to faecal bacteria have shown increased
493 sensitivity to PFA.

494 Additionally, biomarkers sensitive to the PFA_H treatment were found to be
495 resistant to either Chl (18 biomarkers) or UV (26 biomarkers) treatments (Fig. S4e,f).
496 The 18 PFA_H treatment-sensitive biomarkers resistant to Chl included taxa affiliated
497 to faecal indicators such as Chromobacteriaceae, *Chromobacterium*,
498 Flavobacteriaceae and *Flavobacterium* (Fig. S4e). Similarly, the 26 PFA_H
499 treatment-sensitive biomarkers resistant to UV included faecal indicators such as
500 Aeromonadales and Aeromonadaceae (Fig. S4f). These observations further
501 confirmed the higher disinfection efficiency of PFA. Moreover, PFA was also found
502 to be more efficient when increasing the PFA dose since 11 biomarkers sensitive to
503 PFA_H were found resistant to PFA_L (Fig. 6, Fig. S4c).

1 504 Previous studies have noted that the reaction pathways of *E. coli* inactivation by
2 505 PFA included both direct and indirect free radical reactions, in which the PFA, $\cdot\text{OH}$
3
4 506 and peroxide radicals accounted for 73 %, 20 % and 6 %, respectively (Ding et al.,
5
6 507 2023). Thus, PFA exhibit higher efficiency and effectiveness than the free radicals. It
7
8 508 has been shown that PFA is predominantly responsible of cell inactivation by
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10 509 penetrating the microbial cell walls and membranes to damage functional proteins. In
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12 510 contrast, the $\cdot\text{OH}$ and peroxide radicals effectively oxidize the cell interior
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14 511 (Koivunen and Heinonen-Tanski, 2005; Luukkonen and Pehkonen, 2017). PFA has
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16 512 been shown to exhibit high reactivity with cysteine and methionine residues through
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18 513 an oxygen transfer pathway (Du et al., 2018; Wang et al., 2023). It has been shown
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20 514 that the number of broken and cross-linked bacterial cells increases after PFA
21
22 515 disinfection causing cell damage like wrinkles, chapped and rough surface on *E. coli*,
23
24 516 *S. aureus*, and *B. subtilis* (Ding et al., 2023). The intracellular oxidative stress has
25
26 517 been shown to be higher after PFA treatment (Wang et al., 2023). Thus, the PFA
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28 518 destroys the cell walls and membranes of microorganisms, resulting in the release of
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30 519 the cellular content.

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33 520 The regrowth and recovery of pathogenic bacteria and antibiotic resistant bacteria
34
35 521 (ARBs) carrying antibiotic resistance genes (ARGs) can potentially lead to
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37 522 subsequent contamination of the surface and ground water reservoirs (Shekhawat et
38
39 523 al., 2020). Previous studies showed that bacteria were not able to regrow 24 hours
40
41 524 after PFA disinfection of wastewater without irradiation (Ragazzo et al., 2013).
42
43 525 Similarly, *E. coli* and *enterococci* cells showed irreversible inactivation (Rocher et al.,
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45 526 2021). Additionally, PFA has been shown to be beneficial in controlling antibiotic
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47 527 resistance using antibiotic resistant *E. coli* as typical indicator (Campo et al., 2020).
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50 528 **3.4 Functional predictions**

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54 529 In order to estimate the functional capabilities of the microbial communities,
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56 530 functional profiles were determined by PICRUSt2. The nearest sequenced taxon
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58 531 indices (NSTI) indicated that PICRUSt2 prediction was accurate (mean NSTI = 0.11
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60 532 \pm 0.05 SD). Although predictive, with the limitation that no direct evidence of a

1 533 function is provided, such approach provides an overview of the microbial processes
2 534 (Menendez-Serra et al., 2019; Bourhane et al., 2022, 2023). The comparison of the
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4 535 PICRUSt2 predicted functions of bacterial communities showed that the wastewater
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6 536 samples from the same WWTP clustered together at both low and high KEGG
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8 537 hierarchy levels (Fig. 7a, e). Most of the KEGG orthologs at level 2 had
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10 538 enrichment/depletion variations among the three WWTPs. They were more abundant
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12 539 in WWTP2 and WWTP3 than in WWTP1 (Fig. 7e).

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14 540 PCA analysis considering each WWTP revealed that the bacterial communities
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16 541 clustered according to the disinfectant used for the treatment (Fig. 7b, c, d), showing
17
18 542 the functional shifts within the bacterial communities caused by the different
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20 543 disinfectants. The heatmap of the abundant functional patterns demonstrated that the
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22 544 wastewater owns a specific functional pattern according to the origin (Fig. 7e).
23
24 545 Noteworthy, the functional pattern was also modified by the treatment depending on
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26 546 the disinfectant used. Consistently with the microbial community differences
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28 547 observed (Fig. 2), the physical-chemical characteristics prevailing at the WWTP are
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30 548 the main factors shaping the microbial community structure, composition and
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32 549 functions. Such observation was in agreement with previous studies reporting that
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34 550 microbial changes are linked with effluent quality and system performance (Cao et al.,
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36 551 2020; ElNaker et al., 2019).

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38 552 The relative abundance of genes involved in cell cycle pathway of *Caulobacter*
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40 553 [PATH: ko04112] significantly decreased in PFA_H and Chl compared with BT
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42 554 (ANOVA, $p < 0.001$; Fig. S5). It demonstrates the effects of PFA and chlorine on
43
44 555 *Caulobacter* life cycle, which was consistent with a previous study showing that cell
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46 556 damages (e.g. discharge of cellular materials) were observed after chlorination in
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48 557 *Caulobacter* (Kim et al., 1982). Similarly, the relative abundance of genes associated
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50 558 to cell motility pathway significantly decreased in PFA_H compared with BT, being
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52 559 at lower abundance in PFA_H and PFA_L than in UV and Chl (ANOVA, $p < 0.001$;
53
54 560 Fig. S6). This result demonstrated the effect of PFA on decreasing the motility and
55
56 561 activity of microorganisms, which was consistent with the reduction of the microbial
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58 562 diversity and the reduction of *E. coli* and other faecal microorganisms counts
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1 563 observed in PFA treatment (Fig. 1, Fig. 5, Fig. 6). Such observation further confirmed
2 564 the effect of PFA on microbial community, which likely relies on the disinfection
3
4 565 efficiency. It has been shown that the application of 4 mg/L PFA for 16 h has not
5
6 566 toxicological effect using the *V. fischeri* assay (Chhetri et al., 2014), suggesting that
7
8 567 PFA has a short decomposition time. PFA was also reported causing less disinfection
9
10 568 by-products than other commonly used peracids (Luukkonen and Pehkonen, 2017). In
11
12 569 this study, the disinfection was performed with 1 and 4 mg/L PFA for 15 min
13
14 570 followed by a quenching step to stop the reaction. Although the residual toxicity test
15
16 571 was not conducted in this study, PFA achieved high disinfection efficacy while
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18 572 producing very low residual toxicity according to previous reports (Rocher et al.,
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20 573 2021).

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22
23 574 Regarding the metabolism associated pathways, the relative abundance of genes
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25 575 involved in the biosynthesis of secondary metabolites and metabolism of amino acids
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27 576 was significantly different (ANOVA, $p < 0.05$) according to the disinfection treatment
28
29 577 among all three WWTPs (Fig. 7e, S7-9). Previous studies have also reported that
30
31 578 enzymes from the same species may have different activities depending on the
32
33 579 treatment and conditions (Delforno et al., 2017; Emmanuel et al., 2019; Yang et al.,
34
35 580 2020).

36
37 581 The genes involved in penicillin and cephalosporin biosynthesis pathway [PATH:
38
39 582 ko00311] were significantly depleted on UV and Chl in comparison with PFA_H and
40
41 583 PFA_L treatments (ANOVA, $p < 0.05$; Fig. S7). Similarly, the abundance of genes
42
43 584 involved in betalain biosynthesis pathway [PATH: ko00965] was significantly more
44
45 585 abundant in PFA_H than UV (ANOVA, $p < 0.05$; Fig. S8). Betalain has been reported
46
47 586 for its antimicrobial activity on a broad spectrum of microbes such as
48
49 587 multidrug-resistant bacteria, single-species and dual-species biofilm-producing
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51 588 bacteria, dengue virus and fungi (Wijesinghe and Choo, 2022). Betalains primarily
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53 589 occur in Caryophyllales order plants. The enzymes involved in betalain biosynthesis
54
55 590 have been reported in fungi, *Gluconacetobacter diazotrophicus* and Cyanobacteria
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57 591 (Contreras-Llano et al., 2019; Guerrero-Rubio et al., 2020). However, LEfSe did not
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59 592 identified Cyanobacteria as PFA-resistant biomarkers (Fig. S4), suggesting that

1 593 bacteria producing antimicrobial compounds (betalains), likely present in treated
2 594 wastewater, were more sensitive to UV and chlorine than to PFA. Since UV and
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4 595 chlorination have been reported for their strong elimination efficiency of antibiotic by
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6 596 photolysis (Ding et al., 2020; Serna-Galvis et al., 2017), such a slight deficiency does
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8 597 not indicate that PFA is incompetent. It nevertheless alerts the risk of antibiotics
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10 598 release from wastewater into water system that potentially alters microbial ecosystems,
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12 599 that in turn represents a sanitary issue for human health (Baquero et al., 2008). The
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14 600 genes involved in glutathione metabolism pathway [PATH: ko00480] were
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16 601 significantly more abundant in PFA_H (ANOVA, $p < 0.05$; Fig. S9). The glutathione
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18 602 metabolism has been suggested to play important role in bacterial defence against
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20 603 chlorine-based disinfection (Du et al., 2015), demonstrating that PFA has likely
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22
23 604 similar effect on bacteria.

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25 605 These results suggested that PFA_H, even efficient on disinfecting most of the
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27 606 microorganisms (including pathogens), was not effective on inactivating the
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29 607 microorganisms carrying penicillin and cephalosporin, and betalain biosynthesis
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31 608 pathways. However, further analyses determining direct functions, such as shotgun
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33 609 metagenomics, are needed in order to describe in depth the effect of disinfection on
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35 610 the functional capabilities of microbial communities.

36 37 38 611 **4. Conclusions**

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41 612 Although the operational cost of PFA treatment has been estimated to be
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43 613 approximately 2.5 times more expensive than that of chlorine (Luukkonen et al.,
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45 614 2015), the PFA treatment owns several assets to be used as treatment for safe reuse of
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47 615 wastewater as it does not produce by-product. Also it has been shown that its cost can
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49 616 be drastically reduced for treatment plants with larger capacity (Ragazzo et al., 2013).
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51 617 But, even if further analyses are still required to comprehensively evaluate the costs, it
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53 618 is also necessary to evaluate the disinfection efficiency of PFA treatment in
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55 619 comparison to other commonly used treatments.

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57 620 Our study elucidated the effect of PFA, UV and chlorine on microorganism
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59 621 disinfection, microbial community diversity, structure and functions crossing three

1 622 different WWTPs. It provides compelling evidence that different disinfectants
2 623 resulted in specific microbial communities and functions, which effects differed
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4 624 according to the physical-chemical characteristic of the wastewater origin (WWTP).
5
6 625 Our findings indicated that PFA was efficient in *E. coli* disinfection of wastewater,
7
8 626 being able to decrease the microbial community diversity. Additionally, specific
9
10 627 biomarkers of each WWTP were revealed, which likely relies on the processing
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12 628 treatments methods. Also, our study detected sensitive/resistant biomarkers for each
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14 629 disinfectant, PFA exhibiting higher number of sensitive biomarkers than chlorine and
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16 630 UV, PHA_H having the highest number. Our findings underline the fact that PFA is
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18 631 efficient on wastewater disinfection in comparison with the traditional treatments
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20 632 involving UV and chlorine disinfectants. Because PFA was particularly effective in
21
22 633 removing known faecal microorganisms, it has high potential for safe reuse of
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24 634 wastewater.
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27 635

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42
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44
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46
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48
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61

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2
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4
5 654 (NCBI) and is available under the Bioproject ID PRJNA907723.
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971 **Figure captions**

972 Fig. 1. Efficiency of the disinfection treatments on the different WWTPs. The
973 efficiency of the disinfection treatments was estimated by *E. coli* counts. BT, before
974 treatment; The treatments are UV, chlorine, performic acid at low (PFA_L) and high
975 (PFA_H) concentrations.

976
977 Fig. 2. Comparison of microbial communities by Principal Coordinates Analysis
978 (PCoA) based on weighted UniFrac distances including the three WWTPs (a); PCoA
979 based on Bray-curtis distances for WWTP1 (b), WWTP2 (c), and WWTP3 (d). The
980 arrows indicate the significant impact of the parameter (*E. coli* counts) on the
981 microbial community composition (* $p < 0.05$, ** $p < 0.01$, Permutations = 999).
982 Circles: WWTP1; diamond: WWTP2; triangles: WWTP3. Red: chlorine; blue:
983 performic acid at low concentration (PFA_L); orange: performic acid at high
984 concentration (PFA_H); green: before treatment (BT); purple: UV.

985
986 Fig. 3. Composition of microbial communities at the phyla level in each treatment and
987 WWTP. BT, before treatment; The treatments are UV, chlorine, performic acid at low
988 (PFA_L) and high (PFA_H) concentrations.

989
990 Fig. 4. Bacterial biomarkers (bacterial taxa) for the different WWTPs before treatment
991 (BT). The Cladogram shows the bacterial taxa identified by linear discriminant
992 analysis effect size (LEfSe). The biomarkers are colored according to their WWTPs'
993 specificity indicating that they are significantly more abundant in a WWTP than the
994 others. The wastewater samples without any treatment were used in the analysis. D1:
995 Phylum; D2: Class.

996
997 Fig. 5. Linear discriminant analysis effect size (LEfSe) identifying treatment-sensitive
998 (external) and treatment-resistant (inner) biomarkers according to treatment PFA_H,
999 PFA_L, Chlorine and UV. The comparisons were performed between the wastewater

1 1000 before (BT) and after each treatment from the three WWTPs. The length of the bars
2 1001 corresponds to the LDA score. The known pathogens are represented in red. D1:
3
4 1002 Phylum; D2: Class; D3: Order; D4: Family; D5: Genus; D6: Species.
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8 1004 Fig. 6. Disinfection efficiency on pathogen microorganisms according to the different
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10 1005 treatments. The comparisons were performed between each pair of the treatments
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12 1006 from all three WWTPs by linear discriminant analysis effect size (LEfSe) identifying
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14 1007 treatment-sensitive biomarkers. The colored dots correspond to microbial genera that
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16 1008 sensitive to PFA_H (red), PFA_L (orange), Chlorine (purple) and UV (blue)
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18 1009 treatment respectively.
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21 1010
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23 1011 Fig. 7. Functional comparison of bacterial communities predicted by PICRUSt2. The
24
25 1012 PCAs show the predicted functions, based on KEGG categories (level 3), of the three
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27 1013 WWTPs (a), and for the different disinfectants treatments in WWTP1 (b), WWTP2
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29 1014 (c), WWTP3 (d). (e) Heatmap of the standardized abundance of predicted functions
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31 1015 based on KEGG categories (level 2). BT, before treatment; the treatments are UV,
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33 1016 chlorine, performic acid at low (PFA_L) and high (PFA_H) concentrations.
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1 **Safe reuse of wastewater: effect of disinfection methods on microbial community**

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13 **Highlights**

- 14 ● PFA was an efficient disinfectant comparing to chlorine and UV
- 15 ● Microbial communities from treated water were specific to the disinfectant used
- 16 ● Biomarkers sensitive to PFA and resistant to chlorine and/or UV were identified
- 17 ● Bacterial predicted functions diverge according to WWTP and disinfectants

18

19 **Abstract**

20 Performic acid (PFA), a new generation of disinfectant, has shown outstanding
21 performance for wastewater treatment. However, the effect of PFA on wastewater
22 microbial community changes as well as the differences in comparison to the
23 commonly disinfectants used are poorly documented. We provide an overview of the
24 PFA effect and that of chlorine and UV, two commonly used disinfectants, on
25 microbial community diversity and structure. The study was performed using
26 wastewaters from three municipal wastewater treatment plants (WWTPs) processed
27 by activated sludge, lagoon and reed beds, and biofilter. We focused on disinfection
28 of pathogen microorganisms, identifying sensitive and resistant bacteria to
29 disinfectants that can serve as biomarkers for evaluating the disinfectant performances.
30 As well, we investigated the modifications of microbial functions after treatment by
31 functional predictive analysis. The more efficient *E. coli* inactivation was obtained
32 with initial concentration of 4 mg/L of PFA with 15 min disinfectant contact time that
33 achieved 95.71-99.99% inactivation efficiency. Although the origin of wastewater
34 was the main factor determining the microbial community diversity and composition,
35 the microbial communities were specific to the disinfectant used. Linear discriminant
36 analysis Effect Size (LEfSe) identified 18 and 10 biomarkers, including pathogenic
37 microorganisms affiliated to *Chromobacterium*, *Flavobacterium*, *Aeromonas*,
38 Enterobacteriaceae, *Escherichia_Shigella* and Fusobacteriaceae, which were sensitive
39 to PFA, while being resistant to chlorine and/or UV. Consistently, the predicted
40 bacterial functional patterns diverged according to the wastewater origin and the
41 disinfectant used. Pathogen microorganisms were more sensitive to PFA than the
42 other treatments, demonstrating its potential for implementing a safe reuse of
43 wastewater.

44

45 **Keywords:** Wastewater reuse; Performic acid; Disinfection efficiency; Disinfectant
46 sensitive biomarker; Microbial community; Microbial function

47 **1. Introduction**

48 Water scarcity is becoming a global concern in recent decades. The
49 overexploitation of water is one of the major threats to the EU freshwater
50 environment, especially for agricultural irrigation (EC, 2018). More than 40,000
51 million m³ wastewater is treated each year in the EU, only less than 3% being reused
52 (EC, 2022). Thus, there is place to improve water reuse, which represents a potential
53 on easing water supply pressure, also contributing to a sustainable agriculture.

54 Domestic wastewater owns microorganism associated to human diseases
55 (Diamond et al., 2022; McCall et al., 2020). It is thus critical to establish efficient and
56 economical disinfection frameworks in wastewater treatment in order to supply safe
57 water. Tertiary treatment aims to remove stubborn pollutants (organics, turbidity,
58 nitrogen, phosphorus, metals, and pathogens), including also an additional
59 disinfection step (Pei et al., 2019). The tertiary treatment provides additional
60 assurance of effluent quality, protecting thus wildlife in the discharged water system.
61 It also ensures the safety of wastewater reuse for various purposes, such as irrigation
62 and industrial applications (Gerba and Pepper, 2019).

63 Several disinfection approaches are commonly implemented in tertiary treatment
64 for the inactivation of pathogen microorganisms, such as chlorination, ultraviolet (UV)
65 irradiation, ozonation and filtration (USEPA, 2004). Chlorine and its various forms
66 are powerful oxidants for the inactivation of pathogens (Furst et al., 2018), but it
67 forms toxic by-products that might impact the receiving environment threatening the
68 human health (Bull et al., 1995; Nieuwenhuijsen et al., 2000; Victoria, 2002). The UV
69 irradiation has been considered as an alternative to chlorine for wastewater treatment
70 due to its short contact time, and it has the advantage that toxic by-products are not
71 produced (Zyara et al., 2016). However, UV irradiation has the disadvantage to be
72 expensive because of high-energy demand, high costs of the equipment and its
73 management. The investment cost is estimated to be 10 times more expensive than
74 that of chlorine and PFA treatments (Ragazzo et al., 2013). Also, the practical
75 application of UV irradiation carries many limitations because it is dependent on

76 water quality parameters, such as turbidity, colour and suspended solid (Oguma et al.,
77 2001; Zimmer and Slawson, 2002).

78 Performic acid (PFA), a high oxidative peracid, has been considered as an ideal
79 alternative for industrially wastewater disinfection due to its outstanding
80 characteristics (Gehr et al., 2009; Ragazzo et al., 2013). PFA is a wide-spectrum
81 disinfectant with deodorizing ability and low corrosive effect, exhibiting toxicity to
82 viruses, bacteria and bacterial spores, mycobacteria as well as microscopic fungi, but
83 not to higher forms of life (Gehr et al., 2009; Luukkonen and Pehkonen, 2017). The
84 PFA treatment cost has been estimated at 0.0114 €/m³ (Luukkonen and Pehkonen,
85 2017), while that of chlorination at 0.004 €/m³ (Zagklis and Bampos, 2022). Although
86 the operation cost of PFA treatment is estimated to be 2.5 times more expensive than
87 that of chlorine (Luukkonen et al., 2015). It has the advantage to produce by-products
88 (hydrogen peroxide and formic acid) that are nontoxic to aquatic fauna at the
89 disinfection doses (Gehr et al., 2009; Ragazzo et al., 2013). Thus, its reasonable cost
90 and splendid performance at ambient temperature demonstrate that PFA has potential
91 applicability for large wastewater treatment plants (WWTPs) (Luukkonen et al.,
92 2015).

93 Several studies have reported on the effect of the commonly used disinfectants on
94 microorganisms. Most studies have considered the decrease of bacterial cell counts
95 and species richness, the inactivation of viruses, and the removal of microorganisms
96 carrying antibiotic resistance genes (ARGs) (Kantor et al., 2019; Kulkarni et al., 2018;
97 Ng et al., 2019; Stamps et al., 2018; Zaouri et al., 2020). Nevertheless, the effect of
98 disinfectant treatments on the microbial community structure and functions is scarce,
99 especially for the PFA treatment (Kantor et al., 2019; Numberger et al., 2019; Stamps
100 et al., 2018). The release of potential pathogens and the modification of microbial
101 community in the receiving environment have been reported during reclaimed water
102 discharging process (Numberger et al., 2019). Thus, it is important to consider the
103 effect of wastewater treatments on the microbial community. Precisely, the objective
104 of our study was to examine the effect of PFA treatment on the microbial diversity,
105 microbial structure and function. The disinfection efficiency of PFA treatment against

106 pathogens was also evaluated and compared to that of other conventional treatments.

107 We hypothesize that the microbial communities will be modified differently
108 according to the treatment because they involve different action mechanisms.

109 In order to examine the possibility to safety reuse treated wastewater for irrigation,
110 industrial applications, groundwater recharge and other agricultural activities, this
111 study aimed to compare PFA, UV and chlorine treatments regarding their effect on 1)
112 the microbial community diversity and structure, and 2) the disinfection efficiency for
113 the inactivation of pathogens. For this purpose different WWTPs were treated in order
114 to identify sensitive and resistant bacteria to each disinfectant, representing potential
115 microbial biomarkers according to treatment. The bacterial functions were predicted
116 and correlated with the wastewaters resulting from the treatments.

117 2. Materials and methods

118 2.1 Sampling sites and physical-chemical characterization of wastewaters

119 Three domestic WWTPs with different treatment processes, activated sludge,
120 lagoon and reed bed and biofilter were selected. Brief information of the sampling
121 WWTPs is listed in the Table S1.

122 The main physical-chemical parameters were determined on the wastewater
123 samples. Conductivity was measured by 3510 WTW multiparameter-probe. Chemical
124 oxygen demand (COD) was determined using colorimetric method by Hach reactants
125 and Hach DR3900 spectrophotometer. Total suspended solid (TSS) was determined
126 after filtration through 0.45 μm on 47 mm glass fiber filter and drying at 105 $^{\circ}\text{C}$ until
127 constant mass. Total organic carbon (TOC) and total nitrogen (TN) were determined
128 by a Shimadzu TOC VCN analyzer with a TN unit (chemiluminescence measurement
129 of NO_x) according to (Behera et al., 2017). TOC was obtained by the difference of
130 total carbon and inorganic carbon analyses.

131 2.2 Sample collection and disinfection

132 The effluent wastewater samples were collected from each WWTP with sterile
133 Scott bottles. All the samples were collected in the morning and transported to the
134 laboratory at 4 °C. The disinfections were generated by three disinfectants (PFA,
135 chlorine and UV) just right after the water samples arrived to the laboratory. Each
136 disinfection treatment was performed in true biological triplicates. Thus, a total of 45
137 samples (3 WWTP x triplicate x 5 treatments (PFA_L, PFA_H, Chlorine, UV and
138 untreated wastewater as control)) were analysed. All samples were maintained at 4 °C
139 prior further processing.

140 The optimal disinfection concentration of all the disinfectants used was
141 determined in a pre-experiment using *E. coli* and total coliforms as indicators. The
142 initial doses range were determined based on official wastewater treatment manual
143 and previous studies, also adjusted according to the wastewater treatment plants
144 conditions (Anastasi et al., 2013; EPA, 2011; Gehr et al., 2009; Ragazzo et al., 2013;
145 USEPA, 2003). More specifically, previous reports indicated that UV was effective at
146 10 mJ/cm² although a higher dose (30-40 mJ/cm²) would be required for complete
147 disinfection (Andreadakis et al., 1999), while hypochlorite was found effective at 2
148 mg/L (Collivignarelli et al., 2017) and PFA in 1-4 mg/L range (Chhetri et al., 2014).

149 2.2.1 PFA preparation and disinfection

150 For preparing PFA, 10 mL of formic acid was mixed with 1 mL sulfuric acid
151 (98%, Aldrich) as a catalyst on ice and wait about 10 min to cool. 14.2 mL of
152 hydrogen peroxide (H₂O₂, 50% wt, Aldrich) was then added slowly into the mixture.
153 Then, the mixed solution was maintained for 60 min to get ready to use. The whole
154 process was performed in ice bath and mixed with a magnetic stirrer. 100 mg of the
155 mixture was then mixed with 0.1M H₂SO₄ solution and titrated to determine the
156 concentrations of H₂O₂ and PFA. The H₂O₂ titration was conducted with potassium
157 permanganate solution (KMnO₄, 0.02 mol/L, Aldrich). An excess of potassium iodide
158 was then added and oxidized by PFA. Iodine was then titrated with sodium thiosulfate

159 (Na₂SO₃, 0.02 mol/L, Aldrich). The PFA solution was kept at -20 °C and titrated each
160 time right before use.

161 Because PFA is rapidly decomposed (Karpova et al., 2013), two doses of PFA, a
162 lower initial concentration at 1 mg/L with 15 min disinfectant contact time (PFA_L)
163 corresponding to a dose known to inactivate most microorganisms (Karpova et al., 2013)
164 and a higher initial concentration at 4 mg/L with 15 min disinfectant contact time
165 (PFA_H) corresponding to the optimal dose for disinfection (Karpova et al., 2013;
166 Chhetri et al., 2014), were used in the disinfection. Na₂SO₃ solution (0.1 mol/L) was
167 used for quenching the reaction.

168 **2.2.2 Chlorination**

169 A solution of 100 mg/L of total chlorine was prepared from pure calcium
170 hypochlorite (Aldrich) and stored at 4 °C. The prepared solution was titrated by
171 iodometric titration in acetic acid medium before use. The chlorination was performed
172 with the initial concentration at 2 mg/L for 30 min (Chl). Na₂SO₃ solution was used
173 for quenching the reaction.

174 **2.2.3 UV disinfection**

175 The laboratory scale UV disinfection was performed using low-pressure mercury
176 vapour lamp to perform the disinfection at the dose of 10 mJ/cm². The irradiance rate
177 was measured by UV radiometer (Avantes Avaspec 2048L equipped with a 600 µm
178 core optical fiber and a corrector cosine (4.9 mm diameter)). The solutions were
179 irradiated by a home-made device consisting of four Phillips TL UV 8W 30 cm
180 fluorescent lamps. The device was placed on top of the solutions and the height was
181 adjusted until obtaining the desired irradiance. The lamp was preheated for more than
182 15 min before disinfection. 250 mL of wastewater was treated each time in the
183 specific glass beaker. The exposure time was calculated by the desired dose,
184 irradiance rate and transmittance value of the wastewater sample.

185 2.3 Microbial analysis

186 The *Escherichia coli* (*E. coli*) counts were determined by a standard
187 beta-glucuronidase positive method (APHA, 2005).

188 The water samples were filtered by sterile disposable filtration units (MicroFunnel,
189 Pall Corporation, USA) with 0.2 µm Supor membrane to concentrate the microbial
190 biomass. The iQ-Check Free DNA Removal Solution (FDRS, Bio-Rad Laboratories,
191 USA), which degrades free DNA by enzymatic treatment, was used to ensure that the
192 samples are devoid of free DNA prior to DNA extraction. For the free DNA
193 elimination, we followed standard protocol. In short, the membranes were folded into
194 cones and transferred into Eppendorf tubes containing 40 µl FDRS and 460 µl
195 distilled Milli-Q water, followed by 30 min incubation at 37 °C. To inactivate the
196 FDRS and for DNA extraction, 1 mL lysis solution (R1) from Aquadien™ Bacterial
197 DNA Extraction and Purification Kit (Bio-Rad Laboratories, USA) were then added
198 and incubated for 15 min at 95 °C with 1,300 rpm in a shaking heating block
199 (Eppendorf ThermoMixer C, Eppendorf, Germany). We followed the standard
200 protocol for the Aquadien™ Bacterial DNA Extraction and Purification Kit for the
201 following steps.

202 The V3-V4 region of 16S rRNA gene was amplified using the primer pair 341F
203 (5' - CCTAYGGGRBGCASCAG - 3') and 806R (5' -
204 GGACTACNNGGGTATCTAAT - 3') (Gao et al., 2020) connecting with barcodes.
205 PCR amplification, library preparation and sequencing were conducted by Novogene
206 Europe (Cambridge, UK) using their proprietary methods. To ensure an accurate
207 recognition of the samples, the index pairs were specific for each sample. Quality
208 control was performed at each step of the procedure. The PCR products were purified
209 using AMPureXP magnetic beads, pooled in equimolar and the libraries generated
210 with NEBNext® Ultra™ DNA Library Prep Kit for Illumina and quantified via
211 Qubit and Q-PCR. Paired-end 250 bp sequencing was then performed on Illumina
212 NovaSeq 6000 sequencer (Illumina, San Diego, CA, USA).

213 2.4 Bioinformatics analysis and visualization

214 Paired-end reads were assigned to samples based on the sample-specific barcode
215 and truncated by cutting off the barcode and primer sequences, and were merged
216 using FLASH (V1.2.7) (Magoc and Salzberg, 2011). Qiime2 (v 2019.4) was used for
217 the data processing and analysis. The sequence quality control and amplicon
218 sequencing variants (ASVs) assignment were performed using DADA2 plugin.
219 Taxonomy assignment was completed using the q2-feature-classifier plugin against
220 SILVA database (v132) with a clustering threshold of 97% similarity. The ASVs
221 assigned to Eukaryota, Chloroplast, Mitochondria and unassigned ASVs were
222 removed and the filtered dataset was then rarefied at the depth of 71673 using
223 *alpha-rarefaction.py* script. The rarefied data were then used for the following
224 analyses without threshold. The complete dataset was deposited in the Sequence Read
225 Archive (SRA) of the National Center for Biotechnology Information (NCBI) and is
226 available under the Bioproject ID PRJNA907723.

227 For the alpha-diversity, the Shannon, Simpson, observed features, Chao1, ace
228 indices were analyzed using *alpha_diversity.py* in Qiime2. Statistical analysis
229 (ANOVA, Tukey HSD) and visualization were performed in GraphPad Prism.
230 Beta-diversity was calculated in Qiime2 and R based on weighted Unifrac and
231 Bray-Curtis dissimilarity. Permutational multivariate analysis of variance
232 (PERMANOVA) was performed with 999 times permutations using the
233 *beta_significance.py* script in Qiime2 and the “adonis” function in the vegan package
234 in R. Principal coordinate analyses (PCoA) were performed and visualized by
235 EMPERor (Vazquez-Baeza et al., 2013) and the Ape and ggplot2 packages in R. The
236 significances of physical-chemical parameters associated with microbial community
237 were identified by “envfit” function in the “vegan” package in R.

238 Linear discriminant analysis Effect Size (LEfSe, v 1.0) (Segata et al., 2011) was
239 performed to identify the biomarkers between the groups, which emphasizes both
240 statistical significance and biological relevance. LEfSe analysis was performed using
241 the ASV table, at the ASV level spanning all the taxonomic levels. The

242 non-parametric factorial Kruskal-Wallis sum-rank test was performed first to detect
243 features with significant differential abundance between the classes (groups). The
244 pairwise Wilcoxon test was then applied to check if the subclasses agree with the
245 class level trend, which was performed only among subclasses in different classes
246 with the same name. The resulting subset of vectors was then used to build a Linear
247 Discriminant Analysis model. The alpha-value for both Kruskal-Wallis and Wilcoxon
248 tests were at 0.05 and the logarithmic LDA threshold score for discriminative features
249 was 2.

250 The PICRUSt2 was used for the prediction of microbial community functions
251 following the following 4 steps: (1) sequence placement, (2) hidden-state prediction
252 of genomes, (3) metagenome prediction, (4) pathway-level predictions. The
253 pathway-level predictions were performed using a customized mapping file based on
254 the KEGG database (KEGG Brite orthologs to pathways, updated on 22 Nov. 2020,
255 Table S2). The descriptions were then added to the Enzyme Classification (EC)
256 numbers, KEGG orthologs (K) with default mapping table, and a customized mapping
257 table including descriptions of higher hierarchical levels was used for the KEGG
258 pathways (ko, Table S3). STAMP v 2.1.3 was used for visualizing PICRUSt2 output
259 data. ANOVA and Kruskal-Wallis H-test were used for the multiple groups' statistical
260 analysis. Tukey-Kramer and Welch's at 0.95 were performed as Post-hoc tests.

261 3. Results and discussion

262 3.1 Disinfection treatment efficiency

263 The disinfection efficiency of PFA, UV and chlorine was determined for the three
264 different wastewater treatment plants by determining the reduction of the *E. coli*
265 content, which represents a key parameter for characterizing reclaimed water quality
266 in wastewater reuse (EC, 2018; EPA, 2004). The initial *E. coli* cell concentration
267 (before treatment BT, Fig. 1) was 2.8×10^4 , 2.5×10^4 and 5.8×10^3 CFU/100 mL for
268 WWTP1, 2, and 3, respectively. In WWTP1 and WWTP2, the *E. coli* cell
269 concentration significantly decreased in all the treated wastewaters ($p < 0.001$, Fig. 1).

270 Indeed, the WWTPs' effluents showed different physical-chemical characteristics
271 (Table S1). The conductivity was lower in WWTP1 effluent (718 $\mu\text{S}/\text{cm}$) than in
272 WWTP2 (1000 $\mu\text{S}/\text{cm}$) and WWTP3 (1254 $\mu\text{S}/\text{cm}$) effluents (Table S1). Consistently,
273 WWTP1 effluent exhibited the lower contents of COD (36 mg/L), TSS (2 mg/L),
274 TOC (18 mg/L), and TN (3.5 mg/L), while WWTP2 effluent showed the higher COD
275 (80 mg/L) and NT (60.6 mg/L) contents (Table S1).

276 Noteworthy, the PFA treatment was more efficient with WWTP1, which exhibited
277 the lowest TSS content (Table S1, Fig. 1). This result is consistent with previous
278 studies showing that the disinfection efficiency of UV and peracetic acid increased
279 with low TSS (Dominguez Henao et al., 2018; Walters et al., 2014).

280 The Chl and both low and high doses of PFA (PFA_L and PFA_H) treatments
281 showed similar *E. coli* content reduction for WWTP1 and WWTP2 ($p > 0.05$),
282 although the *E. coli* concentration (277 CFU/100 mL) was higher for the PFA_L
283 treatment of WWTP2. All these three treatments were significantly more efficient in
284 decreasing the *E. coli* concentration than the UV treatment ($p < 0.001$). The Chl and
285 PFA_H treatments had the best efficiency, reducing the *E. coli* concentration below
286 40 CFU/100 mL. A reduction of *E. coli* counts by 3-4 \log_{10} magnitude was observed
287 with the PFA_H treatment for WWTP1 and WWTP2, while for WWTP3 the
288 reduction of *E. coli* counts was 1 \log_{10} magnitude (Fig. 1). The treatment of WWTP3
289 showed different trends, except for the PFA_H treatment that was also the most
290 efficient for reducing the *E. coli* content. These observations suggested that the
291 disinfection efficiency, evaluated by the decrease of *E. coli* concentration, was
292 dependent on the water physical-chemical parameters. The treatment with the high
293 dose of PFA (PFA_H) provided better performance than chlorine and UV,
294 irrespective of the wastewater origin, observation that was in agreement with a
295 previous report (Ragazzo et al., 2020).

296 The UV and PFA treatments were less efficient in decreasing the *E. coli*
297 concentration for the WWTP3 wastewater than for WWTP1 and WWTP2, even if the
298 TSS of WWTP2 and WWTP3 were at a similar level (16 and 18 mg/L, respectively).
299 It is likely that the high TSS content of WWTP3 might relate high *E. coli*

300 concentration since WWTP3 exhibited also the highest conductivity (1254 $\mu\text{S}/\text{cm}$,
301 Table S1), which has been shown to be related to the *E. coli* concentration (Foschi et
302 al., 2021). The inactivation efficiency of *E. coli* has been shown to be dependent on
303 the WWTP treatment processes (Biswal et al., 2014). Although UV disinfection
304 significantly decreased *E. coli* concentration, it was not efficient as Chl and PFA in
305 WWTP1 and WWTP2. Among the disinfection treatments, only the PFA_H was
306 effective for reducing the *E. coli* concentration. Such observation was in agreement
307 with previous studies showing that PFA was more efficient in reducing *E. coli*
308 concentration than the disinfectants commonly used for municipal wastewater
309 treatment (Maffettone et al., 2020; Wang et al., 2023).

310 **3.2 Effect of disinfection treatment on microbial community**

311 A total of 5,724,372 raw reads was obtained and assigned to 17,151 amplicon
312 sequence variants (ASVs). The sequences were then rarefied at the minimum 71,673
313 reads per sample. The final dataset, including 3,153,612 reads dispatched within
314 14,838 ASVs, was used for the microbial community analysis.

315 **3.2.1 Impact of disinfection treatments on microbial community diversity**

316 The diversity indices (Shannon, Simpson, observed richness, Chao1 and ace) were
317 significantly different (two-way ANOVA, $p < 0.001$) according to the WWTP origin
318 and the treatment, WWTP origin explaining most of the variation (62.8 %, $p < 0.001$;
319 Table S4). The microbial community of the wastewater from WWTP2 exhibited
320 significantly higher diversity indices than those from WWTP1 and WWTP3 (two-way
321 ANOVA, $p < 0.001$; Fig. S1). The microbial community of wastewater from WWTP3
322 had significantly higher Shannon index than that from WWTP1 (two-way ANOVA, p
323 < 0.001 ; Fig. S1).

324 Noteworthy, the treatments for a given WWTP decreased the microbial diversity
325 (Shannon index and the observed richness, two-way ANOVA, $p < 0.05$; Fig. S2),
326 except for WWTP2 (Fig. S2). Interestingly, the WWTP2, lagoon and reed beds
327 process, showed the higher COD, TOC and TN contents (Table S1). It has been

328 shown that the efficiency of a treatment **in decreasing** the microbial diversity is
329 dependent on the wastewater physical-chemical characteristics such as COD, TOC
330 and TN (Tian and Wang, 2020; Wu et al., 2022), the treatment processes (Sauter et al.,
331 2023), and even on the different type of reed beds systems (Kowal et al., 2021). Thus,
332 it is likely that the high content of organic matter (COD, TOC and TN) might explain
333 the fact that the microbial diversity was not decreased by the treatments in WWTP2.

334 The WWTP3 showed higher diversity (Shannon and observed richness) indices
335 before treatment (BT) than the two other WWTPs (**two-way ANOVA, $p < 0.05$** ; Fig.
336 S2), which may explain the lower *E. coli* inactivation observed with WWTP3
337 wastewater (Fig 1) despite that it showed the lowest *E. coli* concentration. Such
338 observation was consistent with a previous report showing that the *E. coli* inactivation
339 is dependent on the microbial diversity (Qian et al., 2022).

340 For the WWTP1, the Shannon index was significantly lower (**two-way ANOVA,**
341 **$p < 0.001$** ; Fig. S2) for PFA_H than for before treatment (BT), and for the three other
342 treatments. Similarly, the observed richness for PFA_H was significantly lower than
343 for Chl (**two-way ANOVA, $p < 0.05$** ; Fig. S2). The WWTP3 treated by PFA_H and
344 Chl showed significantly lower Shannon index and observed richness than BT
345 (**two-way ANOVA, $p < 0.05$** ; Fig. S2).

346 **3.2.2 Impact of disinfection on microbial community structure**

347 The principal coordinate analysis (PCoA), based on either the weighted UniFrac
348 or Bray-Curtis dissimilarity matrices (Fig. 2), further confirmed that the microbial
349 communities of the wastewaters were different according to the WWTPs, revealing
350 also the treatments effects. The PCoA clustered the microbial communities according
351 to their origin (i.e. WWTP), separating the different WWTPs along the axis 1, which
352 explained 66.7% of the variation (Fig. 2a). This observation was supported by the
353 permutational multivariate ANOVA (PERMANOVA) indicating that the WWTP
354 origin was the main factor explaining the differences between the microbial
355 communities (pseudo-F = 79.68, $p = 0.001$). Within a WWTP cluster, the microbial
356 communities clustered by treatment (Fig. 2a). Thus, in order to reveal the effect of the

357 disinfection treatments on the microbial community, PCoA were performed for each
358 WWTP (Fig. 2b, 2c, 2d). For WWTP2 and WWTP3, wastewater samples from the
359 same treatment tightly clustered together (Fig. 2c, 2d). Envfit (Fits an Environmental
360 Vector or Factor onto an Ordination) analysis also revealed that the composition of
361 wastewater microbial communities significantly varied by treatment in WWTP2 ($r^2 =$
362 $0.94, p < 0.001$) and WWTP3 ($r^2 = 0.89, p < 0.001$), but not in WWTP1.

363 Before the treatments, COD, TOC and TN were identified as the main factors
364 driving the microbial communities of WWTP2 (Envfit; COD: $r^2 = 0.9712, p = 0.004$;
365 TOC: $r^2 = 0.9688, p = 0.004$; NT: $r^2 = 0.9682, p = 0.009$), while the conductivity
366 (Envfit; $r^2 = 0.9930, p = 0.001$) and TSS (Envfit; $r^2 = 0.9844, p = 0.001$) were the
367 main factors driving the microbial communities from WWTP3. After the disinfection
368 treatments, the same factors were found to drive the microbial communities from
369 WWTP2 and WWTP3 irrespective of the treatment: COD (Chl: $r^2 = 0.9568, p = 0.004$;
370 UV: $r^2 = 0.9955, p = 0.004$; PFA_L: $r^2 = 0.9664, p = 0.003$; PFA_H: $r^2 = 0.9251, p =$
371 0.003), TOC (Chl: $r^2 = 0.9522, p = 0.004$; UV: $r^2 = 0.9946, p = 0.004$; PFA_L: $r^2 =$
372 $0.9601, p = 0.003$; PFA_H: $r^2 = 0.9139, p = 0.004$) and NT (Chl: $r^2 = 0.9503, p =$
373 0.020 ; UV: $r^2 = 0.9927, p = 0.004$; PFA_L: $r^2 = 0.9547, p = 0.009$; PFA_H: $r^2 =$
374 $0.9051, p = 0.026$) for WWTP2; conductivity (Chl: $r^2 = 0.9936, p = 0.001$; UV: $r^2 =$
375 $0.9907, p = 0.008$; PFA_L: $r^2 = 0.9968, p = 0.001$; PFA_H: $r^2 = 0.9866, p = 0.002$)
376 and TSS (Chl: $r^2 = 0.9798, p = 0.006$; UV: $r^2 = 0.9943, p = 0.009$; PFA_L: $r^2 = 0.9883,$
377 $p = 0.006$; PFA_H: $r^2 = 0.9678, p = 0.006$) for WWTP3.

378 These results suggested that the origin (i.e. WWTP) was the main factor
379 determining the composition of the microbial community. The different disinfection
380 treatments resulted in specific microbial communities, which effects were different
381 according to the wastewater origin (WWTP).

382 **3.2.3 Microbial composition differences between WWTPs: identification of** 383 **specific biomarkers**

384 The distinction of the microbial communities according to their origin (i.e.
385 WWTP) was confirmed by the presence of different dominant phyla (Fig. 3).

386 Proteobacteria (60.5%) was the most dominant bacterial phylum in all three WWTPs,
387 consistent with previous studies in both domestic and industrial WWTPs (Sharma et
388 al., 2021; Tang et al., 2020a). The relative abundance of the Proteobacteria
389 significantly decreased from WWTP2 (70.7%) to WWTP3 (61.9%), and to WWTP1
390 (48.9%) (ANOVA, $p < 0.001$).

391 The second dominant phylum in the three WWTPs was Bacteroidetes (11.4%),
392 followed then by Actinobacteria (8.1%), Nitrospirae (4.3%) and Nanoarchaeaeota
393 (3.7%). The relative abundance of the Actinobacteria significantly decreased from
394 WWTP1 (15.1%) to WWTP2 (7.1%), and to WWTP3 (2.3%) (ANOVA, $p < 0.01$).
395 Nitrospirae was significantly more abundant in WWTP3 (11.9%) than in WWTP1
396 (0.6%) and WWTP2 (0.6%) (ANOVA, $p < 0.001$). Nitrospirae includes nitrifying
397 taxa, nitrite-oxidizing bacteria (NOB) and comammox bacteria (Koch et al., 2019).
398 Nitrospira, the largest class, have been found to be the dominant nitrifiers in
399 wastewater treatment plants (Forrez et al., 2011). Nitrospira, an aerobic
400 chemolithoautotrophic nitrite-oxidizing bacterium, plays important roles in
401 nitrification and oxidizes nitrite to nitrate (Daims et al., 2015; Daims and Wagner,
402 2018). It has been identified as keystone taxon, an ideal partner for establishing
403 microbial relationships under various conditions, in aquatic environment (Veloso et
404 al., 2023).

405 The linear discriminant effect size (LEfSe) analysis revealed microbial taxa
406 specifically more abundant according to the origin of the WWTP. Such taxa found
407 significantly more abundant to a specific condition by LEfSe analysis has been
408 proposed to serve as biomarkers (Segata et al., 2011). The LEfSe comparing the
409 microbial communities before treatment (BT) revealed biomarkers for each WWTP
410 (Fig. 4). For WWTP1, Actinobacteria, Elusimicrobia, Epsilonbacteraeota,
411 Latescibacteria, Omnitrophicaeota and Spirochaetes phyla, and Parcubacteria and
412 Campylobacteria class were identified as biomarkers (Fig. 4). Actinobacteria, known
413 for carrying antibiotic resistant genes (ARGs) (Tang et al., 2020b), have been reported
414 dominant in activated sludge processed municipal wastewaters (Kang et al., 2020; Xu
415 et al., 2017). Bacteria affiliated to Parcubacteria have been reported to be involved in

416 sulphamethoxazole antibiotic degradation (Song et al., 2019). *Campylobacter* species
417 are known as bacterial indicators for faecal contamination by animals (Ogden et al.,
418 2009).

419 For WWTP2, the LEfSe analysis revealed Proteobacteria, Cyanobacteria phyla
420 and Oxyphotobacteria class as biomarkers (Fig. 4). Cyanobacteria are considered to
421 be well adapted to high organic and low oxygen contents (Saqrane et al., 2020).
422 WWTP2 process is based on lagoon system and reed beds, providing probably
423 appropriate growth condition for Cyanobacteria. Cyanobacteria are known to release
424 cyanotoxins (Saqrane et al., 2020). Nevertheless, after disinfection treatments, the
425 relative abundance of Cyanobacteria in WWTP2 decreased to the same level of that
426 found in WWTP1 and WWTP3 (Fig. S3).

427 For WWTP3, the biofiltration based process, the LEfSe analysis revealed
428 Armatimonadetes, Verrucomicrobia, Acidobacteria, Nitrospirae and
429 Gemmatimonadetes phyla, and the Acidobacteria, Nitrospira and Planctomycetacia
430 classes as biomarkers (Fig. 4). Such observation was consistent with previous studies
431 showing that these taxa were found dominant in three different types of biofilters
432 (Gulay et al., 2019; Lautenschlager et al., 2014; Li et al., 2020). Nitrospirae and
433 Verrucomicrobia have been demonstrated to be involved in the transformation of
434 organic nitrogen (Tang et al., 2021).

435 After the disinfection treatments, most of the detected biomarkers differed from
436 those of the non-treated WWTPs (Fig. S3). However, the Actinobacteria,
437 Alphaproteobacteria and Acidobacteria classes were still identified as biomarkers for
438 WWTP1, WWTP2 and WWTP3 respectively, irrespective of the disinfection
439 treatment (Fig. 4, Fig. S3). Such result further confirmed that the origin of
440 wastewaters is an important factor shaping the microbial community (habitat filtering).
441 Thus, it is likely that the WWTP specific biomarkers are reflecting not only the
442 differences on wastewater composition, but also the processing methods involved in
443 the wastewater treatment systems (activated sludge, lagoon and reed beds, and
444 biofilter, for WWTP1, WWTP2 and WWTP3 respectively).

445 3.3 Identification of biomarkers sensitive and resistant to the disinfection 446 treatments

447 The LEfSe analysis comparing the bacterial communities before (BT) and after
448 each treatment revealed biomarkers, taxa significantly more abundant in a specific
449 condition. Such biomarkers reflect thus the disinfection efficiency of each treatment
450 towards microbial taxa (Fig. 5). Two groups of biomarkers were detected: i)
451 treatment-sensitive biomarkers corresponding to taxa significantly found more
452 abundant in BT (i.e. depleted in the wastewater treated by a disinfectant), and ii)
453 treatment-resistant biomarkers corresponding to taxa significantly found more
454 abundant in treated water.

455 The numbers of treatment-sensitive biomarkers decreased as follow PFA_H,
456 PFA_L, UV, and chlorine. For PFA_H, 39 treatment-sensitive biomarkers were
457 detected including ASVs related to Flavobacteriaceae, Chromobacteriaceae,
458 *Chromobacterium* and *Escherichia_Shigella* (Fig. 5), members of which have been
459 reported as dominant taxa in sewage and WWTP (Ahmed et al., 2017; Leroy-Freitas et
460 al., 2022; Yousra et al., 2017). For the PFA_L, 11 treatment-sensitive biomarkers
461 including the previous discussed taxa Chromobacteriaceae, *Chromobacterium* and
462 *Escherichia_Shigella* were revealed. For chlorine and UV, only 2 and 4
463 treatment-sensitive biomarkers were detected, respectively. Additionally, 11, 3, 3 and
464 1 treatment-resistant biomarkers were detected for the UV, chlorine, PFA_H and
465 PFA_L, respectively (Fig. 5). Paracaedibacteraceae, identified as treatment-resistant
466 biomarker for both Chl and UV, has been reported to be significantly enriched after
467 hydrogen peroxide treatment (Piel et al., 2021). *Acetoanaerobium* and *Acetobacterium*
468 were identified as treatment-resistant biomarker of Chl and UV, respectively. *Ottowia*,
469 identified as UV resistant biomarker, was isolated from different types of wastewater
470 treated by activated sludge (Spring et al., 2004). Obscuribacterales, identified as
471 treatment-resistant biomarkers for both PFA_L and PFA_H, have been found in
472 activated sludge of aerobic reactor, aquifer, soil and animal (McGorum et al., 2015;
473 Salmaso et al., 2018; Soo et al., 2014). The genome analyses of bacteria belonging to

474 Obscuribacterales revealed their ability to perform both aerobic and anaerobic
475 respiration, store polyphosphate, utilize nitrate, as well as their potential to use
476 carbohydrates and/or fatty acids, but their harmful potential has not been reported so
477 far (Dueholm et al., 2022; Salmaso et al., 2018).

478 Such results, showing more sensitive biomarkers and less resistant biomarkers for
479 PFA than for the other treatments, demonstrated a more efficient disinfection of
480 wastewater by the PFA treatment than the treatments involving UV and chlorine
481 disinfectants.

482 In order to better understand the differences in effectiveness of disinfectants, we
483 further performed LEfSe analysis comparing all pairs of two treatments (Fig. 6).
484 Twelve taxa were sensitive to PFA_L and resistant to chlorine, including
485 microorganisms affiliated to i) Aeromonadales, Aeromonadaceae and *Aeromonas* that
486 have been reported as typical taxa in WWTP (Gao et al., 2023; VandeWalle et al.,
487 2012), and ii) Enterobacteriales and Enterobacteriaceae (Fig. 6, Fig. S4a), members of
488 which represent faecal indicators (Kausar et al., 2019). Also, 10 taxa were sensitive to
489 PFA_L and resistant to UV corresponding, in addition to the above-mentioned taxa, to
490 *Chromobacterium* and Fusobacteriaceae (Fig. 6, Fig. S4b), also known as faecal
491 indicators (Hutinel et al., 2022). Thus, PFA exhibits superior disinfection efficacy over
492 chlorine and UV because taxa related to faecal bacteria have shown increased
493 sensitivity to PFA.

494 Additionally, biomarkers sensitive to the PFA_H treatment were found to be
495 resistant to either Chl (18 biomarkers) or UV (26 biomarkers) treatments (Fig. S4e,f).
496 The 18 PFA_H treatment-sensitive biomarkers resistant to Chl included taxa affiliated
497 to faecal indicators such as Chromobacteriaceae, *Chromobacterium*,
498 Flavobacteriaceae and *Flavobacterium* (Fig. S4e). Similarly, the 26 PFA_H
499 treatment-sensitive biomarkers resistant to UV included faecal indicators such as
500 Aeromonadales and Aeromonadaceae (Fig. S4f). These observations further
501 confirmed the higher disinfection efficiency of PFA. Moreover, PFA was also found
502 to be more efficient when increasing the PFA dose since 11 biomarkers sensitive to
503 PFA_H were found resistant to PFA_L (Fig. 6, Fig. S4c).

504 Previous studies have noted that the reaction pathways of *E. coli* inactivation by
505 PFA included both direct and indirect free radical reactions, in which the PFA, \bullet OH
506 and peroxide radicals accounted for 73 %, 20 % and 6 %, respectively (Ding et al.,
507 2023). Thus, PFA exhibit higher efficiency and effectiveness than the free radicals. It
508 has been shown that PFA is predominantly responsible of cell inactivation by
509 penetrating the microbial cell walls and membranes to damage functional proteins. In
510 contrast, the \bullet OH and peroxide radicals effectively oxidize the cell interior
511 (Koivunen and Heinonen-Tanski, 2005; Luukkonen and Pehkonen, 2017). PFA has
512 been shown to exhibit high reactivity with cysteine and methionine residues through
513 an oxygen transfer pathway (Du et al., 2018; Wang et al., 2023). It has been shown
514 that the number of broken and cross-linked bacterial cells increases after PFA
515 disinfection causing cell damage like wrinkles, chapped and rough surface on *E. coli*,
516 *S. aureus*, and *B. subtilis* (Ding et al., 2023). The intracellular oxidative stress has
517 been shown to be higher after PFA treatment (Wang et al., 2023). Thus, the PFA
518 destroys the cell walls and membranes of microorganisms, resulting in the release of
519 the cellular content.

520 The regrowth and recovery of pathogenic bacteria and antibiotic resistant bacteria
521 (ARBs) carrying antibiotic resistance genes (ARGs) can potentially lead to
522 subsequent contamination of the surface and ground water reservoirs (Shekhawat et
523 al., 2020). Previous studies showed that bacteria were not able to regrow 24 hours
524 after PFA disinfection of wastewater without irradiation (Ragazzo et al., 2013).
525 Similarly, *E. coli* and *enterococci* cells showed irreversible inactivation (Rocher et al.,
526 2021). Additionally, PFA has been shown to be beneficial in controlling antibiotic
527 resistance using antibiotic resistant *E. coli* as typical indicator (Campo et al., 2020).

528 3.4 Functional predictions

529 In order to estimate the functional capabilities of the microbial communities,
530 functional profiles were determined by PICRUST2. The nearest sequenced taxon
531 indices (NSTI) indicated that PICRUST2 prediction was accurate (mean NSTI = 0.11
532 \pm 0.05 SD). Although predictive, with the limitation that no direct evidence of a

533 function is provided, such approach provides an overview of the microbial processes
534 (Menendez-Serra et al., 2019; Bourhane et al., 2022, 2023). The comparison of the
535 PICRUSt2 predicted functions of bacterial communities showed that the wastewater
536 samples from the same WWTP clustered together at both low and high KEGG
537 hierarchy levels (Fig. 7a, e). Most of the KEGG orthologs at level 2 had
538 enrichment/depletion variations among the three WWTPs. They were more abundant
539 in WWTP2 and WWTP3 than in WWTP1 (Fig. 7e).

540 PCA analysis considering each WWTP revealed that the bacterial communities
541 clustered according to the disinfectant used for the treatment (Fig. 7b, c, d), showing
542 the functional shifts within the bacterial communities caused by the different
543 disinfectants. The heatmap of the abundant functional patterns demonstrated that the
544 wastewater owns a specific functional pattern according to the origin (Fig. 7e).
545 Noteworthy, the functional pattern was also modified by the treatment depending on
546 the disinfectant used. Consistently with the microbial community differences
547 observed (Fig. 2), the physical-chemical characteristics prevailing at the WWTP are
548 the main factors shaping the microbial community structure, composition and
549 functions. Such observation was in agreement with previous studies reporting that
550 microbial changes are linked with effluent quality and system performance (Cao et al.,
551 2020; ElNaker et al., 2019).

552 The relative abundance of genes involved in cell cycle pathway of *Caulobacter*
553 [PATH: ko04112] significantly decreased in PFA_H and Chl compared with BT
554 (ANOVA, $p < 0.001$; Fig. S5). It demonstrates the effects of PFA and chlorine on
555 *Caulobacter* life cycle, which was consistent with a previous study showing that cell
556 damages (e.g. discharge of cellular materials) were observed after chlorination in
557 *Caulobacter* (Kim et al., 1982). Similarly, the relative abundance of genes associated
558 to cell motility pathway significantly decreased in PFA_H compared with BT, being
559 at lower abundance in PFA_H and PFA_L than in UV and Chl (ANOVA, $p < 0.001$;
560 Fig. S6). This result demonstrated the effect of PFA on decreasing the motility and
561 activity of microorganisms, which was consistent with the reduction of the microbial
562 diversity and the reduction of *E. coli* and other faecal microorganisms counts

563 observed in PFA treatment (Fig. 1, Fig. 5, Fig. 6). Such observation further confirmed
564 the effect of PFA on microbial community, which likely relies on the disinfection
565 efficiency. It has been shown that the application of 4 mg/L PFA for 16 h has not
566 toxicological effect using the *V. fischeri* assay (Chhetri et al., 2014), suggesting that
567 PFA has a short decomposition time. PFA was also reported causing less disinfection
568 by-products than other commonly used peracids (Luukkonen and Pehkonen, 2017). In
569 this study, the disinfection was performed with 1 and 4 mg/L PFA for 15 min
570 followed by a quenching step to stop the reaction. Although the residual toxicity test
571 was not conducted in this study, PFA achieved high disinfection efficacy while
572 producing very low residual toxicity according to previous reports (Rocher et al.,
573 2021).

574 Regarding the metabolism associated pathways, the relative abundance of genes
575 involved in the biosynthesis of secondary metabolites and metabolism of amino acids
576 was significantly different (ANOVA, $p < 0.05$) according to the disinfection treatment
577 among all three WWTPs (Fig. 7e, S7-9). Previous studies have also reported that
578 enzymes from the same species may have different activities depending on the
579 treatment and conditions (Delforno et al., 2017; Emmanuel et al., 2019; Yang et al.,
580 2020).

581 The genes involved in penicillin and cephalosporin biosynthesis pathway [PATH:
582 ko00311] were significantly depleted on UV and Chl in comparison with PFA_H and
583 PFA_L treatments (ANOVA, $p < 0.05$; Fig. S7). Similarly, the abundance of genes
584 involved in betalain biosynthesis pathway [PATH: ko00965] was significantly more
585 abundant in PFA_H than UV (ANOVA, $p < 0.05$; Fig. S8). Betalain has been reported
586 for its antimicrobial activity on a broad spectrum of microbes such as
587 multidrug-resistant bacteria, single-species and dual-species biofilm-producing
588 bacteria, dengue virus and fungi (Wijesinghe and Choo, 2022). Betalains primarily
589 occur in Caryophyllales order plants. The enzymes involved in betalain biosynthesis
590 have been reported in fungi, *Gluconacetobacter diazotrophicus* and Cyanobacteria
591 (Contreras-Llano et al., 2019; Guerrero-Rubio et al., 2020). However, LEfSe did not
592 identified Cyanobacteria as PFA-resistant biomarkers (Fig. S4), suggesting that

593 bacteria producing antimicrobial compounds (betalains), likely present in treated
594 wastewater, were more sensitive to UV and chlorine than to PFA. Since UV and
595 chlorination have been reported for their strong elimination efficiency of antibiotic by
596 photolysis (Ding et al., 2020; Serna-Galvis et al., 2017), such a slight deficiency does
597 not indicate that PFA is incompetent. It nevertheless alerts the risk of antibiotics
598 release from wastewater into water system that potentially alters microbial ecosystems,
599 that in turn represents a sanitary issue for human health (Baquero et al., 2008). The
600 genes involved in glutathione metabolism pathway [PATH: ko00480] were
601 significantly more abundant in PFA_H (ANOVA, $p < 0.05$; Fig. S9). The glutathione
602 metabolism has been suggested to play important role in bacterial defence against
603 chlorine-based disinfection (Du et al., 2015), demonstrating that PFA has likely
604 similar effect on bacteria.

605 These results suggested that PFA_H, even efficient on disinfecting most of the
606 microorganisms (including pathogens), was not effective on inactivating the
607 microorganisms carrying penicillin and cephalosporin, and betalain biosynthesis
608 pathways. However, further analyses determining direct functions, such as shotgun
609 metagenomics, are needed in order to describe in depth the effect of disinfection on
610 the functional capabilities of microbial communities.

611 4. Conclusions

612 Although the operational cost of PFA treatment has been estimated to be
613 approximately 2.5 times more expensive than that of chlorine (Luukkonen et al.,
614 2015), the PFA treatment owns several assets to be used as treatment for safe reuse of
615 wastewater as it does not produce by-product. Also it has been shown that its cost can
616 be drastically reduced for treatment plants with larger capacity (Ragazzo et al., 2013).
617 But, even if further analyses are still required to comprehensively evaluate the costs, it
618 is also necessary to evaluate the disinfection efficiency of PFA treatment in
619 comparison to other commonly used treatments.

620 Our study elucidated the effect of PFA, UV and chlorine on microorganism
621 disinfection, microbial community diversity, structure and functions crossing three

622 different WWTPs. It provides compelling evidence that different disinfectants
623 resulted in specific microbial communities and functions, which effects differed
624 according to the physical-chemical characteristic of the wastewater origin (WWTP).
625 Our findings indicated that PFA was efficient in *E. coli* disinfection of wastewater,
626 being able to decrease the microbial community diversity. Additionally, specific
627 biomarkers of each WWTP were revealed, which likely relies on the processing
628 treatments methods. Also, our study detected sensitive/resistant biomarkers for each
629 disinfectant, PFA exhibiting higher number of sensitive biomarkers than chlorine and
630 UV, PHA_H having the highest number. Our findings underline the fact that PFA is
631 efficient on wastewater disinfection in comparison with the traditional treatments
632 involving UV and chlorine disinfectants. Because PFA was particularly effective in
633 removing known faecal microorganisms, it has high potential for safe reuse of
634 wastewater.

635

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969

970

971 **Figure captions**

972 Fig. 1. Efficiency of the disinfection treatments on the different WWTPs. The
973 efficiency of the disinfection treatments was estimated by *E. coli* counts. BT, before
974 treatment; The treatments are UV, chlorine, performic acid at low (PFA_L) and high
975 (PFA_H) concentrations.

976

977 Fig. 2. Comparison of microbial communities by Principal Coordinates Analysis
978 (PCoA) based on weighted UniFrac distances including the three WWTPs (a); PCoA
979 based on Bray-curtis distances for WWTP1 (b), WWTP2 (c), and WWTP3 (d). The
980 arrows indicate the significant impact of the parameter (*E. coli* counts) on the
981 microbial community composition (* $p < 0.05$, ** $p < 0.01$, Permutations = 999).
982 Circles: WWTP1; diamond: WWTP2; triangles: WWTP3. Red: chlorine; blue:
983 performic acid at low concentration (PFA_L); orange: performic acid at high
984 concentration (PFA_H); green: before treatment (BT); purple: UV.

985

986 Fig. 3. Composition of microbial communities at the phyla level in each treatment and
987 WWTP. BT, before treatment; The treatments are UV, chlorine, performic acid at low
988 (PFA_L) and high (PFA_H) concentrations.

989

990 Fig. 4. Bacterial biomarkers (bacterial taxa) for the different WWTPs before treatment
991 (BT). The Cladogram shows the bacterial taxa identified by linear discriminant
992 analysis effect size (LEfSe). The biomarkers are colored according to their WWTPs'
993 specificity indicating that they are significantly more abundant in a WWTP than the
994 others. The wastewater samples without any treatment were used in the analysis. D1:
995 Phylum; D2: Class.

996

997 Fig. 5. Linear discriminant analysis effect size (LEfSe) identifying treatment-sensitive
998 (external) and treatment-resistant (inner) biomarkers according to treatment PFA_H,
999 PFA_L, Chlorine and UV. The comparisons were performed between the wastewater

1000 before (BT) and after each treatment from the three WWTPs. The length of the bars
1001 corresponds to the LDA score. The known pathogens are represented in red. D1:
1002 Phylum; D2: Class; D3: Order; D4: Family; D5: Genus; D6: Species.

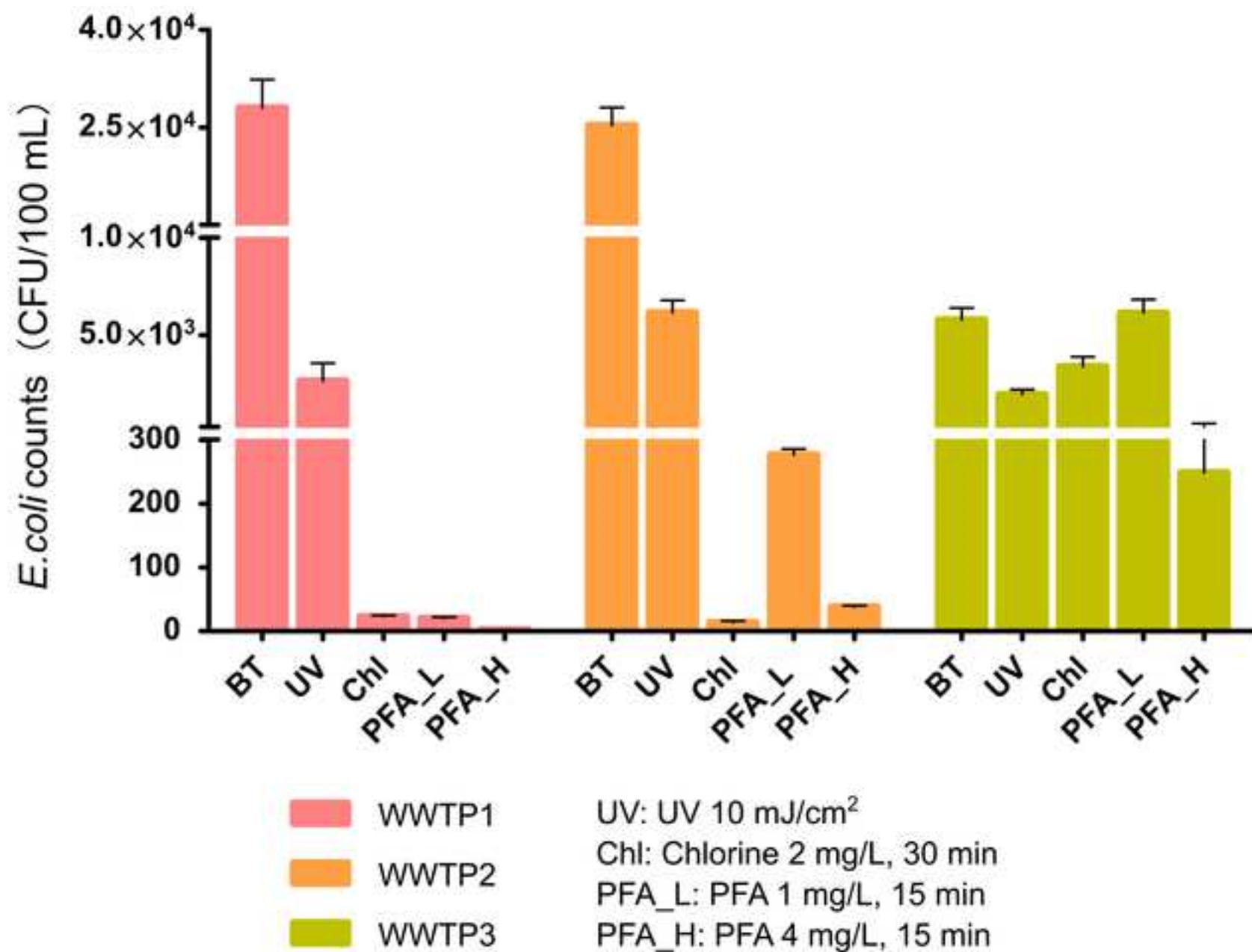
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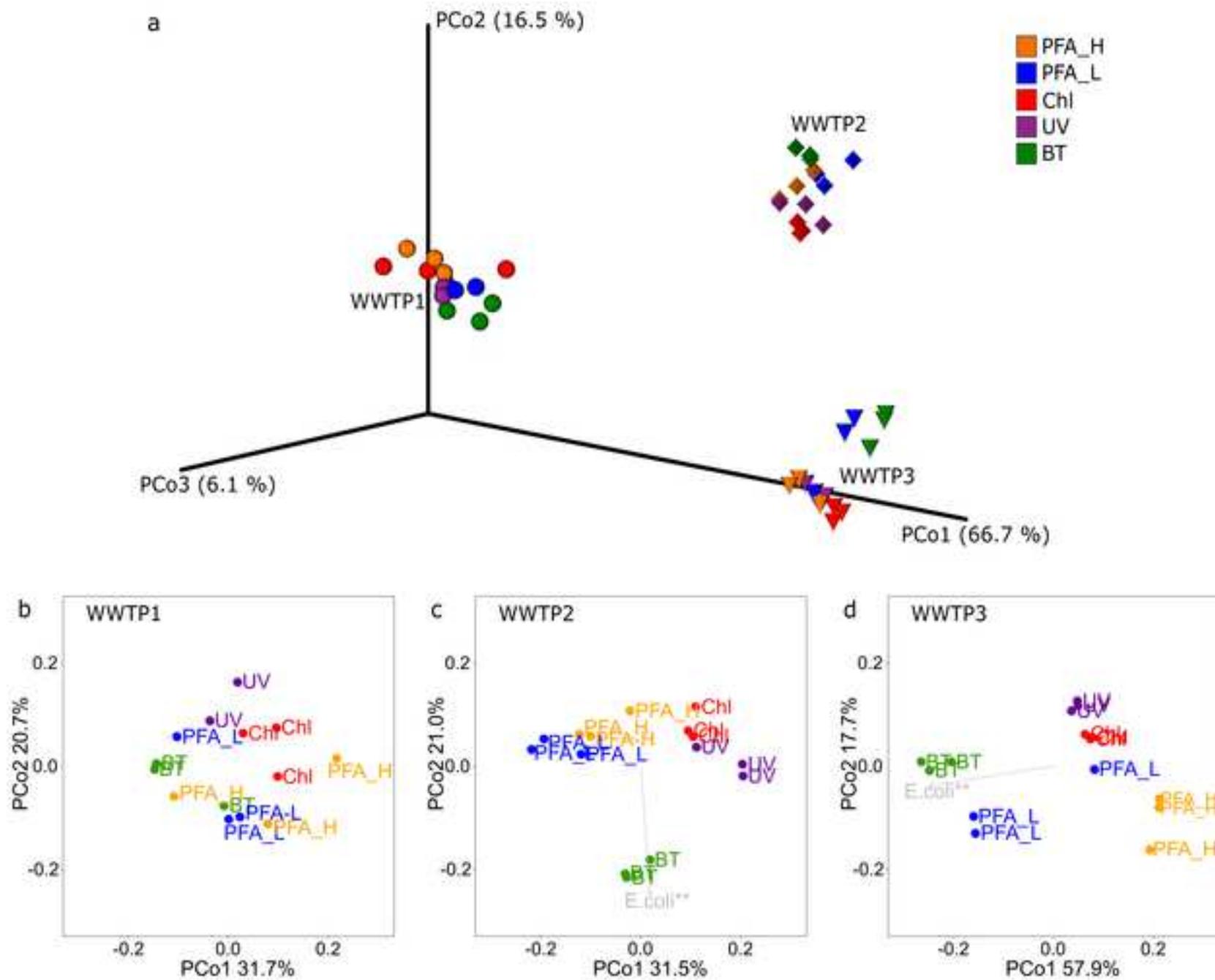
1004 Fig. 6. Disinfection efficiency on pathogen microorganisms according to the different
1005 treatments. The comparisons were performed between each pair of the treatments
1006 from all three WWTPs by linear discriminant analysis effect size (LEfSe) identifying
1007 treatment-sensitive biomarkers. The colored dots correspond to microbial genera that
1008 sensitive to PFA_H (red), PFA_L (orange), Chlorine (purple) and UV (blue)
1009 treatment respectively.

1010

1011 Fig. 7. Functional comparison of bacterial communities predicted by PICRUSt2. The
1012 PCAs show the predicted functions, based on KEGG categories (level 3), of the three
1013 WWTPs (a), and for the different disinfectants treatments in WWTP1 (b), WWTP2
1014 (c), WWTP3 (d). (e) Heatmap of the standardized abundance of predicted functions
1015 based on KEGG categories (level 2). BT, before treatment; the treatments are UV,
1016 chlorine, performic acid at low (PFA_L) and high (PFA_H) concentrations.

1017





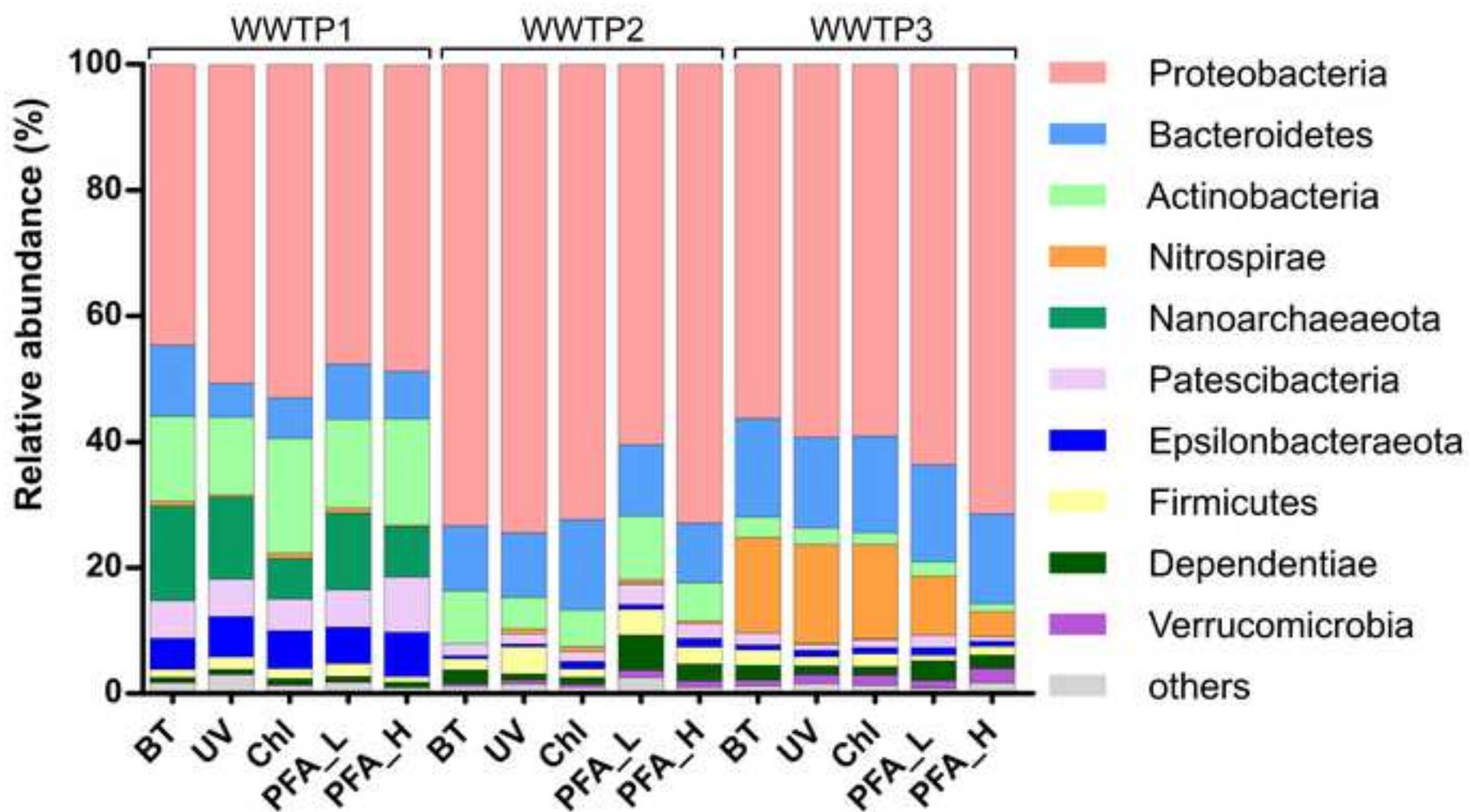


Figure 4

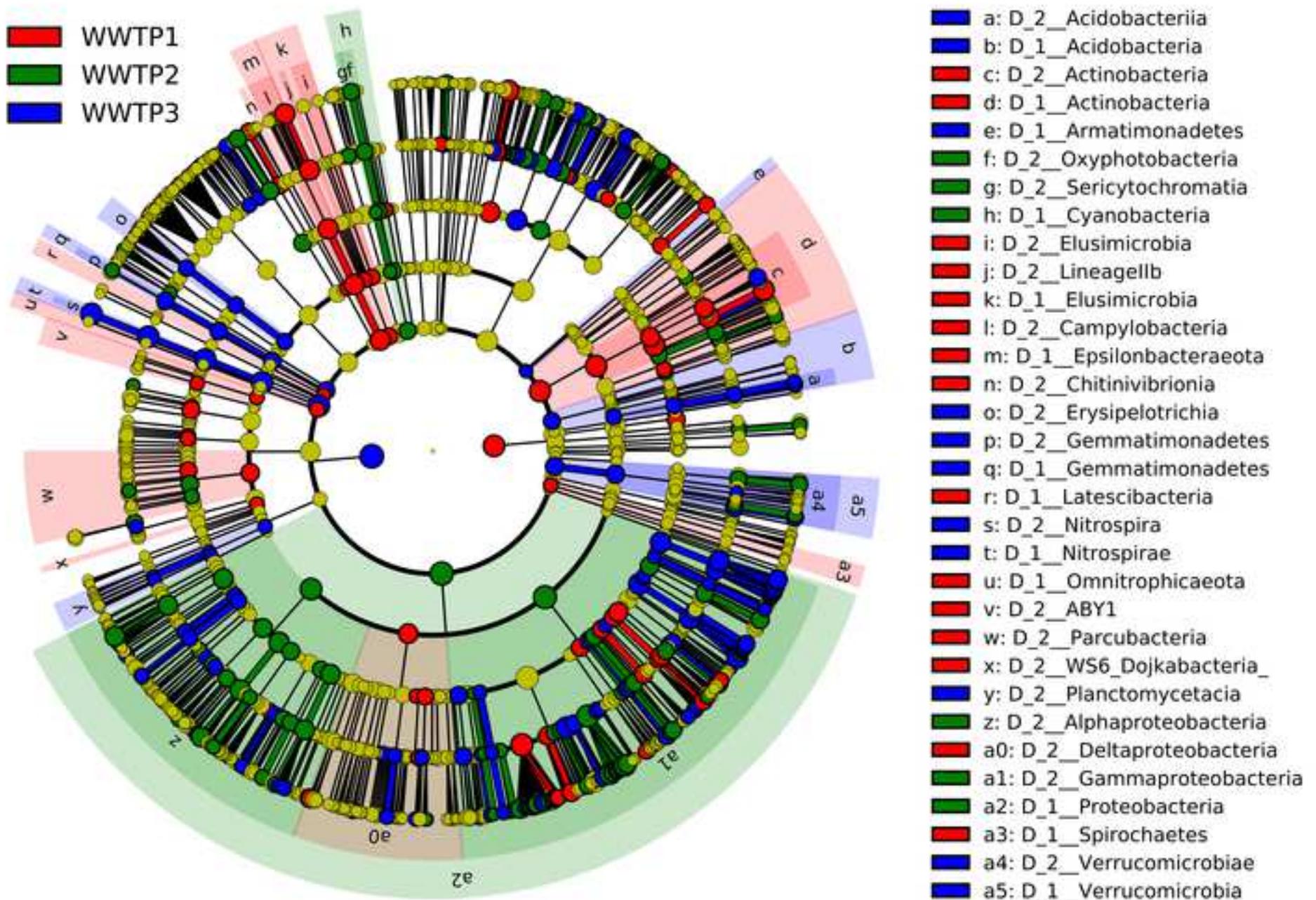
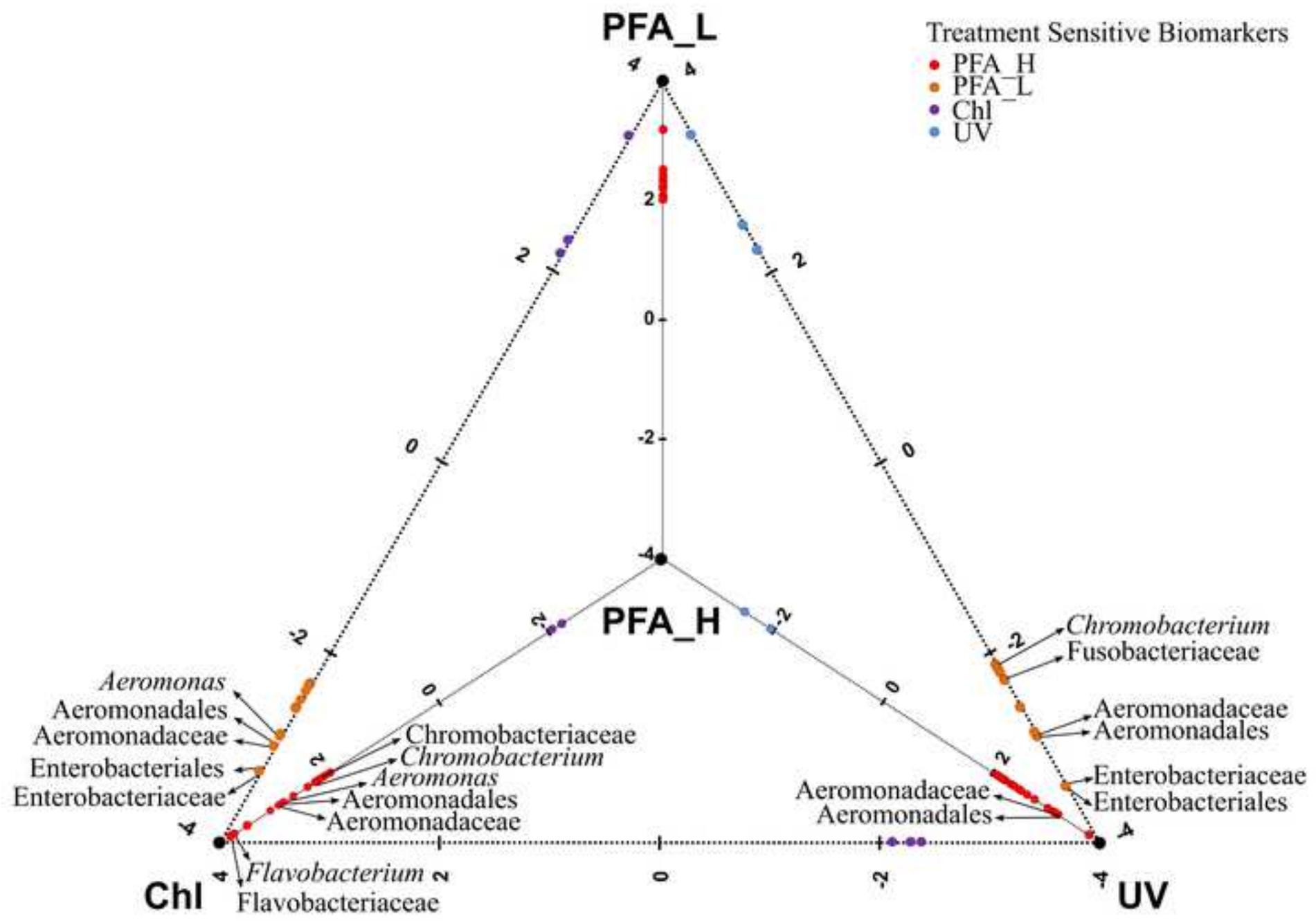
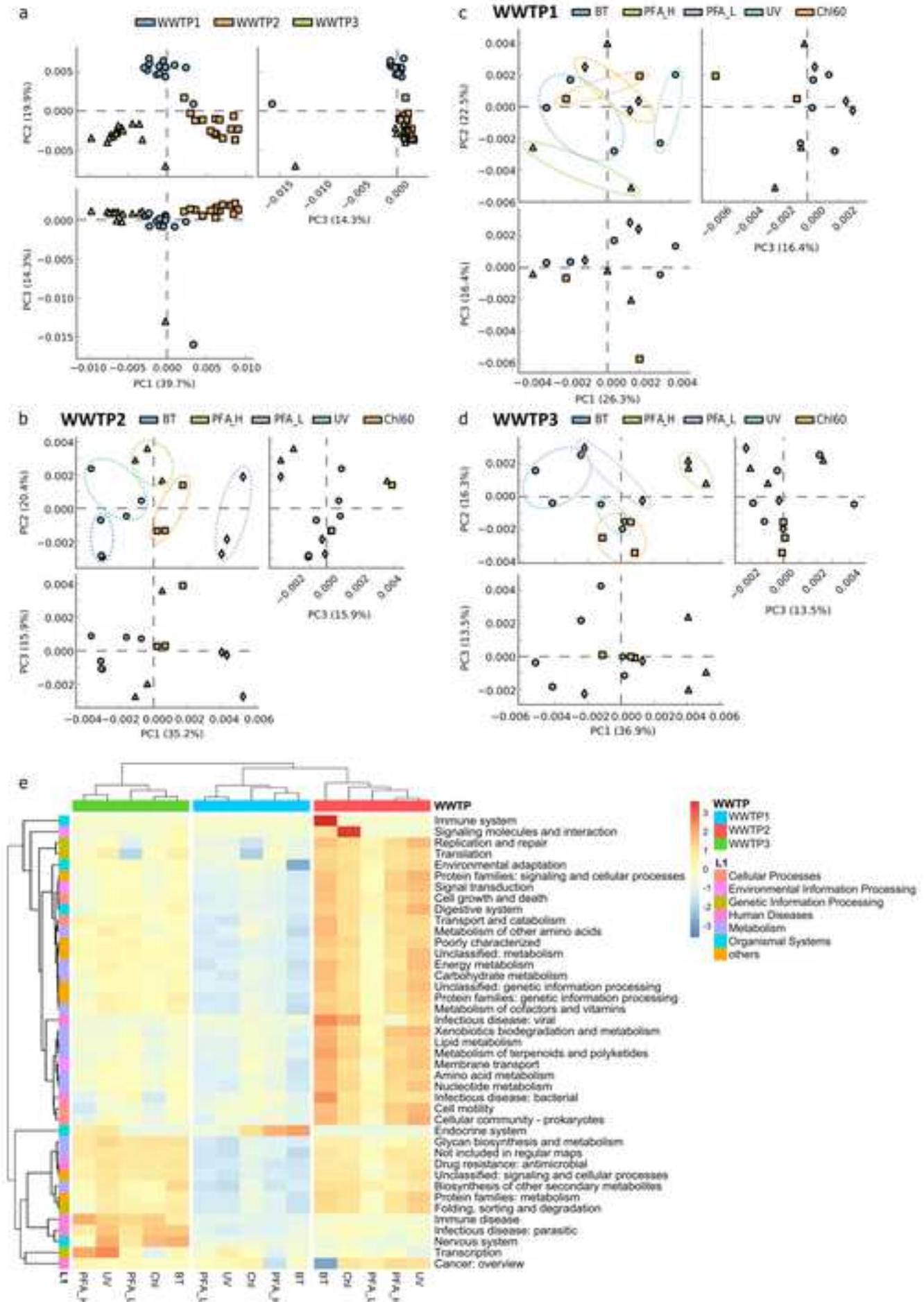
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Figure 6



















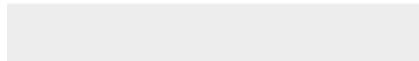
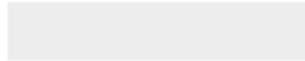






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Authors Contributions: **BW:** Conceptualisation, Investigation, Resources, Formal analysis, Writing – original draft. **RD:** Conceptualisation, Investigation, Resources, Formal analysis, Writing – original draft, Supervision, Project administration, Funding acquisition. **TG:** Conceptualisation, Writing – original draft, Supervision, Project administration, Funding acquisition. **CCL:** Conceptualisation, Investigation, Resources, Formal analysis, Writing – original draft, Supervision, Project administration, Funding acquisition.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: