Graphical Abstract

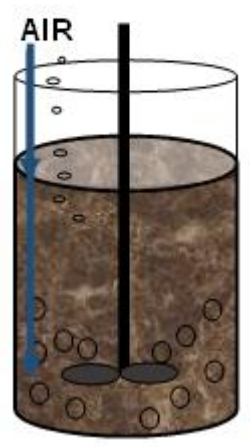
Evaluation of industrial wastewater bio-treatment
Microbiological adaptation

Chemical parameters

DOC, TN
TSS and VSS
Ions

Toxicity

Chronic toxicity



Sludge + CBW

Microbiological techniques

Classical analysis

Plate count

Optical microscopy

DNA extraction

New approaches

qPCR

Metagenomics

*Manuscript
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1 Practical approach to the evaluation of industrial wastewater treatment
2 by the application of advanced microbiological techniques

3

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11

12 **Abstract**

13 In cork industry, the operation of boiling raw cork generates large volumes of
14 wastewater named Cork Boiling Wastewater (CBW). The main characteristics are the
15 low biodegradability and medium to low acute toxicity, resulting in the necessity of
16 designing advanced biological treatments by possible conventional activated sludge
17 adaptation. In order to evaluate the variation of bacterial population along that process,
18 a study based on optical microscopy, plate count, DNA extraction, qPCR and massive
19 sequencing techniques was performed. Results showed a diminution of the total and
20 volatile solids (TSS and VSS), jointly with a decrease in DNA concentration, general
21 bacteria (16S) and ammonia-oxidizing bacteria (AOB). After a few hours of testing,

diverse microbiological species died while others showed a possible adaptation of the biological system, accompained by a dissolved organic carbon (DOC) reduction. In addition, toxicity tests based on activated sludge showed the development of chronic toxicity through the contact time. Combination of classical and advanced microbiological techniques, such as quantitative real time Polymerase Chain Reaction (qPCR) and metagenomics, was essential to predict the variation of species during the experiment and to conclude if effective biological adaptation could be finally attained for the target complex wastewater.

Keywords: Biological treatment, Cork boiling wastewater, metagenomic, qPCR, chronic toxicity

1. Introduction

Cork is the outer bark of *Quercus suber*, a common tree species in the Mediterranean region. Due to its properties, cork has a large variety of applications, such as the production of stoppers for wine and other alcoholic drinks bottles. Cork industrial processing involves a boiling step focused on the cleaning, disinfection and moistening of the raw material. This step generates about 400 L of wastewater per ton of cork[1]. The produced effluent, named cork boiling wastewater (CBW), presents low biodegradability and medium acute toxicity values (inhibition percentages between 30-60%)[2]. CBW is normally being reused between 20 to 30 times in the own industry, becoming highly concentrated in corkwood organic extracts including relevant contaminants (phenolic acids, tannins, 2,4,6-trichloroanisol, pentachlorophenol, etc.)[3] and so, it results recalcitrant enough to make conventional biological systems unable to

46 treat it. In consequence, the need to search for solutions based on advanced processes to
47 tackle the treatment of this type of wastewater has arisen in the last years[4].

48 Nowadays, biological treatment based on activated sludge is one of the most common
49 and preferred technology employed for wastewater treatment mainly due to its low
50 operating costs compared to physic-chemical oxidation processes, and the high
51 versatility and ability to get adapted not only to different kinds of wastewater (more or
52 less complex) but also to changes in the plant operating parameters. Therefore, big
53 efforts should be done on the design and definition of new decontamination techniques
54 based on advanced biological treatments when possible.

55 From a general point of view, the possible adaptation of a conventional biological
56 system for attaining the complete depuration of CBW presents several problems related
57 to their low biodegradability and somehow significant acute toxicity coming from the
58 specific contaminants contained in this wastewater, such as polyphenolic compounds[5].
59 The importance and role of the micro-fauna community in the purification process of
60 activated sludge biological reactors has been well documented in literature[6-11]. The
61 microbial structure of biomass depends on the type of technological system used but, it
62 is possible to favor different species in the bio-structure of activated sludge that
63 supports stability and high efficiency process against specific wastewater with special
64 complex characteristics[12-13]. Therefore, control and/or influence on variations in the
65 microbial community structure appears as key parameter to find out an efficient
66 advanced biological treatment for facing complex industrial wastewaters.

67 Up to date, knowledge of microbial community compositions and their dynamics in the
68 wastewater treatment system usually comes from studies employing traditional culture
69 methods, community fingerprinting techniques, and molecular approaches. Optical

70 microscopy observation and culture-based studies help to identify the predominant
71 microbial populations present in WWTPs due to the susceptibility of bacteria growing
72 on microbiological media[14]. In addition, quantitative real time Polymerase Chain
73 Reaction (qPCR) has been demonstrated to be a useful tool for quantitative analysis of
74 specific microorganisms present in environmental samples. Nevertheless, the design of
75 advanced biological treatments able to tackle with complex industrial wastewater, such
76 as CBW, would require the identification of arisen microbial populations present in the
77 adapted biomass responsible of specific wastewater purification actions (including the
78 degradation of specific recalcitrant compounds). With this objective, emerging
79 molecular biology and new genetic techniques are starting to be applied nowadays.
80 Novel techniques based on genomic sequencing are being developed with the aim of
81 determining dominant population in complex microbial communities[15]. These new
82 methods will also be useful for studying the diversity of microbial life in organisms
83 inhabiting common environments, (referring to the totality of genomes found) such as
84 activated sludge and marine samples[16].

85 In this work, the use of microbiological techniques based on the combination of simple
86 optical microscopy observation, culture-based studies with quantitative PCR[17,18,19] and
87 microbial genomics (applying metagenomics accomplished at high taxonomic
88 resolution)[16], have been carried out in order to study the adaptation process followed by
89 microbial population from conventional WWTP activated sludge when the system is fed
90 with CBW. The main objective was to define and predict the effectivity of the
91 developed adapted biological system as well as clearly identify the limitations that it
92 should face to attain this complex wastewater decontamination.

93

94 **2. Materials and methods**

95 Real cork boiling wastewater used in this study was collected at a cork processing plant
96 located in San Vicente de Alcántara (Extremadura, Spain). Conventional activated
97 sludge was provided by the municipal WWTP of El Toyo (Almería, Spain). Main
98 characteristics of CBW and Mixed liquor received from the urban treatment plant are
99 presented in Table 1 (Results and Discussion section).

100

101 **2.1 Aerobic Biological experiments**

102 Biological assays simulating a Batch (Bio)Reactor were performed in a 5 L stirred flask
103 reactor at laboratory scale. The system consisted of a cylindrical PVC reactor tank (17.5
104 cm depth and 16.5 cm internal diameter) provided with a porous air diffuser placed at
105 the bottom of the reactor. The average temperature was 25 ºC, and aeration was directly
106 injected in order to keep dissolved oxygen concentration close to saturation values
107 (~8 mg L^{-1}), providing also a correct agitation of the whole system.

108 Initially, activated sludge (mixed liquor) taken from the WWTP was maintained in
109 aeration for 24 hours in order to attain endogenous phase. Then, and after adjusting
110 CBW pH to about 7, the required volume of wastewater to get an initial Dissolved
111 Organic Carbon (DOC) value of 200 mg L^{-1} in combination with the mixed liquor in
112 endogenous phase was added to the flask. With such initial DOC, influent operating
113 conditions employed in the real WWTP were replicated with the aim of avoiding shock
114 effects on the biological system provoked by higher organic loads and trying to simulate
115 the possible dilution of this complex wastewater when managed in a conventional
116 biological treatment.

117 The complete procedure for microbial population adaptation to CBW took 19 days (458
118 hours). During this period of time, two feeding of the system (operated as a Bath
119 (Bio)Reactor) with CBW were performed. Samples were taken throughout the
120 experiment at different contact times. Total Solids (TSS) and Volatile Suspended Solids
121 (VSS), optical microscopy, plate count, DNA extraction, and Polymerase Chain
122 Reaction (qPCR) were performed in each sample. Massive sequencing techniques were
123 also applied for a group of selected samples.

124

125 **2.2. Analytical determinations**

126 For analytical purposes, samples from the biological treatment were centrifuged and
127 filtered through 0.22 μm syringe-driven Millex nylon membrane filters (after 30
128 minutes of activated sludge settlement). Organic matter was measured as chemical
129 oxygen demand (COD) using Merck®Spectroquant kits, and as dissolved organic carbon
130 (DOC) in a Shimadzu TC-TOC-TN analyzer (model TOC-V-CSN). Total dissolved
131 nitrogen was measured in the same TC-TOC-TN analyzer coupled to a TNM-1 unit.
132 TSS and VSS were determined according to American Standard Methods[20]. Ions and
133 carboxylic acids were quantified by ion chromatography using Metrohm 872 Extension
134 Modules 1 and 2 configured for gradient analysis. Cations and amines were determined
135 using a Metrohm 850 Professional IC configured for isocratic analysis.

136

137 **2.3 Microbiological analyses**

138 Each taken sample was measured by triplicate in each one of the microbiological
139 analytical techniques reported in this work. Statistical analysis was done by one-way

140 ANOVA and results were highly reproducible ($p < 0.05$). The results showed in graphs
141 are the average of both replicates with standard deviation as error bars.

142

143 **2.3.1 Heterotrophic Plate count (HPC)**

144 The heterotrophic plate count (HPC) or standard plate count technique was employed to
145 detect heterotrophic bacteria in CBW samples according to standard methods[21]. Briefly,
146 plate counting method was done through 10-fold serial dilutions (d1 to d6) in
147 phosphate-buffered saline solution (PBS). 500 μL of each dilution was dropped onto
148 Triptone Glucose Yeast Agar (TGYE) nutrient medium. It was prepared using 5 g L^{-1} of
149 Tryptone (Oxoid, UK), 1 g L^{-1} Glucose (JT Baker, USA), 2.5 g L^{-1} yeast extract and
150 15 g L^{-1} bacteriological agar (Panreac, Spain). The pH of the medium was 7±2.
151 Colonies were counted after incubation for 7 days at 25 °C.

152

153 **2.3.2 Optical microscopy**

154 A Nikon Eclipse 50i Microscope coupled with a Nikon DS camera provided with a
155 super high pressure mercury lamp was used for microscopy observation of biological
156 samples. A drop of each sample was carefully deposited on a glass slide and covered
157 with a cover slip, and samples were observed in clear field mode.

158

159 **2.3.3 DNA extraction and Real Time Polymerase Chain Reaction (qPCR)**

160 DNA was extracted from 1 mL of each sample in duplicate using a Fast DNA™ SPIN
161 Kit for Soil (MP Biomedicals, Solon, OH 44139 USA) according to manufacturer´s kit

162 instructions and a FastPrep® FP24 Classic Instrument. Prior to extraction, 1 mL of each
163 sample was centrifuged for 2 min at 12000 r.p.m. After that, manufacturer´s
164 specifications were followed. DNA concentration in each sample was quantified using a
165 NanoDrop spectrophotometer (NanoDrop Lite, Thermo scientific).

166 DNA amplifications were performed with a 7500 Fast Real Time PCR System (Applied
167 Biosystems, USA) in 96-barcode well plates. Real-time qPCR was used for
168 quantification of general bacteria 16S rDNA and ammonia oxidizing bacteria (AOB)
169 16S rDNA. DNA amplification in both cases was performed as described by Harms et
170 al. 2003[22] and primers and probes sequences as well as components and qPCR programs
171 used are described in Supplementary Material. Primers and probes were purchased from
172 Sigma-Aldrich (USA). Ten-fold dilution of DNA extracts was necessary to prevent
173 PCR inhibition, due to the complexity of the water matrix. Negative control was
174 checked to validate DNA-samples amplification results.

175 The concentration of bacteria was estimated using an external qPCR calibration curve
176 previously reported[23]. With this standard calibration curve, qPCR Cycle threshold (Ct)
177 values were correlated with bacterial concentration in terms of colony forming units
178 (CFU mL^{-1}).

179

180 **2.3.4 Massive sequencing technique (Metagenomic)**

181 Microbial genomics have been also applied to analyze dominant populations in the
182 microbial community present in the samples taken from the biological adaptation
183 process to CBW. Metagenomics analyzes were performed on a MiSeq equipment of the
184 Illumina massive sequencing platform, based on the reversible terminators method of
185 the DNA polymerization reaction, using fluorescently labeled nucleotide analogs. When

186 preparing of the library, two pairs of primers designed against V3 and V4 hypervariable
187 regions of 16S rRNA gene were used. Subsequently a series of raw sequenced data was
188 generated. Finally, a basic 16S based-characterization of bacterial population was
189 carried out.

190 The procedure started with quality control of the DNA samples, where the DNA was
191 analyzed to ensure samples had sufficient integrity and quantity for optimal
192 amplification. For that, 1 μL of each gDNA sample was tested by agarose gel
193 electrophoresis (1%). Afterwards, the samples were quantified by fluorimetry in order
194 to verify the concentration of DNA.

195 Later, a library construction was performed using the Illumina 16S Metagenomic
196 Sequencing Library preparation protocol. The generated DNA fragments were
197 sequenced with MiSeq Reagent Kit v2 in the Illumina MiSeq platform, using 250bp
198 paired-end sequencing reads. Finally, the analysis of the generated raw sequence data
199 was carried out using the Illumina 16S Metagenomics workflow version 1.0.1. In order
200 to identify and classify the different taxonomic levels, the DNA sequences were
201 confronted with the GreenGenes database (released by the GreenGenes Database
202 Consortium). The algorithm used to classify each sequence was the RDP -Ribosome
203 Database Project-. The accuracy required for each sequence to be classified at a given
204 taxonomic level range goes from the 98.24% to assign a species to 100% for a sequence
205 to be classified by Kingdom, Phylum or Class level.

206

207 **2.3.5 Chronic toxicity evaluation**

208 Chronic toxicity tests, using activated sludge from the flask reactors in which the
209 biological treatment took place, were carried out in a BM-T respirometer (Surcis S.L.).

210 This equipment consists of a 1 L capacity vessel, equipped with a thermometer, pH
211 control system and an oxygen probe, (Protos 3400, Knick Elektronische Messgeräte
212 GmbH & v Co. KG) in order to measure the activated sludge activity based on the
213 oxygen uptake rate (OUR). Tests were performed comparing OUR obtained from the
214 slope of the measured dissolved oxygen when the aeration had stopped. With this aim,
215 OUR from conventional activated sludge obtained from the WWTP was used as a
216 control. Afterwards, OUR was measured throughout the experimental set up several
217 times during the treatment. Inhibition values were calculated following equation 1. A
218 decrease in OUR, shows chronic toxicity due to a reduction in the respiration rate of
219 activated sludge and also in the concentration of active microbial population.

220 $$\% \, Inhibition = \left[\frac{OUR_{ref} - OUR_{sample}}{OUR_{ref}}\right] * 100 \qquad \textit{Eq. 1}$$

221

222

223 **3. Results and discussion**

224 **3.1 Chemical evaluation of aerobic biological treatment at laboratory scale**

225 With the aim of following the changes in the mixed liquor when a conventional
226 activated sludge treatment is fed with CBW, evaluation of the chemical parameters has
227 been carried out at laboratory scale. The characterization of activated sludge from the
228 WWTP located at El Toyo (Almería, South East of Spain) and of target CBW is
229 presented in table 1. Consequently, DOC, COD, TN, TSS, VSS were analyzed in
230 samples taken twice a day during the aerobic biological treatment of CBW.

231 After reception, mixed liquor (containing activated sludge) taken from the secondary
232 treatment of the WWTP was left with continuous aeration until achieving endogenous
233 phase (24 hours). Then, a mixture of 188 mL of CBW per liter of mixed liquor
234 (corresponding to the addition of 200 mg L^{-1} of DOC) was placed in the 5 L aerobic
235 biological reactor. The system was operated for 19 days (458 hours) under continuous
236 aeration and agitation conditions (saturated dissolved oxygen was maintained). In
237 addition, after detecting an important decrease of DOC (75%), a second feeding with
238 CBW (200 mg L^{-1} of DOC) was performed after 9 days of treatment (218 hours).

239 Table 1: Cork boiling wastewater and mixed liquor characterization

Parameters (mg L^{-1})	Mixed Liquor	CBW
DOC	12.0	1059
COD	32.0	2968
TSS	7.1	-
VSS	5.6	-
Total nitrogen	0	9.5
Na^{+}	483.8	32.8
K^{+}	46.7	353.2
Cl^{-}	745.7	90.8
NO_3^{-}	0.7	2.7
NO_2^{-}	N.d.	N.d.
SO_4^{2-}	275.9	2.5
PO_4^{2-}	22.5	41.9
NH_4^{+}	N.d.	N.d.
Mg^{2+}	67.7	13.46
Ca^{2+}	102.6	67.9
Trimethylamine	N.d.	N.d.
CH_3COO^{-}	N.d.	N.d.
Short term biodegradability	-	0.1 (non-biodegradable)
Acute toxicity (inhibition)	-	48%

240 N.d. non detected

241

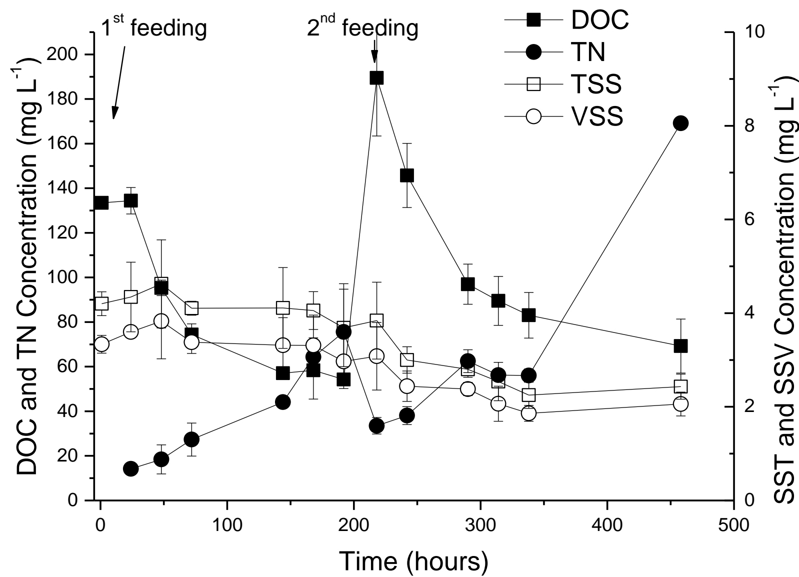
242 In figure 1a, the evolution of DOC, TN, TSS and VSS during the aerobic biological
243 treatment of CBW is shown. As it can be observed, TSS and VSS decreased along the
244 contact time, ending at 2.4 and 2.1 mg L^{-1} for TSS and VSS respectively, which is
245 equivalent to approximately 60% removal in both cases. This effect is just the contrary

246 of what should be expected to occur with both parameters when a biological treatment
247 operating as batch bioreactor works properly, that is, an increase of TSS and VSS.

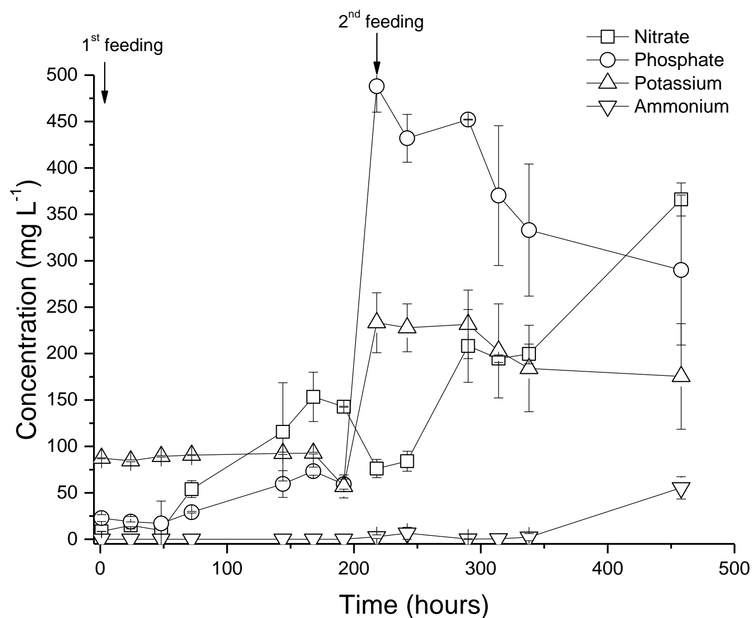
248 DOC also decreased remaining in values between 54 and 70 mg L^{-1} after quite long
249 treatment times compared to normal operation. In contrast, TN increased along the
250 experiment, attaining 169 mg L^{-1} at the end of the experiment, which is also a clear
251 symptom of malfunction of the adapted biological system, considering nitrogen is the
252 fourth most abundant element in cellular biomass.

253 In figure 1b, ions concentration evolution in supernatant of samples is shown. It is
254 important to highlight that phosphate, nitrate, potassium and ammonium increased more
255 than a 200% in all cases, showing concentrations of 290, 366, 175 and 55 mg L^{-1}
256 respectively at the end of the contact time. In the case of phosphate and potassium, both
257 are present at high concentrations in the intracellular fluid of cells, so the detection of an
258 increase in the mixed liquor clearly means that a significant number of bacteria were
259 dying due to the breakdown of the cytoplasmatic membrane. On the contrary, in the
260 case of nitrate, the increase in its concentration was the consequence of the successful
261 nitrification process, though the next step, which would be the elimination of nitrate via
262 denitrification process, did not occur since as anoxic cycles were not programmed.
263 Nevertheless, nitrification process also started to fail after the second feeding with CBW
264 as an accumulation of ammonium was detected. This can be explained as the
265 consequence of a successful oxidation of some organic molecules containing nitrogen
266 (accompanied by the partial elimination of DOC) provoking an increase on ammonium
267 concentration, which was not able to be metabolized to nitrate due to a deficient
268 nitrification process.

269



270 a)



271 b)

272 Figure 1. a) DOC, TN, TSS and VSS evolution along the lab-scale experimental test. b)
273 Ions concentration evolution along the lab-scale experiment.

274

275 The reduction of TSS and VSS, together with a significant release of specific ions on
276 the mixed liquor, evidenced the bacterial flocs and membranes break and the
277 destabilization of the biological system in general. As a consequence, a few new

278 microbial species appeared in the activated sludge, as it is shown and discussed along
279 this work.

280

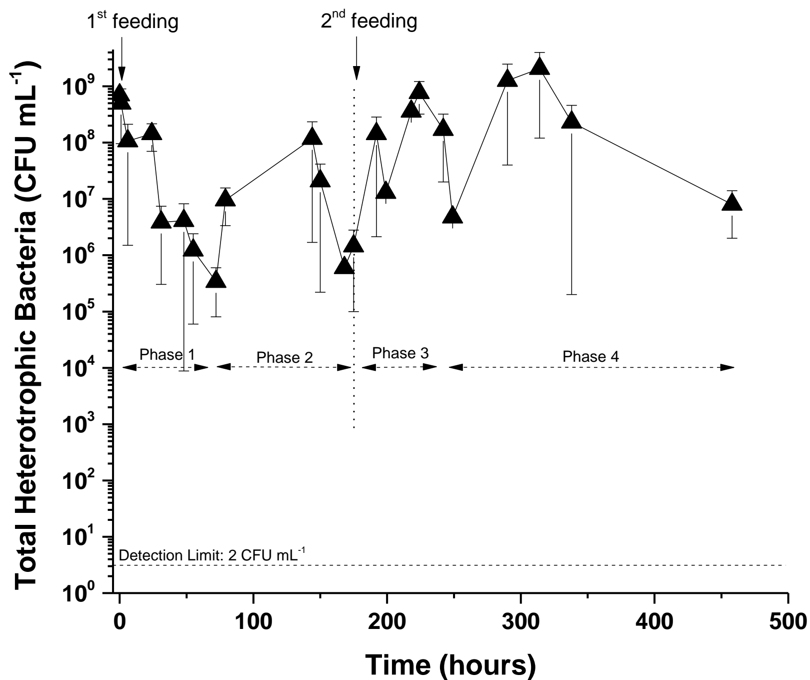
281 **3.2. Evaluation of aerobic biological treatment by plate count technique**

282 Total heterotrophic bacteria (THB), along the biological treatment at laboratory scale,
283 were followed at different contact times and results are shown in figure 2. Natural
284 occurring concentration of THB detected in activated sludge and CBW was
285 $4.5x10^9$ CFU mL^{-1} and $1.12x10^8$ CFU mL^{-1}, respectively. During the experimental
286 contact time, concentration of THB fluctuated between $10^9 – 10^5$ CFU mL^{-1}, with
287 several stages of reduction and increase of bacterial concentration. The following phases
288 can be distinguished in figure 2; i) First phase (0-31 hours of treatment), a clear
289 reduction on THB concentration (ac. 4.5 log) was detected. ii) In a second phase, THB
290 concentration increase and decrease 2.5 log at 150 h of contact time. In addition, during
291 these two phases, the fluctuation on THB was significant in terms of colonies
292 morphology, which suggests a microbiological “adaptation” process to the more
293 complex water matrix. Predominant colonies in phase 1 almost disappeared at the
294 begging of phase 2, changing to filamentous bacteria according to direct observation
295 with optical microscope. During the following phases, 3 and 4 corresponding to the
296 second feed of CBW, fluctuations on THB concentration were also found, however the
297 colonies morphology continued to be predominantly filamentous bacteria, not detecting
298 any other significant change in terms of colonies typology-growing. It is important to
299 highlight that there is not still clear evidence in related literature explaining which
300 parameters or operating conditions provoke a significant increase in filamentous

301 bacteria causing important depuration problems on conventional wastewater treatment

302 plants[24].

303



304

305 Figure 2. Total heterotrophic bacteria (THB) detected by plate count along the
306 biological experiment.

307

308 Therefore, plate count technique revealed an important change in overall bacterial

309 community diversity throughout the experiment as well as significant distortions in

310 relative abundance of microorganisms provoked by the "adaptation" process of the

311 biological system to CBW.

312

313 **3.3. Evaluation of aerobic biological treatment by optical microscopy identification**

314 Microfauna observation has traditionally been used as a bioindicator to evaluate the

315 performance of biological wastewater treatment systems, providing useful information

316 regarding the biological activity of the activated sludge[25]. Microbiological communities
317 were microscopically monitored "*in vivo*" during aerobic biological treatment at
318 laboratory scale. Before the beginning of the experiment, activated sludge from the
319 mixed liquor taken from the WWTP, was studied by optical microscopy analysis as a
320 reference, showing highly compacted flocs with regular shape and medium size,
321 covering approximately 60-70% of the glass slide surface. In general, the microfauna
322 living in activated sludge has a precise composition that depends on the specific nature
323 of the WWTP. In this work, the microfauna identified in the activated sludge was
324 mainly protozoa (fragellates and ciliates as *Opercularia* or *Colpidium*), metazoan
325 (*Rotifers)*, testate amoeba (*Arcellas*), and naked amoeba (see Figure 3a).

326 In concordance with the results shown in previous sections, many variations on
327 microbial population were also observed using microscopy technique. In the samples
328 taken after one hour of biological treatment, population similar to the reference was
329 observed, only detecting an increase on the number of naked amoeba and big fragellates
330 (*Peranema*). After 48 hours of treatment time (in contact with CBW), the number of
331 rotifers, and *Arcella* remained constant but there was also an increase of ciliated
332 protozoa, *Aspidisca*, *Acineria uncinata*, *Litonotus lamella* and *Coleps hirtus* appeared as
333 new species. The number of ciliate *Opercularia* also increased after this time. The
334 presence of ciliated protozoa in activated sludge plants are frequently reported[8, 26-28],
335 and, in general, they improve the efficiency of depuration. However, not all ciliate
336 species are favorable to the system. According to Mara and Horan et al. 2003[29], some
337 ciliates as *Opercularia* can be indicative of activated sludge's bad quality (non-
338 sustainable microbial population system). At the same time, carnivorous species as
339 *Litonotus lamella* appeared in the system, and was maintained in number until the end

340 of the experiment. As shown by Lee et al. 2004[30], this specie is an indicator of poor
341 sedimentation of the activated sludge.

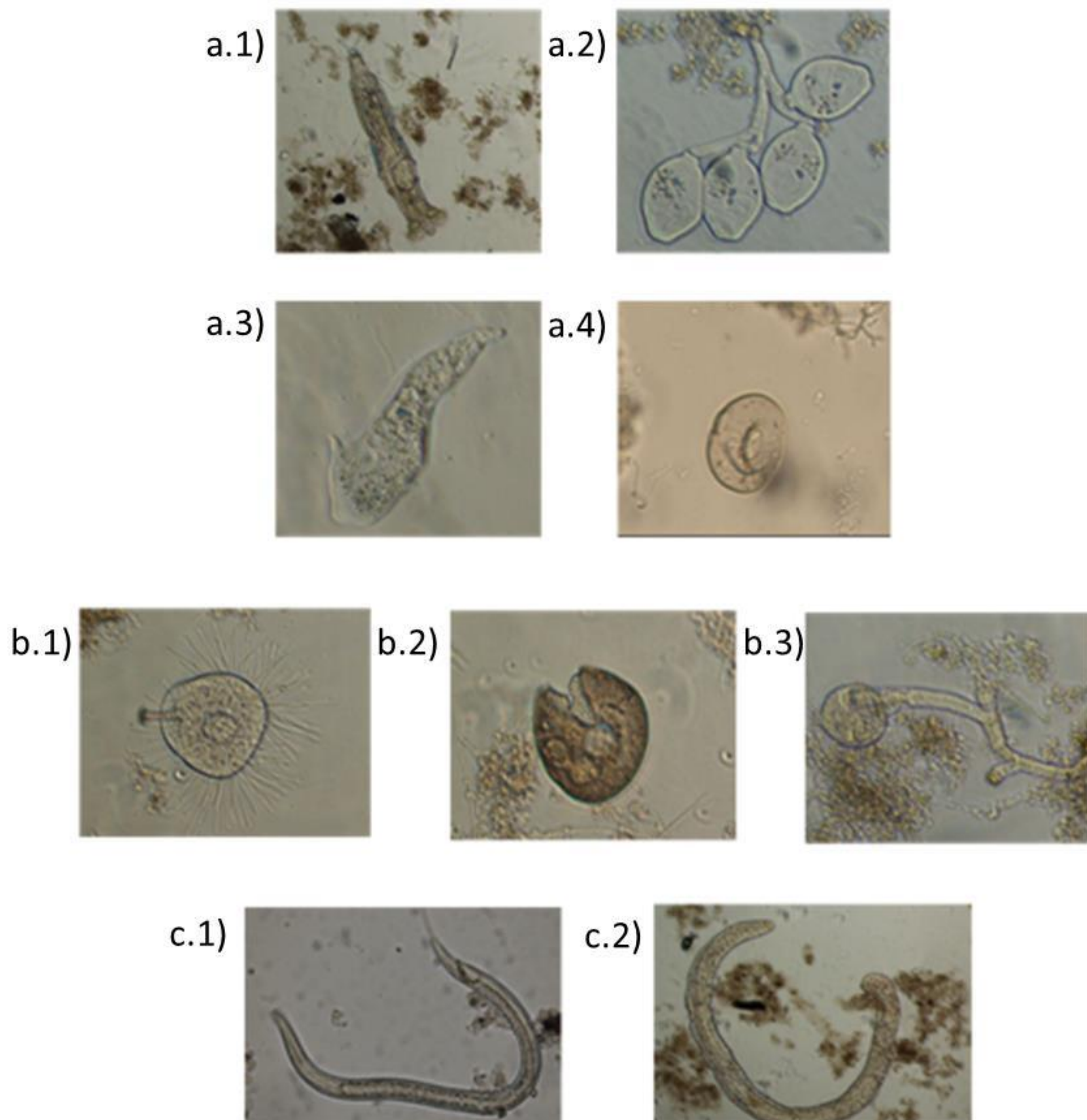
342 After 72 hours of treatment time, dead rotifers and some dead ciliated *Phodophyra*
343 appeared (see Figure 3b.1). In particular, ciliates and rotifers are the first to be affected
344 by toxic organics present in wastewater and thus, they serve as bio-indicators against
345 toxic or recalcitrant compounds or adverse operating conditions in biological reactors[31].
346 At that time, *Arcellas* started breaking-up (see Figure 3b.2). *Arcella testae amoeba* is
347 commonly present in activated sludge where the quality of the effluent is good[29], and it
348 is directly associated to successfully nitrifying process[27]. In addition, *Opercularia*
349 specie completely disappeared after 180 hours of treatment time (Figure 3b.3).

350 After 218 hours of biological treatment and a second addition of CBW, the total number
351 of small fragellates increased. Meanwhile, the presence of filamentous bacteria was
352 growing in the system. At that time, the flocs were damaged, and complete
353 deflocculation was observed. Stable flocs were not formed again from that moment until
354 the end of the experiment.

355 It is worth to mention that after 290 hours of treatment time, there was an unreasonable
356 growth of amoebas, becoming the predominant species in the system until the end of the
357 experiment. This specie is also frequent in WWT activated sludge. However, according
358 to Madoni (1994)[25], the existence of a dominant specie or group in the micro-fauna
359 population is indicative of an imbalance in the ecosystem due to the presence of limiting
360 factors that prevent the development of other species. Generally, the most common
361 limiting factors are the presence of a shocking load of a toxic discharge, a strong sludge
362 extraction or lack of aeration (dissolved oxygen limitation).

363 Finally, after 314 hours of treatment time, *Aspidisca* increased in a 50%, which is
364 indicative of an old activated sludge and high organic loading rate, meaning a bad
365 effluent quality[30] and so a bad depuration performance. At the end of experiment,
366 *Oligoqueto* species appeared, indicating a mineralization of sludge and therefore, an
367 oxidation of the system (Figure 3c).

368



369

370 Figure 3. a) Predominant microfauna observed by 40X phase contrast optical
371 microscopy in activate sludge from WWTP used in this work. 1) *Rotífer*, 2)

372 *Opercularia*, 3) Naked *Amoeba* and 4) *Arcella*. b) Broken species observed in a 40X
373 phase contrast optical microscopy after 180 hours of contact; 1) *Phodophyra*, 2) *Arcella*
374 and 3) *Opercularia*. c) 1) Nematodo and 2) Oligoqueto species observed in a 40X phase
375 contrast optical microscopy.

376

377

378 **3.4. Evaluation of aerobic biological treatment by DNA extraction and PCR**
379 **techniques**

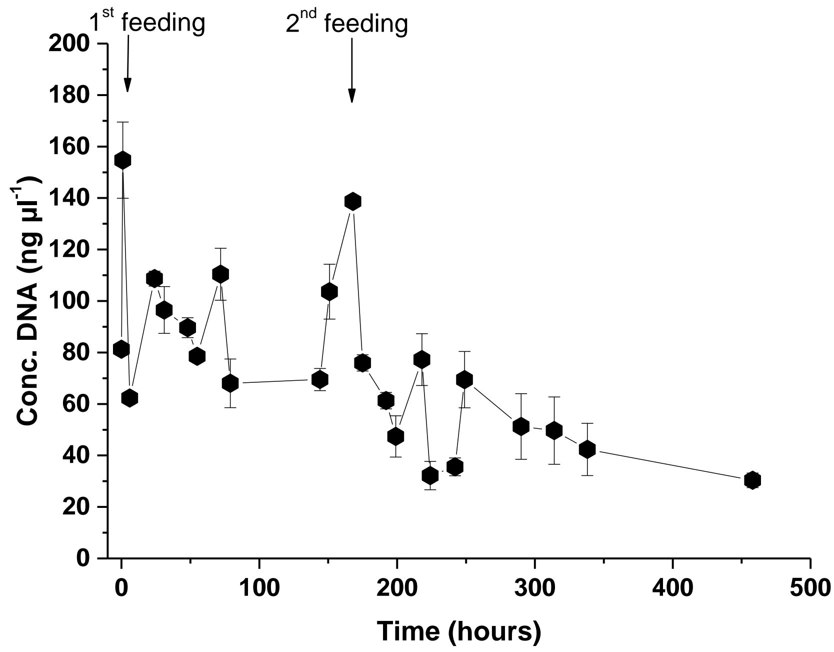
380 DNA concentration was measured in each sample taken throughout the biological
381 treatment. In all cases, DNA concentration ranged between 30 and 150 ng μL^{-1}. In
382 figure 4a, the evolution of the concentration of DNA versus treatment time can be
383 observed. Initial concentration of 81 ± 4 ng μL^{-1}, corresponding to the activated sludge
384 used in the inoculation of the reactor, increased to 154.7 ± 14.8 ng μL^{-1} after the first
385 addition of CBW. However, after the second feeding with CBW, DNA concentration
386 decreased during the treatment time until attaining a minimum concentration of about
387 30 ± 2.85 ng μL^{-1} at the end of experiment.

388 These results provide clear evidence regarding the reduction of microorganism
389 concentration through the biological treatment of CBW due to the chronic inhibition
390 effect that this complex wastewater causes on conventional activated sludge. In
391 addition, the decrease in DNA concentration is in concordance with the decrease in TSS
392 and VSS values also measured throughout the treatment time.

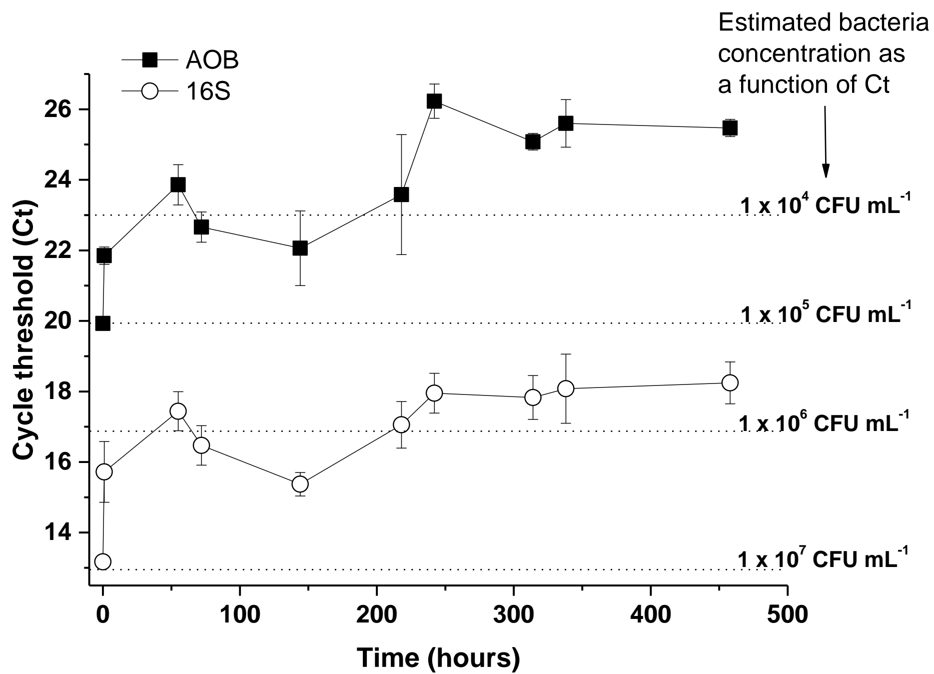
393 Considering that all DNA present in the samples was analyzed from a wide general
394 point of view, it would be highly interesting to try to identify those specific species that
395 could be damaged during the biological treatment of CBW and therefore, could affect

396 important degradation systems such as nitrification processes. This is why real-time
397 qPCR assays were also performed in order to investigate the evolution of total bacteria
398 (16S) and ammonia oxidizing bacteria (AOB) concentration in a set of samples
399 previously selected during the experiment. These samples were selected because they
400 showed significant changes in the concentration of ions, as well as important variations
401 at microscopic level. Figure 4b shows the evolution of 16S and AOB concentrations
402 throughout the experiment. A difference of approximately 2-log on concentration
403 between total bacteria and AOB was observed during the biological treatment though
404 the relation profile between both types of bacteria. These results coincide with those
405 reported by Harms et al. 2003[22]. In this work, average concentration of bacteria (16s
406 rDNA) and AOB were estimated at $4.3 \pm 2.0 \times 10^{11}$ cells L^{-1} and $1.2 \pm 0.9 \times 10^{10}$ cells L^{-}
407 ^{1}, a variation of ca. 1.5 log between both DNA targets analyzed from mixed liquor
408 suspended solids collected from a WWTP over a year. On the other hand, in our results,
409 a significant reduction during the treatment time was observed (approximately 1.5 log
410 of both bacterial targets reduction), which also suggests that the equilibrium of the
411 sludge, including the nitrification system, was altered.

412



413 a)



414 b)

415 Figure 4. a) DNA concentration evolution and b) Total bacteria (16S) and ammonia
416 oxidizing bacteria (AOB) concentration along the lab-scale aerobic biological treatment.

417

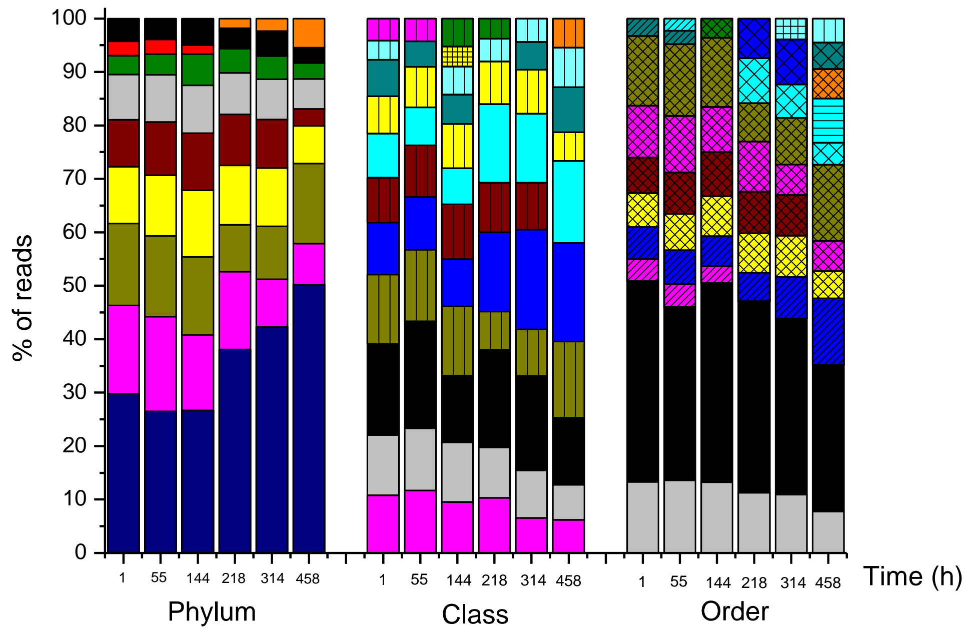
418 Then, it can be stated that the reduction of AOB concentration negatively affected the
419 biological system by worsening the nitrification process´ efficiency. So, this is an
420 example of how CBW would inhibit a conventional biological treatment. But, in this
421 specific case, and taking into account the results observed by optical microscopy, too,
422 the important changes detected in the microbial population of the activated sludge after
423 the contact with CBW, occurred in other types of bacteria. Consequently, most powerful
424 genetic techniques must be applied if we want to clearly identify the most affected
425 microbial species during the experiment, and therefore, understand why the adapted
426 biological system arisen is not able to successfully tackle with the degradation of the
427 organic charge coming from this complex wastewater.

428

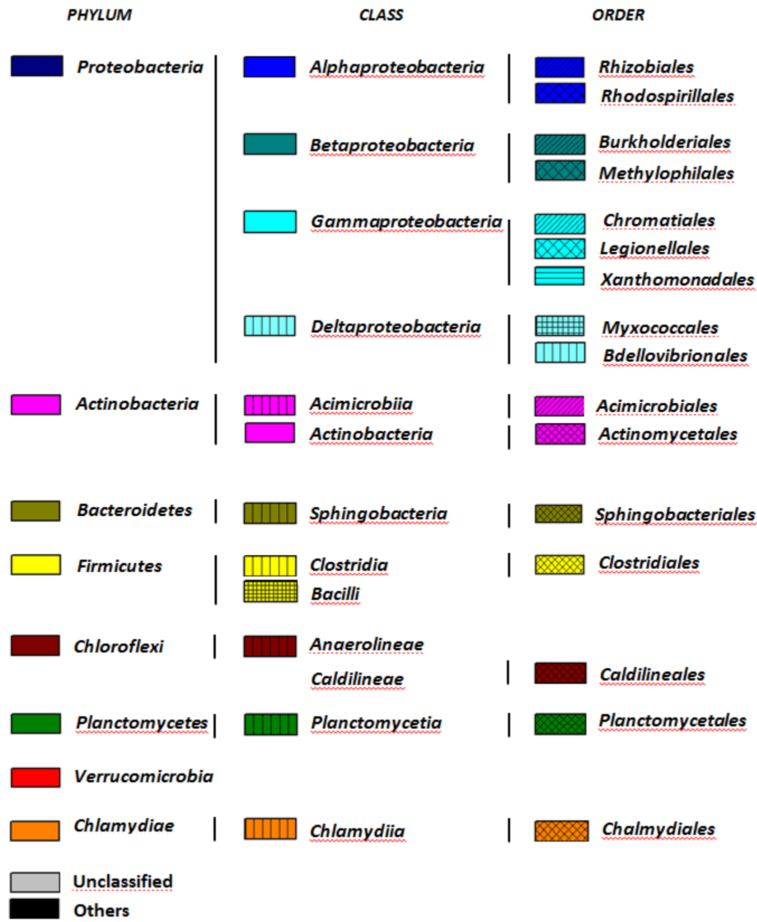
429 **3.5. Evaluation of aerobic biological treatment by Massive Sequencing analysis**

430 After PCR amplification, all samples were sequenced in order to find a phylogenetic
431 variation throughout the biological lab-scale experiment. For that, all species in samples
432 were categorized in kingdom, phylum, class, order, family, genus and specie. The
433 results showed that bacteria were the predominant kingdom, being greater than 99.6 %
434 in all cases.

435 The bars reported in figure 5 showed the phylogenetic classification in phylum, class,
436 and order discovered in the experiment. *Proteobacteria* was the most significant
437 phylum, growing from 30% to 50% during the experiment. *Actinobacteria* was the
438 second most important phylum in samples, decreasing by 50% throughout the lab-scale
439 experiment, finding in 7.72% at 458 hours, evidencing the sludge fragmentation.
440 *Actinobacteria* has been reported to be involved in microbiological phosphate removal
441 processes and in bulking and foaming problems of active sludge[32].

442 

a)

443 

b)

444 Figure 5. a) Relative abundance of phylum, classes and order along the treatment line.

445 b) Legend of the graph.

446

447 The percentage of *Chloroflexi* phylum increased from 8.74% to 10.72% in 144 hours,
448 decreasing to 3.18% at the end of the experiment. Traditionally, members of the
449 *Chloroflexi* phylum have been associated with extreme habitats such as microbial mats
450 in marine and hypersaline environments[33]. Nevertheless, the abundance of *Chloroflexi*
451 species has been found in industrial and municipal wastewater treatment plants with
452 different process designs. Due to their filamentous nature, we can consider that they can
453 negatively affect wastewater treatment plants, however, they are only occasionally
454 involved in bulking or foaming problems[34].

455 The *Bacteroidetes* phylum remained practically constant throughout the experiment
456 between 10% and 15%. This type of bacteria, like *Chloroflexi*, has been found in
457 industrial and municipal wastewater treatment plants in different process configurations
458 (nitrification/denitrification and enhanced biological phosphorous[35]). These bacteria
459 contribute to the filamentous index, and increase the possibility of bulking. Bacteria
460 belonging to *Firmicutes*, predominantly *Clostridia* class in this phylum was detected in
461 all samples, with initial values of 10.66% after 1 hour of experiment and 7.01% at the
462 end of experiment.

463 Various other minority phylum classifications like *Planctomycetes*, *Verrucomicrobia*
464 and *Chlamydiae* were present with small percentages. Dias and Bhat[36] reported that the
465 nature and function of the less dominant species can play a very important role in
466 stabilizing the system. No significant variation of percentage during the experiment was
467 noted for *Plantomycetes*. However, *Verrucomicrobia* values started in 2.69% after 1
468 hour and disappeared at 218 hours. *Chlamydiae*, an obligate parasite that is inside
469 animal cells and is surrounded by a functionally active double layered cytoplasmic

470 membrane[37], appeared at 218 hours and increased to 5.47% at the end of the
471 experiment, indicating a possible rupture of cells wall.

472 The activated sludge class classification was dominated by *Proteobacteria* from the
473 alpha, beta, gamma or delta subclass, being alpha and gamma classes the most abundant
474 subdivisions of *Proteobacteria* in samples. *Planctomycetia* was present only after 144
475 and 218 hours of experiment and *Chlamydiia* was present only at the end of the
476 experiment.

477 In literature, the prevalence and/or selection of *Proteobacteria* have been also reported
478 when analyzing the microbiota dynamics of municipal wastewater before and after
479 several disinfection process (including UVC, ozone, photocatalytic ozone and different
480 solar photocatalysis)[38, 39] of organic micropollutants, human pathogen indicators,
481 antibiotic resistant bacteria and related genes in urban wastewater. Water Research 135
482 (2018) 195-206). Although the objectives of the aforementioned works are quite
483 different from the present study, it is important to stress the high level of survival in a
484 non-favorable environment of this group of bacteria. In addition, they showed a high
485 resistance to oxidative stress including an active DNA repair mechanisms[38].

486 The classification order showed the variability in samples. *Rhizobiales*, *Clostridiales*,
487 *Actinomycetales* and *Sphingobacteriales* were present in all samples. At the end of
488 experiment *Caldilineales* order disappeared while *Chalmydiales* (5.47%),
489 *Xanthomonadales* (8.28%), *Bdellovibrionales* (4.50%) and *Methylophilales* (4.98%)
490 appeared after 458 hours.

491 The number of unclassified phylum, class and order obtained suggest that a wide variety
492 of novel species may inhabit complex activated sludge communities.

493

494 **3.6. Chronic toxicity**

495 In order to support microbiological analysis reported in this work, chronic toxicity
496 based on conventional activate sludge respirometry and generated along the biological
497 lab-scale experiment was evaluated. Toxicity respirometric tests showed increases on
498 the inhibition percentage from 23% after one hour of treatment time to 73% after 144
499 hours. Inhibition values for the rest of selected samples (2, 24 and 72 hours of treatment
500 time) were of 23, 27 and 41% respectively. These values clearly showed the
501 development of a detrimental effect on the activity of microbial population as the
502 biological treatment time increased. So, chronic toxicity measurements have also
503 demonstrated the impossibility of applying an adapted biological system for the
504 remediation of CBW as a highly complex wastewater.

505

506 **5. Conclusions**

507 Complete study on the modification of bacterial population present in activated sludge
508 during the adaptation process of a conventional aerobic biological treatment to CBW
509 has been reported. The variation of microorganisms species throughout the biological
510 adaptation showed a diminution of the total DNA concentration, general bacteria (16S)
511 and AOB (jointly with a reduction of TSS and VSS), being worthy to mention the
512 appearance of filamentous bacteria after 31 hours of contact with CBW. Diverse
513 microbiological species died during the experimental time while a few others arose
514 showing a possible adaptation of the biological system to this complex wastewater
515 accompanied of a DOC reduction. Nevertheless, the important increase of total nitrogen,
516 and specific ions concentration coming from the intracellular fluid coming from cells
517 membranes breakage at the end of the experiment suggested the malfunction of the

518 adapted biological system. This effect accompained of chronic toxicity measured by
519 respirometric assays (increasing from 23% to 73%), confirmed the non-viability of the
520 adapted biomass.

521 These results demonstrate the importance and the added value obtained when classical
522 microbiological analysis (plate counting and optical microscopy) are complemented
523 with new advanced techniques such as qPCR and metagenomics. In addition, it has been
524 concluded that a wide microbial analysis approach is crucial to define and predict the
525 behavior of new biological systems, specifically created or adapted from conventional
526 activated sludge, in order to face the remediation of complex/industrial wastewaters
527 such as CBW.

528 In this specific case, it has been demonstrated that alternative oxidative processes are
529 required to, at least, allow the partial remediation of CBW to make it more
530 biocompatible and so to design a successful adapted biological system for completing
531 the treatment with competitive operating costs.

532

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540

541 **Appendices**

542 Appendice shows the primer´s sequence (5´-3´), probes (table A1) and program (table
543 A2) used for the amplification of bacterial 16S rDNA and AOB 16S rRNA genes.

544

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659 genes in urban wastewater. Water Research, 135, 195-206.

Supplementary Material
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Assay	Target	Primer/probe	Sequence (5'-3')
16S rDNA	Total bacterial (16S rDNA)	1055 f 1392 r 16STaq1115	5'-ATGGCTGTCGTCAGCT-3' 5'-ACGGGCGGTGTGTAC-3' 5'-(6-FAM)-CAACGAGCGCAACCC-(TAMRA)-3'
AOB	Ammonia-oxidizing bacterial (16s rDNA)	CTO 189fA/B CTO 189fC RT1r TMP1	5'-GGAGRAAAGCAGGGGATCG-3' 5'-GGAGGAAAGTAGGGGATCG-3' 5'-CGTCCTCTCAGACCARCTACTG-3' 5'-(6-FAM)-CAACTAGCTAATCAGRCATCRGC-CGCTC-(TAMRA)3'

Table A1. 5'-3'sequence of the primers and probes used for the amplification of bacterial 16S rDNA and AOB 16S rRNA gene (Harms et al., 2003).

	16S rDNA	AOB
	Components	
Primers	1055f (0.4μL) 1392r (0.4μL)	CTO 189fA/B (0.05 μL) CTO 189fC (0.025μL)
Probe	TaqMan 16S Taq1115 (0.0625 μL)	TaqMan TMP1 (0.03125 μL)
Master Mix	Platinum SuperMix-UDG (12.5 μL)	Universal PCR Master Mix (12.5 μL)
Buffer	$MgCl_2$ (2.5μL)	RTlr (0.075 μL)
Water	8.1375μL	(11.31875 (μL)
DNA	1 μL (Dilution 1:10)	1μL (Dilution 1:10)
Total volume/ MicroWell	25 μL	25 μL
	PCR program	
Stabilization and denaturalization	3 min at 50ºC 10 min at 95ºC	2 min at 50ºC 10 min at 95ºC
Cycles	45 cycles: 95ºC during 30 sec and 72ºC during 20 sec	40 cycles: 95ºC during 30 sec and 60ºC during 60 sec

Table A2. Mixture of components and qPCR program for amplification of bacterial 16S rDNA and AOB 16S rRNA genes (Harms et al., 2003).