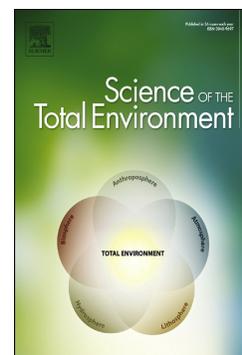


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How do WWTPs operational parameters affect the removal rates of EU Watch list compounds?

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Abstract

This work aims at achieving a better understanding of the mechanisms and the operative conditions regulating the removal of a set of relevant micropollutants in conventional activated sludge (CAS) systems to maximize their removal and, if possible, biodegradation. Eight compounds from the EU Watch list (clothianidin, thiacloprid, methiocarb, E1, E2, EE2, diclofenac and erythromycin) were spiked at 2 µg/L in CAS systems and their behaviour was studied in 6-hour batch tests. The role of sorption was also investigated. Information on the removal of the pesticides clothianidin, thiacloprid and methiocarb is here presented for the first time to the best of the authors' knowledge. With the aim of enhancing the removal of the selected compounds in wastewater treatment, four parameters were explored: biomass concentration, temperature, pH and redox conditions. For each parameter, a low and a high value were chosen, based on the ranges usually applied in wastewater treatment plants (WWTPs). Results show that biomass concentration is the most relevant parameter among the ones investigated, followed by the redox conditions. The operational conditions that maximised removal rates were: 5 g/L of biomass, aerobic conditions, 25

27 °C and pH 7.5. High variability in removal rates was observed for compounds such as E1,
28 erythromycin and methiocarb. The pesticides clothianidin and thiacloprid did not prove
29 to be easily degradable. The highest removal rates were recorded for the hormones,
30 particularly E2, with a transformation rate of at least 96% under all conditions. Sorption
31 proved to be a relevant removal route for EE2, for which the highest sorption rates
32 were recorded, and diclofenac, where the adsorption mechanisms was hypothesised for
33 its prevalence at lower pH values.

34 **1. Introduction**

35 The occurrence and fate of organic micropollutants, such as endocrine disrupting
36 compounds (EDCs), pharmaceuticals and pesticides, has raised great environmental
37 concern during the last decades (Daughton and Ternes, 1999). While the majority of
38 these emerging contaminants is still unregulated in most countries, legal frameworks
39 have been recently implemented in order to phase out the release into the
40 environment of those compounds that threat the aquatic ecosystems or to regulate
41 their presence in water bodies (EC, 2013, 2000). The Directive 2013/39/EU sets
42 concentration limits for a number of chemicals with prejudicial environmental effects,
43 the so-called priority pollutants, but also sets a new decision-making tool for future
44 prioritisation exercises through the Watch list system (Commission Implementing
45 Decision 2015/495 (EC, 2015) and 2018/840 (EC, 2018)). The Watch list, in force since
46 March 2015, includes compounds that could pose a risk to or via the aquatic
47 environment, but for which high-quality monitoring data are not enough to carry out
48 appropriate risk assessment (EC, 2015). The compounds included in the Watch list
49 should be monitored in all EU Member States for a maximum of 4 years after which
50 they might be regulated. In June 2018 a new version of the Watch list was published.

51 Five compounds (oxadiazon, triallate, BHT, EHMC and diclofenac) were removed from
52 the 2018 Watch list, three compounds were added (two antibiotics and a pesticide)
53 while the majority of compounds remained unaltered due to the lack of occurrence
54 data for risk assessment (EC, 2018). According to the EU Decision, the Watch list
55 substances are intended for monitoring in freshwater only. Nonetheless, WWTPs, even
56 though essential to contain anthropogenic contamination, are among the most relevant
57 point sources of contamination. Therefore, unravelling the fate of emerging pollutants
58 and, possibly, enhancing their removal when passing through WWTP are crucial,
59 ultimately ending out in substantial improvement of water quality and environmental
60 standards (Gusmaroli et al., 2019).

61 Conventional activated sludge wastewater treatment plants (CAS-WWTPs) represent
62 the most common treatment for major urban areas. They are highly efficient in influent
63 wastewater purification in terms of organic matter, suspended solids and/or nutrient
64 but they are not specifically designed to remove micropollutants (Buttiglieri and
65 Knepper, 2008; Carballa et al., 2017). Advanced treatment processes could be
66 employed, but limitations are posed due to maintenance and operational costs (Luo et
67 al., 2014; Schröder et al., 2016). Improving the biodegradation process can be,
68 therefore, a suitable solution due to its lower cost and its potential for complete
69 micropollutants removal. Nonetheless, information is largely missing for many emerging
70 micropollutants in terms of operative parameters affecting their removal in CAS
71 systems.

72 The main mechanisms that might play a role in micropollutants removal are
73 biodegradation, sorption onto sludge, air stripping and photo-transformation. For many

74 emerging organic micropollutants, including the pollutants selected in this study, both
75 air stripping and photo-transformation can be neglected (Sipma et al., 2010). Air
76 stripping depends indeed on the Henry's law constant (H_c): with H_c lower than 10^{-4} and
77 the fraction H_c/K_{ow} lower than 10^{-9} , there is a low volatilisation potential (Rogers, 1996).
78 For hydrophobic compounds, the main removal mechanism is usually sorption, while
79 hydrophilic compounds are more prone to biodegradation (Alturki et al., 2010). The
80 extent of sorption is measured by the solid–water distribution coefficients (K_d), defined
81 as the ratio between the concentrations of a substance in the solid and in the aqueous
82 phase at equilibrium. This parameter, in turn, includes two mechanisms: absorption,
83 which consists of hydrophobic interactions – whose extent is measured with K_{ow} – and
84 adsorption, regulated by electrostatic interactions, characterized by pKa. Compounds
85 with a log K_{ow} below 2.5 exhibit a low sorption potential, between 2.5 and 4.0 a medium
86 sorption potential and higher than 4.0 a high sorption potential (Rogers, 1996).
87 Reviewing literature, it becomes evident that fewer studies deal with absorption than
88 adsorption. Moreover, several works use the term sorption, or even adsorption, to refer
89 to both mechanisms, without making distinction between the two processes. Hence, in
90 some cases, it is difficult to tell the two mechanisms apart and to compare the results
91 with previous works. On the whole, sorption onto activated sewage sludge might be less
92 relevant for many micropollutants, as shown by their relatively low sorption coefficients
93 (K_d) (Sipma et al., 2010).

94 The compounds chosen for this study were clothianidin, thiacloprid, methiocarb,
95 erythromycin, diclofenac, estrone (E1), estradiol (E2) and ethinylestradiol (EE2). These
96 were originally included in Decision 2015/495, which is currently undergoing its fourth
97 year of monitoring, and, out of the eight compounds, seven of them have been

98 reconfirmed in the 2018 Watch list too, with the only exception of diclofenac (EC,
99 2018). The chosen compounds are representative of different classes: clothianidin,
100 thiacloprid and methiocarb are pesticides, erythromycin is an antibiotic, diclofenac is a
101 NSAID pharmaceutical, E1 and E2 are natural hormones whereas EE2 is a synthetic
102 hormone. In addition, these compounds exhibit different biodegradability, from
103 recalcitrant to highly biodegradable. Some of these compounds, such as clothianidin,
104 thiacloprid and methiocarb, lack information regarding their behaviour and removal
105 mechanisms in WWTPs, while others, like erythromycin, diclofenac and EE2, are marked
106 by huge variability in terms of occurrence and removal in full-scale WWTPs (Barbosa et
107 al., 2016).

108 In order to shed light on the behaviour and fate of the selected contaminants in CAS
109 systems, batch experiments were conducted and the extent of micropollutants
110 biodegradation and sorption was assessed. Four operational parameters were
111 evaluated: temperature, pH, mixed liquor suspended solid (MLSS) concentration and
112 redox conditions, in ranges usually found in temperate climate conventional WWTPs.

113 **2. Materials and methods**

114 **2.1. Reagents and solutions preparation**

115 Erythromycin, methiocarb, thiacloprid, clothianidin, E1, E2, EE2 and diclofenac (sodium
116 salt) were purchased from Sigma Aldrich (St. Louis, MO, USA). Individual stock solutions
117 were prepared on a weight basis by dissolving 10 mg of the desired compound in 10 mL
118 of methanol (stock 1, concentration of 1000 mg/L). Once prepared, they were stored at
119 -20 °C in the dark, to preserve the compounds from possible photodegradation
120 (Capriotti et al., 2014). Due to the well-known limited stability of antibiotics solutions,

121 the erythromycin stock solution was renewed each 3 months (Gros et al., 2013), while
122 for the rest of compounds stocks were renewed each 6 months. Then, 100 μL of each
123 compound was added to a vial containing a few drops of methanol and the mix was
124 evaporated under a gentle nitrogen stream until complete dryness. The solution was
125 then reconstituted in 10 mL of water (MIX 1, 10 mg/L) and placed into an ultrasonic
126 bath for 15 minutes to ensure complete dissolution. This mix was then further diluted in
127 10 mL of water to obtain the final spiking solution (MIX 2, 0.5 mg/L). Lastly, 1 mL of
128 MIX2 was spiked in the jacketed reactors (see paragraph 2.4) to obtain the experiments
129 design initial concentration of 2 $\mu\text{g/L}$.

130 **2.2. Biomass source and preparation**

131 The biomass was regularly withdrawn from Celrà WWTP (Catalonia, Spain). Celrà WWTP
132 has a capacity of 18,900 PE by design, a load of 10,009 PE (“iAgua - EDAR de Celrà,”
133 n.d.), a hydraulic retention time of 48 h and a sludge retention time of 20-22 days
134 (Collado et al., 2014). Celrà WWTP has 80% flow industrial contribution from a nearby
135 industrialized area with several pharmaceutical industries, which have their own
136 wastewater treatment process before discharging their effluents to the WWTP of study
137 (Collado et al., 2014). Moreover, the WWTP is equipped for phosphorous and nitrogen
138 removal (“iAgua - EDAR de Celrà,” n.d.).

139 Before the beginning of each test, the biomass was aerated for one hour in order to
140 minimise the amount of rapidly degradable organic matter still present. The initial
141 mixed liquor suspended solids (MLSS) content of the sludge, usually related to biomass
142 content, was determined according to Standard Methods (APHA, 2012). To determine
143 the role of adsorption to sludge in micropollutants removal, experiments with

144 inactivated biomass were also carried out. Inactivated biomass was obtained by
145 autoclavation at 120°C for 20 minutes before each experiment.

146 2.3. Experimental design

147 The list of experiments is presented in Table 1. Four parameters were explored:
148 temperature, MLSS, redox conditions and pH. A lower and higher levels were defined
149 for each parameter: 12 and 25°C for temperature, 1 and 5 g/L for MLSS, anoxic and
150 aerobic for redox conditions and 6.5 and 7.5 for the pH. Such ranges were chosen to
151 include typical WWTP operational parameters in temperate climates, and in accordance
152 with Celrà WWTP. All experiments were performed in duplicate.

153 **Table 1** – List of experimental conditions.

Exp.	Temp (°C)	MLSS (g/L)	Redox	pH
1	12	1	anoxic	6.5
2	25	1	anoxic	6.5
3	12	5	anoxic	6.5
4	25	5	anoxic	6.5
5	12	1	oxic	6.5
6	25	1	oxic	6.5
7	12	5	oxic	6.5
8	25	5	oxic	6.5
9	12	1	anoxic	7.5
10	25	1	anoxic	7.5
11	12	5	anoxic	7.5
12	25	5	anoxic	7.5
13	12	1	oxic	7.5
14	25	1	oxic	7.5
15	12	5	oxic	7.5
16	25	5	oxic	7.5

154

155 2.4. Experimental setup, sampling procedure and analyses

156 The experiments were carried out in jacketed reactors connected to a thermostatic
157 bath to maintain the temperature constant throughout the experiment. The batch tests

158 were conducted under continuous stirring (200 revolutions per minute) by means of
159 magnetic stirrers. The biomass was diluted with tap water in order to achieve the
160 desired concentration and a total working volume of 250 mL. To mimic real influent
161 wastewater conditions 0.25 mL of trace elements solution (Kampschreur et al., 2007),
162 10 mL of buffer/inorganic solution, 1 mL of organic solution and 1 mL of either nitrate
163 or ammonia solutions were added before the start of each test, following a procedure
164 adapted from Collado et al., 2013 and Kassotaki et al., 2019. The composition of such
165 solutions can be consulted in SM. pH was set to either 6.5 or 7.5 by addition of acid
166 (HCl) or base (NaOH) at 0.5 M and monitored throughout the batch tests with a
167 portable pH-meter, making adjustments when necessary. Aerobic conditions (>2.5 mg
168 O_2/L) were ensured by continuous, gentle, air supply through an air diffuser, while
169 anoxic conditions were achieved by dosing $NaNO_3$ (see Table S4 in SM).

170 To check the background concentration of micropollutants and to evaluate the matrix
171 effect, samples were collected before starting the experiments. Typically, 4 mL
172 approximately were taken from the reactor with a syringe and immediately filtered
173 through syringe-driven filter units (Millex Syringe PVDF 33mm, pore size 0.45 μm , by
174 Merck Millipore) in order to separate the liquid phase from the sludge, thus interrupting
175 the reaction. The samples were stored in 2-mL vials and immediately frozen until
176 analysis.

177 At time zero, micropollutants were spiked into the system (design initial concentration:
178 2 $\mu g/L$) and, after allowing 30 seconds for mixing, time-zero samples were taken
179 following the procedure described above. Micropollutants were also sampled at elapsed
180 time 10 minutes, 1 hour, 2 hours and 6 hours. The analyses were carried out by

181 measuring the concentration of micropollutants in the liquid phase by means of online
182 SPE-UHPLC-MS/MS according to the method described in Gusmaroli et al., 2018.
183 Shortly, the samples were loaded onto a Hypersil GOLD aQ (20 x 2.1 mm, 12 µm particle
184 size, Thermo Fisher Scientific) column at a flow rate of 1750 µL/min for
185 preconcentration and then the analytes were eluted from the chromatographic column
186 at flow rate of 400 µL/min. Clothianidin, erythromycin, methiocarb and thiacloprid were
187 analysed under positive ionization mode by means of a Kinetex Biphenyl (1.7 µm
188 particle size, 100 x 2.1 mm i.d., Phenomenex). The mobile phase consisted of MeOH and
189 water with 0.1% formic acid and separation was achieved in 8 minutes. The hormones
190 and diclofenac were analysed under negative ionization (NI) mode. As organic mobile
191 phase, a mixture of MeOH and acetonitrile (ACN) was used during the initial stages of
192 the gradient, though the compounds eluted when the percentage of ACN reached
193 almost 100%. The aqueous mobile phase consisted of LC/MS grade water with
194 ammonium fluoride 1 mM. The time of analysis in NI mode lasted 10.5 minutes.
195 Detection was performed by means of a TSQ Vantage mass spectrometer (Thermo
196 Fisher Scientific) equipped with an electrospray turbo spray ionization source, recording
197 two Selected Reaction Monitoring (SRM) transitions for each compound. To obtain
198 information on process efficiency in the preparation of the spiking mix, the
199 concentration of the investigated compounds in the spiking mixture was checked for
200 each experiment. Recoveries of the spiking mix ranged from 55 to 117% for all
201 compounds. The results are presented as normalized concentrations in order to
202 homogenize the concentration levels and allow inter-day comparisons. Normalisation
203 was obtained by dividing each concentration by the concentration value recorded at
204 time 0. The resulting value was then multiplied by 100, thus rescaling all data to a 0-

205 100% scale. Removals were calculated as the difference between initial and final
206 normalised concentrations. Compounds exhibiting removal rates below 25% were
207 classified as poorly degradable, between 25 and 65% were considered medium and
208 above than 65% were classified as highly degradable. The analyses were conducted only
209 on the liquid phase, therefore the term removal is intended as the combination of
210 biological and physical phenomena, whereas sorption refers to the decrease in
211 concentration observed in the experiments carried out with inactivated biomass. Thus,
212 biodegradation was calculated as the difference between the total removal and
213 sorption.

214 Besides the selected micropollutants, other analyses were carried out at the beginning
215 and at the end of each test: mixed liquor suspended solids (MLSS) and mixed liquor
216 volatile suspended solids (MLVSS), total organic carbon (TOC), total nitrogen (TN) and
217 ammonia, nitrite and nitrate concentrations. TOC samples were filtered through
218 syringe-driven nylon filter units with pore size 0.45 μm and samples for nitrogen species
219 analyses were filtered through 0.22 μm nylon filters (Millex Syringe, 33 mm, by Merck
220 Millipore). TOC and TN were analysed with a TOC/TN analyser, while the other nitrogen
221 species were analysed by ion chromatography (APHA, 2012).

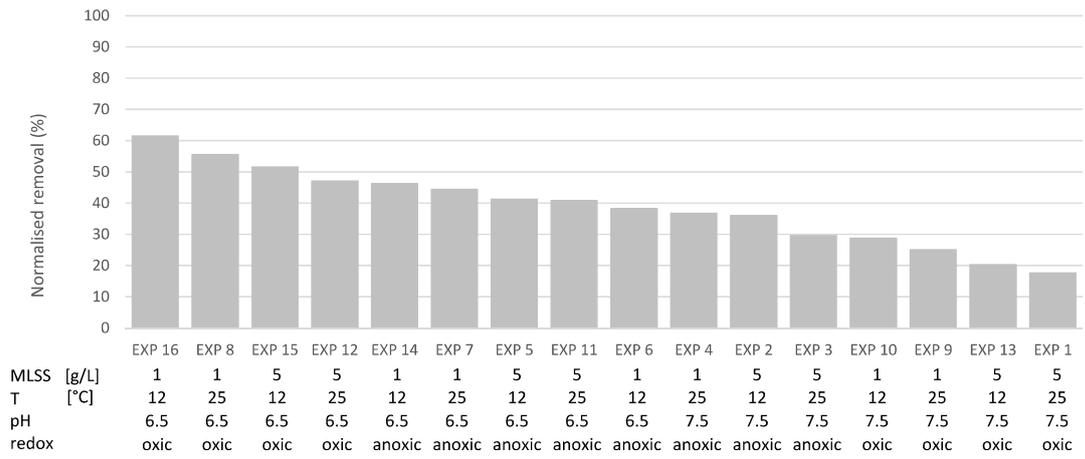
222 **3. Results and discussion**

223 **3.1. Effect of experimental conditions on the overall removal**

224 If we approach the removal of all the micropollutants considered in this study, that is to
225 say, considering the whole set, the highest removals were obtained during experiment
226 16, in which all the parameters were at the higher level: aerobic conditions, 25°C, 5 g/L
227 of biomass and pH 7.5. Please note that removal, described in this section and in

228 section 3.2, is intended as the combined effect of biodegradation and sorption to
229 sludge. Figure 1 shows the average removals, for all compounds, attained in each
230 experiment. Detailed results for each test are shown in Figure S1 of Supplementary
231 Materials.

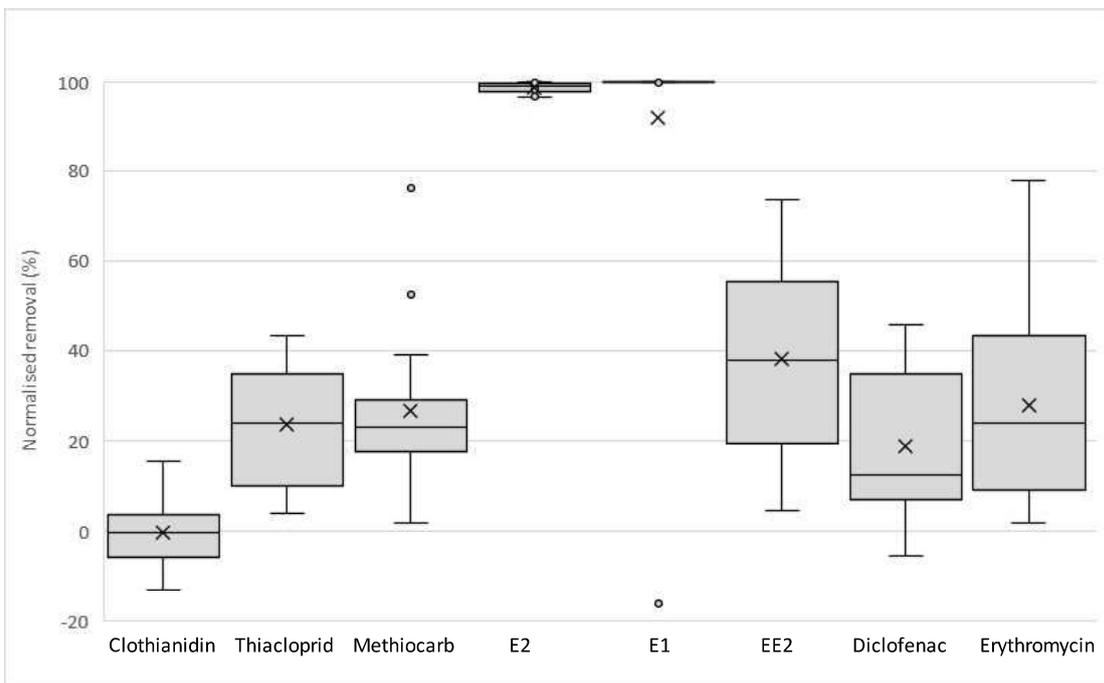
232 The most relevant factor affecting micropollutants removal is MLSS concentration,
233 which yields higher rates at high concentrations, as expected. The second most relevant
234 operational parameter is the redox condition, as working under aerobic conditions
235 enhances the removal. The third one is temperature, which seems to give better results
236 at high level (25°C) followed by pH, a parameter that, at least apparently, has little
237 effect on the overall micropollutants removal in the considered range and the chosen
238 compounds. The analysis of nutrients confirmed that temperature helps accelerating
239 the kinetics of the process as those tests at 25 °C present higher increase/decrease of
240 nutrients (Table S5 in SM). Moreover, the depletion of NH_4^+ that is converted to NO_3^-
241 highlighted that nitrification takes place in aerobic conditions, and more sharply in
242 experiments 8 and 16, at 5 g/L MLSS and 25 °C.



243

244 **Figure 1** - Normalised average removal of all compounds. Data were normalised by
 245 dividing the concentrations obtained at each sampling time by the concentration of
 246 time-0 samples. The obtained values were then multiplied by 100 and are here
 247 presented as percentages.

248 **3.2. Effect of the experimental conditions on the removal of single compounds**

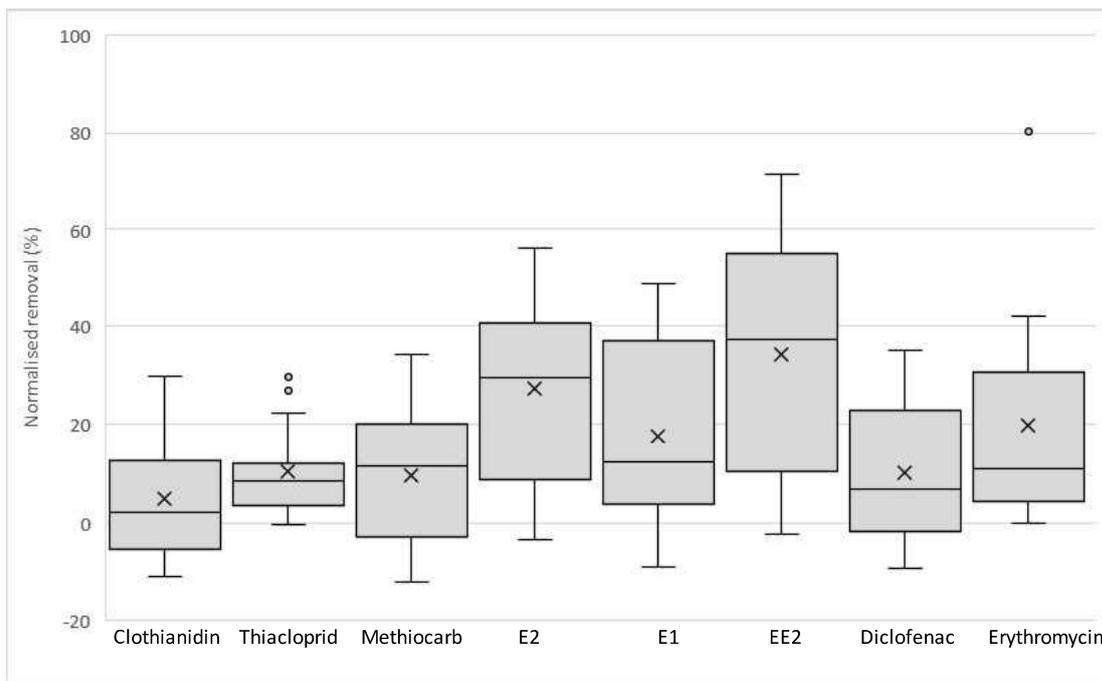


249

250 **Figure 2** - Total removal by compound

251

252



253

254 *Figure 3 – Sorption by compound*

255

256 With regards to the removal by compound, Figure 2 displays the statistical distribution.

257 For compounds such as E1, erythromycin and methiocarb, the ranges sweep from zero

258 to very high or complete removals, highlighting the importance of the choice of the

259 right operational parameters, since even minute variations produce a significantly

260 different response.

261 To the best of the authors' knowledge, literature data concerning the occurrence of

262 clothianidin, thiacloprid and methiocarb in WWTPs are extremely limited in number and

263 no publications exploring their fate and behaviour are available. The lowest removal

264 was observed for clothianidin with a null to low removal (up to 15%, Figure 2 under all

265 experimental conditions, in consistency with the extremely rare literature data

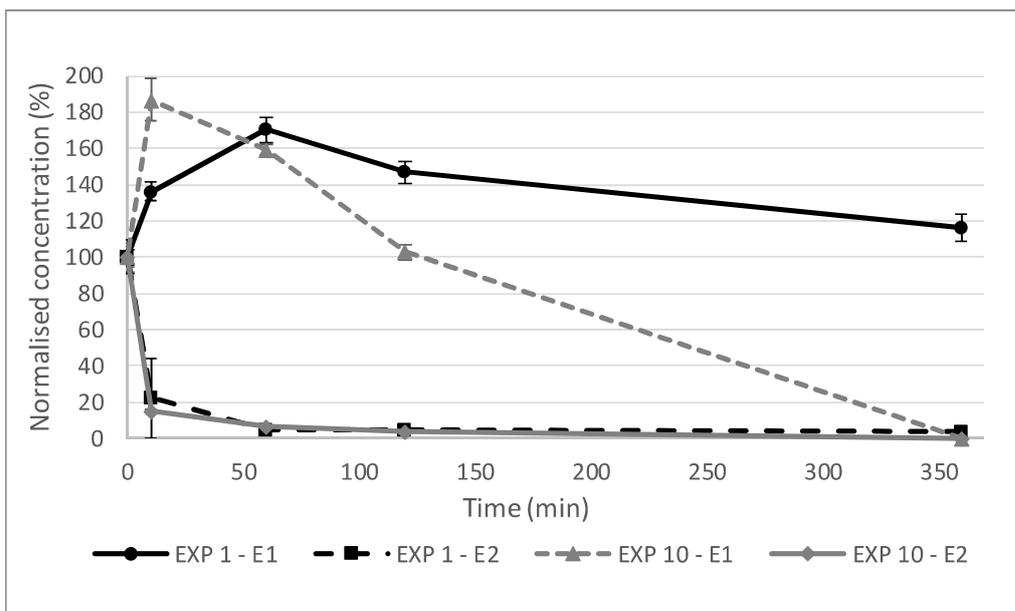
266 concerning its occurrence in a real-scale WWTP (Sadaria et al., 2016).

267 Thiachlopid removal ranged between 3.8 and 43.2% (Figure 2) Among the investigated
268 compounds, thiachlopid is the only one that showed higher removals when operating at
269 the lower level of temperature. This could hint that adsorption is a significant removal
270 pathway, as it is an exothermic process, but the removal rates are so low that it is hard
271 to tell the mechanisms apart. The lowest removal occurred at 25°C, under anoxic
272 conditions, at 1 g/L MLSS and pH 7.5, while the highest degradation rate was obtained
273 under anoxic conditions, at 12°C, high biomass concentration and pH 7.5. Information
274 on thiachlopid biodegradation is completely absent in literature and its occurrence in
275 WWTPs has been studied in an extremely limited number of works. To the best of the
276 authors' knowledge, it was never detected in wastewater (Gusmaroli et al., 2019;
277 Rubirola et al., 2017; Sadaria et al., 2016).

278 The removal of methiocarb spanned through a wide range. At its highest, it was
279 removed up to 76%, under aerobic conditions, at 5 g/L MLSS, pH 7.5 and low
280 temperature (Figure 2). The second and third best removals were achieved when
281 operating under aerobic conditions, high levels of MLSS and temperature and at pH 7.5
282 and 6.5. Nevertheless, its removal was unsatisfactory in most cases, the lowest
283 recorded value equalling 1.9% when operating under anoxic conditions at low
284 concentrations of biomass. The factor accounting for most differences in methiocarb
285 degradation, in the tested conditions, is MLSS. Overall, the removal of this compound
286 does not present a clear pattern and no comparisons are possible due to the absence of
287 references in the literature. Its occurrence has been poorly investigated too: in two
288 recent studies, it was not detected in wastewater (Gusmaroli et al., 2019; Rubirola et
289 al., 2017) and, when detected, its concentration ranged between 1.26 and 105.31 ng/L.
290 A negative removal from wastewater of over 7000% was observed (Campo et al., 2013),

291 a value probably resulting from sampling limitations (neither sludge retention time nor
292 hydraulic retention time were taken into consideration) (Barbosa et al., 2016). More
293 studies are needed to shed light on the factors that enhance the removal and
294 biodegradability of clothianidin, thiacloprid and methiocarb.

295 Among the investigated micropollutants, the compound with the highest
296 biotransformation rate was E2, with removals always above 96% (Figure 2). In
297 particular, a rapid removal of E2 ranging from 60 to 95% occurs in the first 10 minutes,
298 the former at 12 °C, 1 g/L, pH 6.5 and under aerobic conditions and the latter at 25 °C, 5
299 g/L, aerobic conditions and pH 6.5. This is due to the quick oxidation of E2 into E1, a
300 phenomenon already described in literature (Ternes et al., 1999). Despite this
301 difference in terms of E2 biotransformation rate, anyway, by the first 60 minutes of
302 contact time, at least 93.8% in respect to its initial concentration was biodegraded
303 under all experimental conditions. The easy biotransformation of E2 has been widely
304 reported in literature, observed both in full-scale WWTPs (Baronti et al., 2000; de Mes
305 et al., 2005) and in laboratory experiments (López-Fernández et al., 2013), and it has
306 been observed in all redox conditions (aerobic, anaerobic and anoxic) (Li et al., 2011).



307

308 **Figure 4** - E1 and E2 removal profiles in experiments 1 and 10

309 The compound showing the second highest removal rates was E1. This compound
 310 showed medium to high removal rates except when working at 12 °C and at pH 6.5
 311 under anoxic conditions, which led to null to low removals, up to 19.1%. Medium
 312 removals, around 40%, were observed at 12 °C, 1 g/L biomass under aerobic conditions.
 313 For this compound, an increase up to 190% in respect of its initial concentration was
 314 recorded in almost all cases during the first minutes of reaction due to the above-
 315 mentioned conversion of E2 into E1 (see Figure 4). Afterwards, the concentration
 316 decreases either gradually or abruptly, depending on experimental conditions. High
 317 temperature, biomass concentration and pH led to the fastest removals, regardless of
 318 redox conditions. Temperature appears to be one of the factors affecting the kinetics of
 319 E1 removal the most. In fact, at 25 °C, the removal was always complete (>99%). An
 320 improvement in estrogens biodegradability at higher temperatures has been observed
 321 in full-scale WWTPs (de Mes et al., 2005) as well as batch tests (Li et al., 2005; Zeng et

322 al., 2009). Studies have demonstrated that E1 eventually even undergoes mineralization
323 in WWTPs, with 70-80% conversion of E2/E1 into CO₂ in 24 hours (Layton et al., 2000).
324 EE2 removals ranged from 5 to 73.6%, proving the least biodegradable amongst the
325 investigated hormones, in consistency with literature (de Mes et al., 2005; Ting and
326 Praveena, 2017). The lowest removals were obtained at low concentrations of biomass
327 and under anoxic conditions. EE2 behaviour, not only in WWTPs but also in aerobic
328 batch experiments, is reportedly inconsistent. Literature data include an observed
329 persistency over a 5-day period (Norpoth et al., 1973) but also, contrarywise, a
330 complete removal achieved by nitrifying sludge after 6 days (Vader et al., 2000).
331 According to previous works, EE2, when biotransformed, is supposedly degraded by co-
332 metabolism, differently from natural estrogens. The lower biodegradability of EE2 could
333 be caused by its ethynyl group, which sterically hinders metabolism and other reactions
334 (Racz and Goel, 2009). The two hydroxyl groups present in EE2 molecular structure
335 would be susceptible to microbial attack; however, the ethynyl group located on the
336 same C atom which possesses the hydroxyl group makes ring cleavage more difficult
337 and, in turn, causes EE2 to be recalcitrant (Zuo et al., 2013). Nitrifying sludge is held
338 accountable for the conversion of EE2 into more hydrophilic metabolites by ammonium
339 monooxygenase and the phenomenon is usually not observed in sludges with low
340 nitrifying activity (Vader et al., 2000). The maximum EE2 removal (>70%) was achieved
341 under aerobic conditions, at high levels of temperature, biomass concentration and
342 almost regardless of pH, in accordance with the above-mentioned literature data.
343 However, surprisingly, similar removals were obtained under anoxic conditions at 12°C,
344 5 g/L MLSS, pH 7.5 and with a faster degradation profile (60% removed during the first

345 10 minutes of contact time). This result seems to contradict previous studies in which
346 EE2 was removed at a significant rate only under aerobic conditions (Joss et al., 2004).

347 Diclofenac was hardly removed, its biodegradation rates ranging from null to moderate,
348 with a maximum removal of 45.7%, obtained under aerobic conditions at 25°C, 5g/L
349 MLSS, pH 6.5. Diclofenac's poor biodegradability during biological wastewater
350 treatment has been previously reported (Lee et al., 2012; Pérez and Barceló, 2008;
351 Quintana et al., 2005; Vieno and Sillanpää, 2014). The microbial processes that play a
352 role in diclofenac biodegradation and biotransformation have been investigated
353 thoroughly, but the picture is still unclear. High variability can be found in literature,
354 with elimination rates of up to about 80% (Yang et al., 2011) and even negative
355 removals (Clara et al., 2005; Zorita et al., 2009); however, values in the range of 20–
356 50%, similarly to those found in the current study, are more common (Vieno and
357 Sillanpää, 2014). Apparently, aerobic conditions favoured its degradation, while
358 operating at pH 7.5 seems to have a negative effect on its removal. In consistency with
359 this finding, Suárez and co-workers observed no biodegradation under anoxic
360 conditions (Suarez et al., 2010). The dependence on pH suggests that adsorption is a
361 relevant removal route for diclofenac. In fact, the carboxylic acid moiety of diclofenac is
362 negatively charged at neutral pH (the pK_a of diclofenac is 4.15) and therefore the
363 compound repels the negatively charged sludge surface. At lower pH values, on the
364 contrary, the equilibrium shifts towards the electronically neutral form, thus allowing
365 adsorption onto sludge (Vieno and Sillanpää, 2014). This phenomenon has been
366 reported in literature (Ternes et al., 2004). Nevertheless, biodegradation is still
367 considered as its main degradation pathway (Vieno and Sillanpää, 2014).

368 Erythromycin was removed between 1.7 and 77%. The circumstances under which it
369 was not removed were anoxic conditions, 12°C, 1 g/L MLSS and pH 7.5. On the contrary,
370 the highest removal was obtained under aerobic conditions, at high levels of
371 temperature, pH and MLSS. The most relevant parameter was the redox conditions,
372 followed by MLSS concentration and temperature, the three of which yielded higher
373 removals when operating at the higher level. On the contrary, the contribution of pH
374 was limited and the low level, corresponding to pH 6.5, gave better performances.
375 Erythromycin was generally found to be recalcitrant during biological treatment in
376 several studies conducted in real wastewater effluents (Guerra et al., 2014; Kim et al.,
377 2014; Pasquini et al., 2014; Yang et al., 2011) or even at higher concentrations in
378 effluents than in influents (Gusmaroli et al., 2019; Krzeminski et al., 2019; Verlicchi et
379 al., 2012). Nevertheless, wide ranges of variability, including removals up to 80%, are
380 documented in literature (Verlicchi et al., 2012). In a study carried out by Suarez et al.,
381 erythromycin was successfully removed ($\approx 90\%$) in aerobic reactors, while only 20%
382 removal occurred in anoxic reactors, thus hinting that nitrifying bacteria may have a
383 higher affinity to this antibiotic (Suarez et al., 2010).

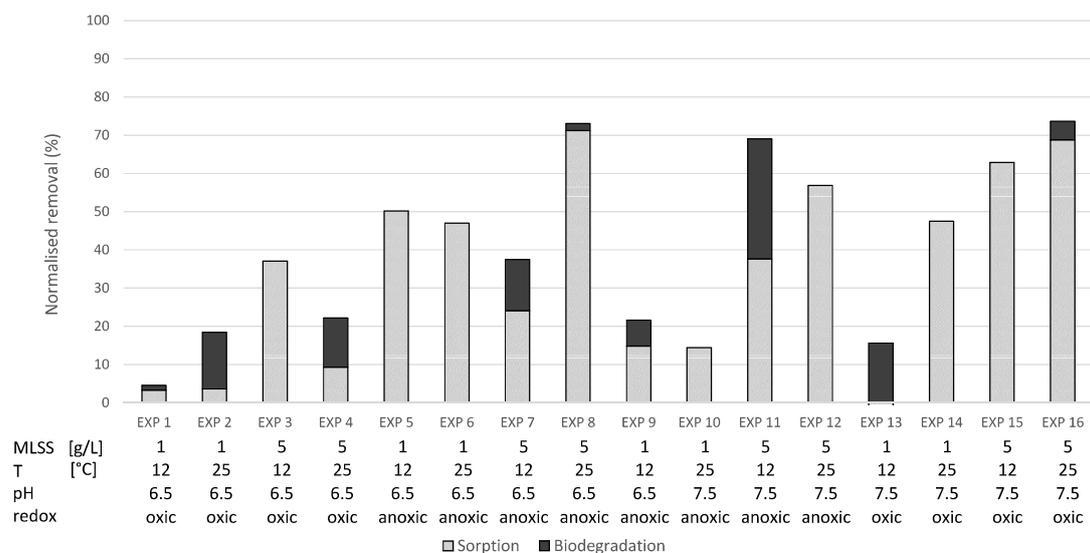
384 **3.3. Effect of experimental conditions on sorption**

385 Considering the whole set of studied micropollutants, removal by sorption was
386 generally low to medium, ranging from an average of 5% in the case of clothianidin to
387 the above-mentioned 34% for EE2 (see Figure 5). In a few cases, adsorption rates were
388 slightly higher than the total removal. This could be ascribed to the fact that the
389 conventional activated sludge was thermally treated for inactivation and this could
390 somewhat alter its properties. There is evidence that heat-inactivation of biomass
391 increases surface area by 6-25%, probably due to the loosening of the attachment

392 between extracellular polymeric substances and bacteria in the flocs (Racz et al., 2012).
393 After inactivation, the biomass is no longer biologically active and therefore the
394 decrease in micropollutants concentration is entirely ascribable to sorption.
395 Biodegradation was determined as the difference between total removal and sorption.
396 When considering the factors that have the most impact onto the extent of sorption,
397 the most important operational parameter proved to be the redox conditions. In fact,
398 working under aerobic conditions maximized sorption, as seen elsewhere (Suarez et al.,
399 2010). The second most relevant parameter was the MLSS concentration. As one could
400 easily predict, highest concentrations of biomass (5 g/L MLSS) gave better removals by
401 sorption, since the presence of more substrate offered more surface area for the
402 phenomenon to take place. The dependence of sorption on sludge concentration has
403 also been reported in previous studies (de Mes et al., 2005). These results might prove
404 useful when evaluating the parameters that maximise removal by sorption, as well as
405 for modelling purposes and the formulation of constants.

406 **3.4. Effect of the experimental conditions on sorption of single compounds**

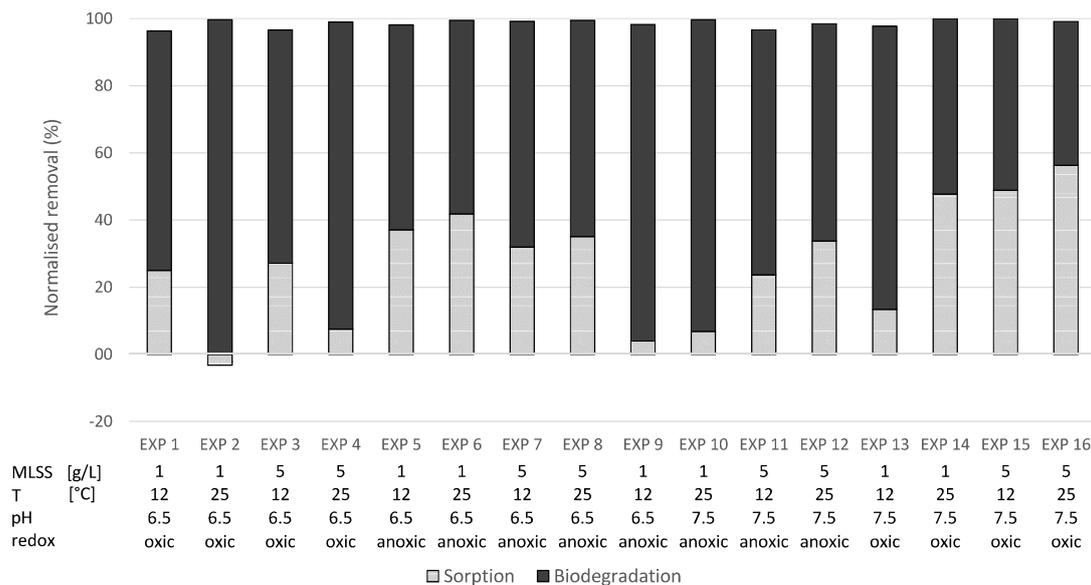
407 It is visible from Figure 3 that the highest sorption rates were achieved for the
408 hormones. EE2 was the compound with the highest average sorption rates over the 16
409 experiments, with a value of 34% in respect to the spiked concentration, and a
410 maximum of 71.2% (experiment 8), the second highest sorption rate among all
411 compounds and batch tests. Except when working at low temperature and
412 concentration of biomass, sorption accounted for the majority or even the entirety of
413 the removal for EE2, as shown in Figure 5, in accordance with literature data (Suarez et
414 al., 2010).



415

416 **Figure 5** - Removal profiles of EE2 throughout the experiments, detailing the
 417 contributions of sorption and biodegradation.

418 Working at higher temperatures increased the sorption of EE2, in contrast with previous
 419 findings (Feng et al., 2010). Since adsorption is an exothermic process, this could mean
 420 that the process involved is absorption instead, which is known to increase with the
 421 increase of temperature. Another factor suggesting such mechanism is the increase of
 422 sorption rates with the pH, indicating a lesser relevance of pKa in the sorption process
 423 when operating in this range of pH. On the contrary, its K_{ow} value of 3.67 - 4.15 (Hansch,
 424 C., Leo, A., Hoekman, 1995; Lai et al., 2000) indicates a medium-high absorption
 425 potential. However, further studies are necessary to confirm this hypothesis and, as
 426 mentioned in section 1, sometimes confusion arises from the misuse of terminology. E2
 427 and E1 were sorbed 27.3% and 17.4% on average, respectively. However, as shown in
 428 Figure 6, biodegradation accounts for most of E2 removal. The charts for the rest of
 429 compounds are displayed in SI.



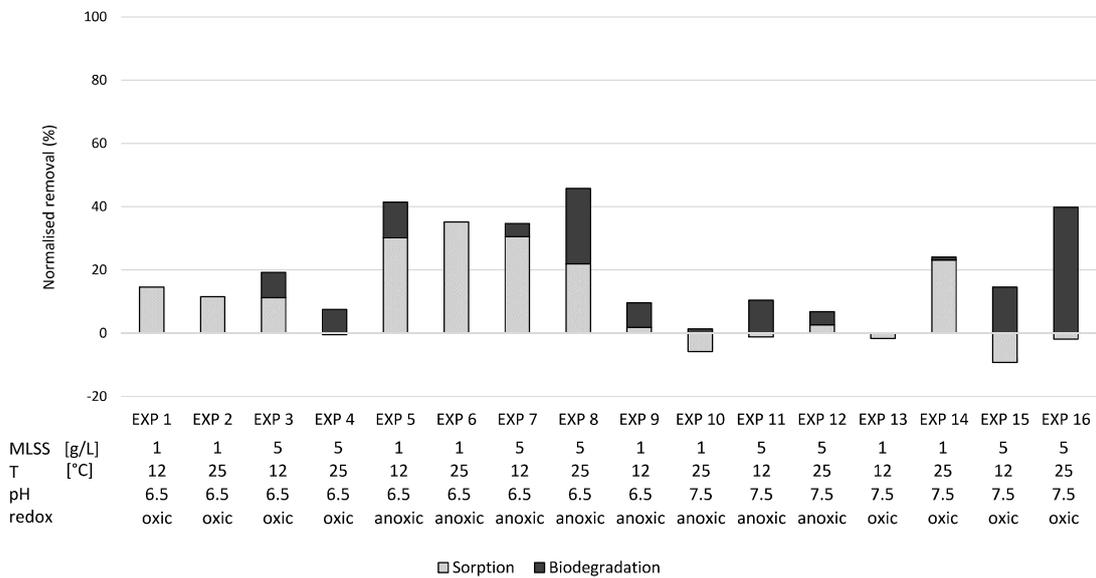
430

431 **Figure 6** - Removal profiles of E2 throughout the experiments, detailing the contributions
 432 of sorption and biodegradation.

433 It is noteworthy that the same order in sorption rates, that is $EE2 > E2 > E1$, was observed
 434 by Andersen and co-workers as well (Andersen et al., 2005) and reflects the decrease of
 435 their average K_{ow} values ($E2 K_{ow}$: 3.1 – 4.13 (Holthaus et al., 2002; Zorita et al., 2009); $E1$
 436 K_{ow} : 2.25 - 3.69 (Besha et al., 2017; Zorita et al., 2009)). Both for E2 and E1, the sorbed
 437 fraction was comprised between 30 and 50% of the total removal in almost all
 438 experiments. Previous studies dealing with sorption of hormones onto conventional
 439 activated sludge gave diverse and sometimes contradictory results (Hamid and
 440 Eskicioglu, 2012; Silva et al., 2012).

441 As for diclofenac, as commented in paragraph 3.2, adsorption was indeed relevant at
 442 pH 6.5, probably due to electrostatic interaction between protonated diclofenac and
 443 the negative sludge surface. In batch tests conducted under aerobic conditions at low
 444 pH, where the highest removal rates were attained, the contribution of sorption was
 445 between 48% and 101.9% of the whole removal rates (see **Figure 7**). This mechanism

446 was also hypothesised in other studies carried out in the same pH range (Ternes et al.,
 447 2004; Urase et al., 2005).



448

449 **Figure 7** - Removal profiles of diclofenac throughout the experiments, detailing the
 450 contributions of sorption and biodegradation.

451 The single highest sorption was recorded for erythromycin, 80.4%, accounting for
 452 approximately the whole removal attained in experiment 16 (25°C, 5 g/L MLSS, pH 7.5
 453 and under aerobic conditions, Figure 3. Nevertheless, its average sorption rate was
 454 19.4%. Medium sorption rates were obtained when operating at pH 6.5 and aerobic
 455 conditions, accounting for 100% of the removals achieved in those experiments.
 456 Although the overall removal rates increased at pH 7.5, sorption at this pH was
 457 generally negligible or low, with the exception of experiments 15 and 16 (5 g/L of MLSS,
 458 aerobic conditions and pH 7.5), where sorption rates of 36.1 and 80.4% were obtained,
 459 respectively, and accounted for the totality of the removal. Erythromycin has a low K_{ow}
 460 value and the relevant sorption mechanism is therefore supposed to be adsorption.
 461 Since its pKa is 8.9 (McFarland et al., 1997), erythromycin is predominantly in its
 462 protonated form at both working pH values (Wunder et al., 2011), prone to electrostatic

463 interactions with the negative charges on the sludge. At higher pH, the protonated
464 fraction starts decreasing, thus explaining the decrease in sorption rates. On the whole,
465 the sorption behaviour pattern of erythromycin is marked by inconsistent literature
466 data. For example, no biotransformation or sorption were reported from a WWTP in
467 France (Pasquini et al., 2014). On the contrary, in a study conducted in nitrifying and
468 denitrifying reactors, erythromycin removal was always complete, more than 80% of
469 which under aerobic conditions and less than 10% under anoxic conditions due to
470 sorption (Suarez et al., 2010).

471 Clothianidin, thiacloprid and methiocarb were marked both by negligible sorption rates,
472 ranging from 5.1 to 10.5%, and lack of literature data. In the only study assessing
473 clothianidin in a real-scale WWTP known to the authors, it was reportedly not sorbed to
474 primary sludge nor removed during secondary treatment (Sadaria et al., 2016).

475 **4. Conclusions**

476 Batch tests under different operational conditions were carried out in order to shed
477 light on the fate and behaviour of a set of eight micropollutants chosen from the EU
478 Watch list (Decision 2015/495 and 2018/840). Total removal as well as the role of
479 sorption were assessed. The chosen compounds are marked by inconsistency in
480 literature or, as in the case of pesticides, by lack of information on their biodegradation
481 and sorption in conventional activated sludge. Considering the totality of compounds,
482 the maximum removal was attained under aerobic conditions at high temperature (25
483 °C), high concentration of biomass (5 g/L) and high pH (7.5). The parameters with the
484 most influence on total removal proved to be the MLSS concentration and redox
485 conditions. The highest removal rates were recorded for E2, with a removal of at least

486 96% attained in all experiments. Clothianidin and thiacloprid proved recalcitrant. For
487 compounds such as E1, erythromycin and methiocarb, the ranges sweep from zero to
488 very high or complete removals, highlighting the importance of the choice of the right
489 operational parameters, since even minute variations produce a significantly different
490 response. EE2 exhibited the highest sorption rates (34% on average under all
491 conditions), followed by E2 and E1, which were nonetheless removed primarily via
492 biodegradation. Diclofenac was removed at a maximum rate of 45.7% and mostly
493 through adsorption, for which a strong dependence on pH was noticed. These results
494 highlight the importance of the choice of operational parameters during wastewater,
495 which have indeed a great impact on micropollutants removal. More studies, including
496 the evaluation of constants and modelling, are needed to shed light on the mechanisms
497 involved.

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