

26 **1. Introduction**

27 The rise in oil price, the fossil fuels depletion, and, even more markedly, the environmental
28 and climate problems associated with their combustion, are promoting the development of
29 renewable fuels. Among the different alternatives currently available, biodiesel highlights as
30 one of the most promising ones since it is biodegradable, less toxic than fossil fuels and
31 provides similar energy density than the mineral one, but improving its lubricating properties
32 (Revellame et al., 2010; Xue et al., 2006). In addition, its ignition point is considerable higher
33 than the diesel one, making it easy and safe to manipulate it (Anuar and Abdullah, 2016;
34 Shahid and Jamal, 2011).

35 Chemically, biodiesel is a mixture of monoalkyl esters of long chain fatty acids, commonly
36 called fatty acid methyl esters (FAME). Industrially, it is currently obtained by
37 transesterification of vegetable oils or animal fats with methanol, obtaining a product known
38 as “first generation” biofuel (Atabani et al., 2012; Shahid and Jamal, 2011). However, the
39 competitive potential of biodiesel is limited due to the high cost of these lipid feedstocks. This
40 fact, as well as ethical issues related to the competition between energy and food industry,
41 have triggered the search for inedible, inexpensive and, if possible, residual raw materials,
42 making up the “second generation” biodiesel (Hajjari et al., 2017). The use of oleaginous
43 microorganisms, those that accumulate lipid droplets in their cells, reaching dry lipid
44 percentages up to 25 % (Koutb and Morsy, 2011), is an attractive alternative. However, the
45 high consumption nutrients and the specific needs of their growth (light, temperature) can
46 discourage its cultivation for this specific aim. On the other hand, the microorganisms used in
47 biological treatments of a wastewater treatment plants (WWTPs) have relevant concentration
48 of triglycerides, and constitute the solid phase of sludge streams usually considered as a waste
49 (Kumar et al., 2016; Mondala et al., 2009).

50 Due to the urbanisation and industrialisation, quantities of sewage sludge produced increase
51 year on year, being considered as the main waste of these plants. It is forecasted that

52 approximately 13 million tonnes of sludge will be produced in the European Union in 2020
53 (Comission, 2010). Its treatment and disposal implies an important cost, in both, economic and
54 environmental terms (Dufreche et al., 2007). Therefore, sewage sludge is an available and
55 cheap feedstock that has attracted attention during the last decade (Dufreche et al., 2007;
56 Kumar et al., 2016; Olkiewicz et al., 2014). Particular characteristics of these sewage sludge
57 (high humidity, heterogeneous and few reproducible composition, etc.), makes difficult its fast
58 commercialisation, being no possible the direct application of conditions previously optimised
59 for the first generation biofuels. Thus, many efforts are nowadays focused on the study and
60 standardisation of this process.

61 In this context, the optimisation of lipid extraction is a major challenge that determines the
62 economy of the process (Kargbo, 2010). Thus, several researchers have proposed different
63 alternatives, such as the liquid-liquid extraction, the solid-liquid extraction and the *in situ*
64 transesterification (Dufreche et al., 2007; Kwon et al., 2012; Mondala et al., 2009; Olkiewicz et
65 al., 2014; Pokoo-Aikins et al., 2010; Revellame et al., 2010; Siddiquee and Rohani, 2011;
66 Willson et al., 2010). The two first ones, liquid-liquid and solid-liquid extractions, require the
67 use of organic solvents, without agreement about the optimum ones, although interesting
68 results using toluene, chloroform, hexane, methanol and ethanol are published (Dufreche et
69 al., 2007; Kwon et al., 2012; Pokoo-Aikins et al., 2010; Siddiquee and Rohani, 2011). However,
70 reported results are difficult to compare because many different conditions were tested and,
71 to the best of our knowledge, there is not a systematic study comparing the different available
72 techniques. Consequently, general conclusions are difficult to withdraw, being difficult to
73 predict the behaviour of other sludges.

74 As to the transesterification, acid catalysis is the most frequently used procedure, mainly using
75 sulphuric acid, obtaining higher biodiesel yields in comparison with results with basic catalyst
76 (Olkiewicz et al., 2016). Despite that classical transesterification of pure oils is industrially
77 carried out using basic materials, when the raw material is a waste, the presence of free fatty

78 acids in a basic medium promotes the saponification, obtaining a non-desired product that
79 hinders the separation and purification of the biodiesel fraction. Recent studies also propose
80 the enzymatic catalysis or the non-catalytic transesterification, when reaction is done under
81 subcritical conditions (Kwon et al., 2012; Pourzolfaghar et al., 2016).

82 Analysing all these previous results, one of the main conclusion is that acid transesterification
83 is very efficient, and results are mainly conditioned by the lipid extraction step. As
84 consequence, some authors propose different alternatives to enhance this step, being the
85 sonication one of the most promising pretreatment. Sonication technology is based on the
86 introduction of high intensity sound waves in the sludge, creating bubbles that implode,
87 breaking the cell walls and releasing the intracellular content, including the lipids, into the
88 medium. This technology has been previously used for obtaining biodiesel from algae or biogas
89 from sludge (Ruffino et al., 2015; Tran et al., 2012; Wolski, 2012). However, the few studies
90 applied to this aim are not conclusive enough (Olkiewicz et al., 2015; Olkiewicz et al., 2012)
91 Taking into account this entire context, biodiesel yields reported in the literature using
92 secondary sludge as raw material vary greatly from one study to another. Therefore, we
93 consider that a systematic comparison of the results obtained applying the three lipid
94 extraction techniques to a specific secondary sewage sludge is of key interest for both
95 understanding the process and being able to propose efficient technologies for this purpose .
96 Once the raw material is the same for all the treatments, and after the transesterification of
97 the obtained lipids, tracking down conclusions would be easy and useful.

98 The main aim of this work is to present a deep comparison among biodiesel yields obtained by
99 applying the three different techniques –liquid-liquid extraction, solid-liquid extraction, and *in*
100 *situ* transesterification –to the same type of secondary sludge. Three different raw materials
101 were used, from oxic, anoxic and anaerobic zone (sampled directly from the corresponding
102 reactor) and results were compared with those obtained from the floating sludge (common
103 pretreatment for these sludges). Industrially, only floating sludge adds up (taking samples

104 directly from the reactors before being concentrated by decantation or floating is economically
105 and technically unviable). However, the individual study of each sludge fraction allows
106 analysing if the sludge nature has any effect in the final efficiency, suggesting an independent
107 pre-concentration of the most interesting fraction to maximize the biodiesel yield. The effect
108 of catalyst concentration as well as the role of sludge pretreatment by sonication was also
109 analysed.

110

111 **2. Materials and Methods**

112 **2.1. Chemicals**

113 n-Hexane (97%) and sulphuric acid (96%) were purchased from Sigma-Aldrich. Sodium chloride
114 (99.5%) and methanol (≥ 99.8) were purchased from Panreac. A mixture of 37 reference fatty
115 acid methyl esters (FAMES) was supplied by Supelco (ref. 47885-U), and it was used for
116 identification and quantification purposes (in the GC-MS and GC-FID analyses).

117

118 **2.2. Sample collection and preparation**

119 Secondary sludge samples were collected from the municipal wastewater treatment plant
120 (WWTP) in Villapérez-Oviedo (Asturias, NW Spain). The block diagram of this plant, which has a
121 capacity to process 8500 L/s, is summarized in Figure 1, indicating the steps where the four
122 different types of secondary sludge (oxic, anoxic, anaerobic and floating ones) are sampled.
123 Considering the global process of this WWTP, primary sludge was discarded because of their
124 low potential capacity (this sludge mainly correspond to solid particles, inorganic chemicals
125 and free fatty acids that can suffer saponification). Samples were taken weekly during one
126 month (4 batches) and stored at 4°C prior to use.

127 The sludge from the oxic, anoxic and anaerobic zones were individually pre-treated following
128 with the aim to reduce the water content and to prepare the samples for the extraction and
129 transesterification. Sludges were settled for 24 h, after which the supernatant was removed.

130 The resulting sludge was centrifuged at 3000 rpm for 10 min using a Kubota 6500 centrifuge.
131 Dewatered sludge was dried at 100 °C for 24 h and the desiccated sludge was crushed into a
132 fine powder (with particle size ranging from 150 to 255 µm) in order to prepare a
133 homogeneous suspension for the following steps. These dried sludge samples were used to
134 the solid-liquid extraction and *in situ* transesterification studies. In the case of liquid-liquid
135 extraction, the sludges were only subjected to the settling process. This procedure has been
136 previously reported in the literature, observing a relevant decrease in water content (less than
137 5 % in the final sample) (Melero et al., 2015; Mondala et al., 2009). In the case of floating
138 sludge, and due to its low water content, the first step (settling) was not performed: they were
139 directly used for the liquid-liquid extraction and centrifuged, dried and crushed before the
140 solid-liquid extraction or *in situ* transesterification.
141 All the samples were characterised before and after the pretreatment, in order to analyse the
142 morphological changes introduced by the initial pre-processing. Total solid content (TS) was
143 analysed according to the standard method 2540G (Rice et al., 2012). Lipid contents were
144 measured by gas chromatography using a mass spectrometer detector (GC-MS) - in a Shimadzu
145 Q2010 Plus – after total extraction with chloroform, following the typical procedure reported
146 in the literature (Siddiquee and Rohani, 2011). Data reported in the *Results section*
147 corresponds to the average value obtained after two analyses with each sample, without
148 observing any variability among sludge from different batches. All the standard deviations are
149 included in the results, being in all the cases lower than 1 %.

150

151 **2.3. Liquid-liquid extraction**

152 Liquid-liquid technique consists of using an organic phase to extract the lipid phase from the
153 suspended flocs. Among the different solvents proposed in the literature, chloroform and
154 toluene were discarded because of environmental concerns (despite their high extraction
155 capacity). From the non-polar organic solvents, hexane was chosen based on economic and

156 technical reasons, having a low cost, high immiscibility with water and a high capacity to
157 extract non-polar saponifiable lipids, the base to obtain the biodiesel. This solvent is
158 extensively studied for these treatments (Melero et al., 2015; Olkiewicz et al., 2014; Siddiquee
159 and Rohani, 2011). In addition to hexane, other more polar organic mixture of solvents were
160 also proposed for the lipid extraction of secondary sewage, adding methanol or acetone to the
161 hexane, suggesting that the mixture of them helps to disrupt the lipid membrane of
162 microorganisms (Dufreche et al., 2007; Zhang et al., 2016; Zhu et al., 2012). However, the use
163 of these solvents was discarded because of the considerable decrease in the selectivity
164 towards saponifiable material reported in previous works (Dufreche et al., 2007).
165 According to the Scheme 2, in the case of liquid-liquid extraction, the sludges were used after
166 the sedimentation step, with the exception of floating sludge, which was used without any
167 previous pre-treatment. Sequential liquid-liquid extraction of lipids was performed in a
168 separatory funnel at ambient temperature using a hexane/sludge ratio of 2:1, according to the
169 best conditions determined by Dufreche and co-workers (Dufreche et al., 2007). Extraction was
170 repeated four times, mixing the organic phases obtained in each step. Hexane was removed
171 using a rotatory evaporator at 70 °C and the samples were dried at 105 °C for one hour and
172 stored in a desiccator. The lipids obtained were weighed in order to determine the extraction
173 yield based on the dried sludge used.

174

175 **2.4. Solid-liquid extraction**

176 Lipid extraction from dried sludge was performed using a Soxhlet apparatus using hexane as
177 solvent. Ethanol and methanol were also proposed for this aim, but they were discarded
178 because of their high polarity, which could enhance the extraction of non-saponifiable lipids,
179 increasing the purification costs of all the process (Pokoo-Aikins et al., 2010). The lipid
180 extraction was carried out with a hexane/dried sludge ratio 10:1 for four hours and 8-9
181 extraction cycles per hour, according to the procedure previously reported by Willson and

182 co-workers (Willson et al., 2010). After extraction, the hexane was removed following the
183 same procedure as in the liquid-liquid extraction, the final oil phase was weight, and the
184 extraction yield estimated based on the dried sludge used:

$$185 \quad \text{Lipids (\%)} = \frac{\text{Oil phase weight (g)}}{\text{Dry sludge weight (g)}} \cdot 100$$

186

187 **2.5. Lipids transesterification and FAMES analysis**

188 Biodiesel production from extracted lipids was carried out following the modified Christie's
189 method through acid catalysis (Christie, 2003). 20 mg of lipids were dissolved in 1 mL of
190 hexane and 2 mL of sulphuric acid in methanol (1% v/v). Resulting mixture was heated at 55 °C
191 for 24 h, after which 5 ml of 5 % sodium chloride were added and the FAMES were extracted
192 twice with 5 mL of hexane.

193 FAME analysis was carried out using a Shimadzu gas chromatography 2010 equipped with a
194 flame ionization detector (GC-FID) and a capillary column CP-Sil 8CB (30 m x 0.25 mm x
195 0.25µm), using helium as carrier gas. 1 µL of sample was injected at 260 °C, with a split ratio
196 equal to 30. The FID detector was set at 260 °C. The GC oven temperature program was: 100
197 °C as initial temperature for five minutes, increased to 240 °C at slope of 4 °C/min, and held at
198 this temperature for 30 min. A mixture of 37 reference fatty acid methyl esters (FAMES) was
199 used for instrument calibration and the qualitative correspondence between standards and
200 experimental samples was corroborated by GC-MS using a Shimadzu QP2010 Plus, using the
201 same column and analysis method than in the GC-FID.

202

203 **2.6. *In situ* transesterification**

204 Reaction were carried out based on the procedure proposed by Revellame and co-workers
205 (Revellame et al., 2010). 1.5 g of dried sludge were mixed with 37.5 mL of sulphuric acid in
206 methanol (4% v/v) and the mixture was introduced into sealed glass reactions vials. The
207 mixture was kept at 55 °C in a stirring bath (100 oscillations per minute –opm-) to ensure the

208 total suspension of the solids during the reaction. After 24 hours at these conditions, and once
209 the sample reach ambient temperature, it was centrifuged at 3000 rpm for 5 min. The
210 supernatant was recovered and the solid residue was re-suspended in 5 mL of methanol and
211 centrifuged at 3000 rpm for 5 min. The volume of both supernatants was reduced to a final
212 volume of around 9 mL using a rotary evaporator. Finally, the FAMEs were extracted four
213 times with 5 mL of hexane, which was removed using a rotatory evaporator at 70 °C. Samples
214 were analysed by GC-FID, using the same procedure explained for the other
215 transesterifications.

216

217 **3. Results and Discussion**

218 **3.1. Sludge characterization**

219 Characterization results are summarized in Table 1. It must be noted that samples from oxic,
220 anoxic and anaerobic areas are taken directly from the reactor, and no after any settling
221 process. In good agreement with the suspended solids concentrations at which these stages
222 are configured, total solids (TS) values are very similar in all the cases, with concentrations
223 around 0.3 %. On the contrary, sludge obtained after the floating treatment is almost nine
224 times more concentrated, reaching the typical value of sludge streams after this kind of
225 treatments (around 3 %). These differences in the TS are less remarkable after pretreatment,
226 reaching values higher than 4 % in all the cases and obtaining the maximum with the floating
227 sludge, despite of the absence of decantation step (5.4 %).

228 Concerning to the lipids content, results obtained after total extraction using chloroform as
229 solvent, and following the procedure previously explained, indicate that sludge from the
230 floating sludge zone achieved the greatest lipid content (19.4%), whereas the minimum
231 amount was detected in the analysis of the anaerobic region (16.2%). In any case, the
232 differences were not very significant, with fluctuations close to 3%. It must be remarked than
233 total lipid content cannot completely transformed into FAMEs-biodiesel, because it consists

234 not only of acyl-glycerols, free fatty acids and some waxes (saponifiable lipids), but also of
235 many other types of sterols, alkyl benzenes, etc. that are not suitable for biodiesel (Jardé et al.,
236 2005; Pastore et al., 2013). This fact introduces the concept of saponifiable lipids. This
237 parameter is indirectly calculated based on the final amounts of FAME produced when the
238 100 % of conversion is ensured. As different conversions were obtained by each technique, a
239 previous value cannot be estimated.

240

241 **3.2. Comparison of biodiesel production by liquid-liquid, solid-liquid and *in situ* techniques**

242 Preliminary analyses were carried out in order to identify the most effective technique to
243 maximize the biodiesel yield from each fraction of sludge. As it was mentioned in the
244 introduction section, there is not good agreement about the optimum procedure, suggesting
245 that the kind of sludge plays a key role in the final yields.

246 In a first approach, the liquid-liquid lipid extraction was considered, using hexane as organic
247 solvent. Considering that liquid-liquid technique is a two-step process, results from the
248 extraction and after the transesterification can be reported separately. Thus, preliminary
249 results obtained after four consecutive cycles of one hour of extraction at room temperature
250 are summarized in Table 2. As it is observed, very low lipid percentages were obtained,
251 conditioning the results of the subsequent transesterification step. In good agreement, only a
252 small amount of FAMEs was detected when floating sludge was used as raw material (0.86 %
253 of FAMEs yield). Despite that previous results in the literature are not conclusive, these values
254 are far from the optimum ones reported in previous papers. One of the highest value using
255 same procedure is reported by *Olkiewicz and co-workers*, reaching almost 27 % of lipids
256 (*Olkiewicz et al.*, 2014). However, it must be taken into account that these authors consider
257 primary sludge as raw material. The higher amount of free lipids in this kind of sewage makes
258 easier the lipid extraction, whereas the extraction of lipids from a cell membrane is more
259 difficult. In good agreement, results reported by *Dufreche and co-workers* suggest, for similar

260 type of sludge, a maximum extraction using hexane of 1.94 % (Dufreche et al., 2007). This
261 result is obtained working with a solvent/solid ratio of 40:1 at 100 °C, considerable more
262 severe conditions in comparison with the 2:1 ratio and room temperature used for this work.
263 Solid-liquid extraction was also studied, considering extraction for four hours in hexane once
264 the sludge was previously dried. These preliminary results are also reported in Table 2,
265 indicating the amount of lipids obtained with each kind of sewage and the percentage of
266 FAMEs after the transesterification step. A clear improvement is observed in all the cases,
267 highlighting the values obtained with the floating sludge (more than one order of magnitude in
268 all the cases). Besides, results obtained with the floating sludge and the solid-liquid extraction
269 method are comparable with those previously reported in the literature by *Dufreche and co-*
270 *workers* (Dufreche et al., 2007), with a lipid yield after extraction with hexane around 1.9%.
271 These results are justified because the mass transfer is improved by this technique by two
272 complementary effects: on the one hand, the huge amount of cycles maximize the contact
273 between solvent and sludge; on the other hand, the high temperature enhances the lipid
274 extraction (Dufreche et al., 2007). Very similar results were obtained for the lipid extraction
275 step when using sewage from oxic and anaerobic areas, (0.9 and 1.0 %, respectively). The
276 higher extraction obtained with the anoxic sewage is explained by the expected higher amount
277 of free fatty acids (FFAs) present in this area, reaching an extraction level comparable with the
278 floating sludge (1.6 and 1.8 %, respectively).

279 However, results obtained after the transesterification step (a relevant high FAME
280 concentration with the anaerobic sample despite of the lower lipid extraction) suggest a
281 relevant difference between lipids obtained from each fraction. Considering that all the values
282 correspond to the same reaction conditions (24 h, 1 mL of methanol, 2 mL of sulphuric acid),
283 the difference lower than 0.7 % in the lipid yield between anaerobic and floating sludge does
284 not justify the relative increase of more than 48 % in the FAMEs yield (15.9 and 23.6 %, with
285 anaerobic and floating sludge, respectively).

286 For understanding these differences, FAME profile of the biodiesel produced was analysed,
287 obtaining the distribution shown in Figure 2a. In general, the esters of acids with 16 and 18
288 carbon atoms predominate regardless of the fraction studied, in good agreement with the
289 distribution reported for other authors (Mondala et al., 2009; Olkiewicz et al., 2014; Revellame
290 et al., 2010; Siddiquee and Rohani, 2011). According to the identification, main fractions
291 correspond to palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0) and oleic acids (C18:1),
292 with less than 3 % of other polyunsaturated fatty acids. This fact represents an advantage over
293 the use of vegetable-based raw materials, since these compounds are very vulnerable to
294 autoxidation, decreasing the oxidative stability of biodiesel (Saluja et al., 2016). If the
295 distribution among results obtained with different sewage fractions is compared, the C16/C18
296 proportion (considering all the acids together) is almost constant with all the fractions (from
297 1.05 to 1.15) observing a slightly majority of the shortest ones. The exception is the case of the
298 anaerobic zone, with a prevalence of longer FAMES (C16/C18 ratio of 0.9). On the other hand,
299 if all the esters are analysed as function of their unsaturated/saturated character, similar
300 results were obtained for all the sludge fractions (45% of unsaturated and 55 % of saturated),
301 except with the sewage from the anaerobic region, with more than 60 % of saturated
302 compounds. These results suggest that saturated lipids are easier to transform into FAMES,
303 having a more saponifiable character. In fact, considering that no time evolution was observed
304 after 24 hours, complete conversion of all the saponifiable lipids is supposed and the
305 percentage of these lipids can be calculated based on the following expression:

306
$$Saponifiable (\%) = \frac{FAMES (g)}{Lipid (g)} \cdot 100$$

307 Experimental results are congruent with a percentage of saponifiable lipids in the dry sludge
308 close to 0.2 % in the case of anaerobic sludge, 0.1% for the anoxic ones and 0.05 % for the
309 aerobic sewage; in the same order as the amount of FAMES obtained. This sequence is in good
310 agreement with previous results reported by Olkiewicz and co-workers (Olkiewicz et al., 2012).

311 A study of the influence of extraction time and the amount of lipid obtained was carried out
312 trying to optimise the conditions to maximise this yield. Different extraction times were
313 considered, from 1 to 8 hours, being the final results obtained plotted in Figure 3. This study
314 was only carried out with the floating sludge because of their more promising results obtained
315 in the preliminary tests. As it was previously observed in similar studies (Olkiewicz et al., 2014),
316 the yield of lipids fast decreases when working with extraction times shorter than 4 hours,
317 whereas longer times do not mean any relevant improvement in the total yield of lipid
318 extracted. Considering that the maximum amount of lipids of this raw material is 19.6 %
319 (previously determined by chloroform extraction), it can be concluded that using hexane as
320 extraction solvent only around the 9 % of the total lipids can be extracted (1.75 g lipids / 100 g
321 dry sludge). Results of transesterification step are congruent with the evolution of lipids
322 extracted with the time, obtaining an increasing trend with a maximum production of 0.4 g
323 FAMES / 100 g dry sludge, which corresponds to the transesterification of 26.8 % of the total
324 lipid extracted. These results were obtained after 24 h of reaction, but the temporal profile
325 (almost flat in the last hours) discards any relevant effect of longer reaction times.

326 The last technique studied is the *in situ* extraction and transesterification, using sulphuric acid
327 in methanol solvent (4 % v/v). Taking into account that *in situ* procedure does not distinguish
328 between extraction and transesterification steps, results directly show the final FAME
329 concentration obtained as function of the sludge fraction used. There is a relevant
330 improvement in the final yield ($wt_{FAMES}/wt_{dry\ sludge}$) obtained with all the fractions, reaching a
331 minimum of 1.7 % with sludge from oxic area and a maximum of 2.1 % for floating sludge (in
332 comparison with the 0.4 % obtained at optimum conditions with the solid-liquid procedure).
333 These results are obtained with 4% of acid catalyst, being congruent with the typical values
334 reported in the literature for this type of raw material. With the aim to identify if these
335 conditions are the optimal, the effect of catalyst loading was studied, in the range of 1 to 8 %,
336 being the results plotted in Figure 4. As in the previous case, only floating sludge were

337 considered for this study because of their preliminary results at a fixed concentration. As it can
338 be observed, there is an exponential relation between the acid catalyst and the final yield
339 obtained when this concentration is lower than 4 %, whereas values higher than 5 % do not
340 have any relevant effect. This behaviour, previously observed in the literature by several
341 authors, (Dufreche et al., 2007; Mondala et al., 2009; Olkiewicz et al., 2014) (Revellame et al.,
342 2010), was justified by the secondary reactions (formation of estolides, polymers of fatty acid
343 esters) that are promoted by high temperatures or strong acid conditions. So, 4 % of sulphuric
344 acid is defined as the optimum one and, after 24 h of reaction, 2.1 % of FAME yield was
345 obtained. This result corresponds to a global extraction and transesterification of more than
346 10.7 % of the total lipid content of these floating sludges.

347 In order to compare the potential quality of biodiesel obtained by solid-liquid and *in situ*
348 procedures, the composition of the fatty acid obtained is plotted in Figure 2b. As it could be
349 expected, similar carbon range were obtained, but with significant differences between these
350 FAMES and those obtained using solid-liquid method. After the *in situ* treatment, a higher
351 concentration of unsaturated FAME is detected, in good agreement with the polar character of
352 methanol (Dufreche et al., 2007; Willson et al., 2010). Globally, more than 66 % of unsaturated
353 compounds were obtained in all the cases after the *in situ* treatment (mainly
354 monounsaturated), being the highest percentage obtained with the floating sludge. On the
355 contrary, this value was lower than 55 % with the solid-liquid procedure. It is reported that an
356 excess of saturated FAMES has a negative effect on the global quality of fuel obtained. Thus,
357 when this amount exceeds 60 %, the biodiesel has a bad behaviour at low temperatures, being
358 able to form crystals from the solidification of the saturated components (Olkiewicz et al.,
359 2014). Other relevant conclusion of these analyses is the similar distribution of acids obtained
360 despite the type of sludge; suggesting that this methodology is more robust and profile
361 obtained is less conditioned by the nature of the initial raw material .

362 According to all these results, the *in situ* process is chosen as the optimum one, obtaining not
363 only the highest conversion (more than 5 times higher than the maximum one obtained with
364 the solid-liquid extraction) but also the highest FAME quality and more reproducible results.
365 However, results obtained with the solid-liquid technique cannot be discarded, so this
366 procedure is also considered in the following experiments, with the aim of identify if final
367 yields can be improved by the pretreatment of the initial samples.

368

369 **3.3. Effect of sludge pretreatment on the final FAME yield**

370 Considering that previous results suggest that the limiting step is the lipid extraction,
371 pretreatment of initial samples was considered, with the aim to break the complex structure of
372 microorganisms (mainly the cell wall) and make easier the contact between solvent and lipids.
373 Despite the scarce references to these procedures, with only some studies carried out by
374 Olkiewicz (Olkiewicz et al., 2015), sonication was chosen as pretreatment technique because of
375 the promising results reported for the lipid extraction from microalgae (Dong et al., 2014;
376 Gerde et al., 2012; Lee et al., 2010; Ranjan et al., 2010). Thus, the effect of sonication was
377 studied for the floating sludge fraction (best results in the preliminary analyses), using a
378 resonance of 10 kHz and analysing the influence of time from 10 to 120 minutes. Results are
379 reported in Figure 5, plotting the final FAMEs yields obtained after the solid-liquid and *in situ*
380 processes.

381 According to Figure 5, maximum FAMEs yield was reached after 50 minutes of sonication, not
382 observing any relevant improvement at longer pretreatment times. These results suggest that
383 the maximum cell fragmentation at these conditions is reached after this time, so the
384 maximum amount of accessible lipids is exposed to the solvent and catalyst. In good
385 agreement, results after transesterification improve more than 65 % respect to the
386 corresponding data without pretreatment: from 0.4 to 0.7 % with the solid-liquid phase, and
387 from 2.1 to 3.46 % with the *in situ* method. This improvement confirms that the extraction is

388 the limiting step of the process and suggests that this pretreatment makes accessible some
389 lipids that cannot be extracted even using a polar solvent (without pretreatment).
390 Previous data are analysed after 24 h reaction time, in both cases. However, if the temporal
391 evolution is compared, the key role of this pretreatment is more evident. Thus, as example,
392 the temporal evolution with and without pretreatment and applying the *in situ* method is
393 plotted in Figure 6. The effect of sonication is clearly observed since the first moments,
394 observing an exponential trend that reaches the maximum after only 16 h (instead of the 24 h
395 needed to reach the maximum in absence of sonication).

396

397 **3.4. Kinetic study**

398 As it was previously mentioned, results of the previous sections were analysed at the end of
399 each method, to make easier the comparison. However, in the case of “in situ”
400 transesterification, samples were analysed during all the process, obtaining the FAMES’
401 temporal evolution. The pseudo-first order dependence on reaction time, as well as the same
402 asymptotic behaviour for the experiments performed in absence and in presence of
403 sonication, and even the improvement detected for the sonicated samples, suggest that the
404 *in situ* transesterification is controlled by mass transfer instead of the chemical reaction. In the
405 same way, no lipids were detected in the liquid phase, also suggesting that once the lipid is
406 transferred to the liquid phase, it is transformed into FAMES. Therefore, it is possible to
407 assume that the asymptotic FAME yield reached in both cases (Fig. 6) correspond to the total
408 concentration of saponifiable lipids (1.5 g saponifiable TG/100 g dry sludge; 4.23 g FAME/100 g
409 dry sludge). Under the mass transfer control assumption and considering the vessel as a
410 stirred batch reactor, the unsteady state mass balance to the triglycerides follows the resulting
411 equation:

$$412 \quad -\frac{dX_{TG,S}}{dt} = k_{SL} \cdot a_p \cdot (X_{TG,S} - K_{eq,TG} \cdot C_{TG,L}) \quad [1]$$

413 Where $X_{TG,s}$ is the concentration of triglycerides in the solid phase (mg of TG per mg of dry
414 solid), and $C_{TG,L}$ their concentration in the liquid phase, whereas $k_{S,L}$ is the solid-liquid mass
415 transfer coefficient, " a_p " is the interfacial area per volume, and $K_{eq,TG}$ is the equilibrium
416 constant for the distribution of the TG between the sludge and the liquid. Considering that the
417 reaction is very fast, as evidenced the above-mentioned analysis, the concentration of
418 triglycerides in the liquid phase is negligible.

419 An integration of the equation [1] under these assumptions, will lead to the following
420 expression:

$$421 \quad \ln \left[\frac{X_{TG,s}}{X_{TG,s,0}} \right] = -k_{SL} \cdot a_p \cdot t \quad [2]$$

422 In this expression, the denominator corresponds to the initial concentration of saponifiable
423 lipids in the sludge, considering that both curves trend to an asymptotic value, this parameter
424 can be calculated from the final concentration of FAME in the liquid phase (1.5 g TG
425 saponifiable/100 g dry sludge).

426 The solid-liquid interfacial area per unit volume of solid is determined using the following
427 expression, where " C_v " is the volume fraction of solids in the slurry and " d_{32} " is the Sauter-
428 mean particle diameter determined by a Zetasizer nano Instrument (Malvern Instruments Ltd.,
429 UK).

$$430 \quad a_p = \frac{6 \cdot C_v}{d_{32}}$$

431 C_v parameter was determined to be 0.09, whereas the d_{32} particle diameter was 170 μm for
432 the floating sludge. It must be remarkable that no significant changes in these parameters
433 were observed comparing the samples before and after the reaction, so the a_p coefficient was
434 considered as constant with a value of 3176.5 m^{-1} .

435 The Scientist® software was used to fit the experimental results to the proposed mechanism,
436 obtaining apparent constant values of 0.037 and 0.12 h^{-1} , for reaction without and with

437 pretreatment, respectively. These results correspond to $k_{s,L}$ values of $1.2 \cdot 10^{-5}$ and $3.8 \cdot 10^{-5}$ m/s,
438 respectively. These values are congruent with the range of values defined in the literatures as
439 typical of processes limited by the mass transfer between solid particles and the liquid phase .
440 (Levenspiel, 1972). In both cases, the correlation index was higher than 0.995, indicating a
441 great correspondence between experimental and fitted data, as it can be observed in Fig 6 as
442 dotted lines. As it was expected, the improvement of sonication is clearly observed in the mass
443 transfer constant values, with a value almost 50 % higher under sonication.

444

445 **4. Conclusions**

446 Promising results were obtained with floating sludge as raw material to obtain biodiesel by *in*
447 *situ* procedure. Liquid-liquid procedure was discarded because of its low efficiency whereas
448 the solid-liquid approach is conditioned by a lower quality of FAMEs obtained. Once reaction
449 was optimized, 2.1 % of FAMEs yield was obtained after 24 h by *in situ* reaction with 4 % of
450 H_2SO_4 . This result increase considerably by using sonication as pretreatment, obtaining a final
451 yield of 3.5 % after 16 h. Results are congruent with a model limited by the mass transfer of
452 lipids from the sludge particle to the liquid, being congruent with the highlighted improvement
453 observed when using sonication as pretreatment..

454

455

456 **Acknowledgements**

457 Authors acknowledge Eva M. Álvarez (Dragados) for providing us the required samples from
458 the Wastewater Treatment Plant (Villaperez-Asturias). This research has been financed by the
459 COGERSA-IDEPA (AsturSludge Project) and the Principality of Asturias (GRUPIN14-078)

460

461

462 **References**

- 463 1. Anuar, M.R., Abdullah, A.Z. 2016. Challenges in biodiesel industry with regards
464 to feedstock, environmental, social and sustainability issues: A critical review. *Renew.*
465 *Sust. Energ. Rev.*, 58, 208-223.
- 466 2. Atabani, A.E., Silitonga, A.S., Badruddin, I.A., Mahlia, T.M.I., Masjuki, H.H.,
467 Mekhilef, S. 2012. A comprehensive review on biodiesel as an alternative energy
468 resource and its characteristics. *Renew. Sust. Energ. Rev.*, 16(4), 2070-2093.
- 469 3. Bong, E.Y., Eshtiaghi, N., Wu, J., Parthasarathy, R. 2015. Optimum solids
470 concentration for solids suspension and solid–liquid mass transfer in agitated vessels.
471 *Chem. Eng. Res. Des.*, 100, 148-156.
- 472 4. Comission, E. 2010. Environmental, economic and social impacts of the use of
473 sewage sludge on land.
- 474 5. Christie, W.W. 2003. *Lipid Analysis*. Third edition ed. The Oily Press, Bridgwater.
- 475 6. Dong, S., Huang, Y., Zhang, R., Wang, S., Liu, Y. 2014. Four Different Methods
476 Comparison for Extraction of Astaxanthin from Green Alga *Haematococcus pluvialis*.
477 *Sci. World J.*, 2014, 7.
- 478 7. Dufreche, S., Hernandez, R., French, T., Sparks, D., Zappi, M., Alley, E. 2007.
479 Extraction of Lipids from Municipal Wastewater Plant Microorganisms for Production
480 of Biodiesel. *J. Am. Oil Chem. Soc.*, 84(2), 181-187.
- 481 8. Gerde, J.A., Montalbo-Lomboy, M., Yao, L., Grewell, D., Wang, T. 2012.
482 Evaluation of microalgae cell disruption by ultrasonic treatment. *Bioresour. Technol.*,
483 125, 175-181.

- 484 9. Hajjari, M., Tabatabaei, M., Aghbashlo, M., Ghanavati, H. 2017. A review on the
485 prospects of sustainable biodiesel production: A global scenario with an emphasis on
486 waste-oil biodiesel utilization. *Renew. Sust. Energ. Rev.*, 72, 445-464.
- 487 10. Jardé, E., Mansuy, L., Faure, P. 2005. Organic markers in the lipidic fraction of
488 sewage sludges. *Water Res.*, 39(7), 1215-1232.
- 489 11. Kargbo, D.M. 2010. Biodiesel Production from Municipal Sewage Sludges.
490 *Energy Fuels*, 24(5), 2791-2794.
- 491 12. Koutb, M., Morsy, F.M. 2011. A potent lipid producing isolate of *Epicoccum*
492 *purpurascens* AUMC5615 and its promising use for biodiesel production. *Biomass*
493 *Bioenergy*, 35(7), 3182-3187.
- 494 13. Kumar, M., Ghosh, P., Khosla, K., Thakur, I.S. 2016. Biodiesel production from
495 municipal secondary sludge. *Bioresour. Technol.*, 216, 165-171.
- 496 14. Kwon, E.E., Kim, S., Jeon, Y.J., Yi, H. 2012. Biodiesel production from sewage
497 sludge: new paradigm for mining energy from municipal hazardous material. *Environ.*
498 *Sci. Technol.*, 46(18), 10222-8.
- 499 15. Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., Oh, H.-M. 2010. Comparison of several
500 methods for effective lipid extraction from microalgae. *Bioresour. Technol.*, 101(1,
501 Supplement), S75-S77.
- 502 16. Levenspiel, O. 1972. *Chemical reaction engineering*. Wiley.
- 503 17. Melero, J.A., Sánchez-Vázquez, R., Vasiliadou, I.A., Martínez Castillejo, F.,
504 Bautista, L.F., Iglesias, J., Morales, G., Molina, R. 2015. Municipal sewage sludge to

505 biodiesel by simultaneous extraction and conversion of lipids. *Energ. Convers.*
506 *Manage.*, 103, 111-118.

507 18. Mondala, A., Liang, K., Toghiani, H., Hernandez, R., French, T. 2009. Biodiesel
508 production by in situ transesterification of municipal primary and secondary sludges.
509 *Bioresour. Technol.*, 100(3), 1203-1210.

510 19. Olkiewicz, M., Caporgno, M.P., Fortuny, A., Stüber, F., Fabregat, A., Font, J.,
511 Bengoa, C. 2014. Direct liquid–liquid extraction of lipid from municipal sewage sludge
512 for biodiesel production. *Fuel Process. Technol.*, 128, 331-338.

513 20. Olkiewicz, M., Fortuny, A., Stüber, F., Fabregat, A., Font, J., Bengoa, C. 2015.
514 Effects of pre-treatments on the lipid extraction and biodiesel production from
515 municipal WWTP sludge. *Fuel*, 141, 250-257.

516 21. Olkiewicz, M., Fortuny, A., Stüber, F., Fabregat, A., Font, J., Bengoa, C. 2012.
517 Evaluation of Different Sludges from WWTP as a Potential Source for Biodiesel
518 Production. *Procedia Eng.*, 42, 634-643.

519 22. Olkiewicz, M., Plechkova, N.V., Earle, M.J., Fabregat, A., Stüber, F., Fortuny, A.,
520 Font, J., Bengoa, C. 2016. Biodiesel production from sewage sludge lipids catalysed by
521 Brønsted acidic ionic liquids. *Appl. Catal. B: Environ.*, 181, 738-746.

522 23. Pastore, C., Lopez, A., Lotito, V., Mascolo, G. 2013. Biodiesel from dewatered
523 wastewater sludge: a two-step process for a more advantageous production.
524 *Chemosphere*, 92(6), 667-73.





- 525 24. Pokoo-Aikins, G., Heath, A., Mentzer, R.A., Sam Mannan, M., Rogers, W.J., El-
526 Halwagi, M.M. 2010. A multi-criteria approach to screening alternatives for converting
527 sewage sludge to biodiesel. *J. Loss Prev. Process Ind.*, 23(3), 412-420.
- 528 25. Pourzolfaghar, H., Abnisa, F., Daud, W.M.A.W., Aroua, M.K. 2016. A review of
529 the enzymatic hydroesterification process for biodiesel production. *Renew. Sust.*
530 *Energ. Rev.*, 61, 245-257.
- 531 26. Ranjan, A., Patil, C., Moholkar, V.S. 2010. Mechanistic Assessment of Microalgal
532 Lipid Extraction. *Ind. Eng. Chem. Res.*, 49(6), 2979-2985.
- 533 27. Revellame, E., Hernandez, R., French, W., Holmes, W., Alley, E. 2010. Biodiesel
534 from activated sludge through in situ transesterification. *J. Chem. Technol. Biotechnol.*,
535 85(5), 614-620.
- 536 28. Rice, E.W., Eaton, R.B.B., Clesceri A.D. L.S.. 2012. Standard methods for the
537 examination of water and wastewater 22nd, Washington.
- 538 29. Ruffino, B., Campo, G., Genon, G., Lorenzi, E., Novarino, D., Scibilia, G., Zanetti,
539 M. 2015. Improvement of anaerobic digestion of sewage sludge in a wastewater
540 treatment plant by means of mechanical and thermal pre-treatments: Performance,
541 energy and economical assessment. *Bioresour. Technol.*, 175, 298-308.
- 542 30. Saluja, R.K., Kumar, V., Sham, R. 2016. Stability of biodiesel – A review. *Renew.*
543 *Sust. Energ. Rev.*, 62, 866-881.
- 544 31. Shahid, E.M., Jamal, Y. 2011. Production of biodiesel: A technical review.
545 *Renew. Sust. Energ. Rev.*, 15(9), 4732-4745.

- 546 32. Siddiquee, M.N., Rohani, S. 2011. Experimental analysis of lipid extraction and
547 biodiesel production from wastewater sludge. *Fuel Process. Technol.*, 92(12), 2241-
548 2251.
- 549 33. Tran, D.-T., Yeh, K.-L., Chen, C.-L., Chang, J.-S. 2012. Enzymatic
550 transesterification of microalgal oil from *Chlorella vulgaris* ESP-31 for biodiesel
551 synthesis using immobilized *Burkholderia* lipase. *Bioresour. Technol.*, 108, 119-127.
- 552 34. Willson, R.M., Wiesman, Z., Brenner, A. 2010. Analyzing alternative bio-waste
553 feedstocks for potential biodiesel production using time domain (TD)-NMR. *Waste*
554 *Manag.*, 30(10), 1881-1888.
- 555 35. Wolski, P.Z., I. (2012). Effect of Ultrasound Field on Dewatering of Sewage
556 Sludge. *Archives of Environmental Protection*, 38(2), pp. 25-31. Retrieved 16 Apr. 2017,
557 from doi:10.2478/v10265-012-0015-7.
- 558 36. Xue, F., Zhang, X., Luo, H., Tan, T. 2006. A new method for preparing raw
559 material for biodiesel production. *Process Biochem.*, 41(7), 1699-1702.
- 560 37. Zhang, X., Yan, S., Tyagi, R.D., Drogui, P., Surampalli, R.Y. 2016. Ultrasonication
561 aided biodiesel production from one-step and two-step transesterification of sludge
562 derived lipid. *Energy*, 94, 401-408.
- 563 38. Zhu, F., Zhao, L., Zhang, Z., Jiang, H. 2012. Preliminary Study at Lipids Extraction
564 Technology from Municipal Sludge by Organic Solvent. *Procedia Environ. Sci.*, 16, 352-
565 356.
- 566
- 567

568 **Figure captions**

569 **Figure 1.** Process of urban wastewater treatment of the WWTP of Villapérez (Oviedo, Spain)

570 **Figure 2.** FAME composition of the biodiesel produced by (a) solid-liquid extraction method

571 and (b) *in situ* transesterification  Oxicsludge,  anoxic sludge,  anaerobic sludge, 

572 Floating sludge

573 **Figure 3.** Process yields at different extraction times using floating sludge as raw material by

574 solid-liquid extraction. Results corresponding to the (a) extraction step and (b)

575 transesterification process

576 **Figure 4.** Effect of catalyst concentration on FAMES yield by *in situ* transesterification with

577 floating sludge as raw material

578 **Figure 5.** Effect of sonication time on FAMES yield, ● Solid-liquid extraction and ■ *in situ*

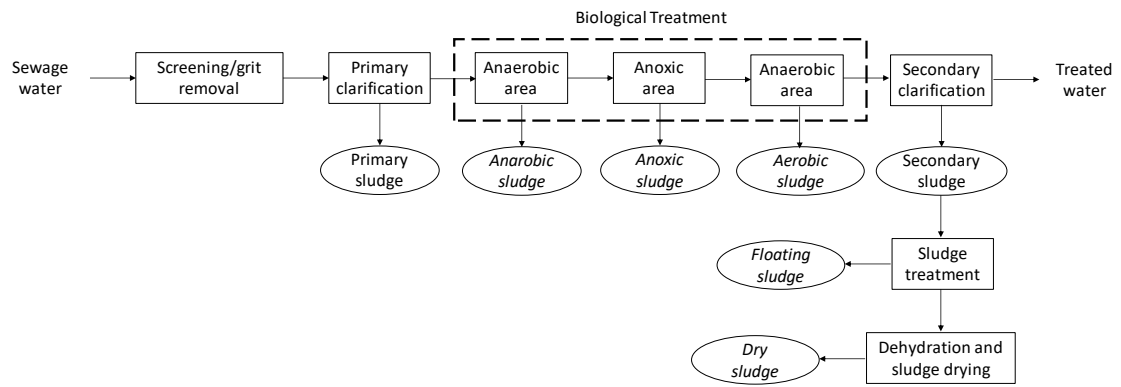
579 transesterification, with floating sludge as raw material

580 **Figure 6.** FAME yield versus reaction time by *in situ* transesterification of floating sludge: ●

581 without pretreatment and ◆ 50 minutes of ultrasound as pretreatment. Dotted lines

582 correspond to model fitting.

583

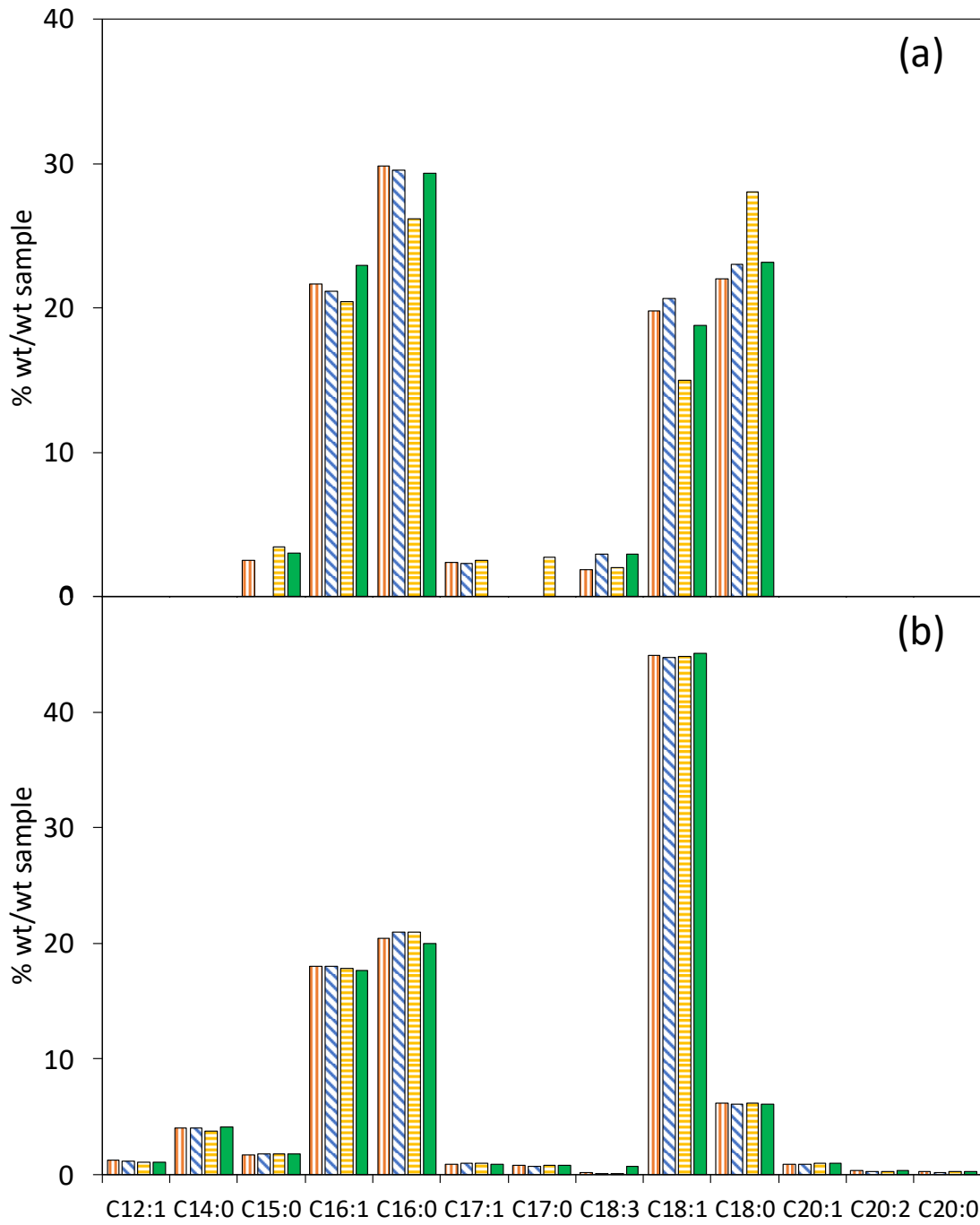


584

585

Figure 1. Process of urban wastewater treatment of the WWTP of Villapérez (Oviedo, Spain)

586



587

588

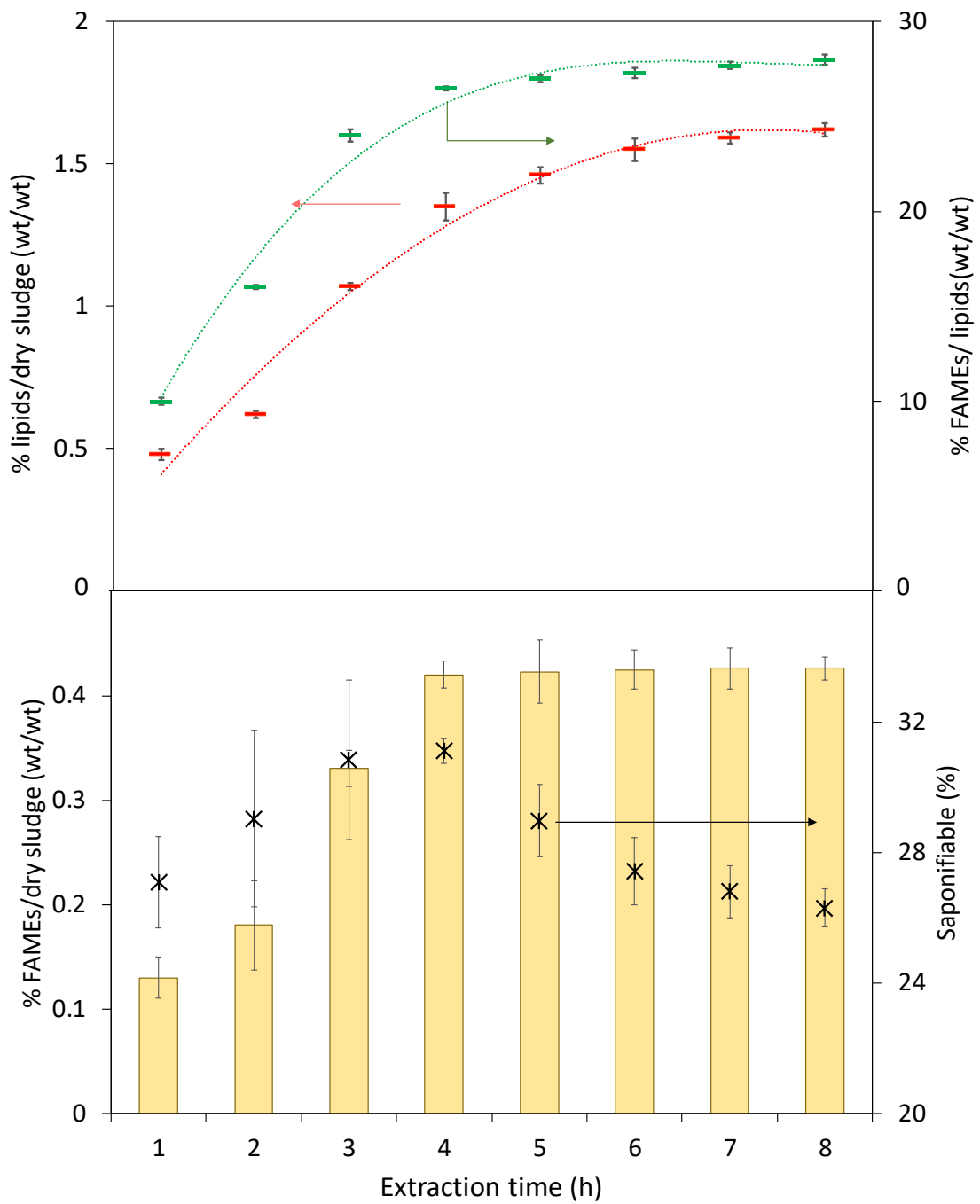
Figure 2. FAME composition of the biodiesel produced by (a) solid-liquid extraction method and (b)

589

in situ transesterification Oxidic sludge, anoxic sludge, anaerobic sludge, Floating sludge

590

591



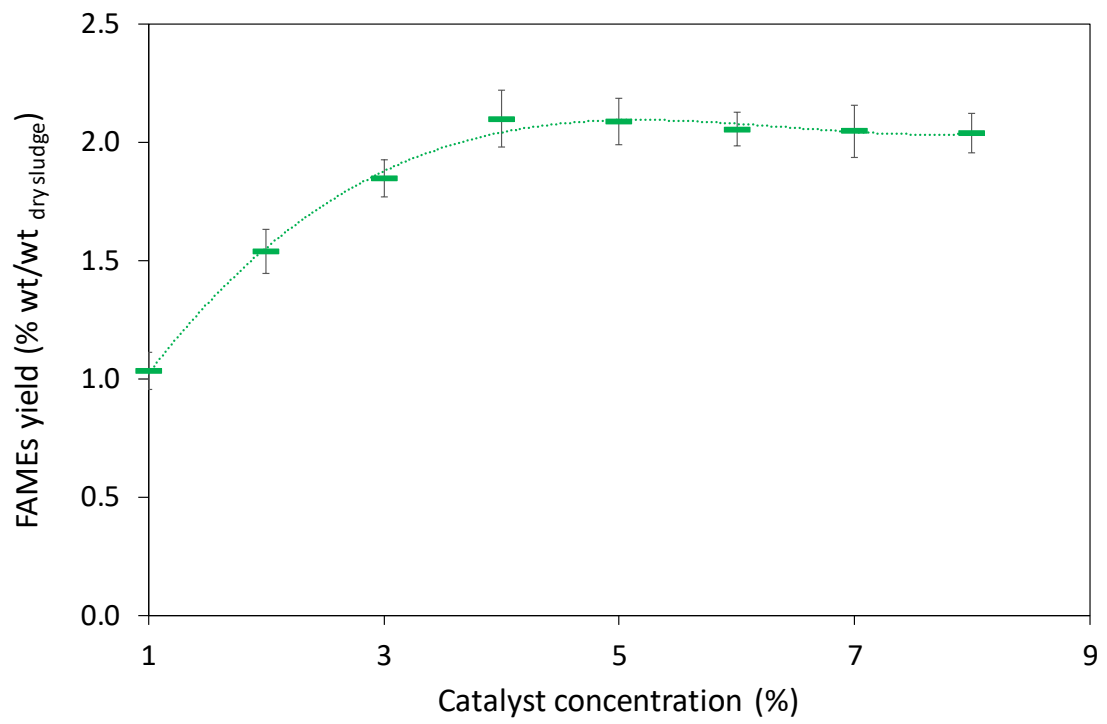
592

593 Figure 3. Process yields at different extraction times using floating sludge as raw material by

594 solid-liquid extraction. Results corresponding to the (a) extraction step and (b)

595 transesterification process

596



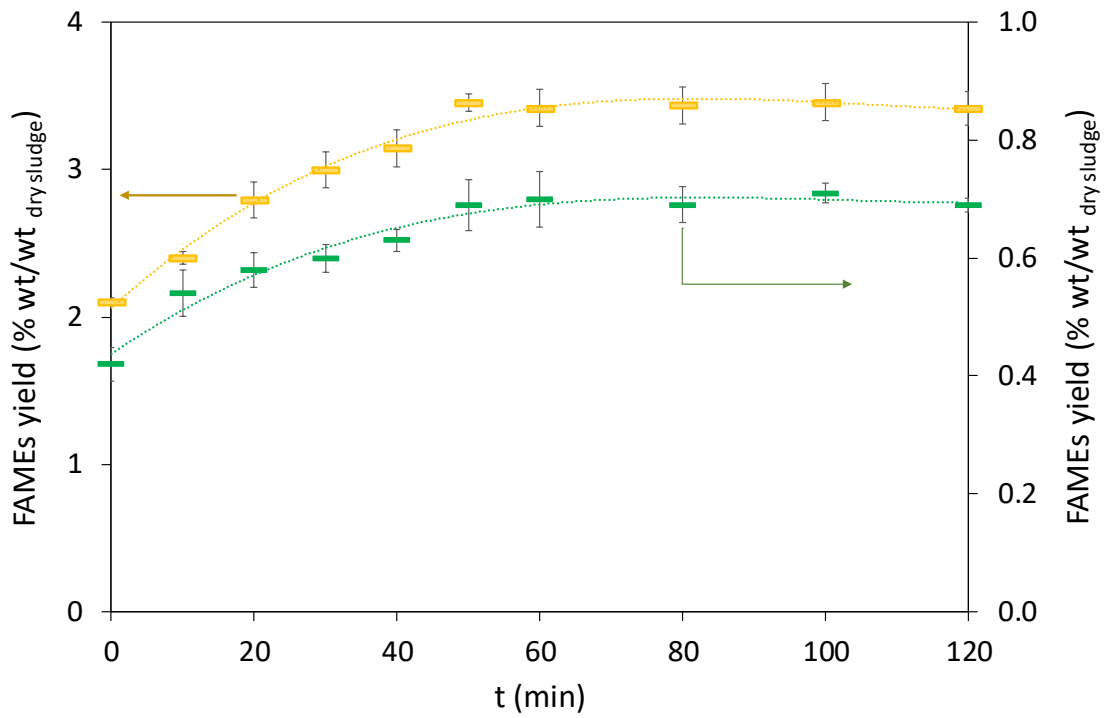
597

598 Figure 4. Effect of catalyst concentration on FAMES yield by *in situ* transesterification with

599

floating sludge as raw material

600



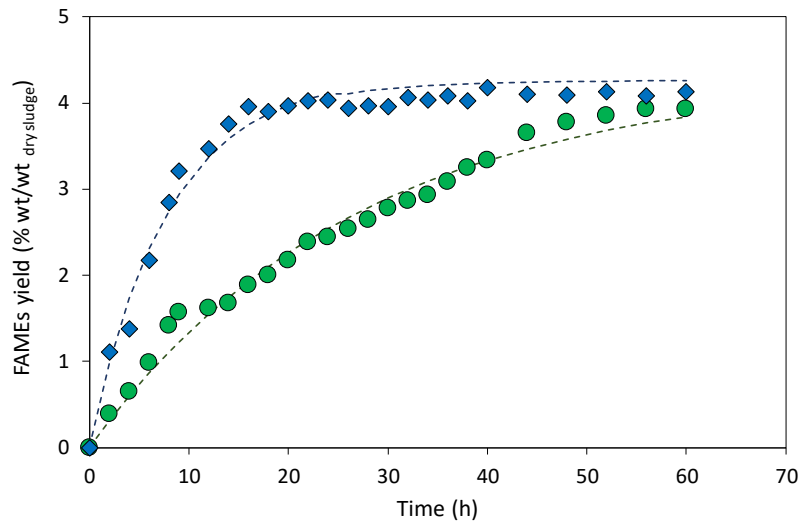
601

602 Figure 5. Effect of sonication time on FAMES yield, ● Solid-liquid extraction and ■ *in situ*

603

transesterification, with floating sludge as raw material

604



605

606 Figure 6. FAME yield versus reaction time by in situ transesterification of floating sludge: ●

607 without pretreatment and ◆ 50 minutes of ultrasound as pretreatment. Dotted lines

608 corresponds to model fitting.

609

Table 1: Moisture content, total solid concentration and lipid content (dry sludge basis) of the considered sludge samples.

Sludge sample	Moisture (%)	Total Solids (%)			Lipids (%)
		Initial	After decantation	After centrifugation	
Oxic zone	99.7 ± 0.1	0.30 ± 0.02	0.79 ± 0.06	4.12 ± 0.02	16.6 ± 0.1
Anoxic zone	99.7 ± 0.1	0.29 ± 0.01	0.55 ± 0.07	4.56 ± 0.05	17.2 ± 0.1
Anaerobic zone	99.7 ± 0.1	0.28 ± 0.01	0.64 ± 0.06	4.90 ± 0.07	16.2 ± 0.1
Floating sludge	97.3 ± 0.1	2.63 ± 0.06	2.64 ± 0.06	5.42 ± 0.06	19.6 ± 0.1

610

611

Table 2. Yields for the extraction and transesterification steps for Liquid-Liquid and Solid-Liquid methods

		Lipid yield	FAMES yield
		% lipids/dry sludge (w/w)	% FAMES/lipids (w/w)
Liquid-Liquid	Oxic zone	0.03 ± 0.04	0
	Anoxic zone	0.05 ± 0.04	0
	Anaerobic zone	0.02 ± 0.03	0
	Floating sludge	0.52 ± 0.05	0.86 ± 0.03
Solid-Liquid	Oxic zone	0.94 ± 0.03	5.32 ± 0.04
	Anoxic zone	1.56 ± 0.04	7.30 ± 0.05
	Anaerobic zone	1.03 ± 0.04	15.90 ± 0.05
	Floating sludge	1.75 ± 0.03	23.55 ± 0.02

612

613