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1 **Increasing the tolerance to polyphenols of the anaerobic digestion of olive wastewater through**
2 **microbial adaptation**

3
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13
14 **Abstract**

15 The valorisation of olive oil mill wastewater (OMW) through anaerobic digestion requires
16 identifying the concentration of polyphenols (PP) that causes failure of the process of digestion. In
17 addition, the advantages of the possible microbial adaptation, in terms of increased methane
18 production, to significant concentrations of PP as well as the kinetics of OMW anaerobic
19 degradation requires evaluation. To fill these knowledge gaps, anaerobic digestion batch tests were
20 carried out on three blends of OMW and inoculum (digestate from a biogas plant fed with agro-
21 wastes) at a PP concentration of 0.5, 1.0 and 2.0 g l⁻¹ in mesophilic conditions. Total inhibition of
22 anaerobic digestion was found at a PP concentration of 2.0 g l⁻¹ (non-adapted inoculum group). A
23 positive effect of the adaptation to the substrate was, instead, observed for the blends with adapted
24 inoculum at a PP concentration of 1.0 and 2.0 g l⁻¹. Methane yields increased by 70% (PP = 1.0 g l⁻¹
25 ¹) and 300% (2.0 g l⁻¹) in the group with adapted inoculum compared to the group with non-adapted
26 inoculum. The results suggest that OMW should not be subject to anaerobic digestion at high PP
27 concentrations (i.e. higher than about 1 g l⁻¹) due to the microbial inhibition detected. Moreover,
28 given the benefits of the adaptation of the microbial population that was more evident at the highest
29 PP concentration tested, it is advisable to allow the progressive adaptation of the digestion to OMW
30 feeding. Thanks to the increased methane yield, because of the improved microbial tolerance to
31 inhibiting compounds, the anaerobic digestion of OMW could be a viable and environmentally
32 sound solution for the treatment of agro-industrial wastewater.

33

34 **Keywords:** anaerobic digestion; oil residues; inhibiting compound; methane yield; microbial
35 adaptation; biogas production.

36

37 **Nomenclature**

38	OMW	Olive Mill Wastewater
39	PP	Polyphenols
40	TS	Total Solids
41	TVS	Total Volatile Solids
42	DM	Dry Matter
43	COD	Chemical Oxygen Demand
44	BMP1	Series of tests carried out with raw digestate as inoculum
45	BMP2	Series of tests carried out with adapted digestate and BMP1 as inoculum
46	T_{50}	Time needed to get 50% of the maximum methane yield
47	T_{90}	Time needed to get 90% of the maximum methane yield
48	B	methane production of the 1 st -order kinetics model
49	B_0, M, y	cumulative methane production of the 1 st -order kinetics, Gompertz or logistic models
50	k	constant of the 1 st -order kinetics or logistic models
51	t	time for digestion of the 1 st -order kinetics, Gompertz or logistic models
52	P	methane potential of Gompertz model
53	R_m	maximum methane production rate of Gompertz model
54	λ	lag phase period of Gompertz model
55	a, b	constants of logistic model
56	WW	Winery Waste
57	PW	Pig Waste
58	OMSW	Olive Mill Solid Waste
59	AW	Abattoir Wastewater
60	LPM	Liquid Poultry Manure

61

62 **1. Introduction**

63 The olive oil industry, which is one of the most traditional agricultural industries in the
64 Mediterranean region, generates large amounts of residues: a very wet, plastic olive cake, the so-
65 called "olive pomace", and a liquid stream, called "olive oil mill wastewater" (OMW), which is
66 produced by the wastewater generated during the different stages of the process in olive oil
67 production and by the water used for cleaning purposes (Moreno et al., 2017). On a broad scale,

68 olive processing produces 50% wastewater, 30% solid residues and 20% olive oil (Komnitsas and
69 Zaharaki, 2012). The major environmental problems associated with olive oil extraction mills are
70 related to both the large volumes of water required and the ineffective management of OMW and
71 olive pomace (Dourou et al., 2016). OMW composition presents a large diversity depending on
72 several parameters, such as the variety of olives and their maturity, the region of origin, and
73 especially the technology used for oil extraction (Roig et al., 2006). However, OMW is always
74 characterised by high concentrations of several organic compounds (e.g. organic acids, tannins and
75 phenolic compounds), which make it difficult to treat due to its resistance to biodegradation
76 (Turano et al., 2002) causing serious environmental concerns when its management is not
77 environmentally sound. For instance, OMW discharged into water courses can lead to water body
78 deterioration with significant damages to the aquatic life (Karaouzas et al., 2011), while pollution of
79 groundwater, soil contamination, production of unpleasant smells, as well as the toxicity of
80 vegetation are also possible in case of uncontrolled disposal and/or insufficient treatment of OMW
81 (Aggelis et al., 2003). Therefore, due to its polluting power and to the increasing severity of the
82 applicable legislation (Gómez et al., 2010), the disposal of residues from oil extraction has become
83 a major concern for olive oil producers. The need to apply suitable management practices, which
84 are able to combine environmental and economic sustainability for olive oil facilities, is now clear.
85 Currently, the methods applied for OMW treatment are either physico-chemical (e.g. simple
86 evaporation, reverse osmosis, ultrafiltration, coagulation, oxidation, thermal drying and advanced
87 oxidation processes) or biological (aerobic treatment, composting, vermicomposting together with
88 other agro-industrial residues), but the majority of these techniques are complex and expensive and
89 the results obtained are often poor (Gomez et al., 2010; Komnitsas and Zaharaki, 2012; Dourou et
90 al., 2016).

91 Anaerobic digestion has been proposed as a promising technology for the valorisation of olive oil
92 residues through biofuel production (i.e. biogas) (Ponsà et al., 2011; Sheng et al., 2013), since this
93 process can be carried out by applying relatively inexpensive and simple reactor designs and
94 operating procedures (Tekin and Dalgic, 2000). However, researchers must overcome many issues
95 (such as low pH and nitrogen content, alkalinity, presence of inhibiting compounds) for this
96 technology to be applied to OMW (Orive et al., 2016). Most researches have attributed the related
97 problems observed during anaerobic digestion to the presence of polyphenols (PP). However, these
98 experiments were often carried out using synthetic wastewater containing cellulose or acetate as
99 main substrate (Madiguo et al., 2016; Wang et al., 1991; Chapleur et al., 2016) or even PP as
100 substrate (Field and Lettinga, 1987; Fedorak and Hrudey, 1984). High concentrations of PP in the
101 anaerobic digestion of OMW lead to very low biogas and methane production rates and consequent

102 reduced treatment efficiency. Moreover, the reduced energy yield reduces the economical
103 convenience of the anaerobic digestion of OMW.

104 In general, typical PP concentrations of OMW are in the range of 0.5 - 24 g l⁻¹ (Borja et al., 2006;
105 Gonzalez-Lopez et al., 1994), but severe methane yield reductions have been noticed already at PP
106 concentrations of about 0.5 - 2 g l⁻¹ (Fedorak and Hruday, 1984; Borja et al., 1996). This means that
107 raw OMW should be either co-digested with other organic substrates or fed with limited loads to the
108 digester.

109 The scientific literature reports of many experimental tests concerning the anaerobic digestion of
110 olive residues, often in co-digestion with other substrates. For instance, as co-substrates,
111 Fontoulakis et al. (2008) used winery waste, Athanasoulia et al. (2012) activated sludge waste and
112 Dareioti et al. (2009) cheese whey and liquid cow manure. Pre-treatments for the removal of PP
113 compounds were also explored focusing on the possibility of increasing methane yield (e.g.
114 biological treatment with yeast or fungi, Gharsallah et al., 1999; Martinez-Garcia et al., 2009; Borja
115 et al., 1995; aerobic treatment, González-González and Cuadros, 2015; chemical treatment,
116 Siciliano et al., 2016).

117 Co-digestion is the most common practice to reduce the negative effects associated with the
118 inhibiting compounds, since pre-treatments are often expensive and not sustainable by the smallest
119 olive oil mills. However, co-digestion requires knowing which is the OMW maximum
120 concentration tolerated by the anaerobic process to obtain an acceptable energy yield.

121 Enhancing the tolerance of the anaerobic processes towards PP through microbial adaptation may
122 be a simple method to increase methane yield during OMW digestion. To explore this possibility, it
123 is firstly necessary to investigate which is the PP limit concentration to avoid the inhibition on the
124 methanogenic activity. Some studies are available on the effects of PP on OMW anaerobic
125 digestion, but often data are scattered due to the different conditions adopted for the process (e.g.
126 for co-digestion: hydraulic retention time, organic loading rate, Fountoulakis et al., 2008; Battista et
127 al., 2013; Dareioti, 2009, 2010) and for the different inocula used (e.g. Gonzàles-Gonzàles and
128 Cuadros, 2015, Fezzani and Chiekh, 2008; Kougias et al., 2010, 2014). The advantages of microbial
129 adaptation to PP must be further evaluated by a quantitative approach; in other words, the increase
130 of methane yield due to the increased tolerance of OMW to PP has to be measured by suitable
131 Biochemical Methane Potential (BMP) tests, in order to evaluate the maximum methane yield that
132 could be obtained by using an acclimated inoculum. The increase of methane yield by microbial
133 adaptation may increase the appeal of anaerobic digestion as one of the most profitable methods for
134 OMW valorisation in the olive oil production sector.

135 Unfortunately, the number of investigations on microbial population adaptation to PP contained in
136 OMW is limited and they are mainly carried out as OMW co-digestion (Kougiyas et al., 2010, 2014;
137 Fezzani and Cheikh, 2008, Gannoun et al., 2007); however, a quantitative evaluation of the
138 potential increase in methane yield due to microbial adaptation has not to the best of our
139 knowledge, been performed. Furthermore, to evaluate the accuracy of the literature models (e.g.
140 first-order kinetics, Gompertz and logistic models) in simulating the methane production rates
141 observed in the BMP test (Durruti et al., 2012) could also turn useful; again no experiments have
142 been conducted on this topic, to the best of our knowledge.

143 In order to fill these knowledge gaps, this paper evaluates the PP concentration in the anaerobic
144 reactor that induces inhibition of OMW anaerobic digestion, by comparing non-adapted and
145 adapted inocula. More specifically, through batch tests under mesophilic conditions at three PP
146 concentrations we: (i) evaluated the concentration of PP that leads to the inhibition of OMW
147 anaerobic digestion; (ii) measured the increase in methane yield due to the microbial adaptation of
148 the inoculum to PP, compared to the non-adapted inoculum, and (iii) tested the accuracy of the
149 literature models (first-order kinetics, Gompertz and logistic models) in simulating the methane
150 yields measured in our tests. More specifically, modelling is an essential tool for a synthetic
151 analysis of the outcome of the experimental activity and is also useful to set the operational
152 parameters during the scaling up of the process to pilot or full-scale plants dimensions.

153

154 **2. Materials and methods**

155 *2.1 Biochemical Methane Potential (BMP) tests*

156 Two series of anaerobic batch tests (designated BMP1 and BMP2) were carried out. The first series
157 (BMP1) consisted of three tests with digestate (taken from an external plant and used as *inoculum*)
158 and OMW blended at different PP concentrations (0.5, 1.0 and 2.0 g l⁻¹, indicated below as PP_{0.5},
159 PP_{1.0} e PP_{2.0}, respectively), each one in triplicate. This BMP1 series was aimed at evaluating the
160 biogas/methane yield reduction at increasing PP concentrations or, in other words, the PP
161 concentration that inhibited the anaerobic process.

162 Subsequently, the digestate produced during BMP1 tests (adapted digestate) was mixed and used as
163 inoculum of the BMP2 test at the same PP concentration. The objective was to evaluate whether an
164 inoculum, already subject to anaerobic digestion in the presence of a significant PP concentration
165 and thus characterised by an adapted microbial population, played a positive effect not only on
166 biogas production, but also on the capability of bacteria to tolerate a higher PP concentration when
167 compared to a non-adapted inoculum. BMP2 tests were carried out at PP concentrations of 1.0 and
168 2.0 g l⁻¹, respectively (two reactors for each PP concentration). For each of the two series of tests, a

169 fourth test (control) was carried out using only inoculum, in order to evaluate endogenous biogas
170 yields. A schematic representation of the BMP design is shown in Fig. 1.

171

172 2.2 *Analytical methods*

173 The digestate used in the BMP1 tests (raw digestate) was taken from a commercial biogas plant, fed
174 mainly with manure and other agro-waste.

175 OMW was extracted by a 3-phase olive oil mill. However, raw OMW showed a low PP
176 concentration (1.05 g l^{-1}), presumably under the expected inhibition limit. Therefore, OMW was
177 concentrated in an oven at 60°C (in any case, below the evaporation temperature of the PP) until the
178 PP concentration in the final volume was about 4-fold the initial concentration (Table 1).

179 The raw digestate and OMW were previously characterised, by measuring the Total Solids (TS, by
180 oven drying the wet biomass at 70°C until reaching weight stabilisation), Total Volatile Solids
181 (TVS, by calcination of the dry matter), pH (a portable pH-meter, XS Instruments) and C/N ratio
182 (Leco series 628, United States). COD and PP concentrations in the OMW were measured. COD
183 was determined on diluted (1:10 v/v) effluents by cuvette cap tests (WTW, code 1.14555,
184 photometer WTW, PhotoLab S12). The measurements were carried out in triplicate. PP
185 concentration was measured by the Folin-Ciocalteu colorimetric method (Folin and Ciocalteu,
186 1927) and expressed as g l^{-1} of gallic acid, measured by a spectrophotometer (PerkinElmer, Lambda
187 35 UV-VIS). Table 1 reports the main physico-chemical parameters of the substrates (OMW and
188 digestate) used in the two series of tests.

189 Table 2 reports the characteristics of the four blends subject to the BMP1 and BMP2 tests.

190

191

Table 1

192

193

Table 2

194

195 All batches of both series were kept for 30 d under mesophilic conditions (35°C). Before the
196 beginning of the test, sodium bicarbonate was added to increase the buffering capacity. Each test
197 was carried out in triplicate (BMP1) or duplicate (BMP2, due to the lack of sufficient amount of
198 acclimated inoculum) in a hermetic bottle, which was continuously stirred by a magnetic bar.

199 Throughout the experiment, biogas and methane production were measured three times a week. The
200 volume of biogas produced was measured according to Calabrò et al. (2016, 2018); the methane
201 content in the biogas was estimated by the fluid displacement method using a 3M NaOH solution
202 (Calabrò et al., 2016, 2018). The average methane yields (evaluated at standard pressure and

203 temperature conditions) of the control tests were subtracted from the corresponding values of the
204 other tests, in order to estimate the net specific methane yield.

205 The time needed to get 50% and 90%, respectively, of the maximum methane yield (hereinafter
206 indicated as “T₅₀” and “T₉₀”) was also evaluated.

207

208 2.3 *Experimental design and statistical analysis*

209 The design of the experiment followed a 14 × 3 (BMP1) or 14 × 2 (BMP2) factorial layout, with 14
210 sampling dates of daily specific methane yields and 3 (BMP1, each batch with 3 replicates) or 2
211 (BMP2, each batch with 2 replicates) PP concentrations (at p < 0.01). This factorial layout, which
212 was analysed by a two-way ANOVA using the PAST software (Hammer et al., 2001), was aimed at
213 evaluating whether there was an interaction between time and PP concentrations.

214 Subsequently, based on the outcome of the two-way ANOVA, using single factor analysis of
215 variance (ANOVA) along with Tukey’s test (designed for the pairwise comparisons), statistical
216 analysis of cumulated specific methane yield from batch anaerobic digestion was performed. The
217 significance threshold was set at p < 0.05. More specifically, pairwise comparisons were applied to:
218 (i) different PP concentrations (separately for the BMP1 and BMP2 batches); (ii) BMP1 and BMP2
219 batches at the same PP concentration (separately for PP = 1.0 and 2.0 g l⁻¹). Moreover, pairwise
220 comparisons by Tukey’s test were also used to evaluate the statistical significance in variability of
221 daily specific methane yields (i.e. the difference in slopes of the cumulated production rates)
222 throughout the anaerobic digestion cycle.

223 The ANOVA analysis of variance assumes that the residuals are normally distributed. Thus, data
224 from batch experiments were also tested for the assumption of normality by using the Aderson-
225 Darling methodology, which is based on the function of empirical distribution. This statistical
226 analysis was performed using Minitab Statistical Software release 17 (Minitab, Inc., State College,
227 Pennsylvania, USA).

228

229 2.4 *Analysis of degradation kinetics*

230 The kinetics and the experimental methane yields were finally simulated by first-order kinetics,
231 modified Gompertz and logistic models (Donoso-Bravo et al. 2010, Ghatak and Mahanta, 2014):

232

233 *1st-order kinetics:*

$$234 \quad B = B_0 \cdot \left[1 - \exp^{-kt} \right] \quad (1)$$

235 where B is methane production, l g⁻¹ [TVS]; B₀ is cumulative methane production (maximum
236 methane yield), l g⁻¹ [TVS]; k is first order kinetic constant, d⁻¹; and t is time for digestion, d.

237

238 *Gompertz (modified equation):*

$$239 \quad M = P \cdot \exp^{-\exp \frac{R_m \cdot e}{P} (\lambda - t) + 1} \quad (2)$$

240 Where M is cumulative methane production, l g⁻¹ [TVS]; P is methane potential, l g⁻¹ [TVS]; R_m is
241 maximum methane production rate, l g⁻¹ [TVS] d⁻¹; λ is lag phase period or the minimum time
242 required to produce biogas, d; and t is time for digestion, d.

243

244 *Logistic model:*

$$245 \quad y = B \frac{a}{1 + b \cdot \exp^{-kt}} \quad (3)$$

246 Where y is cumulative methane production, l g⁻¹ [TVS]; t is time for digestion, d; k is kinetic rate
247 constant, d⁻¹; and a, b are constants.

248

249 **3. Results**

250 *3.1. Batch tests with non-adapted inoculum (BMPI)*

251 The maximum cumulative value (0.419 Nl g⁻¹ [TVS]) of the net specific methane yield was
252 observed in the PP_{0.5} batch. With 2-fold and 4-fold PP concentrations (the PP_{1.0} and PP_{2.0} blends)
253 methane yields were, respectively, 43% (0.244 Nl g⁻¹ [TVS]) and 89% (0.045 Nl g⁻¹ [TVS]) lower
254 (Fig. 2b and Table 3).

255

256

Figure 2

257

258

Table 3

259

260 The batches with the highest PP concentration reached 50% of the maximum methane yield just
261 after 5.6 d, thus showing an early blockage of methanogenic activity; the other blends showed T₅₀
262 of 11.1 (PP_{0.5}) and 14.6 (PP_{1.0}) days, while the methane production was practically completed after
263 20-25 days (Table 3). The methane production shown by the PP_{1.0} batches increased almost
264 constantly throughout the experiment.

265 The cumulative production of biogas was similar to that of methane; the biogas yield was 0.614,
266 0.385 and 0.116 Nl g⁻¹ [TVS] for the PP_{0.5}, PP_{1.0} and PP_{2.0} blends, respectively (Fig. 2a). The highest
267 biogas content, which was averaged during the overall process, was detected for the PP_{0.5} (58%)

268 and PP_{1.0} (47%) blends, while, in contrast to the other blends, a noticeable reduction of the biogas
269 percentage was observed in PP_{2.0} (28%).

270 For all the batches pH values were always in the optimal range (7-8) required for a balanced
271 anaerobic digestion (Gonzàles-Gonzàles and Cuadros, 2015; Fezzani and Cheikh, 2008; Martinez-
272 Garcia et al., 2009). The lowest final pH was measured for PP_{2.0} (Table 4), presumably linked to a
273 higher concentration of acids (not consumed by methanogenic bacteria).

274 The maximum correlation level with our experimental data was observed with the Gompertz model
275 ($r^2 = 0.96$, PP_{2.0} batch, with a maximum value of 0.99 for PP_{0.5} and PP_{1.0}), when simulating the net
276 methane specific yields by the literature models.

277 Table 5 shows the parameters obtained with the modified Gompertz equation determined by the
278 “Solver” function of Excel® (Microsoft® Office®) application, minimising the differences between
279 experimental and modelled cumulative methane production (least squares method). The parameters
280 of the model confirmed the adverse effect of increasing PP concentration both in terms of final yield
281 and maximum production rate.

282

283

Table 4

284

285

Table 5

286

287 3.2. *Batch tests with adapted inoculum (BMP2)*

288 In the BMP2 tests (adapted inoculum), the cumulated net specific methane yield, measured for the
289 two blends, PP_{1.0} and PP_{2.0}, was 0.419 and 0.170 NI g⁻¹ [TVS], respectively (Table 3). T₅₀ and T₉₀
290 were higher than in the BMP1 tests (Table 3).

291 The biogas yield was on average 0.602 and 0.286 NI g⁻¹ [TVS] for the PP_{1.0} and PP_{2.0} blends,
292 respectively (Fig. 3). The test also showed a high content in methane (65%, PP_{1.0}, and 58%, PP_{2.0};
293 Fig. 2).

294 As found for the BMP1 tests, the best simulation of our experimental data was given by the
295 Gompertz model for both the PP_{1.0} e PP_{2.0} batches ($r^2 = 0.99$ in both cases; Table 5). Modelling
296 confirmed the positive effect of inoculum adaptation; in particular, the maximum production rate
297 for BMP2 at a PP concentration equal to 1.0 g l⁻¹ was very close to that of BMP1 at a PP
298 concentration equal to 0.5 g l⁻¹. From a practical point of view, this implies that to roughly obtain
299 0.25 NI g⁻¹ [TVS] of methane in a reactor containing 1.0 g l⁻¹ of PP 31 d are needed with a non-
300 adapted inoculum while only 19 d are sufficient with an adapted one.

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4. Discussion

The analysis of the specific methane yields measured for the two BMP series highlighted noticeable differences among blends at different PP concentrations. For both the BMP1 and BMP2 series, the two-way ANOVA highlighted that the factors "sampling time" and "PP concentration" were statistically significant, when considered separately, but that their reciprocal interaction was not significant (for $p < 0.01$).

In the BMP1 test, the statistical analysis (one-way ANOVA together with Tukey's test for pairwise comparisons) showed that only the difference between PP_{0.5} and PP_{2.0} was statistically significant ($p < 0.05$). The PP_{0.5} batch produced methane at a significantly higher rate ($p < 0.05$) until the 20th day compared to the same production measured at a later stage (days 21-30). Moreover, the methane production rate of the PP_{0.5} batch was significantly ($p < 0.05$) higher compared to PP_{1.0} in the first 20 d, while the difference in production rate between the two batches became not significant ($p < 0.05$; Figure 2b) during the course of the days (days 21-30).

In the BMP2 test, the cumulated net specific methane yield, measured for the two blends, PP_{1.0} and PP_{2.0}, was significantly different between the two batches ($p < 0.05$; Fig. 3). A slow increase in net specific methane yield was detected for both batches during the first ten days of the experiment; this production increased at a higher rate during the course of the following ten days, although not significantly compared to the previous period. The difference in methane production rates of the PP_{1.0} e PP_{2.0} batches was significant ($p < 0.05$) only at the lowest PP concentration (Fig. 4). This outcome suggests a possible adaptation of the microbial population to the inhibiting compounds in the batch.

Figure 4 compares the net specific methane yields of the BMP1 (raw inoculum) and BMP2 (inoculum adapted to PP) tests. The significant increase of the specific methane yield detected in the tests may be attributable to the microbial tolerance to high PP concentrations developed in the previous digestion cycle. More specifically, the net specific methane yield (0.419 NI g⁻¹ [TVS]) of the PP_{1.0} blend in the BMP2 test was higher by 72% compared to the same (0.244 NL g⁻¹ [TVS]) in the BMP1 test (Table 3) although results were not significant. In contrast, the net specific methane yield (0.17 NI g⁻¹ [TVS]) of the PP_{2.0} blend in the BMP2 test was even 4-fold to that observed (0.045 NI g⁻¹ [TVS]) in the BMP1 test and results were significant ($p < 0.05$; Table 3). The average methane percentage also increased by using the adapted inoculum (from 47 to 65%, PP_{1.0}, and from 28 to 58%, PP_{2.0}), which seems to confirm the positive role of the microbial adaptation on the methanogenic process.

336

337

Figure 4

338

339 The analysis of the process parameters modelled by the literature models (Table 5) highlighted that
340 the adapted inoculum increased both the maximum methane production rate (from 0.010 to 0.018
341 $\text{Nl g}^{-1} [\text{TVS}] \text{d}^{-1}$ for the $\text{PP}_{1.0}$ batches and from 0.001 to 0.007 $\text{Nl g}^{-1} [\text{TVS}] \text{d}^{-1}$ for the $\text{PP}_{2.0}$ batches)
342 and the potential in specific production (from 0.317 to 0.615 $\text{Nl g}^{-1} [\text{TVS}]$ for the $\text{PP}_{1.0}$ batches and
343 from 0.235 to 0.478 $\text{Nl g}^{-1} [\text{TVS}]$ for the $\text{PP}_{2.0}$ batches). During the BMP1 test the maximum
344 tolerated PP concentration that did not lead to process inhibition was about 0.5 g l^{-1} , while in the
345 BMP2 test this concentration increased to about 1 g l^{-1} . Lag periods were higher in the BMP2 test
346 (4.61 and 7.73 d, for the $\text{PP}_{1.0}$ and $\text{PP}_{2.0}$ batch, respectively) than in the BMP1 test (Table 5),
347 probably due to non-degraded PP in the digestate derived from BMP1; however, methane
348 production quickly recovered after the adaptation phase for both blends.

349 These results suggest that the adaptation of the inoculum significantly increases the tolerance to the
350 inhibition effects played by phenolic compounds. An incremental feeding of OMW in anaerobic
351 digestion plants, starting from very limited amounts, could enhance microbial adaptation. This
352 could be a possible and cheap solution, compared to the more expensive pre-treatments, to
353 significantly increase the methane yields of OMW with a non-negligible content of PP. This option,
354 which requires the use of large tanks for OMW storage, could optimise the PP concentration in the
355 reactor and keep the microbial population adapted to these substrates. Furthermore, OMW storage
356 in the tanks would allow its availability as substrate for anaerobic digestion throughout the year,
357 thus avoiding the limits linked to its seasonal production (concentrated from October to February).
358 Some interesting considerations were drawn when we compared the results of our batch tests with
359 other experiments reported in the literature regarding anaerobic digestion tests conducted with
360 OMW blends and at mesophilic conditions (Table 6):

- 361 - the highest methane yields of our study (the $\text{PP}_{0.5}$ and $\text{PP}_{1.0}$ blends) without microbial adaptation
362 (205 and $353 \text{ Nml g}^{-1} [\text{COD}_{\text{added}}]$, respectively) were comparable to those observed by Fontoulakis
363 et al. (2008) ($214 \text{ Nml g}^{-1} [\text{COD}_{\text{added}}]$);
- 364 - conversely, our $\text{PP}_{2.0}$ blend (in which inhibition was evident) showed the lowest methane yield
365 among all the studies reported;
- 366 - with microbial adaptation, only the $\text{PP}_{1.0}$ blend showed a methane yield ($275 \text{ Nml g}^{-1} [\text{COD}_{\text{added}}]$)
367 similar to the findings of Kougiyas et al. (2010, 2014) ($277 \text{ Nml g}^{-1} [\text{COD}_{\text{removal}}]$ and 480 Nml g^{-1}
368 $[\text{TVS}]$) in which the experiment evaluated the co-digestion of OMW and swine manure;

369 - the PP concentration that showed severe inhibition (2.0 g l^{-1}) was similar to that determined by
370 Gannoun et al. (2007).

371

372

Table 6

373

374 As previously mentioned, the majority of studies concerning PP inhibitory concentrations were
375 carried out on synthetic wastewater containing specific phenolic compounds. In other experiences,
376 mesophilic anaerobic digestion of OMW was carried out in co-digestion with other organic wastes,
377 or in semi-continuous mode, and without an acclimated inoculum (Azbar et al., 2009; Fountoulakis
378 et al., 2008; Fezzani and Cheikh, 2010; Athanasoulia et al., 2012; Dareioti and Kornaros, 2014).
379 The BMP experiments carried out on an acclimated inoculum were also performed in co-digestion;
380 moreover, different methods were used to acclimate the inoculum. Kougias et al. (2010), who used
381 an inoculum of digested sludge obtained from an anaerobic reactor fed with OMW and pig waste
382 (PW) at the optimal ratio of 40:60 (v/v), measured a maximum methane yield of 480 Nml g^{-1} [TVS]
383 (corresponding to 297 Nml g^{-1} [$\text{COD}_{\text{added}}$]). The same authors found a methane yield of 370 Nml g^{-1}
384 [TVS] (corresponding to 277 Nml g^{-1} [$\text{COD}_{\text{removed}}$]) at the same optimal ratio (Kougias et al.,
385 2014). Khoufi and co-workers (2015) used the anaerobic stock culture from a semi-pilot anaerobic
386 bioreactor fed with OMW as inoculum; the optimal ratio for the co-digestion of OMW and liquid
387 poultry manure (LPM) in batch reactors was 70:30 (v/v), corresponding to a PP concentration of
388 6.83 g l^{-1} , although the methane yield was only 55.1 Nml g^{-1} [TS]. Fezzani and Cheikh (2008)
389 studied the efficiency of the co-digestion of OMW and olive mill solid waste (OMSW); digestion of
390 only OMW produced 11.17 l of biogas per 1 of digester (PP concentration of 6 g l^{-1}) and this biogas
391 production increased to 30.5 l l^{-1} [digester] by co-digestion of OMW with OMSW (PP concentration
392 of the mixture equal to 3 g l^{-1}). Finally, Gannoun and collaborators (2007) carried out a toxicity test
393 in order to evaluate the inhibitory PP concentration in different mixtures of OMW and abattoir
394 wastewater (AW) at the optimal ratio OMW:AW of 40:60 (v/v) and PP concentration of 2.04 g l^{-1} :
395 the biogas yield after 10 days was 0.189 l per g [$\text{COD}_{\text{removed}}$]; the inoculum used was taken from an
396 active mesophilic digester treating OMW.

397 In all the aforementioned cases, the tests were aimed at measuring the optimal ratio between OMW
398 and the other co-substrates; none of these studies identified the inhibitory PP concentration or
399 evaluated the beneficial effect of the acclimated inoculum on the increase of the bio-methane
400 production. Our BMP tests, instead, were successful in identifying the concentration of polyphenols
401 inhibiting the anaerobic digestion of OMW (close to 0.5 g l^{-1} without microbial adaptation) and in
402 quantifying the advantages of the microbial population adapted to phenols (by measuring the

403 increase of methane production - from about 70% to 280% - compared to the non-adapted
404 substrate).

405

406 **5. Conclusions**

407 The batch tests revealed that:

- 408 • A PP concentration of 2.0 g l⁻¹ induced an early blockage of methanogenic activity, while
409 the lowest PP (PP 0.5 g l⁻¹) gave the highest methane yield (0.419 Nl g⁻¹ [TVS]);
- 410 • When the digestate of the first tests was used as inoculum in the second series, the net
411 specific methane yield of the blend with 1.0 g l⁻¹ PP was 72% higher compared to the
412 BMP1 test, while the blend with 2.0 g l⁻¹ PP showed a methane yield that was 4-fold higher;
- 413 • The change described above was attributed to the adaptation of the bacterial population to
414 PP, suggesting that OMW should be fed to anaerobic digestion by initially keeping PP
415 concentrations always lower than 1.0 g l⁻¹ and by then gradually increasing PP
416 concentrations in order to allow the full exploitation of the mechanisms of adaptation;
- 417 • The net methane specific yields were well simulated by using the modified Gompertz
418 equation;
- 419 • Modelling allowed to evaluate potential practical implications of inoculum adaptation to PP;
- 420 • It is feasible to consolidate the idea that a potentially pollutant organic waste (i.e. the olive
421 processing residues) can be turned into a profitable source by anaerobic digestion
422 technology, thus enhancing a sustainable and cleaner production of olive oil.

423

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430

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553 **TABLES**

554

555 Table 1 - Main physico-chemical parameters of the substrates (OMW and digestate) used in the two
556 series of anaerobic digestion tests (BMP1 and BMP2).

557

Parameter	Test					
	BMP1			BMP2		
	<i>Inoculum</i>	<i>Raw</i> OMW ¹	<i>Concentrated</i> OMW ¹	<i>Inoculum</i>	<i>Raw</i> OMW ¹	<i>Concentrated</i> OMW ¹
TS ^{2,*} [%]	5.9±0.05	3.4±0.07	12.7±0.1	7.30±0.05	3.4±0.04	10.1±0.1
TVS ^{3,*} [% on DM ⁴]	75.2±0.65	70.4±1.95	72.4±0.5	62.5±1.8	84.3±0.9	70.3±2.2
COD ^{**} [g l ⁻¹]	-	25.6±0.3	109.3±0.42	-	47.1±1.4	107.7±2.72
PP ^{5,**} [g l ⁻¹]	-	1.1±0.12	4.4±0.03	-	1.2±0.08	4.2±0.08
C/N [*]	13.8±0.2	37.2±0.1	40.9±0.15	16.4±0.14	27.1±0.2	29.2±0.3
pH [*]	8.4±0.01	4.2±0.02	4.5±0.01	8.4±0.02	4.3±0.01	4.5±0.02

558 Notes: ¹OMW = Olive Oil Mill Wastewater; ²TS = Total Solids; ³TVS = Total Volatile Solids; ⁴DM = Dry Matter; ⁵PP
559 = Polyphenols; * n = 2 replicates; ** n = 3 replicates.

560

561

563

564 Table 2 - Main physico-chemical parameters of the blends subject to the BMP tests.

565

	Parameter	Test						
		BMP1				BMP2		
		Inoculum	PP _{0.5}	PP _{1.0}	PP _{2.0}	Inoculum	PP _{1.0}	PP _{2.0}
Inoculum	Volume [ml]	200						
	TS, ^{2,*} [g]	11.8±0.08				14.6±0.08		
	TVS ^{3,*} [g]	8.9±0.06				9.2±0.22		
Concentration OMW ¹	Volume [ml]	-	26	60	170	-	63	183
	TS ^{2,*} [g]		3.4±0.02	7.6±0.0	21.6±0.1		6.3±0.0	18.4±0.15
	TVS ^{3,*} [g]		2.4±0.02	5.5±0.0	15.6±0.1		4.5±0.0	12.9±0.33
	COD ^{**} [g]		2.8±0.01	6.6±0.0	18.6±0.0		6.7±0.0	19.7±0.0004
	PP ^{4,**} [g l ⁻¹]		0.5±0.00	1.0±0.0	2.0±0.00		1.0±0.0	2.0±0.032

566 Notes: ¹OMW = Olive Oil Mill Wastewater; ²TS = Total Solids; ³TVS = Total Volatile Solids; ⁴PP = Polyphenols;

567 COD = Chemical Oxygen Demand; * n = 2 replicates; ** n = 3 replicates.

568

570 Table 3 – The net specific methane yield (in NI g⁻¹ [TVS]) after 30 d and number of days for 50%
 571 (T₅₀) and 90% (T₉₀) of maximum yield.

572

Test	Net specific methane yield	Time	
	Maximum	T ₅₀	T ₉₀
BMP1			
PP _{0.5} *	0.419	11.1	20.8
PP _{1.0} *	0.244	14.6	24.4
PP _{2.0} *	0.045	5.6	27.3
BMP2			
PP _{1.0} **	0.419	16.4	26.4
PP _{2.0} **	0.170	18.0	27.8

573 Notes: PP = Polypenols; T₅₀, T₉₀= Time needed to get 50% and 90% of the maximum methane yield; * n = 2 replicates;

574 ** n = 3 replicates.

576 Table 4 – Mean values of initial and final pH in the BMP1 and BMP2 tests (mean of 2 replicates).

577

pH	Inoculum	PP_{0.5}	PP_{1.0}	PP_{2.0}
BMP1				
Initial	8.52±0.01	7.71±0.02	7.28±0.04	7.03±0.02
Final	8.05±0.02	7.89±0.03	7.92±0.05	6.72±0.02
BMP2				
Initial	8.46±0.02	-	7.19±0.04	7.05±0.07
Final	7.92±0.02	-	7.97±0.02	7.80±0.14

578 Note: PP = Polyphenols.

580 Table 5 – The parameters obtained by the modified Gompertz model.

581

Test	P [l g ⁻¹ [TVS]] ¹	R_m [l g ⁻¹ [TVS] d ⁻¹] ¹	λ [d]	R-square [-]
<i>BMP1</i>				
<i>PP_{0.5}</i>	0.468	0.022	1.06	0.999
<i>PP_{1.0}</i>	0.317	0.010	2.73	0.997
<i>PP_{2.0}</i>	0.235	0.001	0.00	0.963
<i>BMP2</i>				
<i>PP_{1.0}</i>	0.615	0.018	4.61	0.994
<i>PP_{2.0}</i>	0.478	0.007	7.73	0.996

582 Notes: ¹TVS = Total Volatile Solids; P = methane potential; R_m = maximum methane production rate; λ = lag phase
583 period.

584

585 Table 6 - Comparison of the maximum methane yields of OMW blends reported in the literature for BMP tests.

Substrates	Inoculum adaptation	PP [g l ⁻¹]	Maximum methane yield		Notes	Authors
			Nml g ⁻¹ [TVS]] ⁷	[Nml g ⁻¹ [CODadded]] ⁸		
OMW ¹ +WW ²	No	n.a.	n.a.	214	35°C	Fontoulakis et al. (2008)
OMW ¹ +WW ²	No	n.a.	n.a.	301	55°C	
OMW ¹ (40%)+PW ³ (60%)	Yes	n.a.	480	297	-	Kougias et al. (2010)
OMW ¹ (40%)+PW ³ (60%)	Yes	n.a.	370*	277 Nml g ⁻¹ [COD _{removed}]]	-	Kougias et al. (2014)
OMW ¹	Yes	6.0*	n.a.	11.17 [l [biogas] l ⁻¹ [digester]]	-	Fezzani and Cheikh (2008)
OMW ¹ + OMSW ⁴ (56 g TS/L.OMW)	Yes	3.0*	n.a.	30.5 [l [biogas] l ⁻¹ [digester]]	-	
OMW ¹ (40%) + AW ⁵ (60%)	Yes	2.04	n.a.	0.189 [l [biogas] l ⁻¹ [COD _{removed}]]	Toxicity test 37°C	Gannoun et al. (2007)
OMW ¹ (70%) + LPM ⁶ (30%)	Yes	6.83*	55.1 [ml g ⁻¹ [TS]]	n.a.	-	Khoufi et al. (2015)
OMW ¹	No	0.5	419	353	BMP1 tests	This study
OMW ¹	No	1.0	243	205		
OMW ¹	No	2.0	45	37		
OMW ¹	Yes	1.0	419	275	BMP2 tests	
OMW ¹	Yes	2.0	170	111		

586 Notes: ¹OMW = Olive Oil Mill Wastewater; ²WW = Winery Waste; ³PW = Pig Waste; ⁴OMSW = Olive Mill Solid Waste; ⁵AW = Abattoir Wastewater; ⁶LPM = Liquid Poultry

587 Manure; ⁷TVS = Total Volatile Solids; ⁸COD = Chemical Oxygen Demand; *Adapted data.

588 **FIGURE CAPTIONS**

589

590 Fig. 1 – Scheme of the BMP experimental design.

591

592 Fig. 2 – Net specific biogas (a) and methane (b) yield in BMP1 tests (mean \pm standard
593 deviation).

594

595 Fig. 3 - Net specific biogas and methane yield in BMP2 tests (mean \pm standard
596 deviation).

597

598 Fig. 4 - Comparison of cumulated specific methane yields measured in BMP1 and
599 BMP2 tests as a function of PP concentrations (mean \pm standard deviation).

600

601 Notes: The different lowercase letters indicate significant differences between PP concentrations within
602 the same BMP series; the different capital letters indicate significant differences between the BMP1 and
603 BMP2 series of blends at the same PP concentration (one-way ANOVA together with Tukey's test for
604 pairwise comparisons at $p < 0.05$).

605







