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► **To cite this version:**

Omar Bouaouine, Isabelle Bourven, Fouad Khalil, Michel Baudu. Reuse of olive mill wastewater as a bioflocculant for water treatment processes. *Journal of Cleaner Production*, 2020, 246, pp.119031 -. 10.1016/j.jclepro.2019.119031 . hal-03488904

HAL Id: hal-03488904

<https://hal.science/hal-03488904>

Submitted on 21 Jul 2022

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Reuse of olive mill wastewater as a bioflocculant for water treatment processes

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Abstract

This study investigates the reuse potential of olive mill wastewater (OMW) high in tannin content and whose complete treatment requires the use of complex and expensive processes. Tannins from OMW could serve as bioflocculant compounds according to the literature. The defatted OMW is tested in a coagulation-flocculation process on synthetic water, and the best turbidity removal rate derived is: $92\% \pm 1\%$ at pH 11 for 100 mg L^{-1} . Water-solubilized material at pH 11 precipitates after acidification at pH 6. This precipitate fraction (PP), solubilized at pH 11, is found to contain more active constituents with a turbidity removal rate of $82\% \pm 2\%$ for a 60 mg L^{-1} dose yet reveals an increase in absorbance at 254 nm. This increase in absorbance can be corrected by using lime for a pH adjustment to 11 with an absorbance removal at 254 nm of $50\% \pm 1\%$. The bioflocculant material is characterized by means of physicochemical methods. Acid-base titration, enzymatic treatment and colorimetric dosage all confirm that tannins and/or flavonoids and cellulose constitute the active groups involved in the coagulation-flocculation process.

Keywords: bioflocculation; olive mill wastewater; tannins; flavonoids; cellulose; reuse.

22 **1. Introduction**

23 Olive mill wastewater (OMW) is a byproduct of the three-phase process of olive oil
24 extraction, with a global annual production reaching 8.4 million m³ (Doula *et al.*, 2017). This
25 black liquid is composed of the olive fruit vegetation water, the water used for washing and
26 treatment, plus a portion of the olive pulp and residual oil (Scioli *et al.*, 1997). Contamination
27 from this waste can be reduced by adsorbing polyphenols onto activated carbon (Annab *et al.*,
28 2018). However, a complete treatment of OMW had only been possible using expensive
29 advanced oxidation processes (Ochando-Pulido *et al.*, 2017) or electrocoagulation (Flores *et*
30 *al.*, 2018). Recovery methods have been proposed, such as the production of H₂ yield by
31 means of supercritical water gasification (Casademont *et al.*, 2018) or by reaching much
32 greater depths in agricultural practices (Pardo *et al.*, 2017) depending on the potential
33 processed wastes (Campani *et al.*, 2017).

34 OMW is a foul-smelling acidic liquid composed of water (83-92%), organic matter (4-16%)
35 and minerals (1-2%) (Perez *et al.*, 1998). The organic load, as reflected in both the high
36 biological oxygen demand (BOD) (up to 100 g L⁻¹) and chemical oxygen demand (COD) (up
37 to 200 g L⁻¹), contains mostly sugars, polyphenols and fats (Hamdi *et al.*, 1993). The use of
38 OMW as organic compost without any treatment could cause a degradation of the soil or
39 water, due in particular to its phenolic constituents (antimicrobial activity) as well as to the
40 low pH range (4-5) (Elhajjouji *et al.*, 2007). OMW contains a wide variety of compounds,
41 such as polyphenol, polysaccharides, tannins and proteins (Michael *et al.*, 2014) and therefore
42 is potentially involved in the coagulation-flocculation mechanism.

43 The coagulants and flocculants currently required for water treatment, i.e. metal salts and/or
44 synthetic polymers, may exert several environmental impacts, namely: i) a possible increase
45 of the metal concentration in water (with implications for human health); and ii) extensive
46 sludge production. This production of metal hydroxides lies at the origin of many

47 environmental investigations, due to the volumes involved and the high treatment-related
48 costs if landfill burial is the chosen option. For these reasons, alternative coagulants and
49 flocculants have been considered for environmental applications (Kudryavtsev and
50 Kudryavtsev, 2016). Biopolymers may be quite attractive since they are vegetal products, and
51 their life cycle could incorporate agricultural reuse or improved integration into the
52 environment with biological degradation.

53 Some of the reported biological materials, including biopolymers (starches, chitosan,
54 alginates), have shown their potential in the flocculation process. The biochemical families
55 extracted from plants can also be used; these families include polysaccharides from *Ocimum*
56 *basilicum* (Shamsjenati *et al.*, 2015), starches from cereal (Choy *et al.*, 2016), proteins from
57 *Cocos nucifera* (Fatombi *et al.*, 2013), *Moringa oleifera* (Dolto *et al.*, 2018), polyphenols
58 from *Opuntia ficus indica*, acorn (Bouaouine *et al.*, 2018a), *Cassia obtusifolia*
59 (Subranmoniam *et al.*, 2014) and grape seed (Jeon *et al.*, 2009). Some polyphenols form
60 complexes with macromolecules and, more specifically, with proteins via hydrogen bonds or
61 hydrophobic interactions (Bruneton *et al.*, 2009). Polyphenols have already been introduced
62 into water treatment as flocculants. Jeon *et al.* (2009) demonstrated that catechin (flavonoid)
63 and tannic acid (tannin) were involved in the molecular flocculation mechanism. A
64 commercial tannin-based flocculant product enhanced the performance of clarification in a
65 biological treatment unit (Hameed *et al.*, 2018).

66 According to the literature, no information exists to date regarding the efficiency of OMW as
67 a bioflocculant for water treatment. Given this context, the objectives of the present study are
68 threefold: i) highlight the capabilities of OMW as a coagulant/flocculant; ii) enhance current
69 knowledge on the most active constituents potentially involved in water treatment; and iii)
70 propose an application in the field of water treatment. To achieve these objectives, water-
71 soluble chemical compounds have been isolated. These materials were first characterized

72 using overall physicochemical methods (zeta potential, colloidal particle size distribution and
73 spectroscopy). The active molecular groups were then determined by means of acid-base
74 titration, selective enzymatic treatment and colorimetric dosage.

75

76 **2. Materials and methods**

77

78 2.1 Biofloculant preparation

79

80 2.1.1 Preparation of olive mill wastewater materials

81 The olive mill wastewater (OMW) used herein was extracted from a modern semi-oil mill in
82 Fez (Morocco); moreover, no chemical additives were used during production of this olive
83 oil. In an initial step, the lipid residual was extracted from the concentrated suspension by
84 organic solvents. These solvents (60 mL (2/1, v/v chloroform/hexane)) were then introduced
85 into a suspension of OMW (100 mL) and shaken for 2 hours at room temperature. This step
86 was repeated six times in order to ensure maximum recovery of the lipid. The defatted
87 material (defatted OMW) was dehydrated at 80°C for 24 hours and stored at 4°C following
88 freeze-drying (see Fig. 1). The volatile dry weight (% VDW) equaled: 78% ± 1%.

89 2.1.2 Extraction at various pH levels

90 To separate the active constituents, 1 g of defatted OMW material was solubilized in 100 mL
91 of distilled water at pH 6 and 11 adjusted with NaOH (1 M) and HCl (1 M). The Water
92 Solubilized Material (WSM 6 and WSM 11) was then stirred at 200 rpm at room temperature
93 for 24 hours ± 0.2 hours and filtrated at 0.45 µm, before being freeze-dried and stored at 4°C
94 (Bouaouine *et al.*, 2018a). Next, WSM 11 was acidified to pH 6, which allowed precipitating
95 the soluble alkaline extract under acidic conditions. The precipitate filtrated at 0.45 µm and
96 re-solubilized under an alkaline condition (pH 11) was denoted "precipitate" (PP). This PP
97 was also freeze-dried and stored at 4°C (Fig. 1).

98

100 2.2 Physicochemical characterization of defatted OMW materials

101 WSM 6 and 11 and the defatted OMW solution were subjected to 105°C for 24 hours in order
102 to determine the dry weight (DW) of the material, in g L⁻¹. The volatile dry weight (VDW)
103 was deduced after burning the previous sample for two hours at 550°C. VDW% is expressed
104 as a fraction of DW. The residual solid encompasses all insoluble solids present in solution
105 after a 0.45-µm filtration.

106 The UV-1800 Shimadzu spectrophotometer was used for the absorbance measurement
107 campaigns. Absorbances at 210 and 280 nm were read in a glass cuvette after filtration (0.45-
108 µm cellulose acetate) of 10 g dry weight of a defatted OMW fraction in 1 L. The majority of
109 organic molecules absorbed at both 210 nm (carbohydrates, proteins, tannins, etc.) (Her *et al.*,
110 2008) and 280 nm (absorption of aromatic groups from tannins, proteins) could be measured.

111 The Malvern Master Zetasizer 3000 device was used to obtain the zeta potential, in
112 accordance with the same procedure as in Bouaouine *et al.* (2018a). The defatted OMW
113 materials were characterized by means of Fourier transform infrared (FTIR) spectroscopy,
114 while the ionizable groups of defatted OMW or WSM were determined using acid-base
115 titration (Bouaouine *et al.*, 2018a).

116 2.3 Colorimetry

117 The defatted OMW fraction contents, in terms of proteins (Frolund *et al.*, 1996),
118 carbohydrates, polysaccharides (Dubois *et al.*, 1956), uronic acid (Blumenkrantz *et al.*, 1973)
119 and polyphenols (flavonoids, tannins) (Frolund *et al.*, 1996; Salem *et al.*, 2010, respectively),
120 were determined using colorimetric methods, with the parameters as described by Bouaouine
121 *et al.* (2018b), and in supplementary data “table S1”.

123
124 2.4 Coagulation-flocculation experiments

125 2.4.1 Synthetic water

126 Like in many studies of flocculation mechanisms, a synthetic water is proposed with kaolin (1
127 g L⁻¹) (Sigma Aldrich) in order to derive a turbidity (using the Hanna HI 88713 turbidimeter)
128 of 325 ± 25 nephelometric turbidity units (NTU) (Miller *et al.*, 2008). To complete this
129 synthetic water, humic acid (10 g L⁻¹) (Sigma Aldrich) (Yang *et al.*, 2010) and mineral salts
130 were diluted (Table 1).

131

	Cations (µM)					Anions (µM)				pH	
132	Values	K ⁺	Na ⁺	Mg ²⁺	Ca ²⁺	Si ⁴⁺	Cl ⁻	HCO ₃ ⁻	SO ₄ ²⁻	NO ₃ ⁻	6.5 ± 0.4
133	134	29	283	81	108	140	171	283	51	134	

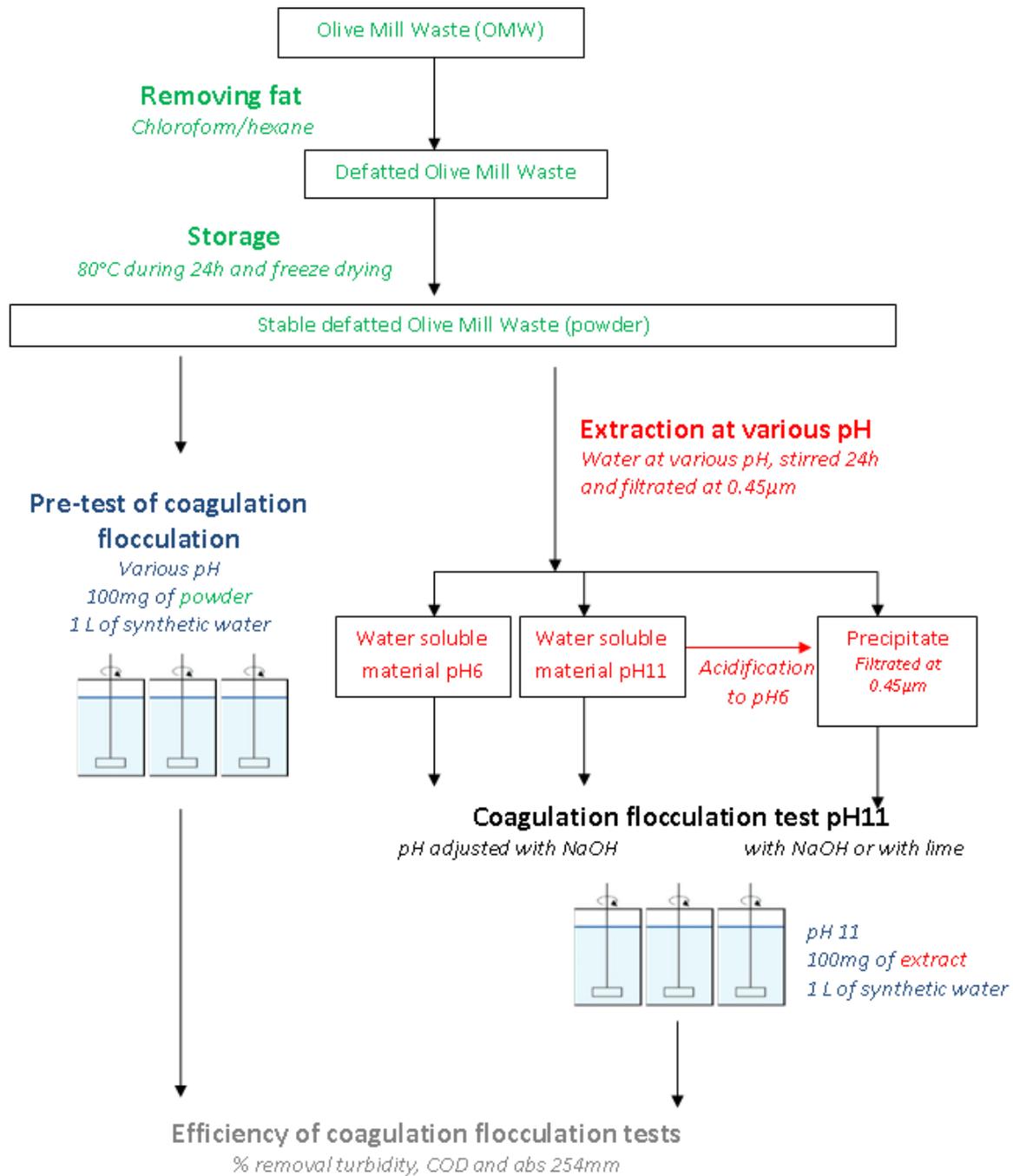
135
136

137 **Table 1:** The ionic balance of synthetic water

138
139 2.4.2 Coagulation-flocculation protocol and efficient measurements

140 The device used for the coagulation-flocculation process was composed of six stainless steel
141 paddles of multiple stirrers (VELP Scientifica JTL6), each containing 1 L of synthetic water.
142 In order to determine the optimal pH, a pH variation from 3 to 12 was applied with NaOH and
143 HCl at a constant treatment rate of 100 mg L⁻¹ of defatted OMW. Afterwards, a series of
144 different doses, ranging from 0 to 160 mg L⁻¹ of defatted OMW, was tested to obtain the
145 optimal treatment conditions. The sample was immediately stirred at a constant speed of 250
146 rpm for 10 min, followed by a slow stirring at 50 rpm for 30 min; once a 2-hour setting time
147 had elapsed, the turbidity measurement was conducted on the supernatant.

148



149

150

Fig. 1: Flocculent production from Olive Mill Waste and corresponding evaluation methodology

151

2.4.3 Implementation for optimization purposes

152

The PP treatment efficiency was compared for the case of a pH 11 adjustment by either NaOH

153

or lime. The treatment efficiency for various PP doses was measured by both turbidity and

154

absorbance at 254-nm. These results were then compared to a standard treatment by

155

aluminum sulfates (AS), with a variation of AS doses from 0 to 160 mg L⁻¹ at a fixed pH of 7

156 being introduced in order to achieve optimal conditions. The three conditions described were
157 compared for optimality.

158 2.5 Enzymatic treatment

159 To identify the active (macro) molecules in the defatted OMW material, hydrolytic enzymes
160 were tested for the degradation of targeted compounds found in the defatted OMW. The
161 enzymes and specific conditions of their actions are summarized in Table 2.

Enzyme	Hydrolyzed biochemical enzyme	pH	Temperature (°C)
α amylase (Sigma Aldrich)	Starch	7.0 ± 0.1	20
Cellulase (Alfa Aesar)	Cellulose	5.0 ± 0.1	50
Tyrosinase (Sigma Aldrich)	Tannin	6.5 ± 0.1	25
Protease alcalase from <i>Bacillus Licheniformis</i> (Sigma Aldrich)	Protein	9.0 ± 0.1	55

162
163 **Table 2:** pH and temperature enzymatic conditions for hydrolyzing precipitate (PP)

164 The enzymatic digestion procedure applied to the precipitate fraction was the same as that
165 detailed by Bouaouine *et al.* (2018b). Put briefly, 18 mg of enzyme were added to a solution
166 of 10 mL of distilled water containing an optimal PP dose of 62.5 mg L^{-1} in order to reach a
167 ratio $> 10\%$ W/W, i.e. enzyme/precipitate. The incubation period under optimal pH and
168 temperature conditions, which depend on the specific enzyme type, lasted two hours in the
169 previous mixture (Table 2). This preparation is then added to 1 L of synthetic water tested
170 during the coagulation-flocculation experiment (see Section 2.4).

171

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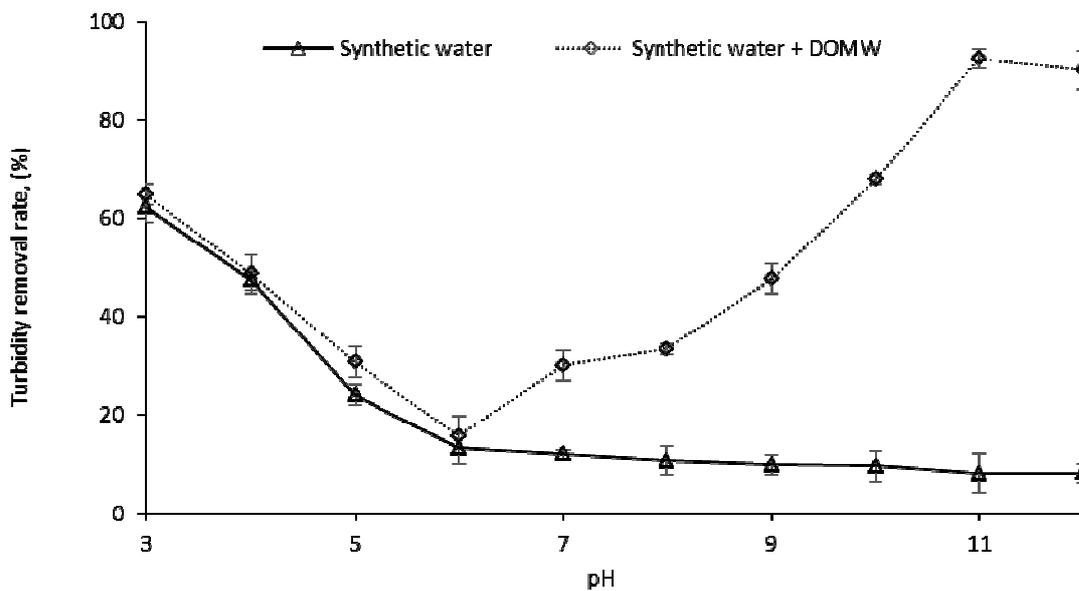
174 **3. Results and discussion**

175

176 3.1 Coagulation-flocculation effect

177 We initially sought to check the capacity of the defatted OMW material as a flocculent. The
178 removal of turbidity at a constant dose of 100 mg L⁻¹, as a function of pH, is proposed in
179 Figure 2.

180



181

182 **Fig. 2:** pH effect on the coagulation-flocculation process at a fixed dose of 100 mg of defatted olive
183 mill wastewater (OMW) material for 1 L of synthetic water; n = 3 (independent assays)

184 The efficiency comparison with other bioflocculants (Table 3) showed a significant reduction
185 in turbidity with the defatted OMW at a low treatment rate. The range of pH values for
186 optimal conditions varies (from 6 to 11) most likely depending on the extracted molecules.

187

188

189

190

Biomaterial	Defatted OMW	<i>Cocos Nucifera</i>	<i>Gossypium Spp</i>	<i>Luffa cylindrica</i>	<i>Phaseolus Mungo</i>	Tannin
Effluent	Kaolin suspensions	Silica suspensions	Phosphorus wastewater	Surface water	Surface Water	Municipal wastewater
Initial turbidity (NTU)	350	48	-	32	482	79
Optimal pH	11	8	6	9.4	7.6	8
Optimal dose (mg L ⁻¹)	60	250	500	8,000	800	30
Turbidity removal (%)	93 ± 1	25	> 70	85	100	83
References		Fatombi <i>et al.</i> , 2013	Babayem <i>et al.</i> , 2015	Soweyman <i>et al.</i> , 2011	Mbogo <i>et al.</i> , 2008	Hameed <i>et al.</i> , 2018

191

Table 3: Comparison of biomaterial performance

192 In the presence of defatted OMW, three pH value ranges can be observed. The optimal
193 coagulation-flocculation pH equals 11.0, while the minimum is around 6. Under an acidic
194 condition (i.e. from pH 3 to 6), turbidity removal rates do not differ significantly for synthetic
195 water alone or in combination with defatted OMW, yet they do decrease with pH value from
196 64% to 15%; these results indicate the absence of any defatted OMW effect. The increase in
197 zeta potential (i.e. reduction of negative charge), with acidification of the solution favors a
198 coagulation mechanism of clay particles (Aljerf, 2018). High-efficiency extraction of
199 bromocresol purple dye and heavy metals as chromium from industrial effluent by adsorption
200 onto a modified surface of zeolite: Kinetics and equilibrium study. Journal of environmental
201 management. 225. 120-132. 10.1016/j.jenvman.2018.07.048. along with a coagulation of
202 colloids, in accordance with DLVO theory (see supplementary data “fig S2” which describes
203 the zeta potential variation as pH function). Afterwards, from pH 6 to 11, when defatted

204 OMW is present in solution, turbidity removal reaches a peak at pH 11 with a rate of: $92\% \pm$
205 2% . Lastly, from pH 11 to 12, the removal rate remains practically stable, from 92% to $90\% \pm$
206 1% . The zeta potential measurement indicated negative values for both defatted OMW and
207 synthetic water regardless of pH (between 3 and 12) (data not shown). The molecules present
208 in defatted OMW start out reactive, while the negative charge of clay particles increases. This
209 negative charge is unfavorable to a coagulation mechanism with a strong repellent effect
210 between colloids. As such, the destabilization of clays and defatted OMW colloids is not a
211 direct neutralization of particles by introduction of cationic charges (ions or colloids) in the
212 synthetic water as shown the zeta potential measurement, but instead a flocculation, and more
213 specifically an adsorption/bridging mechanism with a weak neutralization of clay particles but
214 a floc structuration.

215 To better grasp the influence of pH, defatted OMW solubilization was performed at two pH
216 values, i.e. pH = 11, the maximum flocculation efficiency, and pH = 6, at which flocculation
217 appears to be minimized (Fig. 2). Moreover, in order to isolate the specific active constituents
218 of defatted OMW materials soluble at pH 11, the pH has been subsequently adjusted to 6, so
219 as to form a precipitate (i.e. at pH 6, component WSM 11 is inactive). The flocculant activity
220 of the water-solubilized material at pH 6 (WSM 6) and pH 11 (WSM 11) was tested, as it was
221 for the precipitate (PP). It is observed that both PP and WSM 11 display nearly the same
222 efficiency, with a strong turbidity reduction, yet the effect of WSM 6 remains weak. The most
223 robust activity of defatted OMW at pH = 11 is correlated with: i) qualitative aspects with the
224 solubilization of specific molecules and an ionization at alkaline pH; and ii) a new
225 conformation of macromolecules.

226

227

228 3.2 Identification of functional groups

229
 230 The characteristics of the water-solubilized material at both pH values 6 (WSM 6) and 11
 231 (WSM 11) are presented in Table 4 below.

pH of WSM	Solubilization (RS/Defatted OMW) (%DW)	VDW (%)	Abs _{210 nm} for 1 g dry weight	Abs _{280 nm} for 1 g dry weight
6.0	2.1 ± 0.1	30 ± 2	0.64 ± 0.01	0.13 ± 0.02
11.0	2.7 ± 0.1	44 ± 1	0.94 ± 0.03	0.21 ± 0.01

232
 233 **Table 4:** Characteristics of WSM 6 and WSM 11; WSM: Water-solubilized material;
 234 DW: Dry weight; RS: Residual solid; n = 3 (independent assays)

235
 236 The organic and inorganic matter contained in the defatted OMW is more readily solubilized
 237 in water at pH 11 than at pH 6 since 2.1 and 2.7 g / 100 g of matter are respectively recovered
 238 in the supernatant (factor = 1.3), thus underscoring an enrichment in organic matter (VDW
 239 increases from 30 ± 2% to 44 ± 1% for WSM 6 and WSM 11, respectively). This tendency
 240 also reflects the different nature of organic matter in the WSM fraction: note the increase in
 241 absorbance at 210 and 280 nm relative to pH, with a factor > 1.47 for the two wavelengths. At
 242 pH 11, WSM 11 exhibits higher absorbance at 210 nm, where many organic molecules can
 243 absorb (Her *et al.*, 2008). At 280 nm, only those organic molecules with an aromatic ring (or
 244 conjugated bonds), such as phenol in tannin or protein, are capable of absorption. At 280 nm,
 245 the WSM 11/WSM 6 absorbance factor ratio equals 1.61. This finding implies that WSM 11
 246 contains more molecules enriched in aromatic groups than WSM 6.

247 Acid-base titrations of defatted OMW materials (Table 5) have led to detecting four acidic
 248 constants (pK_as): pK_{a1} (2.5 ± 0.2) corresponds to the carboxyl group from free amino acids
 249 (Vcélakova *et al.*, 2004); pK_{a2} (6.5 ± 0.3) is mainly correlated with -COOH from
 250 polysaccharides and secondary alcohols (Droussi *et al.*, 2009); pK_{a3} (9.0 ± 0.4) may be

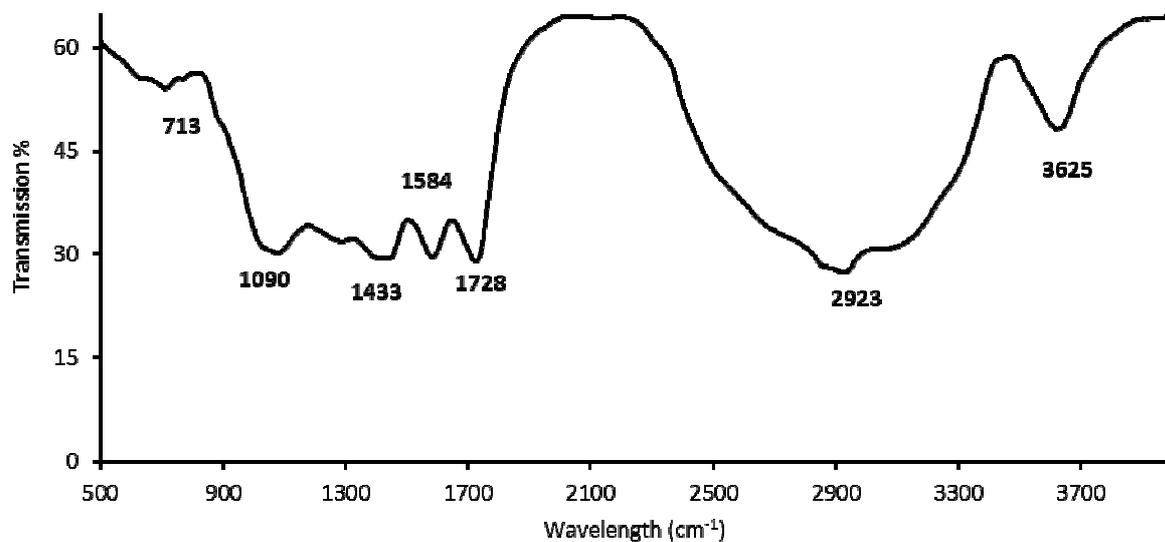
251 associated with the phenol groups (Ragnar *et al.*, 2000) from either lignin (Beliy *et al.*, 2010)
 252 or tannin, e.g. catechin (Martinez *et al.*, 2005); and lastly pKa₄ (11-12) could correspond to
 253 amino acids like tyrosine (Sjoholm *et al.*, 1974) or else to free sugars, such as glucose,
 254 arabinose and fructose (Bhattacharyya and Rohrer, 2012).

255

pKa _s	Defatted OMW	WSM 11	WSM 6	PP
pKa ₁	2.5 ± 0.2			
pKa ₂	6.5 ± 0.3		6.5 ± 0.3	
pKa ₃	9.0 ± 0.4	9.0 ± 0.3		9.0 ± 0.4
pKa ₄	12.0 ± 0.7	11.8 ± 0.4	11.0 ± 0.8	12.0 ± 0.5

256

257 **Table 5:** pKa_s values for all defatted olive mill wastewater (Defatted OMW) used as a raw material,
 258 water-solubilized material (WSM) at pH values of 6 and 11 and precipitate (PP); n=2



259

260 **Fig. 3:** FTIR spectrum of defatted olive mill wastewater (Defatted OMW) material

261

262 According to the literature, a comparison drawn between the results detected in FTIR bands
 263 and acidic constants of ionizable groups has revealed that pKa₁, which corresponds to
 264 carboxyl groups (Vcélakova *et al.*, 2004), as confirmed by the appearance of a peak at 1,100
 265 cm⁻¹ (Bouatay *et al.*, 2014), might possibly be associated with tryptophan. The presence of -

266 COOH groups has already been proven by both pK_{a2} and the band at 710 cm^{-1} (Droussi *et al.*,
267 2009), which may be corroborated by the uronic acid (polysaccharides) (Elhajjouji *et al.*,
268 2007). pK_{a3} is correlated with phenol groups (Droussi *et al.*, 2009), and this presence may
269 pertain to the tannins and/or lignin. Lastly, pK_{a4} , as represented by $-C=O$ groups (Elhajjouji *et*
270 *al.*, 2007) and having appeared in FTIR by the peak absorption at $1,728\text{ cm}^{-1}$ (Moran *et al.*,
271 2008), could correspond to polysaccharides (Fig. 3).

272 The defatted olive mill wastewater (OMW) material displays four acidic constants, whereas
273 WSM 6, WSM 11 and PP only display two pK_{a_s} . pK_{a2} , which represents uronic acids (Wang
274 *et al.*, 1991), is observed to be common between defatted OMW and WSM 6 materials (except
275 for the fact that the removal efficiency of WSM 6 is only 23% with 325 and 250 NTU for
276 turbidity in and out respectively).

277 pK_{a3} , which corresponds to phenol groups, and pK_{a4} may however align with sugars and
278 proteins, with common features across the defatted OMW material, WSM 11 and PP. From
279 these observations, polyphenols, proteins and sugars are all suspected to be correlated with
280 turbidity removal efficiency as reported in the literature (Bouaouine *et al.*, 2018a, Fatombi *et*
281 *al.*, 2013, and Shamsjenati *et al.*, 2015, respectively)

282 3.3 Identification of reactive molecules

283 In order to identify the major constituents involved in the flocculation reaction, we have
284 determined by means of colorimetric assays the main biochemical families, i.e. proteins,
285 polysaccharides, uronic acid, phenol and tannins included in phenol family, for the defatted
286 olive mill wastewater (OMW) materials plus the three preparations (WSM 6, WSM 11 and
287 PP). Table 6 presents the contents of biochemical molecules on WSM 6, 11, PP and their
288 WSM 11 / WSM 6 and PP / WSM 11 ratios.

289

Sample	Protein (SAB eq.)	Polysaccharide (glucose eq.)	Uronic acid (gluc. acid eq.)	Phenol (gallic acid eq.)	Tannin (catechin eq.)
WSM 6 (g.g ⁻¹)	0.145 ± 0.001	0.028 ± 0.001	0.29 ± 0.01	0.165 ± 0.001	0.026 ± 0.001
WSM 11 (g.g ⁻¹)	0.152 ± 0.001	0.023 ± 0.001	0.24 ± 0.01	0.17 ± 0.01	0.028 ± 0.001
PP (g.g ⁻¹)	0.15 ± 0.01	0.023 ± 0.001	0.24 ± 0.01	0.175 ± 0.001	0.05 ± 0.01
WSM11/WSM 6	1.05 ± 0.01	0.82 ± 0.02	0.83 ± 0.02	1.03 ± 0.01	1.33 ± 0.02
PP/WSM 11	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.02	1.03 ± 0.01	1.55 ± 0.01

290

291 **Table 6:** Contents of biochemical molecules on WSM 6, 11, PP and their ratios; n = 3
292 (independent assays)

293

294 Table 6 shows that the [WSM 11 / WSM 6] ratio for protein and phenol nearly exceed 1. For
295 tannin, the [WSM 11 / WSM 6] ratio is greater than 1 (1.33), and this same trend was observed
296 with the ratios between PP and WSM 11 (1.55). The phenol/polyphenol family includes tannins
297 and lignin, which indicates that tannins are particularly well solubilized at pH 11 and
298 concentrated in PP, as compared to other biochemical families. These results confirm that
299 phenol groups (see Table 5) and therefore tannins could be among the most active molecules in
300 the flocculation process and soluble at pH 11.

301 In order to specify the biochemical group for tannins, which includes condensed tannins and
302 flavonoids, the condensed tannins and total flavonoids for WSM 6, WSM 11 and PP were
303 determined (Table 7). The increase in total tannin concentrations between WSM 6 and WSM
304 11 (factor = 1.33) and between WSM 11 and PP (factor = 1.55) confirms the specific
305 solubilization in alkaline pH and the concentration by precipitation, respectively. For
306 condensed tannins, an increase was also found in concentrations between WSM 6 and WSM 11
307 (factor = 1.3) and between WSM 11 and PP (factor = 1.2). This same trend could also be
308 observed for total flavonoids, with a strong increase from WSM 6 to WSM 11 (factor > 1.3),
309 hence an increase between WSM 11 and PP (factor > 2.1).

310

Sample	Tannins, in mg.g ⁻¹ of DW (catechin eq.)	Condensed tannins, in mg.g ⁻¹ of DW (catechin eq.)	Flavonoids, in mg.g ⁻¹ of DW (catechin eq.)
WSM 6	30 ± 1	20 ± 2	12 ± 2
WSM 11	40 ± 1	26 ± 1	16 ± 3
Precipitate (PP)	62 ± 1	31 ± 1	34 ± 2

311

312 **Table 7:** Contents of specific polyphenols: total tannins, condensed tannins and flavonoids from
313 solubilized defatted olive mill wastewater (OMW), as determined by colorimetric methods;

314 DW: dry weight; n=3

315 These results reveal that the molecules responsible for the flocculation mechanism belong to
316 flavonoids and/or condensed tannins. Hameed *et al.* (2018) found the reactivity of commercial
317 tannin for turbidity removal from a municipal wastewater at a rate of 83% for a of 30 mg L⁻¹
318 treatment rate and a pH value around 8. Moreover, Ozacar *et al.* (2002) showed that at pH 11,
319 tannins extracted from *Valonia* were highly efficient, with an 80.5% decrease in turbidity for
320 a synthetic surface water at a dose of 100 mg L⁻¹. Other authors have demonstrated that
321 polyphenols (Jeon *et al.*, 2009), proteins (Ndabigenegesere *et al.*, 1998) and polysaccharides
322 (Miller *et al.*, 2008) represent the active molecules in grape seed, *Moringa Oleifera* and cactus
323 *Opuntia ficus indica*, respectively. For these reasons, other tests are essential in confirming
324 the potential of proteins and polysaccharides (according to the results in Table 6 and Fig. 3) to
325 be the constituents responsible for the flocculation mechanism by defatted OMW.

326 To complete the investigation of flocculent biochemical molecules present in PP, this fraction
327 was processed with four hydrolytic enzymes (Table 2): α amylase, cellulase, protease
328 alcalase, and tyrosinase. If active molecules are being hydrolyzed, then the modified PP
329 shows a lower efficiency for the flocculation of synthetic water. To validate these results, two
330 controls were performed, i.e.: Control 1 (preparation with enzyme alone), and Control 2
331 (preparation without any enzymes, i.e. PP alone). The four Controls 1 and 2 indicated a

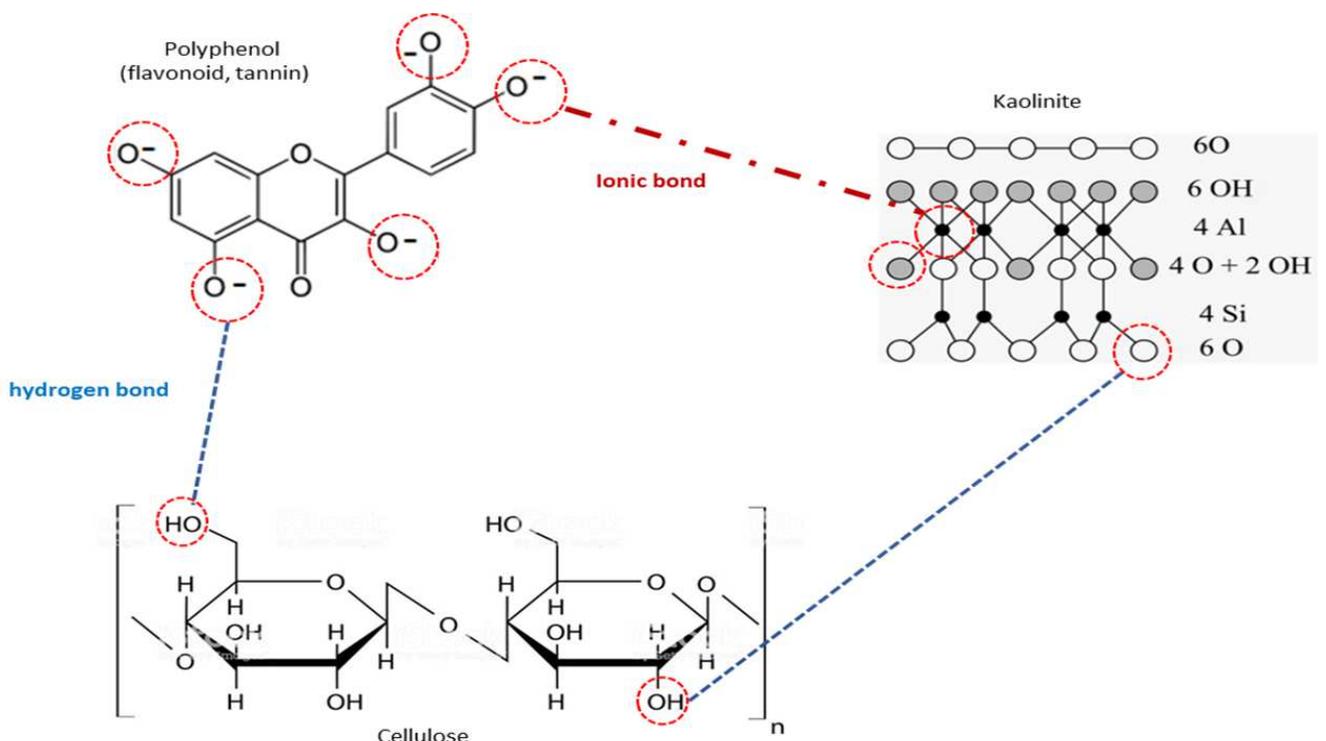
332 turbidity removal efficiency of $15\% \pm 1\%$ (turbidity in 325 NTU and turbidity out 275 NTU)
333 and $83\% \pm 1\%$ (turbidity in 325 NTU and turbidity out 55 NTU), respectively.

334 Flocculation efficiency after degradation by enzymes exhibits hardly any effect for α amylase
335 and protease alcalase, with $79\% \pm 2\%$ and $80\% \pm 3\%$ turbidity removal rates, respectively.
336 With cellulose however, the turbidity removal by coagulation-flocculation was decreased to
337 $60\% \pm 2\%$ and to an even greater extent with tyrosinase: $24\% \pm 4\%$. These results reveal that
338 enzymatic treatments are in agreement with colorimetric dosages (Fig. 3 and Table 6), FTIR
339 and acid-base titrations, thus confirming that tannins (condensed tannins and/or flavonoids)
340 and cellulose, present in defatted OMW materials (Hamdi *et al.*, 1993), do constitute the
341 active groups responsible for the flocculation phenomenon by defatted OMW. Data from the
342 literature confirm the efficiency of cellulose from cotton pulp in coagulation-flocculation
343 (Song *et al.*, 2010). These results thus suggest that tannin and/or flavonoid may act as a
344 coagulant by destabilizing colloids by adsorption on cationic sites (iron oxides, aluminum or
345 calcium) may be present on the clays surface (Xu *et al.*, 1988) . Septian *et al.* (2018) showed
346 that sulfadiazine adsorption was possible onto montmorillonite and kaolinite at pH 8. A
347 mechanism of p-p interaction between clay surface and organic functional groups was
348 observed for polycyclic aromatic hydrocarbons (Yanfen *et al.*, 2018). Binding is attributed to
349 p-p interaction between an oxygen plane on the clay and aromatic ring on the molecule.
350 Following coagulation, the cellulose interacts with polyphenol (Aldred *et al.*, 2009) and may
351 form a large cross-linked polymeric network responsible for flocculation by means of
352 bridging.

353 3.4 The mechanism involved and highlights

354 The strong negative charge of colloids (insoluble material of defatted OMW or bentonite)
355 under an alkaline condition is unfavorable to a coagulation mechanism with strong repellent
356 effects. In accordance with DLVO theory, the aggregation of colloidal aqueous dispersions is

357 improbable when the energy barrier is maximum. Particles remain dispersed throughout the
 358 medium. At larger distances the energy profile became minimum and chemical flocculation is
 359 possible by polymer bridging. A model of chemical interactions between active molecules
 360 from PP and kaolinite is proposed in Figure 4. At pH 11, tannins are ionized and could
 361 establish ionic bonds with the Al of kaolinite (Kuntic *et al.*, 2003). Cellulose can be adsorbed
 362 on kaolinite by hydrogen bonding and contribute to floc structuration and both flocculation by
 363 bridging and sweeping coagulation (Sjoberg *et al* 1999). Adsorption on the basal surface of
 364 kaolinite occurs mainly on the aluminum hydroxide surface and at the edge; polyphenol may
 365 be linked by hydrogen bonds to aluminol or silanol groups and principally to aluminol in
 366 alkaline media (Bergaya and Lagaly, 2013). To explain the interaction taking place between
 367 polyphenols (tannins) and cellulose, two means merit discussion: i) hydrogen bonds, and ii)
 368 hydrophobic interactions (Mateus *et al.*, 2004).



369

370 **Fig. 4:** Chemical interactions involved in coagulation-flocculation between quercetin, cellulose and
 371 kaolinite

372 Coagulation-flocculation reactions with defatted OMW material (Fig. 2) are indicative of the
 373 potential in substituting chemical coagulants/flocculants. Additional research has been
 374 proposed to experiment on integrating the most active material of defatted OMW (PP) in a
 375 water treatment process. The treatment with precipitate adjusted to pH 11 with NaOH or lime
 376 is compared to a standard reference for wastewater treatment, aluminum sulfate (AS) (Table
 377 8).

	Dose (mg L ⁻¹)	COD (mg L ⁻¹)		Turbidity (NTU)	
		in	out	in	out
Al ₂ (SO ₄) ₃ (pH = 7)	80	14.8	0.7 ± 0.3	325	7 ± 3
Precipitate (pH = 11 with NaOH)	60	14.8	21.6 ± 1	325	21 ± 3
Precipitate (pH = 11 with lime)	60	14.8	7.4 ± 0.3	325	23 ± 3

378 **Table 8:** Comparison of the efficiency treatment between aluminum sulfate (AS) and precipitate (PP)
 379 with a pH adjustment by NaOH or lime; n=3 (independent assays)

380 Absorbance of the synthetic water at 254 nm, which corresponds to aromatic groups from
 381 humic substances, equals 1.2 ± 0.2.

382 Table 8 presents the optimal dose for the three treatments, the residual COD and turbidity
 383 under these conditions. The best efficiency with AS was observed at 80 mg L⁻¹, with 95 ± 2%
 384 and 98 ± 1% of absorbance and turbidity drops, respectively. In parallel, the use of PP has
 385 demonstrated competitive results, as adjusting pH with NaOH led to turbidity removal rate of
 386 83 ± 1%, albeit with an increase in absorbance at 254 nm (-46 ± 1%) for a treatment rate of 60
 387 mg L⁻¹. In contrast, pH adjustment by lime has resulted in a higher removal of turbidity (93 +

388 1%) and an improved absorbance removal at $50 \pm 2\%$ for a dose of 60 mg L^{-1} . Lime exerts an
389 effective action on the coagulation-flocculation mechanism (Rharrabati *et al.*, 2018). The
390 divalent cation Ca^{2+} contributes to interparticle bridging between all anionic species:
391 kaolinite, polyphenols, but also the cellulose or humic acids of synthetic water (Choudhary *et*
392 *al.*, 2019). This preparation from defatted OMW (precipitate fraction (PP) obtained after
393 decreasing pH to 6 upon extracting the alkaline water), used in conjunction with a lime-based
394 pH regulation (pH = 11), yields adequate turbidity and abs 250-nm removal rates. This
395 preparation from vegetal waste appears to be a possible alternative to water treatment (natural
396 water or wastewater) by metal salts (iron or aluminum) or synthetic polyelectrolytes, both of
397 which may be toxic.

398 4. Conclusion

399 Olive mill wastewater (OMW) was tested as a bioflocculant in the coagulation-flocculation
400 process for water treatment. After OMW removal steps, a high removal rate ($92 \pm 1\%$) of the
401 synthetic water turbidity was obtained at pH 11 with a treatment dose of 100 mg L^{-1} . The
402 active part of this material can be isolated by solubilization under alkaline conditions and
403 precipitation by means of neutralization (PP). Enzymatic treatment, colorimetric dosage,
404 FTIR and acid-base titration all confirm that tannins and/or flavonoids and cellulose constitute
405 the active groups responsible for the flocculation phenomenon. PP exhibits a high
406 performance in turbidity removal, compared with other vegetal flocculants. Moreover, the
407 adjustment of pH using lime, associated with a treatment rate of 60 mg L^{-1} of PP, has allowed
408 optimizing the process with removal rates of $93 \pm 1\%$ and $50 \pm 2\%$ for turbidity and
409 absorbance at 254 nm, respectively. This study has demonstrated that olive mill wastewater
410 may be reliably reused for water treatment in a flocculation process.

411

412

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570

Graphical Abstract

