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1 **THE ROLE OF EXTRACELLULAR POLYMERIC SUBSTANCES ON**  
2 **AEROBIC GRANULATION WITH STEPWISE INCREASE OF SALINITY**

3  
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10  
11 **ABSTRACT**

12 A granular sequencing batch reactor (GSBR) worked for 164 days to study the effect of salinity  
13 on aerobic granulation. The feeding had an organic loading rate (OLR) of 1.6 kg COD·m<sup>-3</sup>·d<sup>-1</sup>  
14 and a gradual increase of salinity (from 0.30 to 38 g NaCl·L<sup>-1</sup>) to promote a biological salt-  
15 adaptation. First aggregates (average diameter ≈ 0.4 mm) appeared after 14 days. Extracellular  
16 polymeric substances (EPSs) analyses revealed that proteins were mainly higher than  
17 polysaccharides, and microorganisms metabolized EPSs as additional carbon source, mostly in  
18 feast phase, to face the energy demand for salinity adaptation. No significant worsening of  
19 organic matter removal was observed. The initial decrease of nitrification (from 58% to 15%)  
20 and the subsequent increase (up to 25%), confirmed the acclimation of AOBs to saline  
21 environment, while the accumulation of nitrites suggested NOBs inhibition. The nitrogen  
22 removal initially decreased from 58% to 15%, due to the inhibitory effect of salinity, and  
23 subsequently increased up to 29% denoting a simultaneous nitrification-denitrification. The  
24 dimensions of mature granules (higher than 1 mm) probably involved PAOs growth in the inner  
25 anaerobic layers. Nitrites caused a temporary deterioration of phosphorous removal (from 60%  
26 to almost zero), that increased up to 25% when nitrites were depleted.

28 **Keywords:** Aerobic granular sludge; saline wastewater; extracellular polymeric substances;  
29 EPS; hydrophobicity; nutrients removal.

30

## 31 **1. INTRODUCTION**

32 Saline wastewaters represent a high amount of sewage produced all over the world,  
33 since several industrial sectors, such as agro-food, petrochemical, textile and leather  
34 industries, paper-making, chemical manufacturing, pesticides and herbicides industries  
35 use water and inorganics salts in the process chain [1]. These effluents contain salts like  
36 chlorides, carbonates and sulphates and, often, organic and particularly recalcitrant  
37 compounds such as aromatic compounds [2]. The discharge of such wastewater having  
38 at the same time high salinity and high organic content without prior treatment,  
39 adversely affect the environment.

40 Physic-chemical processes are often adopted to treat industrial wastewaters, but they  
41 have some drawbacks linked to high management costs mainly due to the use of some  
42 chemicals and to the energy consumption, as well as the generation of other hazardous  
43 by-products as secondary pollutants to be disposed [3].

44 Biological systems for the treatment of organic matter in saline wastewater are  
45 nowadays increasingly the focus of research. Currently, activated sludge systems are the  
46 main biological processes implemented at full-scale. However, their practical  
47 application to treat complex industrial wastewaters is rather limited because the  
48 microorganisms are known to be inhibited by toxic and recalcitrant compounds and to  
49 be affected by high salinity [4]. Furthermore, high percentages of inorganic salts are  
50 known to strongly inhibit both the heterotrophic and the autotrophic strains, which are  
51 subjected to a huge osmotic pressure causing cellular plasmolysis or inhibition of many  
52 enzymes [5,6]. Consequently, high salt concentrations affect organic matter, nitrogen

53 and phosphorus removal. Currently, aerobic granular sludge (AGS) process is one of the  
54 most promising technologies for biological wastewater treatment. In this system,  
55 compact and fast settling granules allow on the one hand a faster separation of the  
56 biomass in the treatment reactor, and on the other hand a higher biomass concentration  
57 compared to the conventional activated sludge systems, minimizing the plant footprint  
58 [4,7]. The oxygen transfer limitation which occurs within the granules' layers, allows to  
59 obtain different redox conditions (anaerobic, anoxic and aerobic) inside the layered  
60 granular structure, so favoring nitrification, denitrification and phosphate removal [8].  
61 A great interest in AGS for the treatment of synthetic saline wastewater [9–13] and  
62 industrial saline wastewater [2,4,14–17] has been expressed recently. In all these works,  
63 the AGS technology has proven to be very effective in presence of inorganic salts.  
64 Moreover, from a microbiological point of view Ou et al. (2018) [18], founded that  
65 cultivating aerobic granular sludge in a saline environment lead to growth of moderately  
66 halophilic genera, such as *Salinicola* and *Halomonas* with great versatility with respect  
67 to salt tolerance. However, few studies have been conducted about granules' stability  
68 under salinity conditions [19], therefore some information is still lacking concerning the  
69 role and the functionality of extracellular polymeric substances (EPS) in granulation  
70 process in a saline environment. EPS are considered as one of the most relevant factors  
71 in granules formation [20]. They are made of various compounds (proteins,  
72 polysaccharides, humic acids, lipids) [21] with specific biological-physical  
73 characteristics, as a jelly-like aspect and consistence, that confer key properties  
74 determining microbial aggregation and aerobic granular sludge formation.  
75 Bearing in mind the above mentioned issues linked to salt concentration of raw  
76 wastewater, the main objective of this work is to analyze the granulation process in a

77 sequencing batch reactor subjected to a step-wise increase of the influent feeding  
78 salinity. Special attention was paid to the physical properties of the granular biomass  
79 and the extracellular polymeric substances production, since EPS are considered as the  
80 most important factor forming granules and biofilms. Moreover, the acclimation of  
81 microorganisms to the saline environment was corroborated through the monitoring of  
82 the main biological performances of the reactor.

83

## 84 **2. MATERIALS AND METHODS**

### 85 **2.1 Bench scale plant description and operational conditions**

86 The granular sequencing batch airlift reactor was a column-type reactor, with a working  
87 volume of 3.5 l and a height to diameter ratio (H/D) equal to 10. The reactor was  
88 operated on a six hours per cycle time, resulting in a hydraulic retention time (HRT) of  
89 12 hours. A single cycle included 20 minutes of influent feeding, 333 minutes of  
90 aeration, 2 minutes of settling and 5 minutes of effluent discharge. The volume  
91 exchange ratio (VER) was fixed at 50% by placing a solenoid valve at half height of the  
92 filling height. Air was introduced via a fine bubble aerator placed at the base of the  
93 reactor at a flow rate of  $3,3 \text{ cm}\cdot\text{s}^{-1}$  to ensure high shear forces. A programmable logic  
94 controller (PLC) Crouzet model Millenium 3 CD20 handled the SBR cycling  
95 operations. The reactor was fed with a synthetic saline wastewater prepared with a  
96 media composed by NaAc 97.7mM,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  3.7mM,  $\text{K}_2\text{HPO}_4$  20mM,  $\text{KH}_2\text{PO}_4$   
97 10mM, KCl 4.8mM,  $\text{NH}_4\text{Cl}$  30 mM, according to Beun et al., (2002)[22]. This media  
98 was diluted with tap water to obtain an organic loading rate (OLR) next to 1.6 kg  
99  $\text{COD}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$ ) for the whole experimental period. Sodium chloride was separately added  
100 to obtain the desired salinity measured as electrical conductivity. The reactor was

101 seeded with 1.75 l of flocculent activated sludge collected from the municipal  
102 wastewater treatment plant of Enna (Sicily, Italy). Some physical properties of this  
103 sludge were: sludge volume index after 30 min settling ( $SVI_{30}$ ) of  $111 \text{ mL}\cdot\text{gTSS}^{-1}$ , and  
104 mixed liquor total suspended solids concentration next to  $2.12 \text{ gMLTSS}\cdot\text{L}^{-1}$ . During the  
105 first 29 days (Phase 0), the OLR was gradually increased from 0.4 to  $1.6 \text{ kg COD}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$ ,  
106 and the settling time was decreased from 15 min to 2 min to avoid organic and  
107 hydraulic loading shocks, respectively, on the inoculum biomass. Subsequently, the  
108 reactor was subjected to a step-wise increase of salinity. In particular, the following  
109 phases were assessed: Phase 0-29 days ( $0.30 \pm 0.09 \text{ gNaCl}\cdot\text{L}^{-1}$ ), Phase I-15 days ( $1.80$   
110  $\pm 0.74 \text{ gNaCl}\cdot\text{L}^{-1}$ ), Phase II – 30 days ( $4.87 \pm 0.69 \text{ gNaCl}\cdot\text{L}^{-1}$ ), Phase III – 30 days  
111 ( $11.56 \pm 0.31 \text{ gNaCl}\cdot\text{L}^{-1}$ ), Phase IV-30 days ( $24.31 \pm 2.74 \text{ gNaCl}\cdot\text{L}^{-1}$ ), Phase V-30  
112 days ( $37.79 \pm 1.21 \text{ gNaCl}\cdot\text{L}^{-1}$ ).

113

## 114 **2.2 Analytical procedures**

115 The influent wastewater and the effluent have been sampled twice per week and  
116 analyzed according to the Standard Methods [23]. In particular, the following  
117 parameters were measured: total and volatile mixed liquor suspended solids (MLTSS  
118 and MLVSS), total suspended solids discharged with the effluent ( $TSS_{out}$ ) chemical  
119 oxygen demand (COD), total organic carbon (TOC), ammonia nitrogen ( $NH_4-N$ ),  
120 anions (nitrite ( $NO_2^-$ ), nitrate ( $NO_3^-$ ), phosphate ( $PO_4^{3-}$ ), chloride ( $Cl^-$ )). During all the  
121 experimentation, COD was measured by means of chemical titration and, in high  
122 salinity conditions, mercury sulphate was added to eliminate chloride interference. The  
123 ammonium and all the anions concentrations were measured by means of a ionic  
124 chromatography by ICS Dionex 1100. The total organic carbon (TOC) was measured by

125 means of thermos-catalytic oxidation with a high-temperature TOC-VCSH analyzer that  
126 also provides the total carbon (TC) and the inorganic carbon (IC). In addition, the sizes  
127 of granules, settling velocity, sludge volume index after 5 min of settling (SVI<sub>5</sub>) and  
128 after 30 min settling (SVI<sub>30</sub>), hydrophobicity, and extracellular polymeric substances  
129 (EPSs) were analyzed once per week to limit the sludge withdrawal.

130 The size of granules was measured by means of a dynamic microscope with image  
131 analysis capability (QICPIC by Sympatec). Granulation rate was evaluated as the  
132 percentage of particles with a diameter over 600 µm, according to [24]. The settling  
133 velocity was determined by placing individual granules in a graduated cylinder and  
134 measuring the time they took to drop from a fixed height [25]. The hydrophobicity of  
135 the cell surface was determined in accordance with the method described by Rosenberg  
136 et al. (1980). The total Extracellular Polymeric Substances were expressed as the sum of  
137 bound EPSs and soluble microbial products (SMPs), as protein and polysaccharide  
138 fractions. Then, the EPS content was referred to the VSS concentration. The SMPs were  
139 obtained by centrifugation at 5000 rpm for 5 min while the bound EPSs were extracted  
140 by a thermal extraction method [21]. The polysaccharides were determined according to  
141 the phenol–sulphuric acid method with glucose as standard [26], while the proteins were  
142 determined by the Folin method with bovine serum albumin as standard [27]. The  
143 samples for EPSs analysis were taken twice in a reaction cycle. In particular, the first  
144 sample was taken at the end of the feast phase, when most of the substrate was oxidized,  
145 while the second sample was taken at the end of the famine phase. Dissolved Oxygen  
146 (DO) concentration and pH were monitored during the cycle to identify the end of the  
147 feast phase.

148

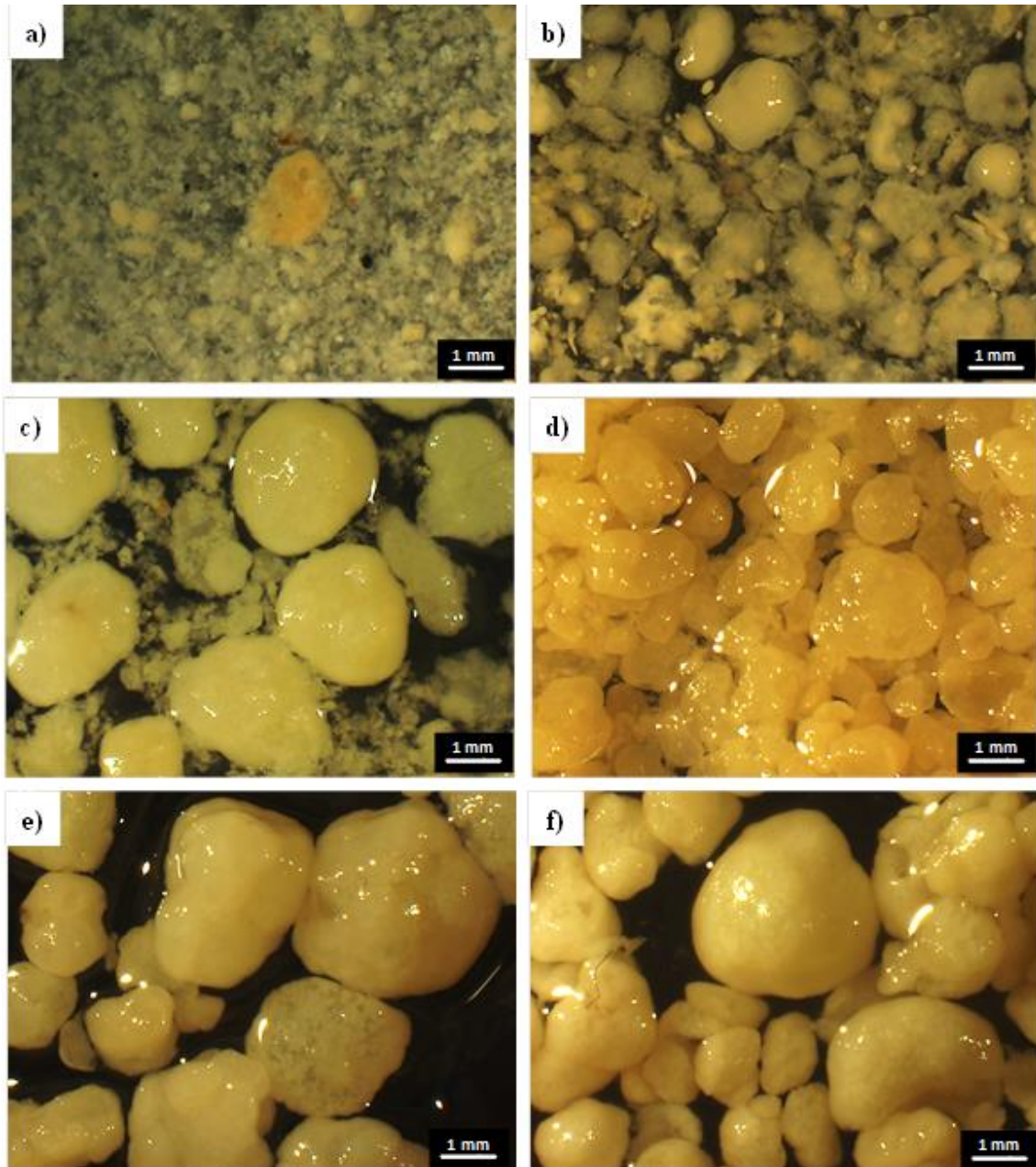


### 149 3. RESULTS AND DISCUSSIONS

#### 150 3.1 Granulation process and physical properties of granules

151 The reactor was seeded with flocculent biomass collected from the wastewater  
152 treatment plant of Enna (Sicily, Italy). In **Figure 1** and **Figure 2a**, the morphology and  
153 the dimensions of granules are shown, respectively. The seeding sludge was  
154 characterized by very small flocs with an average size of 80-100  $\mu\text{m}$ . During the first 14  
155 days (Phase 0), small aggregates started to appear in the reactor. They rapidly reached a  
156 mean diameter next to 0.3-0.4 mm (**Figure 2a**) and the granulation rate was next to 44%  
157 . In Phase I ( $1.80 \pm 0.74 \text{ gNaCl}\cdot\text{L}^{-1}$ ), the granulation process proceeded and yellow  
158 granules (**Figure 1b**) having an average diameter of about 1.1-1.2 mm and an irregular  
159 shape, were clearly visible in the reactor. During Phase II, the granules maintained an  
160 average diameter of 1.3 mm until the day 59, then their size increased up to 1.7-1.8 mm  
161 on day 73, where the granulation rate increased up to 86%. As shown in **Figure 1c**, the  
162 granules were mainly characterized by a rounded and smooth outer surface. However,  
163 some fluffy granules were observed in response to the salt stress, as also noted by  
164 Taheri et al. (2012) [28]. In Phase III, the further increase of salinity up to  
165 approximately  $11.56 \pm 0.31 \text{ gNaCl}\cdot\text{L}^{-1}$ , caused a significant change in the granules  
166 appearance, that became translucent (**Figure 1d**). In particular, on day 102, the average  
167 diameter of the granules decreased from about 1.8 mm to about 1.5 mm, as observed by  
168 Pronk et al. (2014) [4], despite for a higher salinity ( $20 \text{ g Cl}\cdot\text{L}^{-1}$ ). Although a slight  
169 modification of the granules morphology occurred in this period, a further increase in  
170 the granulation rate up to 93% was observed. The reduction of the mean diameter in this  
171 phase didn't involve a decrease in the granulation rate because the mean granules size  
172 was maintained over 0.6 mm. In Phase IV, despite the further increase of salinity (about

173 24.31 ± 2.74 gNaCl·L<sup>-1</sup>), the granules formed during the previous phases were  
174 subjected to a maturation process resulting in the increase of their average diameter up  
175 to about 1.7 mm (**Figure 1e**). At that time, the granules resulted very compact and  
176 structurally stable. The stability of the granules was maintained also in Phase V (37.79 ±  
177 1.21 gNaCl·L<sup>-1</sup>), during which the average diameter increased up to 2 mm the day 157  
178 and the granules appeared compact, rounded and with a regular shape (**Figure 1f**).  
179 During Phase IV and Phase V, the maturation process resulted in a further increase of  
180 the granulation rate up to 99 % for both phases, confirming the formation of stable and  
181 mature aerobic granules. Although some authors [29] found that in a saline environment  
182 the replacement of Ca<sup>2+</sup> by the abundantly available Na<sup>+</sup>, in the EPS matrix, caused the  
183 deterioration of the granule strength resulting in a weaker and swollen granule structure,  
184 in the present study the high salinity resulted in the formation of compact granules in  
185 accordance with Li and Wang, (2008)[9]. The compact structure of the granules could  
186 be due to the high buoyancy of saline wastewater that washed out the slow settling  
187 particles, and the high concentration of cations in the bulk that reduced the electric  
188 double layers on the surface of granules, favoring the microbial aggregation.  
189



190

191 **Figure 1:** Aerobic granules observed at the stereomicroscope (7X magnification): (a)  
 192 Phase 0 – day 14, (b) Phase I – day 38, (c) Phase II – day 73, (d) Phase III – day 102, (e)  
 193 Phase IV – day 131, (f) Phase V – day 157.  
 194

195 The trends of the total and volatile suspended solids in the mixed liquor (MLTSS,  
 196 MLVSS), are shown in **Figure 2b**. At the beginning of Phase 0, a severe biomass  
 197 washout was observed, due to the high hydraulic selection pressure (15 minutes of  
 198 settling). Consequently, the MLTSS decreased from  $2.12 \text{ g}\cdot\text{L}^{-1}$  (value of the inoculum)

199 to  $0.85 \text{ g}\cdot\text{L}^{-1}$ . Then, the concentration of biomass in the new operating conditions  
200 increased up to  $1.3 - 1.4 \text{ g}\cdot\text{L}^{-1}$ . The further reduction in the settling time, did not involve  
201 a severe reduction in the MLTSS as previously occurred, because most of the biomass  
202 was already hydraulically selected. From that day on, the MLTSS concentration was  
203 almost constant, at least until the day 18, when the change of the settling time to 2 min  
204 caused a severe decrease in the MLTSS to  $0.26 \text{ g}\cdot\text{L}^{-1}$  and a subsequent washout of  
205  $\text{TSS}_{\text{out}}$  in the effluent with a concentration of  $0.33 \text{ g}\cdot\text{L}^{-1}$ .

206 In Phase I, the initial increase of salinity of the influent feeding caused a plasmolysis  
207 with a decrease of MLTSS until the day 57 on Phase II. To promote the  
208 microorganisms' acclimation to the saline environment, it was decided to double the  
209 duration of each phase from 15 to 30 days.

210 Subsequently, the MLTSS concentration continuously increased for the remaining part  
211 of Phase II and during Phase III, reaching a steady value approximately close to  $7.5 \text{ g}\cdot\text{L}^{-1}$   
212 <sup>1</sup>.

213 In the same phase, the  $\text{TSS}_{\text{out}}$  in the effluent was almost constant next to  $0.15 \text{ g}\cdot\text{L}^{-1}$  until  
214 the day 85. Then, the change of the granules' morphology occurred in this period  
215 (**Figure 1d**), resulted in an increase of the  $\text{TSS}_{\text{out}}$  washed out from the system with a  
216 peak of approximately  $0.45 \text{ g}\cdot\text{L}^{-1}$  the day 92, without an appreciable decrease in the  
217 MLTSS concentration. After this day, the  $\text{TSS}_{\text{out}}$  in the effluent decreased as a  
218 consequence of a higher stability of the aerobic granules.

219 In the subsequent Phase IV, a reduction of the MLTSS from  $7.5 \text{ g}\cdot\text{L}^{-1}$  to  $5.4 \text{ g}\cdot\text{L}^{-1}$  was  
220 observed. During this phase, the  $\text{TSS}_{\text{out}}$  concentration in the effluent gradually increased  
221 up to  $0.54 \text{ g}\cdot\text{L}^{-1}$  the day 130. This trend was mainly attributable to the higher buoyancy  
222 force in the bulk due to the high salt concentration.

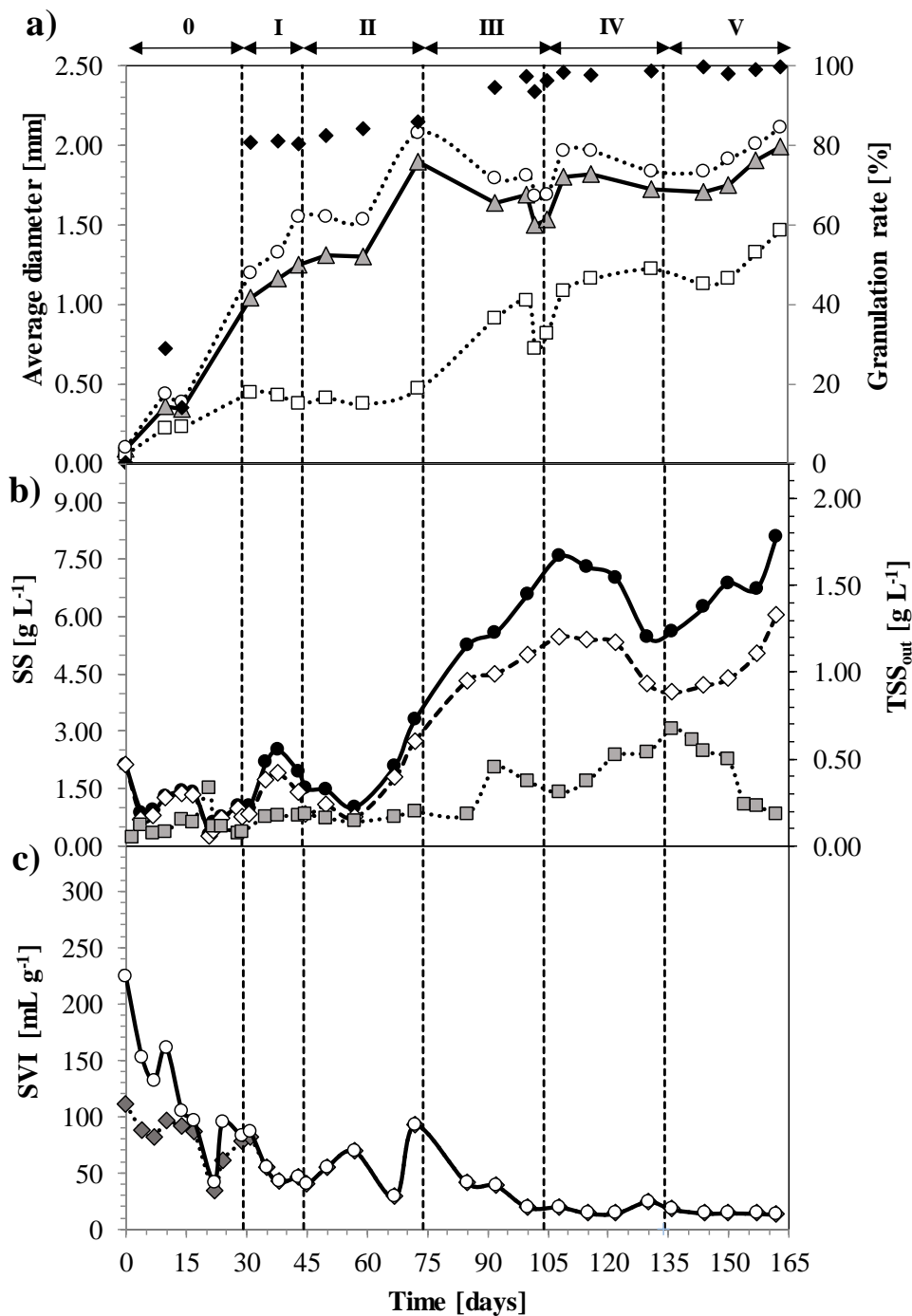
223 Indeed, as also observed by [30], the high salt concentration resulted in the increase of  
224 the bulk density and, as a result, the buoyancy increased as well contributing to reduce  
225 the settling velocity of the granules. In other words, it could be stated that the salinity  
226 exerted an additional positive effect of hydraulic selection pressure on the aerobic  
227 granules.

228 In the last Phase V, no significant changes, compared to the previous period, occurred in  
229 spite of the salinity increase, confirming the achievement of structurally stable granules.

230 The trend of the MLVSS (**Figure 2b**) was similar to that of the MLTSS. However,  
231 starting from the Phase III onward, a gradual reduction in the MLVSS fraction was  
232 observed. This was probably due to a gradual inclusion of salts within the structure of  
233 the aerobic granules. Indeed, because the salts are inorganic compounds, once adsorbed  
234 inside the granule, they contributed to increase the non-volatile fraction.

235 The settling properties of the aerobic granules were evaluated through the SVI after 5  
236 and 30 minutes. The trends of both the SVI<sub>5</sub> and the SVI<sub>30</sub> during the five experimental  
237 periods are shown in **Figure 2c**. As reported by many authors [31,32], the complete  
238 granulation can be identified when the ratio between the SVI<sub>5</sub> and the SVI<sub>30</sub> is close to  
239 one. This condition was observed starting from the day 35 in Phase I, when both the  
240 SVI<sub>5</sub> and the SVI<sub>30</sub> values were close to 55 mL·g<sup>-1</sup>. In Phase II, the SVI<sub>5</sub> and the SVI<sub>30</sub>  
241 values increased to 90 mL·g<sup>-1</sup> suggesting a slight worsening of the settleability. From  
242 the Phase III until the end of the experiment, a gradual decrease of the SVI<sub>5</sub> and the  
243 SVI<sub>30</sub> down to 20 mL·g<sup>-1</sup> was observed, confirming the achievement of stable and  
244 mature aerobic granules.

245



246

247 **Figure 2:** (a) Mean diameter of granules (D50 —▲—), particle diameter corresponding to  
 248 10% (D10 ...□...) and to 60% (D60 ...○...) cumulative undersize particle size distribution,  
 249 granulation rate (◆); (b) total suspended solids (—●—) and volatile suspended solids  
 250 (—◇—) in the reactor, total suspended solids in the effluent (...□...); (c) sludge volume  
 251 index after 5 min (—○—) and 30 min (...◆...).

252

### 253 3.2 Extracellular polymeric substances analysis

254 During the whole experimental period, the EPSs in forms of proteins and  
255 polysaccharides were analyzed (**Figure 3**). EPS are metabolic products accumulating at  
256 the surface of the bacterial cells, which alter the physic-chemical characteristics of the  
257 cellular surface such as the charge and the hydrophobicity [33]. EPSs are mainly  
258 constituted by proteins, polysaccharides, humic acids, and lipids secreted by  
259 microorganisms. Proteins are considered to be the main polymeric substances  
260 responsible to maintain the granules structure. Furthermore, proteins are mainly present  
261 in the inner layer, while polysaccharides concentrate on the outer surface of aerobic  
262 granules [34]. **Figure 3a** shows the trend of the proteins content of the granules referred  
263 to the unit of MLVSS during the experimentation. In Phase 0, without salt addition, it  
264 was registered an increase of protein respect to the inoculum value, because the biomass  
265 taken from the conventional activated sludge plant underwent a biological stress due to  
266 the new operating conditions (batch feeding, shear forces, hydraulic selection pressure,  
267 etc.). Then, when the biomass was acclimating to the new conditions and pseudo  
268 steady-state was achieved, a decrease of the specific proteins concentration was  
269 observed. Subsequently, when the settling time decreased from 4 min to 2 min, the  
270 increase in the hydraulic selection pressure induced a higher physical stress which  
271 caused a significant production of proteins (up to  $180 \text{ mg}\cdot\text{gMLVSS}^{-1}$  the day 17)  
272 fulfilling the function of structure of aerobic granules. Subsequently, the following  
273 decrease of proteins values confirmed the adaptation of granules to new operational  
274 parameters.

275 The days 31 and 45, other two peaks of proteins, close to  $140 \text{ mg}\cdot\text{gMLVSS}^{-1}$  and  $180$   
276  $\text{mg}\cdot\text{gMLVSS}^{-1}$ , were noted, due to the increase of salinity. In this case, the osmotic

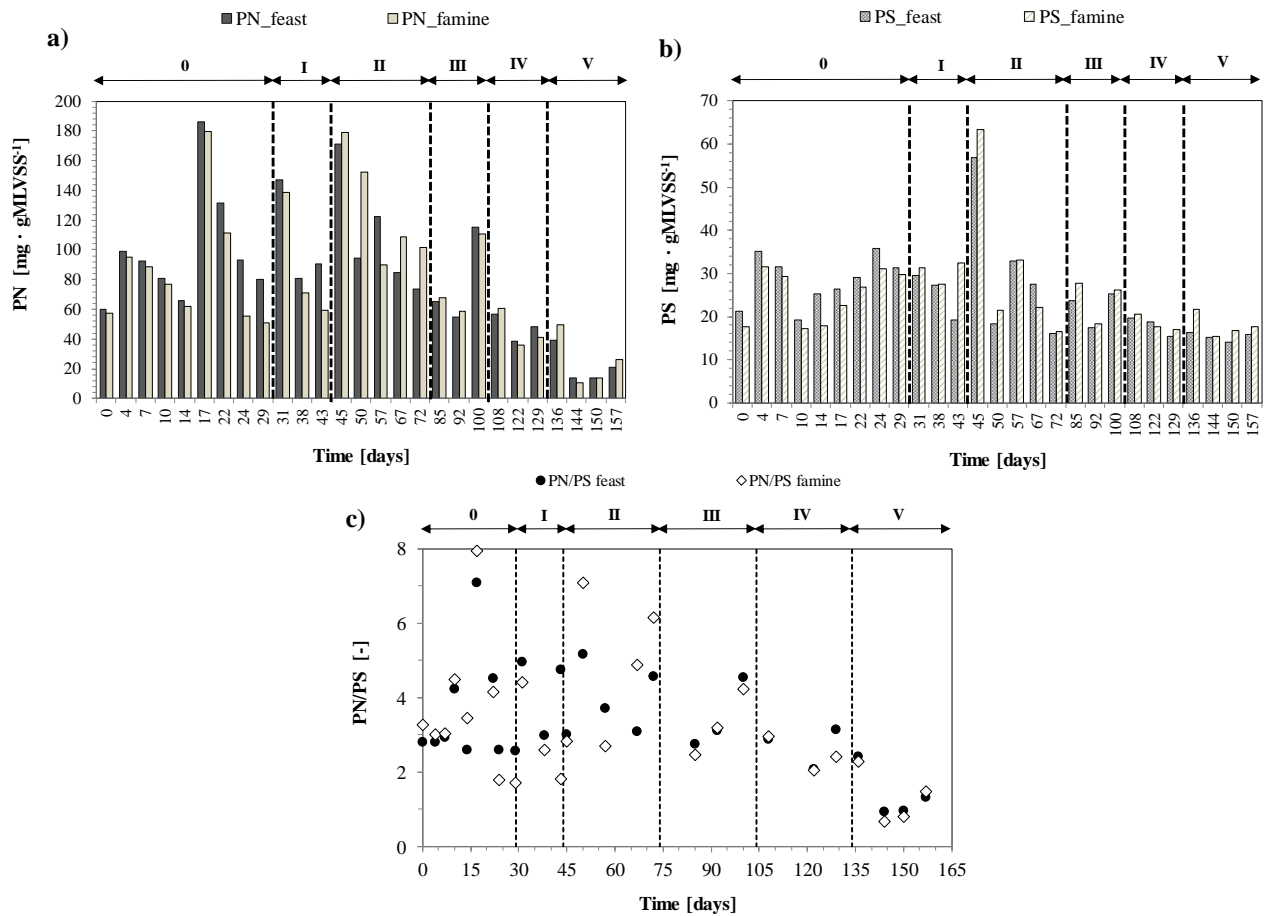
277 stress caused by the salts implied a higher production of proteins by bacteria forming  
278 granules. This could be explained with the need of the microorganisms to balance the  
279 osmotic pressure, hindering their cell lysis and death. In a saline environment, the  
280 bacterial cells tend to produce extracellular polymeric substances as a biological  
281 mechanism of balancing the osmotic pressure from the bulk, as also observed by other  
282 researchers [17,35]. In this way, the protein EPS production in aerobic granules could  
283 assist enhancing intra-granular strength, with the increase of salinity.

284 From the Phase III onwards, the proteins concentration decreased, except a pick value  
285 observed on day 100, that determined the change of the morphology and the structure of  
286 the granules, as discussed previously. The decrease of the proteins content indicated that  
287 the biomass was not more affected by the further salinity increase, suggesting that  
288 granules achieved a high level of maturation and stability, and the acclimation to  
289 salinity successfully occurred.

290 Regarding to the polysaccharides (**Figure 3b**) their concentration ranged between 20 ÷  
291 30 mg·gMLVSS<sup>-1</sup> until the day 43 in Phase I. However, at the beginning of Phase II, the  
292 polysaccharides concentration rose to 60 mg·gMLVSS<sup>-1</sup> in accordance with the increase  
293 of the proteins content. Nevertheless, at each salinity increase the polysaccharides  
294 production was much lower compared to the proteins, suggesting that microorganisms  
295 faced the osmotic pressure mainly through the proteins secretion.

296





297

298 **Figure 3:** (a) EPS proteins content; (b) EPS polysaccharides content; (c) PN/PS ratio, in  
 299 feast and famine conditions.

300

301 As shown in **Figures 3a and 3b**, throughout the whole experimental period, the EPS at  
 302 the end of the feast and the famine phases were also measured. In general, the feast  
 303 phase duration ranged between 20 ÷ 40 min, while the famine phase lasted 290 ÷ 310  
 304 min, on average.

305 During the Phase 0 and the Phase I, the proteins in the feast phase resulted higher than  
 306 in the famine one, because during the phases of formation of granules the  
 307 microorganisms produced a higher concentration of proteins in feast phase contributing  
 308 to the formation of granule's structure. Indeed, during the feast phase, when the organic  
 309 substrate was available in large amounts, bacteria created storage products in forms of

310 proteins, contributing to the formation of granules. In the following famine phase, the  
311 proteins were used as carbon source by microorganisms in aerobic starvation and this  
312 helped to increase cell surface hydrophobicity and to enhance the ability of anti-toxic  
313 shock of granular sludge [34,36,37]. Once the granules were formed, the maturation  
314 process began and lasted for all the remaining phases. For the remaining days of  
315 operation, it was observed that the maturation and the improvement of the stability of  
316 granules implied a less production of proteins in feast phase than in the famine phase.  
317 During the Phase 0 with no salt addition, the polysaccharides in feast phase were always  
318 higher than in famine phase. This was in agreement with other authors [17,38] who  
319 found that the feast/famine strategy led to a storage of polymers (mainly  
320 polysaccharides) when the substrate was present (feast phase) which was used for  
321 growth of microorganisms when the external substrate was depleted (famine phase).  
322 However, also in this case, during the following experimental phases, the  
323 polysaccharides in the famine phase were always higher than in the feast phase. Both  
324 proteins and polysaccharides trends in increasing salinity conditions, were apparently in  
325 contrast with what observed by the same authors in a previous study [17], where the  
326 biomass was previously adapted to salinity. It should be stressed that, in the present  
327 study, the salinity constituted an important environmental variable which affected all  
328 the biological and physical processes involved in the granulation phenomenon. At this  
329 purpose, a fundamental aspect which cannot be ignored is that the microbial community  
330 was probably changed to adapt to the high salinity environment, as founded by Wan et  
331 al. (2014) [35]. So it is possible that halotolerant microorganisms were biologically  
332 selected, as also found by [18], ensuring aerobic granules stability under high salinity  
333 environment. Moreover, these new microorganisms possessed different metabolic

334 kinetics, which were not analyzed in this study, compared to common bacterial strains  
335 of soft wastewater. As observed by Taheri et al., (2012) [28], the half-saturation  
336 constant ( $K_S$ ) for the treatment of saline wastewater is significantly greater than the  
337 value determined for the treatment of salt-free domestic wastewater. So, when bacteria  
338 activate the mechanism to adapt to salinity by means the transport of osmolytes from  
339 and into the cell, they require more energy and, therefore, more carbon source which  
340 causes  $K_S$  to become higher. Therefore, since in this work the exogenous COD was  
341 maintained almost constant for each Phase, it could be assumed that, when the salinity  
342 increased, the microorganisms consumed both the proteins and the polysaccharides as  
343 an additional endogenous carbon source, in a greater extent in the feast phase than in the  
344 famine phase. This could explain the unusual trend of EPS observed during the feast  
345 and famine phases in presence of salinity.

346 Finally, the evolution of the EPS composition in terms of proteins/polysaccharides ratio  
347 (PN/PS) was studied. The PN/PS ratio is considered an important parameter to study the  
348 granulation, since it expresses the combined effect of proteins and polysaccharides on  
349 the aerobic granules formation. In this study, this ratio was very similar for the feast and  
350 the famine phases, as shown in **Figure 3c**. This could mean that both proteins and  
351 polysaccharides vary in a similar way both in feast and in famine conditions. Moreover,  
352 the time course of the PN/PS ratio was decreasing and it resulted very similar to that of  
353 the proteins, since proteins were the major component of the EPS.

354

### 355 **3.3 Hydrophobicity**

356 Hydrophobicity has been cited as an important granules property [34] and many authors  
357 underlined that the formation of the granular sludge was associated with a sharp

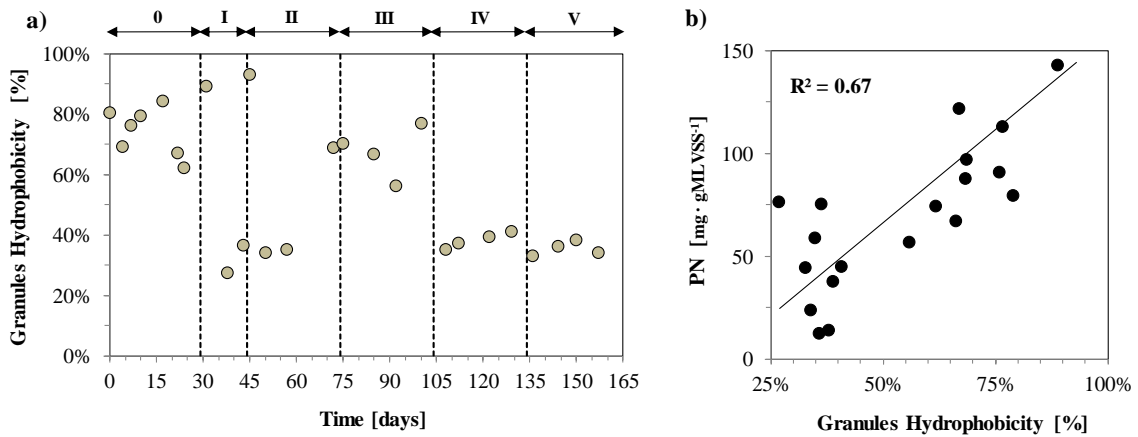
358 increase of the hydrophobicity [39–41]. The increase of surface hydrophobicity would  
359 promote cell-to-cell interaction and further serves as inducing cell force for aggregating  
360 and promoting biogranulation [39]. However, high salt concentrations of wastewater  
361 directly affects sludge hydrophobicity [29], because  $\text{Na}^+$  ions produce a change in the  
362 cell surface properties. In particular, the presence of salt involves the replacement of  
363 divalent  $\text{Ca}^{2+}$  ions with monovalent  $\text{Na}^+$  ions of the EPS, resulting in a minor reduction  
364 of surface electronegativity and therefore in a lower hydrophobicity [4]. **Figure 4a**  
365 shows the trend of the granules hydrophobicity during the whole experimental study. In  
366 general, as observed in other studies [17,37], this work confirmed that the increase in  
367 proteins content, which are well known to be charged positively, was found to decrease  
368 the surface negative charge of bacteria cells and increasing the hydrophobicity. This  
369 reduced the zeta potential and the electrostatic repulsions, favoring bridging and  
370 microbial aggregation.

371 In particular, in Phase 0 the sludge hydrophobicity decreased from the 80% (inoculum  
372 value) to a value close to 64%. Subsequently the time course of the sludge  
373 hydrophobicity was very irregular. In Phase III ( $11.56 \pm 0.31 \text{ gNaCl}\cdot\text{L}^{-1}$ ) the  
374 hydrophobicity was on average higher than in the previous two phases. As discussed  
375 previously, in this phase it was noted a substantial modification of granules morphology  
376 and, probably, of bacterial strains. So, it could be supposed that halotolerant  
377 microorganisms produced amino-acids groups, as protein components, with a higher  
378 positive charge. This could explain the higher values of hydrophobicity in this phase.

379 Subsequently in the remaining Phase IV and Phase V the lower protein production of  
380 mature granules implied a lower hydrophobicity.

381 In each phase it could be observed that the highest value of hydrophobicity sharply  
 382 corresponded with the highest value of proteins previously discussed. This was  
 383 confirmed by a discrete correlation between the granules hydrophobicity and the protein  
 384 content, as shown in **Figure 4b**.

385



386

387 **Figure 4:** (a) Granules hydrophobicity; (b) correlation between granules hydrophobicity  
 388 and protein (PN) content. All the represented values are averages between feast phase  
 389 and famine phase.

390

### 391 3.4 Bench scale plant performances

392 To assess the acclimation to salinity of the microorganisms forming the aerobic  
 393 granules, the main biological processes were monitored through the analysis of the  
 394 organic matter and nutrients removal efficiencies.

395

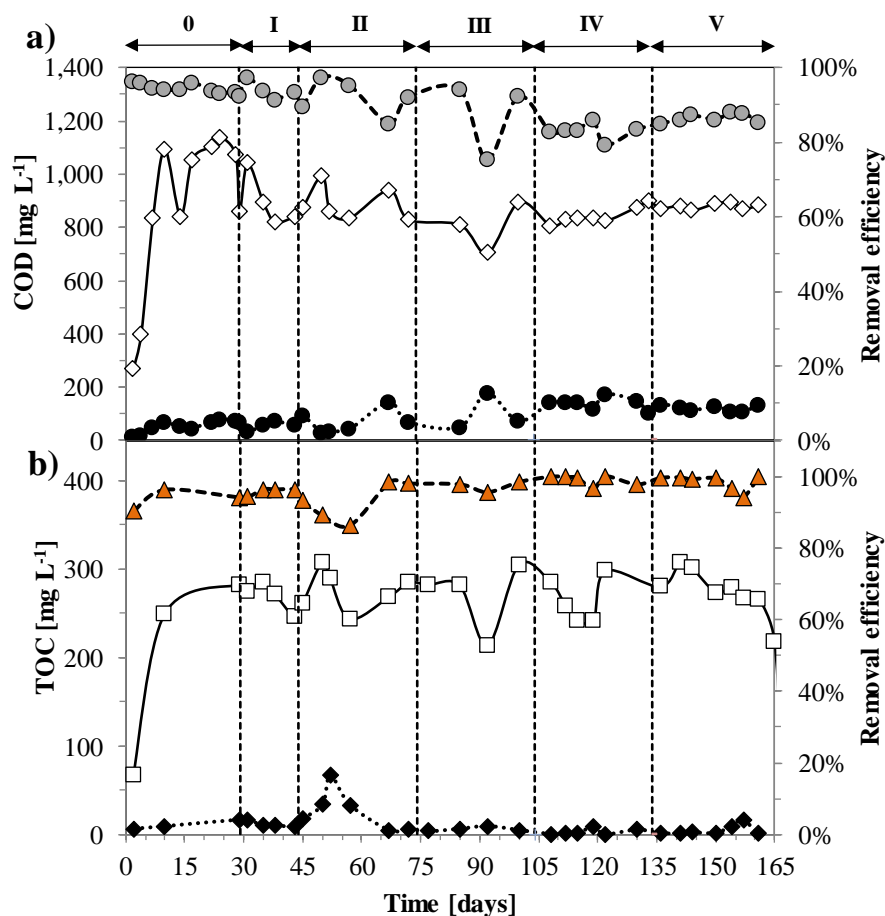
#### 396 3.4.1 Organic matter removal

397 During the Phase 0 and the Phase I the reactor was able to remove more than 95% of  
 398 COD (**Figure 5a**). In Phase II and Phase III, the heterotrophic microorganisms were  
 399 probably affected by the salinity increase as found also by Wang et al. (2016) [42] and  
 400 the removal efficiency dropped to 85% on day 67 and continued to decrease to 75%

401 until the day 92. Subsequently, during Phase IV and Phase V, the COD removal  
402 efficiency was quite stable around a steady value of 85%, denoting the acclimation of  
403 the heterotrophic microorganisms to the saline environment.

404 However, since in a saline environment the COD determination is affected by the  
405 interference of chlorides that often imply a higher determination of COD than the real  
406 value, the authors simultaneously analysed the TOC (**Figure 5b**) as a further parameter  
407 directly referred to the organic matter. The TOC analysis of saline wastewater is not  
408 affected by any interference, therefore this could help to better understand the real  
409 organic matter removal. By comparing both the graphs of Figure 5, the removal  
410 efficiencies of COD and TOC in Phase 0 and in Phase I, resulted quite similar.  
411 Subsequently, when the effluent COD grew from Phase II till the end of the  
412 experimentation, the effluent TOC always remained quite low, denoting that the  
413 worsening of the organic matter removal expressed as COD was, with high probability,  
414 due to an overestimation linked to the analytical method, so constituting a false positive.  
415 Also Wang et al. (2017) [12] observed a similar trend of the effluent COD when the  
416 salinity increased, and also in that case it was probably caused by the interference of  
417 chlorides. Therefore, it should be stressed that in this study the comparison between the  
418 trends of COD and TOC allowed a more precise analysis of organic matter removal of  
419 saline wastewater.

420



421

422 **Figure 5:** (a) COD in the influent ( $\text{---}\diamond\text{---}$ ), COD in the effluent ( $\cdots\bullet\cdots$ ), COD removal  
 423 efficiency ( $\text{---}\bullet\text{---}$ ); (b) TOC in the influent ( $\text{---}\square\text{---}$ ), TOC in the effluent ( $\cdots\blacklozenge\cdots$ ), TOC  
 424 removal efficiency ( $\text{---}\blacktriangle\text{---}$ ).  
 425

### 426 3.4.2 Nitrogen and Phosphorous removal

427

428 **Figure 6a** shows how the gradual increase of salinity until Phase III ( $11.56 \pm 0.31$   
 429  $\text{gNaCl}\cdot\text{L}^{-1}$ ), caused an inhibition of autotrophic biomass, leading to a decrease of  
 430 nitrification from 63% (Phase 0) to 20% (Phase II -  $4.87 \pm 0.89 \text{gNaCl}\cdot\text{L}^{-1}$ ) and thus a  
 431 decrease of nitrogen removal from 58% to 30%. In particular, during Phase III a strong  
 432 inhibition of autotrophic biomass was observed, and nitrogen was mainly removed for  
 433 growth/assimilation by microorganisms (next to 15%), while the nitrification was at its

434 minimum of 5%. During this phase the autotrophic biomass underwent a strong salinity  
435 inhibition. Then, in the Phase IV and V the nitrification activity reprised from 5% up to  
436 25%, highlighting an adaptation of nitrifying microorganisms to the high salinity  
437 environment, and the nitrogen removal increased from 15% to 29%. Observing the  
438 denitrification efficiency, since it is a relative value strictly depending on the  
439 nitrification efficiency, it was relatively high between 80-90% from Phase 0 (no salt  
440 addition) to Phase II ( $4.87 \pm 0.89 \text{ gNaCl}\cdot\text{L}^{-1}$ ), when salt concentration was not too high.  
441 During Phase III, the denitrification activity dropped to 19% due to the further increase  
442 of salinity that exerted a strong inhibitory effect. Then, a gradual reprise of  
443 denitrification activity was observed (up to 71% in Phase V) that, together with the  
444 gradual increase of the nitrification, contributed to increase the total nitrogen removal.  
445 This suggested that, not only nitrifying but also denitrifying microorganisms were  
446 adapted to the saline environment.

447 Although in Phase 0 the washout of biomass occurred in the earliest days of the  
448 operation, both the absence of salinity and the inoculum seed sludge enriched in  
449 nitrifying microorganisms were responsible for the highest nitrification efficiency  
450 (**Figure 6b**). Moreover, granules with smaller sizes have a higher specific surface area  
451 and thereby a higher specific oxidation capacity, as also observed by Pronk et al.,  
452 (2014) [4]. Then, when the settling time was reduced from 15 to 2 min, a significant  
453 worsening of the nitrification with outlet ammonium concentration close to  $45 \text{ mg}\cdot\text{L}^{-1}$ ,  
454 was observed. This was mainly due to the severe washout of the slow growing  
455 autotrophic biomass, occurred following to the increase of the hydraulic selection  
456 pressure. At the end of Phase 0, when the slow settling microorganisms were discharged  
457 and the reactor enriched in fast settling biomass, the nitrification efficiency rose to 40%

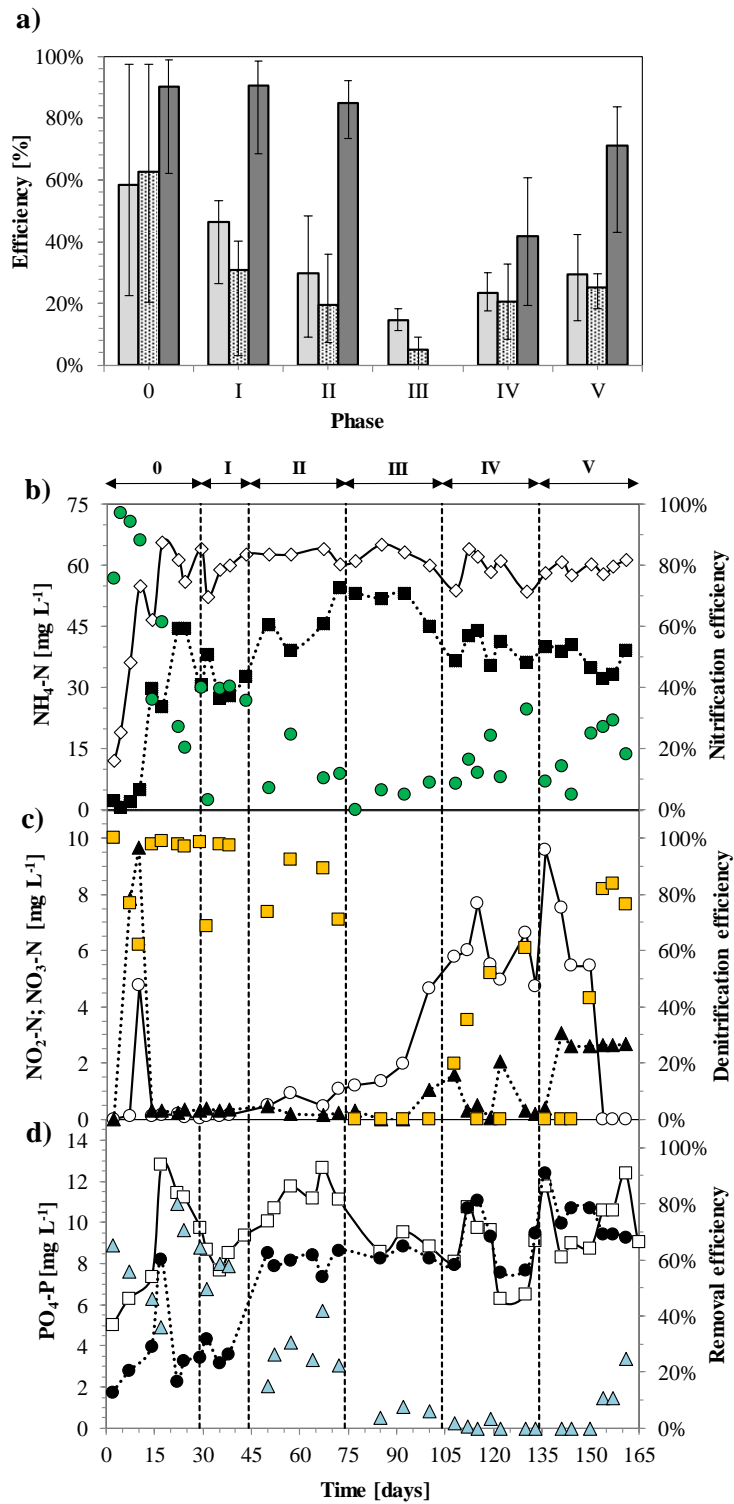


458 on day 29. At the beginning of Phase I, on day 31, the nitrification efficiency suddenly  
459 collapsed, probably due to an initial inhibitory effect of salinity on the autotrophic  
460 bacteria. Subsequently, a gradual reprise of nitrification up to 40% denoted the  
461 acclimation of the autotrophic biomass to  $1.80 \pm 0.74 \text{ gNaCl}\cdot\text{L}^{-1}$ . In Phase II, a  
462 progressive increase of ammonium in the effluent was noted, likely due to the gradual  
463 inhibition of the nitrification process when autotrophic microorganisms were exposed to  
464 about  $4.87 \pm 0.69 \text{ gNaCl}\cdot\text{L}^{-1}$ . In Phase III ( $11.56 \pm 0.31 \text{ gNaCl}\cdot\text{L}^{-1}$ ) it was observed a  
465 strong inhibition of nitrifying biomass and the ammonium was mainly removed for  
466 assimilation and growth of heterotrophic microorganisms. Based on these observations  
467 and on the changes of granules morphology and structure discussed previously, it could  
468 be asserted that Phase III was crucial as transitional stage where granules changed their  
469 physical and biological characteristics to face the saline environment. During Phase IV  
470 ( $24.31 \pm 2.74 \text{ gNaCl}\cdot\text{L}^{-1}$ ) a decrease of ammonium in the effluent was noted and,  
471 consequently, the nitrification efficiency increased up to 33%, probably due to the  
472 growth and specialization of nitrifying bacteria. Moreover, it was observed a contextual  
473 maturation of granules, which appeared more robust and stronger at further salinity  
474 increase. The dense structure of the granules likely helped to reduce the inhibitory  
475 effects of salinity on the bacteria dwelling in the inner layers, providing optimal  
476 environmental conditions for the maintenance of the biological processes.

477 Finally in Phase V ( $37.79 \pm 1.21 \text{ gNaCl}\cdot\text{L}^{-1}$ ), after few initial days of biological  
478 inhibition due to the high salinity concentration, the autotrophic bacteria living in stable  
479 and compact granules nitrified the influent ammonium up to 30%, highlighting the  
480 adaptation of nitrifying biomass to high saline environment.

481 Based on the concentrations of nitrites and nitrates in the effluent and on the  
482 denitrification efficiency (**Figure 6c**), it could be stated that the simultaneous  
483 nitrification denitrification (SND) [43] occurred. In the first days of operation, two  
484 peaks of nitrates and nitrites concentration of 10 and 5 mg·L<sup>-1</sup> were observed,  
485 respectively. This was mainly due to the small dimensions of granules at that time, that  
486 implied a small anoxic layer and a poor denitrification efficiency of about 60%,  
487 accordingly. Then, due to the increase of the aerobic granules size and the development  
488 of an anoxic layer, all the nitrified ammonium was mostly denitrified with a removal  
489 efficiency close to 98%. Subsequently, in Phase I, the increase in the inlet salt  
490 concentration produced an initial decrease of the denitrification efficiency, due to a  
491 partial inhibition of the denitrifiers microorganisms. After that, the denitrification  
492 efficiency rose again up to 98%. During the Phase II, the further salinity increase caused  
493 a worsening of the denitrification efficiency that dropped to 71%, and a nitrite  
494 concentration in the effluent next to 1 mg·L<sup>-1</sup> was observed. In Phase III, a gradual  
495 accumulation of nitrites in the effluent up to 5 mg·L<sup>-1</sup> was observed, and the  
496 denitrification efficiency decreased down to about 17% at the end of the phase. This  
497 was probably due to the change of the granules morphology and structure observed in  
498 this phase. As discussed previously, during Phase III the average size of the granules  
499 was reduced and the anoxic layer become thinner, so limiting the denitrification process.  
500 Moreover, the nitrite accumulation suggested the inhibition of the autotrophic nitrite  
501 oxidizing bacteria (NOB), according to other authors [4,11,15] who noted that NOB are  
502 more sensitive to saline environment respect to the ammonia oxidizing bacteria (AOB).  
503 In Phase IV the nitrite concentration in the effluent was around 6 mg·L<sup>-1</sup> with a  
504 denitrification efficiency increased up to 60%. Moreover, at that time some nitrates

505 were also observed in the effluent. Finally, in Phase V, the nitrites concentration in the  
506 effluent rose to  $9.6 \text{ mg}\cdot\text{L}^{-1}$  due to the salt increase ( $37.79 \pm 1.21 \text{ gNaCl}\cdot\text{L}^{-1}$ ) which may  
507 have strongly inhibited the NOBs. Subsequently, the decrease of nitrites down to zero  
508 and the correspondent increase of nitrates up to an almost constant value of about 3  
509  $\text{mg}\cdot\text{L}^{-1}$  suggested both an improvement of denitrification (from 45% to 90%), due to the  
510 extension of the anoxic layer with the increase of granules dimensions, and the possible  
511 acclimation of NOB to the saline environment. Bearing in mind the results above, the  
512 high robustness of granular sludge led to a decreased sensitivity of aggregated  
513 microorganisms forming granules towards the impact of stress factors such as salinity.  
514



515

516 **Figure 6.** (a) Nitrogen average removal efficiency (□), nitrification average efficiency  
 517 (■), denitrification average efficiency (▣); (b) NH<sub>4</sub>-N in the influent (◇), NH<sub>4</sub>-N in  
 518 the effluent (■), nitrification efficiency (●); (c) NO<sub>2</sub>-N in the effluent (○),  
 519 NO<sub>3</sub>-N in the effluent (▲), denitrification efficiency (■); (d) PO<sub>4</sub>-P in the influent  
 520 (□), PO<sub>4</sub>-P in the effluent (●), phosphorous removal efficiency (△).

521 Finally, also the potential simultaneous phosphorous removal was analyzed (**Figure 6d**).  
522 More specifically, from the beginning of the experimentation until the day 17 (Phase 0),  
523 a gradual decrease of the phosphorous removal efficiency was observed. Particularly, it  
524 decreased from about 60% to less than 35% because steady-state was not achieved and  
525 granules were not well-formed. Therefore, in this phase phosphorous was mainly  
526 removed for growth and assimilation of microorganisms. Subsequently, the granules  
527 formation resulted in an improvement of the phosphorous removal to 70%, because the  
528 higher average diameter of granules may have promoted the development of an  
529 anaerobic core where phosphorus accumulating organisms (PAOs) may have  
530 proliferated. In Phase I ( $1.80 \pm 0.74 \text{ gNaCl}\cdot\text{L}^{-1}$ ), the phosphorous removal efficiency  
531 slightly decreased to 60%. In Phase II, an increase of phosphorous in the effluent was  
532 noted, probably due to the appearance of nitrites in the reactor, and the phosphorous  
533 removal efficiency dropped to 25%. As well known in the literature [4,11], nitrites can  
534 negatively affect the phosphorous uptake activity of PAOs under both anoxic and  
535 aerobic conditions. In this work, the obtained results confirmed that the effect of salt  
536 was detrimental to NOBs, which was reflected in the accumulation of nitrites, as  
537 discussed previously. In turn, phosphates uptake dramatically reduced when nitrites  
538 concentration was above  $1 \text{ mg}\cdot\text{L}^{-1}$ , as observed in Phase III (5%), in Phase IV (almost  
539 0%) and until the day 150 in Phase V. Then, when nitrites concentration dropped to  
540 zero, the phosphorous removal efficiency reprise to increase up to 25% at the end of the  
541 experimentation. These observations indicated a probable development of PAOs  
542 microorganisms in the inner layers of granules. The gradual deterioration of  
543 phosphorous removal efficiency was likely caused by a reversible inhibition of PAOs,  
544 caused by the nitrites accumulation in the saline environment. In order to resume the

545 results of this study, **Table 1** reports a summary of the performances and the main  
 546 granules features throughout the whole experimental period.

547

548 **Table 1.** Summary of the main granules features and performances throughout the  
 549 experimental period.

550

		<b>Phases and time span</b>					
<b>Parameter</b>	<b>Units</b>	<b>Phase 0</b>	<b>Phase I</b>	<b>Phase II</b>	<b>Phase III</b>	<b>Phase IV</b>	<b>Phase V</b>
		<b>0-29</b> <b>(29 days)</b>	<b>30-44</b> <b>(15 days)</b>	<b>45-74</b> <b>(30 days)</b>	<b>75-104</b> <b>(30 days)</b>	<b>105-134</b> <b>(30 days)</b>	<b>135-164</b> <b>(30 days)</b>
<b>MLTSS</b>	g·L <sup>-1</sup>	1.07 ± 0.52	1.66 ± 0.81	1.88 ± 0.79	5.79 ± 0.69	6.83 ± 0.94	6.70 ± 0.92
<b>MLVSS</b>	g·L <sup>-1</sup>	0.99 ± 0.54	1.30 ± 0.60	1.43 ± 0.75	4.61 ± 0.34	5.10 ± 0.58	4.73 ± 0.82
<b>TSS<sub>out</sub></b>	g·L <sup>-1</sup>	0.12 ± 0.08	0.14 ± 0.05	0.17 ± 0.02	0.33 ± 0.14	0.43 ± 0.11	0.42 ± 0.20
<b>Mean diameter</b>	mm	0.26 ± 0.15	1.15 ± 0.10	1.50 ± 0.34	1.61 ± 0.10	1.72 ± 0.13	1.84 ± 0.13
<b>SVI<sub>5</sub></b>	mL·g <sup>-1</sup>	126 ± 55	66 ± 22	55 ± 23	33 ± 12	18 ± 5	15 ± 2
<b>(PN/PS)<sub>feast</sub></b>	-	3.57 ± 1.50	4.22 ± 1.09	3.91 ± 0.94	3.48 ± 0.95	2.70 ± 0.56	1.41 ± 0.69
<b>(PN/PS)<sub>famine</sub></b>	-	3.65 ± 1.86	2.95 ± 1.34	4.73 ± 1.96	3.28 ± 0.89	2.47 ± 0.46	1.31 ± 0.74
<b>Salinity</b>	gNaCl·L <sup>-1</sup>	0.30 ± 0.09	1.80 ± 0.74	4.87 ± 0.69	11.56 ± 0.31	24.31 ± 2.74	37.79 ± 1.21
<b>η<sub>COD</sub></b>	%	95 ± 1	94 ± 3	92 ± 4	87 ± 10	83 ± 2	86 ± 1
<b>η<sub>TOC</sub></b>	%	93 ± 4	95 ± 1	93 ± 5	97 ± 1	99 ± 1	98 ± 2
<b>η<sub>N</sub></b>	%	58 ± 27	46 ± 13	30 ± 14	15 ± 3	23 ± 5	29 ± 11
<b>η<sub>P</sub></b>	%	59 ± 16	58 ± 6	27 ± 9	6 ± 2	1 ± 1	18 ± 7

551

552

#### 553 **4. CONCLUSIONS**

554 Stable granules were obtained after a step-wise salinity increase. EPS analyses revealed  
 555 that proteins were dominant and were consumed mainly in feast phase as additional  
 556 carbon source to face the energy demand for salt adaptation. This particular EPS

557 metabolic pathway enhanced aerobic granulation in presence of high salinity. No  
558 worsening of organic matter removal efficiency was observed. The initial decrease and  
559 the subsequent increase of nitrification confirmed the acclimation of AOBs to saline  
560 environment, while the accumulation of nitrites suggested the NOBs inhibition. The  
561 high mean dimensions of granules may have promoted the formation of an anaerobic  
562 core where PAOs may have grown. The presence of nitrites caused a temporary  
563 deterioration of PAOs phosphorous removal efficiency, that increased when nitrites  
564 were depleted.

565

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570

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