

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

3
4 Riccardo Campo^{a*}, Santo Fabio Corsino^b, Michele Torregrossa^b, Gaetano Di Bella^a.

5 ^a Facoltà di Ingegneria e Architettura, Università degli Studi di Enna “Kore”, Cittadella Universitaria,
6 94100 Enna, Italy

7 ^bDipartimento di Ingegneria Civile, Ambientale, Aerospaziale, dei Materiali, Università degli Studi di
8 Palermo, Viale delle Scienze, 90128 Palermo, Italy

9 *Corresponding author e-mail address: riccardo.campo@unikore.it

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 $1.6 \text{ kg COD}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$
14 and a gradual increase of salinity (from 0.30 to 38 g NaCl·L⁻¹) to promote a biological salt-
15 adaptation. First aggregates (average diameter $\approx 0.4 \text{ mm}$) 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, K_2HPO_4 20mM, KH_2PO_4
97 10mM, KCl 4.8mM, NH_4Cl 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 ($\text{NH}_4\text{-N}$),
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 (SVI₅) and
128 after 30 min settling (SVI₃₀), 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.

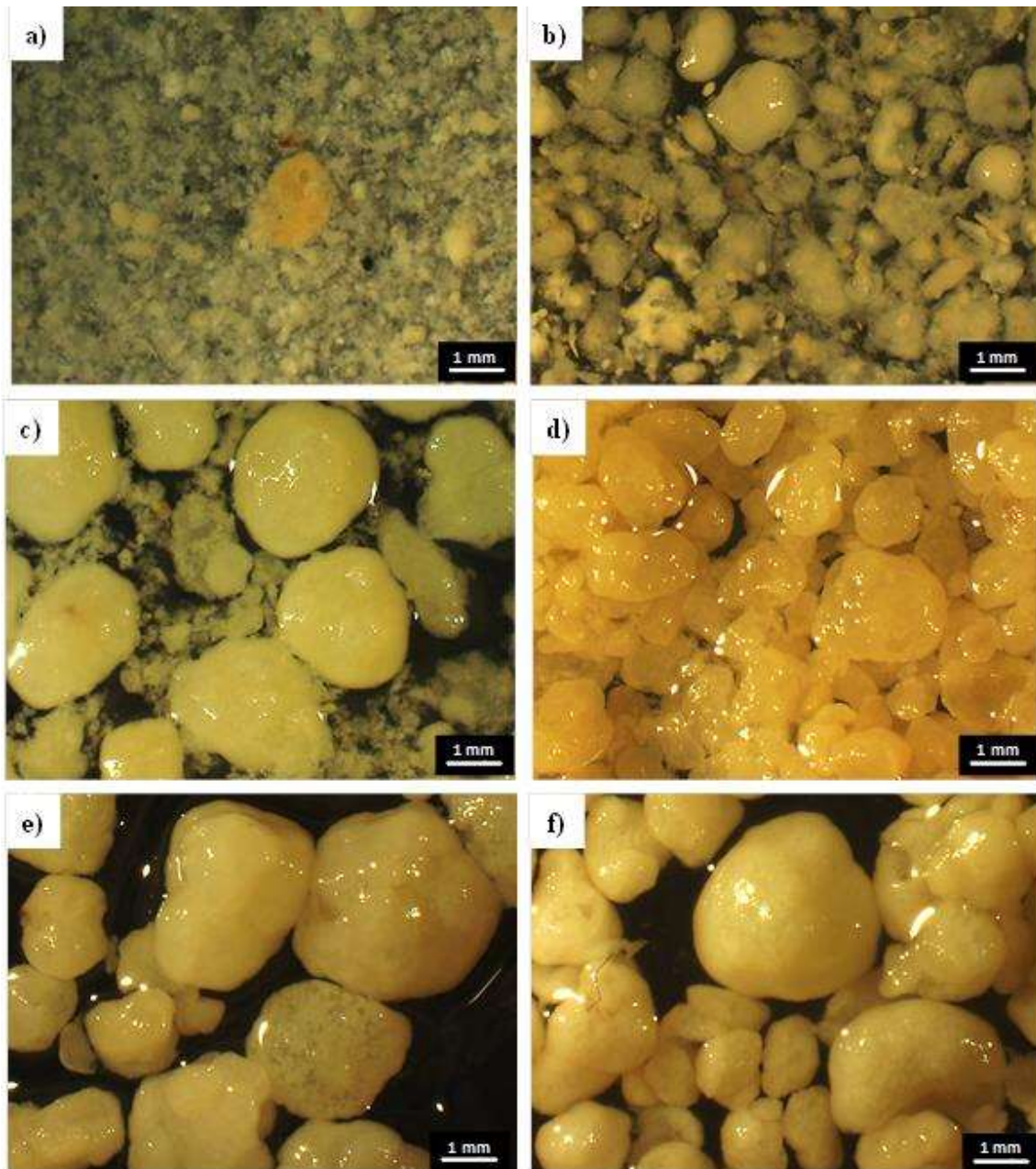
148

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 μm . 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⁻¹), 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⁻¹), 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 Ca²⁺ by the abundantly available Na⁺, 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 TSS_{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 ¹.

213 In the same phase, the TSS_{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 TSS_{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 TSS_{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 TSS_{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.

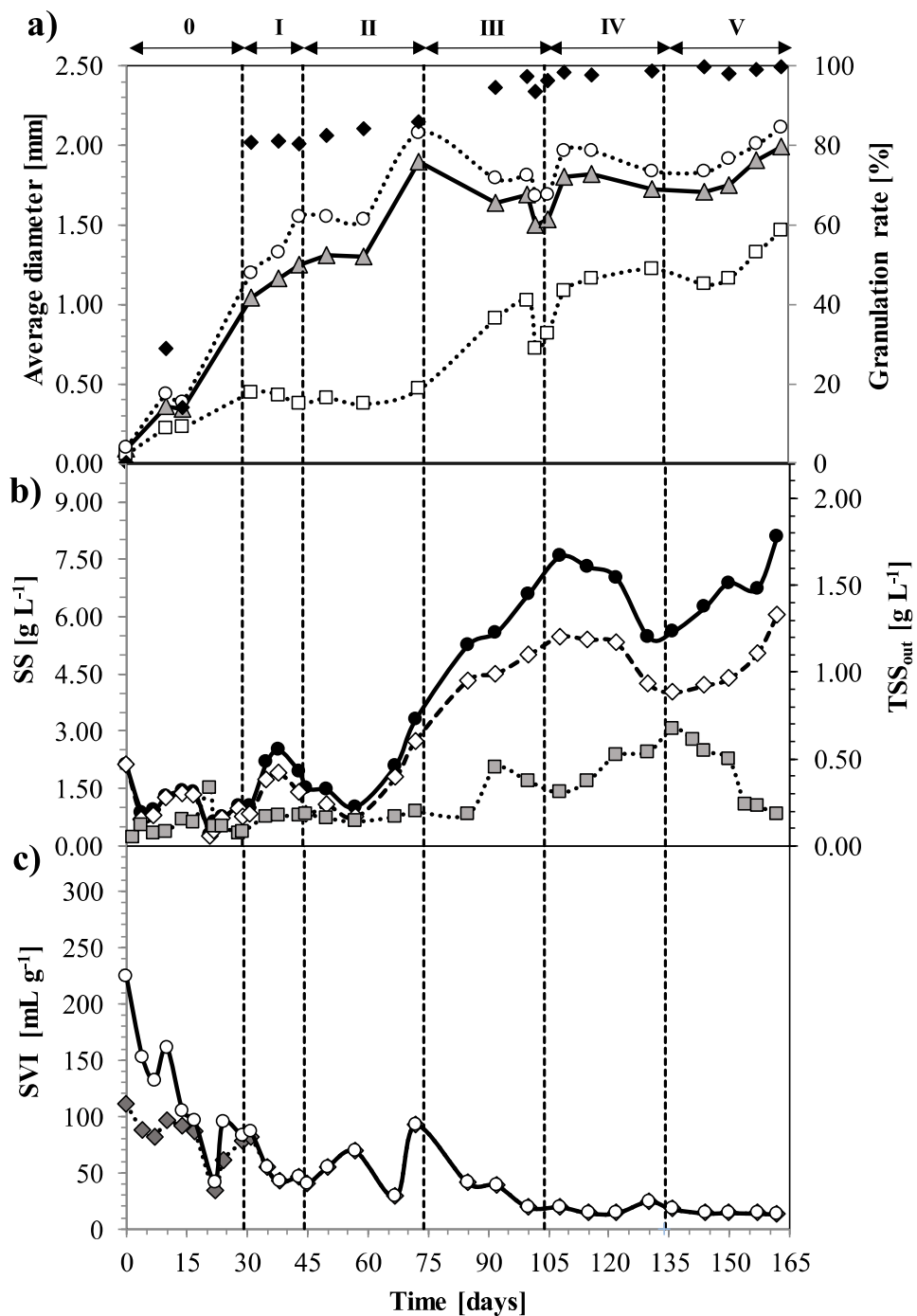
223 Indeed, as also observed by [30], the high salt concentration resulted in the increase of
224 the bulk density and, as a result, the buoyancy increased as well contributing to reduce
225 the settling velocity of the granules. In other words, it could be stated that the salinity
226 exerted an additional 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 SVI₅ and the SVI₃₀ 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 SVI₅ and the SVI₃₀ is close to
239 one. This condition was observed starting from the day 35 in Phase I, when both the
240 SVI₅ and the SVI₃₀ values were close to 55 mL·g⁻¹. In Phase II, the SVI₅ and the SVI₃₀
241 values increased to 90 mL·g⁻¹ suggesting a slight worsening of the settleability. From
242 the Phase III until the end of the experiment, a gradual decrease of the SVI₅ and the
243 SVI₃₀ down to 20 mL·g⁻¹ 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.

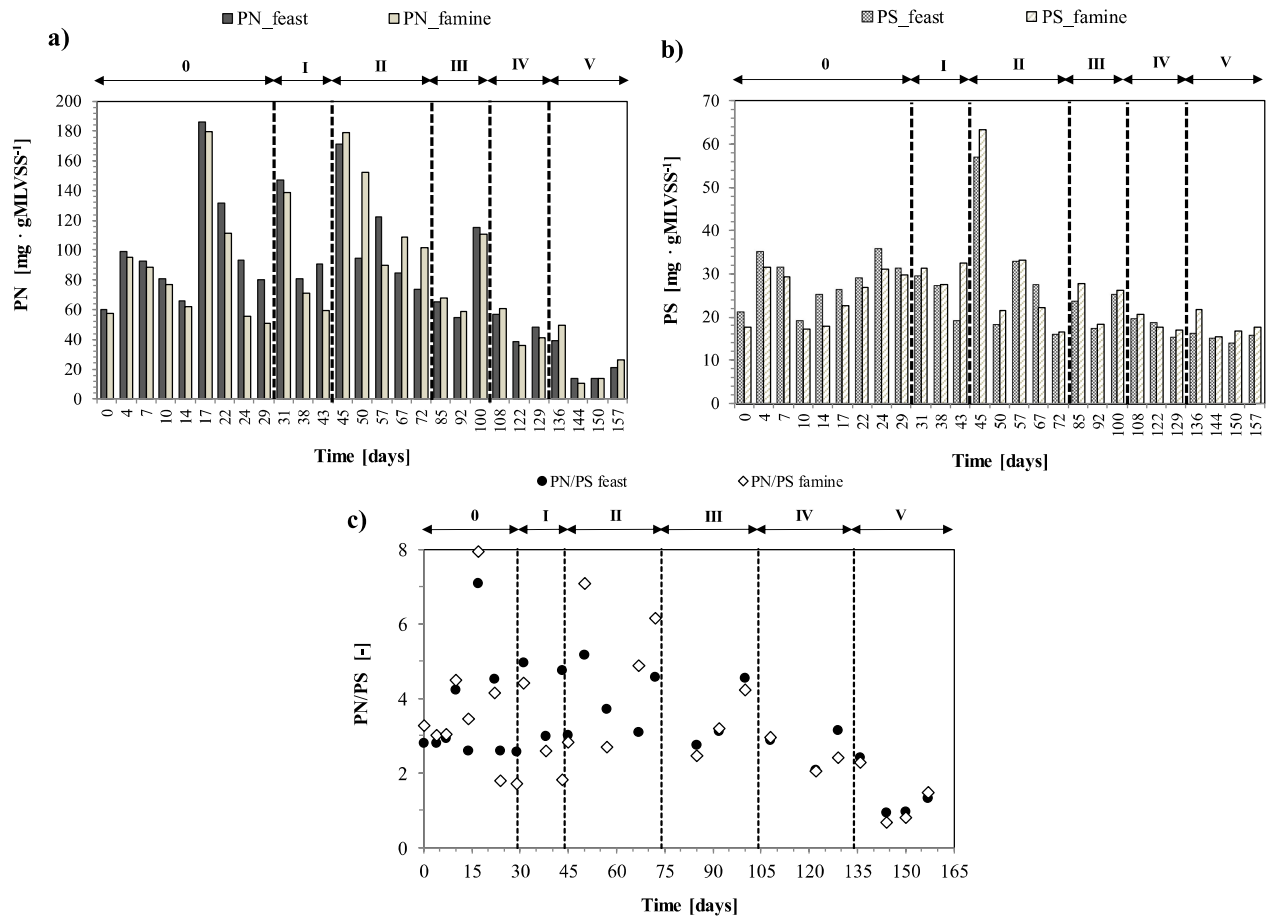
275 The days 31 and 45, other two peaks of proteins, close to $140 \text{ mg}\cdot\text{gMLVSS}^{-1}$ and 180
276 $\text{mg}\cdot\text{gMLVSS}^{-1}$, were noted, due to the increase of salinity. In this case, the osmotic

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⁻¹ until the day 43 in Phase I. However, at the beginning of Phase II, the
292 polysaccharides concentration rose to 60 mg·gMLVSS⁻¹ 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

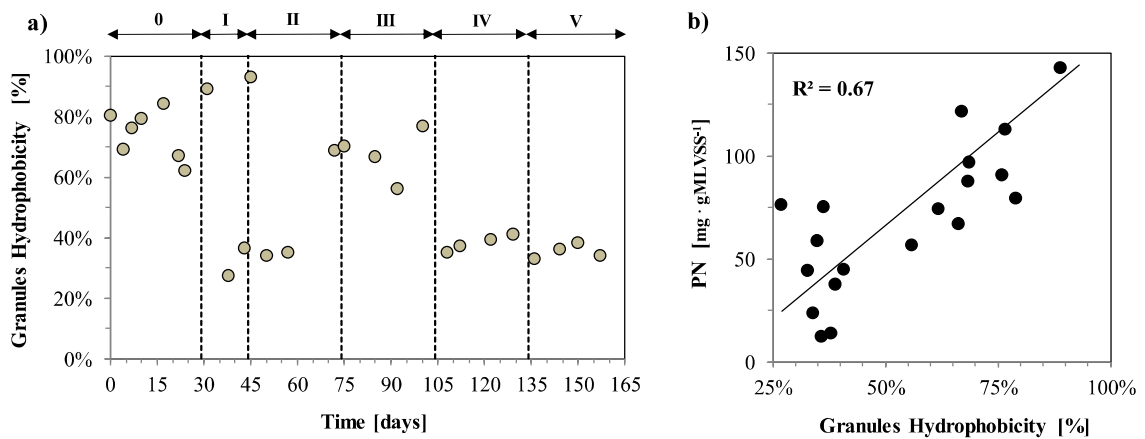
358 increase of the hydrophobicity [39–41]. The increase of surface hydrophobicity would
359 promote cell-to-cell interaction and further serves as inducing cell force for aggregating
360 and promoting biogranulation [39]. However, high salt concentrations of wastewater
361 directly affects sludge hydrophobicity [29], because Na^+ ions produce a change in the
362 cell surface properties. In particular, the presence of salt involves the replacement of
363 divalent Ca^{2+} ions with monovalent 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

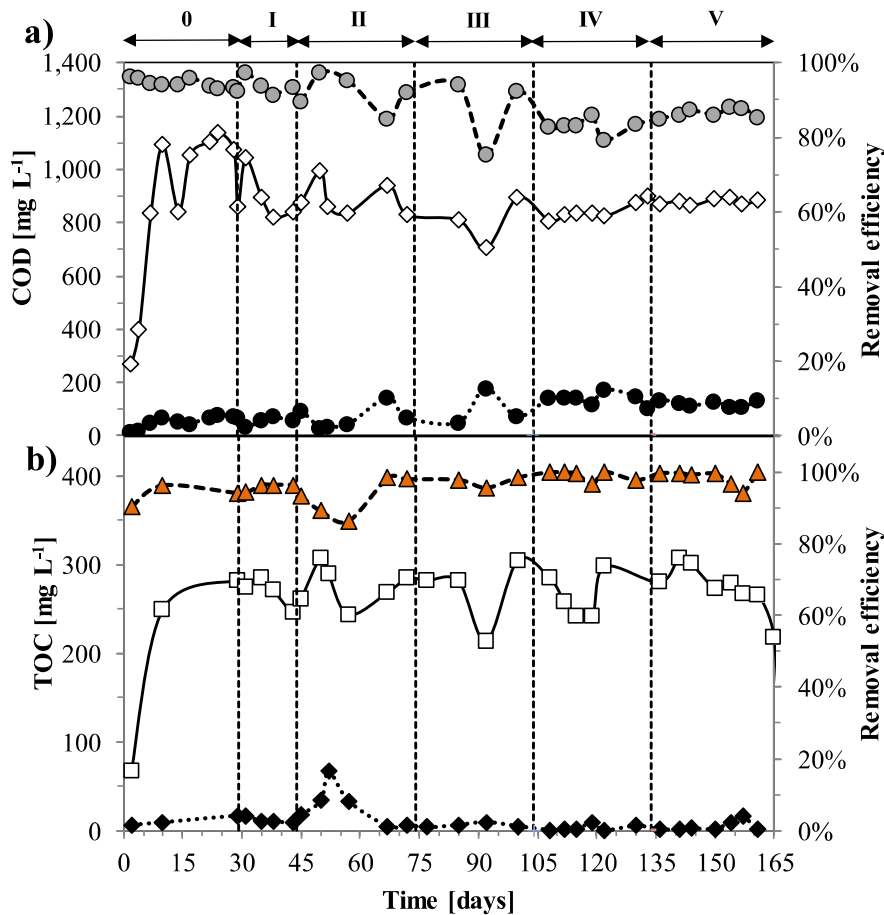
396 3.4.1 Organic matter removal

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 ± 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.

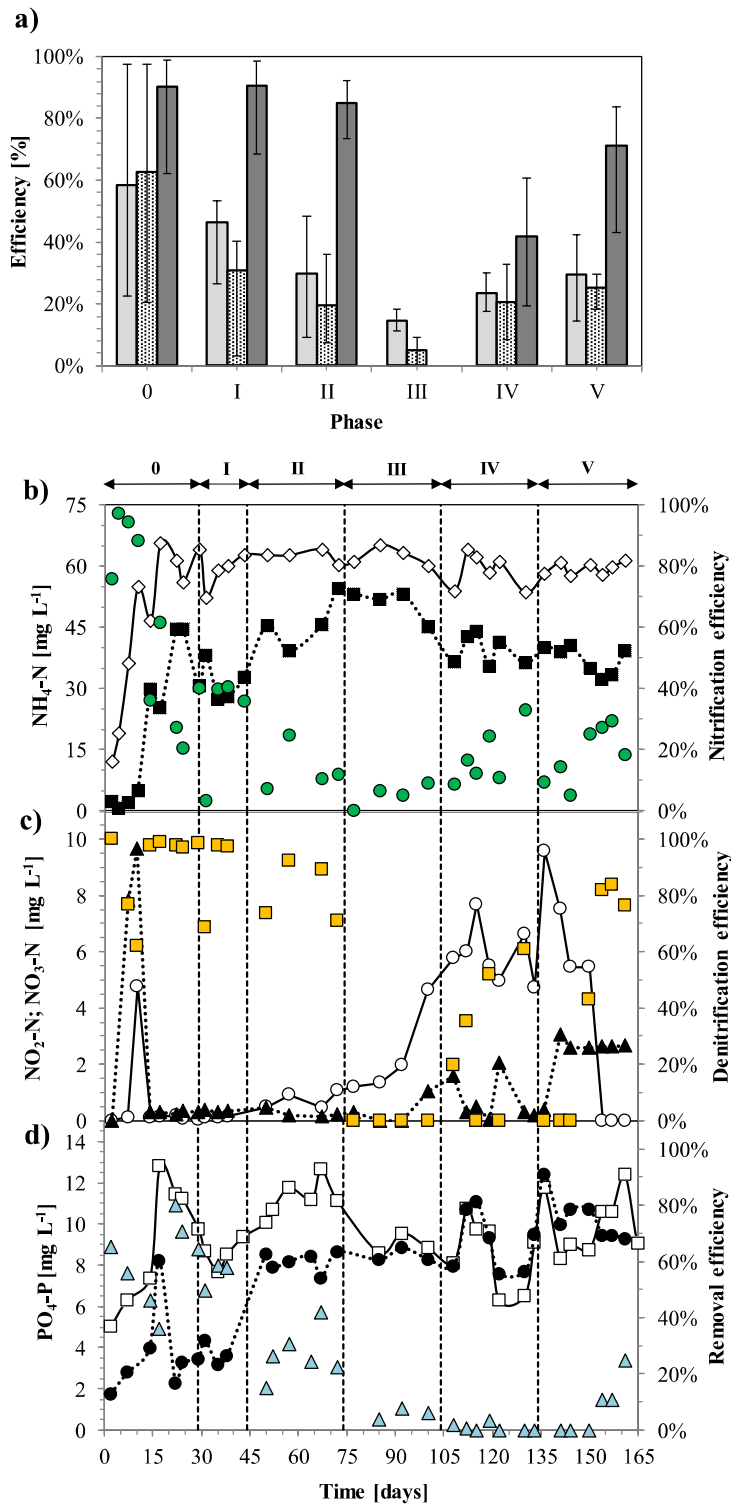
447 Although in Phase 0 the washout of biomass occurred in the earliest days of the
448 operation, both the absence of salinity and the inoculum seed sludge enriched in
449 nitrifying microorganisms were responsible for the highest nitrification efficiency
450 (**Figure 6b**). Moreover, granules with 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⁻¹ 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⁻¹ was observed. In Phase III, a gradual
495 accumulation of nitrites in the effluent up to 5 mg·L⁻¹ 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⁻¹ 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) NH₄-N in the influent (◊), NH₄-N in
 518 the effluent (◼); nitrification efficiency (●); (c) NO₂-N in the effluent (○),
 519 NO₃-N in the effluent (▲), denitrification efficiency (■); (d) PO₄-P in the influent
 520 (□), PO₄-P in the effluent (●), phosphorous removal efficiency (Δ).

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

		Phases and time span					
Parameter	Units	Phase 0	Phase I	Phase II	Phase III	Phase IV	Phase V
		0-29	30-44	45-74	75-104	105-134	135-164
		(29 days)	(15 days)	(30 days)	(30 days)	(30 days)	(30 days)
MLTSS	g·L ⁻¹	1.07 ± 0.52	1.66 ± 0.81	1.88 ± 0.79	5.79 ± 0.69	6.83 ± 0.94	6.70 ± 0.92
MLVSS	g·L ⁻¹	0.99 ± 0.54	1.30 ± 0.60	1.43 ± 0.75	4.61 ± 0.34	5.10 ± 0.58	4.73 ± 0.82
TSS_{out}	g·L ⁻¹	0.12 ± 0.08	0.14 ± 0.05	0.17 ± 0.02	0.33 ± 0.14	0.43 ± 0.11	0.42 ± 0.20
Mean diameter	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
SVI₅	mL·g ⁻¹	126 ± 55	66 ± 22	55 ± 23	33 ± 12	18 ± 5	15 ± 2
(PN/PS)_{feast}	-	3.57 ± 1.50	4.22 ± 1.09	3.91 ± 0.94	3.48 ± 0.95	2.70 ± 0.56	1.41 ± 0.69
(PN/PS)_{famine}	-	3.65 ± 1.86	2.95 ± 1.34	4.73 ± 1.96	3.28 ± 0.89	2.47 ± 0.46	1.31 ± 0.74
Salinity	gNaCl·L ⁻¹	0.30 ± 0.09	1.80 ± 0.74	4.87 ± 0.69	11.56 ± 0.31	24.31 ± 2.74	37.79 ± 1.21
η_{COD}	%	95 ± 1	94 ± 3	92 ± 4	87 ± 10	83 ± 2	86 ± 1
η_{TOC}	%	93 ± 4	95 ± 1	93 ± 5	97 ± 1	99 ± 1	98 ± 2
η_N	%	58 ± 27	46 ± 13	30 ± 14	15 ± 3	23 ± 5	29 ± 11
η_P	%	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

566 **Acknowledgements**

567 This work was performed at the University Kore of Enna within the PhD in Civil
568 Infrastructures for the territory (XXX cycle), where the first author is a Ph.D student,
569 currently.

570

571 **REFERENCES**

- 572 [1] O. Lefebvre, R. Moletta, Treatment of organic pollution in industrial saline
573 wastewater: A literature review, *Water Res.* 40 (2006) 3671–3682.
- 574 [2] C. Ramos, M.E. Suárez-Ojeda, J. Carrera, Long-term impact of salinity on the
575 performance and microbial population of an aerobic granular reactor treating a
576 high-strength aromatic wastewater, *Bioresour. Technol.* 198 (2015) 844–851.
- 577 [3] K.-H. Kim, S.-K. Ihm, Heterogeneous catalytic wet air oxidation of refractory
578 organic pollutants in industrial wastewaters: A review, *J. Hazard. Mater.* 186
579 (2011) 16–34.

- 580 [4] M. Pronk, J.P. Bassin, M.K. De Kreuk, R. Kleerebezem, M.C.M. Van
581 Loosdrecht, Evaluating the main and side effects of high salinity on aerobic
582 granular sludge, *Appl. Microbiol. Biotechnol.* 98 (2014) 1339–1348.
- 583 [5] B. Dalmacija, E. Karlovic, Z. Tamas, D. Miskovic, Purification of high-salinity
584 wastewater by activated sludge process, *Water Res.* 30 (1996) 295–298.
- 585 [6] K. Muda, A. Aris, M.R. Salim, Z. Ibrahim, A. Yahya, M.C.M. van Loosdrecht, et
586 al., Development of granular sludge for textile wastewater treatment, *Water Res.*
587 44 (2010) 4341–4350.
- 588 [7] L.M.M. de Bruin, M.K. de Kreuk, H.F.R. van der Roest, C. Uijterlinde, M.C.M.
589 van Loosdrecht, Aerobic granular sludge technology: An alternative to activated
590 sludge?, *Water Sci. Technol.* 49 (2004) 1–7.
- 591 [8] M.K. de Kreuk, M.C.M. van Loosdrecht, Formation of aerobic granules with
592 domestic sewage, *J. Environ. Eng.* 132 (2006) 694–697.
- 593 [9] Z.H. Li, X.C. Wang, Effects of salinity on the morphological characteristics of
594 aerobic granules, *Water Sci. Technol.* 58 (2008) 2421–2426.
- 595 [10] Z. Li, T. Zhang, N. Li, X. Wang, Granulation of filamentous microorganisms in a
596 sequencing batch reactor with saline wastewater, *J. Environ. Sci.* 22 (2010) 62–
597 67.
- 598 [11] J.P. Bassin, M. Pronk, G. Muyzer, R. Kleerebezem, M. Dezotti, M.C.M. van
599 Loosdrecht, Effect of elevated salt concentrations on the aerobic granular sludge
600 process: Linking microbial activity with microbial community structure, *Appl.*
601 *Environ. Microbiol.* 77 (2011) 7942–7953.

- 602 [12] X. Wang, T. Yang, B. Lin, Y. Tang, Effects of salinity on the performance,
603 microbial community, and functional proteins in an aerobic granular sludge
604 system, *Chemosphere*. 184 (2017) 1241–1249.
- 605 [13] Z. Wang, M.C.M. van Loosdrecht, P.E. Saikaly, Gradual adaptation to salt and
606 dissolved oxygen: Strategies to minimize adverse effect of salinity on aerobic
607 granular sludge, *Water Res.* 124 (2017) 702–712.
- 608 [14] A. Val Del Río, M. Figueroa, A. Mosquera-Corral, J.L. Campos, R. Méndez,
609 Stability of aerobic granular biomass treating the effluent from a seafood
610 industry, *Int. J. Environ. Res.* 7 (2013) 265–276.
- 611 [15] M. Figueroa, A. Mosquera-Corral, J.L. Campos, R. Méndez, Treatment of saline
612 wastewater in SBR aerobic granular reactors, *Water Sci. Technol.* 58 (2008) 479–
613 485.
- 614 [16] A. Val del Río, M. Figueroa, B. Arrojo, A. Mosquera-Corral, J.L. Campos, G.
615 García-Torriello, R. Méndez, Aerobic granular SBR systems applied to the
616 treatment of industrial effluents, *J. Environ. Manage.* 95 (2012) S88–S92.
- 617 [17] S.F. Corsino, R. Campo, G. Di Bella, M. Torregrossa, G. Viviani, Cultivation of
618 granular sludge with hypersaline oily wastewater, *Int. Biodeterior. Biodegrad.*
619 105 (2015) 192–202.
- 620 [18] D. Ou, H. Li, W. Li, X. Wu, Y. Wang, Y. Liu, Salt-tolerance aerobic granular
621 sludge: Formation and microbial community characteristics, *Bioresour. Technol.*
622 249 (2018) 132–138.
- 623 [19] S.F. Corsino, M. Capodici, M. Torregrossa, G. Viviani, Physical properties and

- 624 Extracellular Polymeric Substances pattern of aerobic granular sludge treating
625 hypersaline wastewater, *Bioresour. Technol.* 229 (2017) 152–159.
- 626 [20] X.F. Li, Y.J. Li, H. Liu, Z.Z. Hua, G.C. Du, J. Chen, Correlation between
627 extracellular polymeric substances and aerobic biogranulation in membrane
628 bioreactor, *Sep. Purif. Technol.* 59 (2008) 26–33.
- 629 [21] P. Le-Clech, V. Chen, T.A.G. Fane, Fouling in membrane bioreactors used in
630 wastewater treatment, *J. Memb. Sci.* 284 (2006) 17–53.
- 631 [22] J.J. Beun, M.C.M. Van Loosdrecht, J.J. Heijnen, Aerobic granulation in a
632 sequencing batch airlift reactor, *Water Res.* 36 (2002) 702–712.
- 633 [23] APHA, Standard Methods for the Examination of Water and Wastewater, Stand.
634 Methods. (2005) 541.
- 635 [24] H. Liu, Y. Li, C. Yang, W. Pu, L. He, F. Bo, Stable aerobic granules in
636 continuous-flow bioreactor with self-forming dynamic membrane, *Bioresour.*
637 *Technol.* 121 (2012) 111–118.
- 638 [25] A. Bartrolí, J. Pérez, J. Carrera, Applying ratio control in a continuous granular
639 reactor to achieve full nitrification under stable operating conditions, *Environ. Sci.*
640 *Technol.* 44 (2010) 8930–8935.
- 641 [26] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric
642 Method for Determination of sugars and related substances, *Anal. Chem.* 28
643 (1956) 350–356.
- 644 [27] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with

- 645 the Folin phenol reagent., *J. Biol. Chem.* 193 (1951) 265–275.
- 646 [28] E. Taheri, M.H. Khiadani Hajian, M.M. Amin, M. Nikaeen, A. Hassanzadeh,
647 Treatment of saline wastewater by a sequencing batch reactor with emphasis on
648 aerobic granule formation, *Bioresour. Technol.* 111 (2012) 21–26.
- 649 [29] S.B. Ismail, C.J. de La Parra, H. Temmink, J.B. van Lier, Extracellular polymeric
650 substances (EPS) in upflow anaerobic sludge blanket (UASB) reactors operated
651 under high salinity conditions, *Water Res.* 44 (2010) 1909–1917.
- 652 [30] M.-K.H. Winkler, J.P. Bassin, R. Kleerebezem, R.G.J.M. van der Lans, M.C.M.
653 van Loosdrecht, Temperature and salt effects on settling velocity in granular
654 sludge technology, *Water Res.* 46 (2012) 3897–3902.
- 655 [31] S.S. Adav, D.-J. Lee, K.-Y. Show, J.-H. Tay, Aerobic granular sludge: Recent
656 advances, *Biotechnol. Adv.* 26 (2008) 411–423.
- 657 [32] Y. Liu, J. Tay, State of the art of biogranulation technology for wastewater
658 treatment, 22 (2004) 533–563.
- 659 [33] K.Y. Show, D.J. Lee, J.H. Tay, Aerobic granulation: Advances and challenges,
660 *Appl. Biochem. Biotechnol.* 167 (2012) 1622–1640.
- 661 [34] B.S. McSwain, R.L. Irvine, M. Hausner, P.A. Wilderer, Composition and
662 distribution of extracellular polymeric substances in aerobic flocs and granular
663 sludge, *Appl. Environ. Microbiol.* 71 (2005) 1051–1057.
- 664 [35] C. Wan, X. Yang, D.-J. Lee, X. Liu, S. Sun, C. Chen, Partial nitrification of
665 wastewaters with high NaCl concentrations by aerobic granules in continuous-

- 666 flow reactor, *Bioresour. Technol.* 152 (2014) 1–6.
- 667 [36] Z.W. Wang, Y. Liu, J.H. Tay, The role of SBR mixed liquor volume exchange
668 ratio in aerobic granulation, *Chemosphere.* 62 (2006) 767–771.
- 669 [37] L. Zhu, M.-L. Lv, X. Dai, Y.-W. Yu, H.-Y. Qi, X.-Y. Xu, Role and significance
670 of extracellular polymeric substances on the property of aerobic granule,
671 *Bioresour. Technol.* 107 (2012) 46–54.
- 672 [38] M.C.M. Van Loosdrecht, M.A. Pot, J.J. Heijnen, Importance of bacterial storage
673 polymers in bioprocesses, in: *Water Sci. Technol.*, 1997: pp. 41–47.
- 674 [39] Y.-Q. Liu, Y. Liu, J.-H. Tay, The effects of extracellular polymeric substances on
675 the formation and stability of biogranules, *Appl. Microbiol. Biotechnol.* 65
676 (2004) 143–148.
- 677 [40] L. Zhang, X. Feng, N. Zhu, J. Chen, Role of extracellular protein in the formation
678 and stability of aerobic granules, *Enzyme Microb. Technol.* 41 (2007) 551–557.
- 679 [41] Z. Wang, M. Gao, Z. She, S. Wang, C. Jin, Y. Zhao, et al., Effects of salinity on
680 performance, extracellular polymeric substances and microbial community of an
681 aerobic granular sequencing batch reactor, *Sep. Purif. Technol.* 144 (2015) 223–
682 231.
- 683 [42] R. Wang, P. Zheng, A.-Q. Ding, M. Zhang, A. Ghulam, C. Yang, et al., Effects
684 of inorganic salts on denitrifying granular sludge: The acute toxicity and working
685 mechanisms., *Bioresour. Technol.* 204 (2016) 65–70.
- 686 [43] G. Di Bella, M. Torregrossa, Simultaneous nitrogen and organic carbon removal

687 in aerobic granular sludge reactors operated with high dissolved oxygen
688 concentration, *Bioresour. Technol.* 142 (2013) 706–713.