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

3

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11 ABSTRACT

12 A granular sequencing batch reactor (GSBR) worked for 164 days to study the effect of salinity on aerobic granulation. The feeding had an organic loading rate (OLR) of 1.6 kg COD·m⁻³·d⁻¹ 13 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 ≈ 0.4 mm) appeared after 14 days. Extracellular polymeric substances (EPSs) analyses revealed that proteins were mainly higher than 16 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 use water and inorganics salts in the process chain [1]. These effluents contain salts like 35 36 chlorides, carbonates and sulphates and, often, organic and particularly recalcitrant compounds such as aromatic compounds [2]. The discharge of such wastewater having 37 38 at the same time high salinity and high organic content without prior treatment, adversely affect the environment. 39

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

Biological systems for the treatment of organic matter in saline wastewater are 44 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 known to strongly inhibit both the heterotrophic and the autotrophic strains, which are 50 subjected to a huge osmotic pressure causing cellular plasmolysis or inhibition of many 51 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 most promising technologies for biological wastewater treatment. In this system, 54 55 compact and fast settling granules allow on the one hand a faster separation of the biomass in the treatment reactor, and on the other hand a higher biomass concentration 56 57 compared to the conventional activated sludge systems, minimizing the plant footprint [4,7]. The oxygen transfer limitation which occurs within the granules' layers, allows to 58 obtain different redox conditions (anaerobic, anoxic and aerobic) inside the layered 59 60 granular structure, so favoring nitrification, denitrification and phosphate removal [8]. A great interest in AGS for the treatment of synthetic saline wastewater [9–13] and 61 industrial saline wastewater [2,4,14–17] has been expressed recently. In all these works, 62 63 the AGS technology has proven to be very effective in presence of inorganic salts. Moreover, from a microbiological point of view Ou et al. (2018) [18], founded that 64 cultivating aerobic granular sludge in a saline environment lead to growth of moderately 65 halophilic genera, such as Salinicola and Halomonas with great versatility with respect 66 to salt tolerance. However, few studies have been conducted about granules' stability 67 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 characteristics, as a jelly-like aspect and consistence, that confer key properties 73 74 determining microbial aggregation and aerobic granular sludge formation.

75 Bearing in mind the above mentioned issues linked to salt concentration of raw76 wastewater, the main objective of this work is to analyze the granulation process in a

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

83

84 2. MATERIALS AND METHODS

2.1 Bench scale plant description and operational conditions

The granular sequencing batch airlift reactor was a column-type reactor, with a working 86 87 volume of 3.5 l and a height to diameter ratio (H/D) equal to 10. The reactor was operated on a six hours per cycle time, resulting in a hydraulic retention time (HRT) of 88 12 hours. A single cycle included 20 minutes of influent feeding, 333 minutes of 89 aeration, 2 minutes of settling and 5 minutes of effluent discharge. The volume 90 exchange ratio (VER) was fixed at 50% by placing a solenoid value at half height of the 91 92 filling height. Air was introduced via a fine bubble aerator placed at the base of the reactor at a flow rate of $3.3 \text{ cm} \cdot \text{s}^{-1}$ to ensure high shear forces. A programmable logic 93 controller (PLC) Crouzet model Millenium 3 CD20 handled the SBR cycling 94 95 operations. The reactor was fed with a synthetic saline wastewater prepared with a media composed by NaAc 97.7mM, MgSO₄ · 7H₂O 3.7mM, K₂HPO₄ 20mM, KH₂PO₄ 96 10mM, KCl 4.8mM, NH₄Cl 30 mM, according to Beun et al., (2002)[22]. This media 97 98 was diluted with tap water to obtain an organic loading rate (OLR) next to 1.6 kg $COD \cdot m^{-3} \cdot d^{-1}$) for the whole experimental period. Sodium chloride was separately added 99 to obtain the desired salinity measured as electrical conductivity. The reactor was 100

seeded with 1.75 l of flocculent activated sludge collected from the municipal 101 102 wastewater treatment plant of Enna (Sicily, Italy). Some physical properties of this 103 sludge were: sludge volume index after 30 min settling (SVI₃₀) of 111 mL·gTSS⁻¹, and mixed liquor total suspended solids concentration next to 2.12 gMLTSS·L⁻¹. During the 104 105 first 29 days (Phase 0), the OLR was gradually increased from 0.4 to 1.6 kg COD·m⁻³·d⁻ ¹, and the settling time was decreased from 15 min to 2 min to avoid organic and 106 hydraulic loading shocks, respectively, on the inoculum biomass. Subsequently, the 107 108 reactor was subjected to a step-wise increase of salinity. In particular, the following phases were assessed: Phase 0-29 days (0.30 ± 0.09 gNaCl^{-·}L⁻¹), Phase I-15 days (1.80 109 ± 0.74 gNaCl⁻·L⁻¹), Phase II - 30 days (4.87 ± 0.69 gNaCl⁻·L⁻¹), Phase III - 30 days 110 $(11.56 \pm 0.31 \text{ gNaCl}^{-}\text{L}^{-1})$, Phase IV-30 days $(24.31 \pm 2.74 \text{ gNaCl}^{-}\text{L}^{-1})$, Phase V-30 111 days $(37.79 \pm 1.21 \text{ gNaCl}^{-1}\text{L}^{-1})$. 112

113

114 **2.2 Analytical procedures**

The influent wastewater and the effluent have been sampled twice per week and 115 analyzed according to the Standard Methods [23]. In particular, the following 116 parameters were measured: total and volatile mixed liquor suspended solids (MLTSS 117 and MLVSS), total suspended solids discharged with the effluent (TSS_{out}) chemical 118 oxygen demand (COD), total organic carbon (TOC), ammonia nitrogen (NH₄-N), 119 anions (nitrite (NO₂⁻), nitrate (NO₃⁻), phosphate (PO₄³⁻), chloride (Cl⁻)). During all the 120 experimentation, COD was measured by means of chemical titration and, in high 121 122 salinity conditions, mercury sulphate was added to eliminate chloride interference. The ammonium and all the anions concentrations were measured by means of a ionic 123 124 chromatography by ICS Dionex 1100. The total organic carbon (TOC) was measured by

means of thermos-catalytic oxidation with a high-temperature TOC-VCSH analyzer that also provides the total carbon (TC) and the inorganic carbon (IC). In addition, the sizes of granules, settling velocity, sludge volume index after 5 min of settling (SVI₅) and after 30 min settling (SVI₃₀), hydrophobicity, and extracellular polymeric substances (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 analysis capability (QICPIC by Sympatec). Granulation rate was evaluated as the 131 132 percentage of particles with a diameter over 600 µm, according to [24]. The settling velocity was determined by placing individual granules in a graduated cylinder and 133 measuring the time they took to drop from a fixed height [25]. The hydrophobicity of 134 135 the cell surface was determined in accordance with the method described by Rosenberg et al. (1980). The total Extracellular Polymeric Substances were expressed as the sum of 136 137 bound EPSs and soluble microbial products (SMPs), as protein and polysaccharide fractions. Then, the EPS content was referred to the VSS concentration. The SMPs were 138 obtained by centrifugation at 5000 rpm for 5 min while the bound EPSs were extracted 139 140 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 sample was taken at the end of the feast phase, when most of the substrate was oxidized, 144 145 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.

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

 24.31 ± 2.74 gNaCl⁻·L⁻¹), the granules formed during the previous phases were 173 174 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 \pm 177 1.21 gNaCl⁻·L⁻¹), during which the average diameter increased up to 2 mm the day 157 and the granules appeared compact, rounded and with a regular shape (Figure 1f). 178 179 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 mature aerobic granules. Although some authors [29] found that in a saline environment 181 the replacement of Ca²⁺ by the abundantly available Na⁺, in the EPS matrix, caused the 182 183 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 be due to the high buoyancy of saline wastewater that washed out the slow settling 186 particles, and the high concentration of cations in the bulk that reduced the electric 187 188 double layers on the surface of granules, favoring the microbial aggregation.



190

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

The trends of the total and volatile suspended solids in the mixed liquor (MLTSS, MLVSS), are shown in **Figure 2b**. At the beginning of Phase 0, a severe biomass washout was observed, due to the high hydraulic selection pressure (15 minutes of settling). Consequently, the MLTSS decreased from 2.12 g·L⁻¹ (value of the inoculum) to 0.85 g·L⁻¹. Then, the concentration of biomass in the new operating conditions increased up to 1.3 - 1.4 g·L⁻¹. The further reduction in the settling time, did not involve a severe reduction in the MLTSS as previously occurred, because most of the biomass was already hydraulically selected. From that day on, the MLTSS concentration was almost constant, at least until the day 18, when the change of the settling time to 2 min caused a severe decrease in the MLTSS to 0.26 g·L⁻¹ and a subsequent washout of TSS_{out} in the effluent with a concentration of 0.33 g·L⁻¹.

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

Subsequently, the MLTSS concentration continuously increased for the remaining part of Phase II and during Phase III, reaching a steady value approximately close to 7.5 g·L⁻ 1 .

In the same phase, the TSS_{out} in the effluent was almost constant next to 0.15 g·L⁻¹ until the day 85. Then, the change of the granules' morphology occurred in this period (**Figure 1d**), resulted in an increase of the TSS_{out} washed out from the system with a peak of approximately 0.45 g·L⁻¹ the day 92, without an appreciable decrease in the MLTSS concentration. After this day, the TSS_{out} in the effluent decreased as a consequence of a higher stability of the aerobic granules.

In the subsequent Phase IV, a reduction of the MLTSS from 7.5 g·L⁻¹ to 5.4 g·L⁻¹ was observed. During this phase, the TSS_{out} concentration in the effluent gradually increased up to 0.54 g·L⁻¹ the day 130. This trend was mainly attributable to the higher buoyancy force in the bulk due to the high salt concentration. Indeed, as also observed by [30], the high salt concentration resulted in the increase of the bulk density and, as a result, the buoyancy increased as well contributing to reduce the settling velocity of the granules. In other words, it could be stated that the salinity exerted an additional positive effect of hydraulic selection pressure on the aerobic granules.

In the last Phase V, no significant changes, compared to the previous period, occurred in spite of the salinity increase, confirming the achievement of structurally stable granules. The trend of the MLVSS (**Figure 2b**) was similar to that of the MLTSS. However, starting from the Phase III onward, a gradual reduction in the MLVSS fraction was observed. This was probably due to a gradual inclusion of salts within the structure of the aerobic granules. Indeed, because the salts are inorganic compounds, once adsorbed 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 and 30 minutes. The trends of both the SVI₅ and the SVI₃₀ during the five experimental 236 periods are shown in Figure 2c. As reported by many authors [31,32], the complete 237 granulation can be identified when the ratio between the SVI₅ and the SVI₃₀ is close to 238 one. This condition was observed starting from the day 35 in Phase I, when both the 239 SVI₅ and the SVI₃₀ values were close to 55 mL \cdot g⁻¹. In Phase II, the SVI₅ and the SVI₃₀ 240 values increased to 90 mL·g⁻¹ suggesting a slight worsening of the settleability. From 241 the Phase III until the end of the experiment, a gradual decrease of the SVI₅ and the 242 SVI₃₀ down to 20 mL·g⁻¹ was observed, confirming the achievement of stable and 243 244 mature aerobic granules.



246

Figure 2: (a) Mean diameter of granules $(D50 \rightarrow)$, particle diameter corresponding to 10% $(D10 \rightarrow)$ and to 60% $(D60 \rightarrow)$ cumulative undersize particle size distribution, granulation rate (\bullet); (b) total suspended solids (\rightarrow) and volatile suspended solids (\rightarrow) in the reactor, total suspended solids in the effluent (\cdots); (c) sludge volume index after 5 min (-) and 30 min (\cdots).

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 cellular surface such as the charge and the hydrophobicity [33]. EPSs are mainly 257 constituted by proteins, polysaccharides, humic acids, and lipids secreted by 258 microorganisms. Proteins are considered to be the main polymeric substances 259 260 responsible to maintain the granules structure. Furthermore, proteins are mainly present in the inner layer, while polysaccharides concentrate on the outer surface of aerobic 261 granules [34]. Figure 3a shows the trend of the proteins content of the granules referred 262 263 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 the new operating conditions (batch feeding, shear forces, hydraulic selection pressure, 266 etc.). Then, when the biomass was acclimating to the new conditions and pseudo 267 268 steady-state was achieved, a decrease of the specific proteins concentration was observed. Subsequently, when the settling time decreased from 4 min to 2 min, the 269 270 increase in the hydraulic selection pressure induced a higher physical stress which caused a significant production of proteins (up to 180 mg·gMLVSS⁻¹ the day 17) 271 fulfilling the function of structure of aerobic granules. Subsequently, the following 272 decrease of proteins values confirmed the adaptation of granules to new operational 273 274 parameters.

The days 31 and 45, other two peaks of proteins, close to 140 mg·gMLVSS⁻¹ and 180 mg·gMLVSS⁻¹, were noted, due to the increase of salinity. In this case, the osmotic

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

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

Regarding to the polysaccharides (**Figure 3b**) their concentration ranged between $20 \div$ 30 mg·gMLVSS⁻¹ until the day 43 in Phase I. However, at the beginning of Phase II, the polysaccharides concentration rose to 60 mg·gMLVSS⁻¹ in accordance with the increase of the proteins content. Nevertheless, at each salinity increase the polysaccharides production was much lower compared to the proteins, suggesting that microorganisms faced the osmotic pressure mainly through the proteins secretion.

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

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

During the Phase 0 and the Phase I, the proteins in the feast phase resulted higher than in the famine one, because during the phases of formation of granules the microorganisms produced a higher concentration of proteins in feast phase contributing to the formation of granule's structure. Indeed, during the feast phase, when the organic substrate was available in large amounts, bacteria created storage products in forms of proteins, contributing to the formation of granules. In the following famine phase, the proteins were used as carbon source by microorganisms in aerobic starvation and this helped to increase cell surface hydrophobicity and to enhance the ability of anti-toxic shock of granular sludge [34,36,37]. Once the granules were formed, the maturation process began and lasted for all the remaining phases. For the remaining days of operation, it was observed that the maturation and the improvement of the stability of 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 found that the feast/famine strategy led to a storage of polymers (mainly 319 320 polysaccharides) when the substrate was present (feast phase) which was used for growth of microorganisms when the external substrate was depleted (famine phase). 321 322 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 proteins and polysaccharides trends in increasing salinity conditions, were apparently in 324 325 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 probaby 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 environment. Moreover, these new microorganisms possessed different metabolic 333

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 value determined for the treatment of salt-free domestic wastewater. So, when bacteria 337 338 activate the mechanism to adapt to salinity by means the transport of osmolytes from and into the cell, they require more energy and, therefore, more carbon source which 339 causes K_s to become higher. Therefore, since in this work the exogenous COD was 340 341 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 an additional endogenous carbon source, in a greater extent in the feast phase than in the 343 344 famine phase. This could explain the unusual trend of EPS observed during the feast and famine phases in presence of salinity. 345

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

Hydrophobicity has been cited as an important granules property [34] and many authorsunderlined 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 divalent Ca²⁺ ions with monovalent Na⁺ ions of the EPS, resulting in a minor reduction 363 364 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 proteins content, which are well known to be charged positively, was found to decrease 367 368 the surface negative charge of bacteria cells and increasing the hydrophobicity. This reduced the zeta potential and the electrostatic repulsions, favoring bridging and 369 370 microbial aggregation.

In particular, in Phase 0 the sludge hydrophobicity decreased from the 80% (inoculum 371 value) to a value close to 64%. Subsequently the time course of the sludge 372 hydrophobicity was very irregular. In Phase III (11.56 \pm 0.31 gNaCl⁻·L⁻¹) the 373 hydrophobicity was on average higher than in the previous two phases. As discussed 374 375 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 microorganisms produced amino-acids groups, as protein components, with a higher 377 positive charge. This could explain the higher values of hydrophobicity in this phase. 378 379 Subsequently in the remaining Phase IV and Phase V the lower protein production of 380 mature granules implied a lower hydrophobicity.

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

385



386

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

During the Phase 0 and the Phase I the reactor was able to remove more than 95% of COD (**Figure 5a**). In Phase II and Phase III, the heterotrophic microorganisms were probably affected by the salinity increase as found also by Wang et al. (2016) [42] and the removal efficiency dropped to 85% on day 67 and continued to decrease to 75% until the day 92. Subsequently, during Phase IV and Phase V, the COD removal
efficiency was quite stable around a steady value of 85%, denoting the acclimation of
the heterotrophic microorganisms to the saline environment.

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



Figure 5: (a) COD in the influent (-, COD in the effluent (-, COD removal efficiency (-); (b) TOC in the influent (-, TOC in the effluent (-, TOC removal efficiency (-).

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426 **3.4.2 Nitrogen and Phosphorous removal**

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Figure 6a shows how the gradual increase of salinity until Phase III (11.56 \pm 0.31 gNaCl·L⁻¹), caused an inhibition of autotrophic biomass, leading to a decrease of nitrification from 63% (Phase 0) to 20% (Phase II - 4.87 \pm 0.89 gNaCl·L⁻¹) and thus a decrease of nitrogen removal from 58% to 30%. In particular, during Phase III a strong inhibition of autotrophic biomass was observed, and nitrogen was mainly removed for 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 environment, and the nitrogen removal increased from 15% to 29%. Observing the 437 denitrification efficiency, since it is a relative value strictly depending on the 438 439 nitrification efficiency, it was relatively high between 80-90% from Phase 0 (no salt addition) to Phase II (4.87 \pm 0.89 gNaCl·L⁻¹), when salt concentration was not too high. 440 441 During Phase III, the denitrification activity dropped to 19% due to the further increase of salinity that exerted a strong inhibitory effect. Then, a gradual reprise of 442 denitrification activity was observed (up to 71% in Phase V) that, together with the 443 444 gradual increase of the nitrification, contributed to increase the total nitrogen removal. This suggested that, not only nitrifying but also denitrifying microorganisms were 445 adapted to the saline environment. 446

Although in Phase 0 the washout of biomass occurred in the earliest days of the 447 operation, both the absence of salinity and the inoculum seed sludge enriched in 448 449 nitrifying microorganisms were responsible for the highest nitrification efficiency (Figure 6b). Moreover, granules with smaller sizes have a higher specific surface area 450 451 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 worsening of the nitrification with outlet ammonium concentration close to 45 mg·L⁻¹, 453 was observed. This was mainly due to the severe washout of the slow growing 454 455 autotrophic biomass, occurred following to the increase of the hydraulic selection pressure. At the end of Phase 0, when the slow settling microorganisms were discharged 456 and the reactor enriched in fast settling biomass, the nitrification efficiency rose to 40% 457

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

Finally in Phase V (37.79 ± 1.21 gNaCl⁻·L⁻¹), after few initial days of biological inhibition due to the high salinity concentration, the autotrophic bacteria living in stable and compact granules nitrified the influent ammonium up to 30%, highlighting the adaptation of nitrifying biomass to high saline environment. 481 Based on the concentrations of nitrites and nitrates in the effluent and on the denitrification efficiency (Figure 6c), it could be stated that the simultaneous 482 483 nitrification denitrification (SND) [43] occurred. In the first days of operation, two peaks of nitrates and nitrites concentration of 10 and 5 mg·L⁻¹ were observed, 484 respectively. This was mainly due to the small dimensions of granules at that time, that 485 implied a small anoxic layer and a poor denitrification efficiency of about 60%, 486 accordingly. Then, due to the increase of the aerobic granules size and the development 487 488 of an anoxic layer, all the nitrified ammonium was mostly denitrified with a removal efficiency close to 98%. Subsequently, in Phase I, the increase in the inlet salt 489 concentration produced an initial decrease of the denitrification efficiency, due to a 490 491 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 a worsening of the denitrification efficiency that dropped to 71%, and a nitrite 493 concentration in the effluent next to $1 \text{ mg} \cdot \text{L}^{-1}$ was observed. In Phase III, a gradual 494 accumulation of nitrites in the effluent up to 5 mg \cdot L⁻¹ was observed, and the 495 496 denitrification efficiency decreased down to about 17% at the end of the phase. This was probably due to the change of the granules morphology and structure observed in 497 this phase. As discussed previously, during Phase III the average size of the granules 498 499 was reduced and the anoxic layer become thinner, so limiting the denitrification process. Moreover, the nitrite accumulation suggested the inhibition of the autotrophic nitrite 500 oxidizing bacteria (NOB), according to other authors [4,11,15] who noted that NOB are 501 502 more sensitive to saline environment respect to the ammonia oxidizing bacteria (AOB). In Phase IV the nitrite concentration in the effluent was around 6 mg \cdot L⁻¹ with a 503 504 denitrification efficiency increased up to 60%. Moreover, at that time some nitrates

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



Figure 6. (a) Nitrogen average removal efficiency (\square), nitrification average efficiency (\square), denitrification average efficiency (\square); (b) NH₄-N in the influent ($\neg \neg \vdash$), NH₄-N in the effluent ($\cdots \blacksquare \cdots$); nitrification efficiency (\square); (c) NO₂-N in the effluent ($\neg \neg \neg$), NO₃-N in the effluent ($\cdots \blacksquare \cdots$), denitrification efficiency (\square); (d) PO₄-P in the influent ($\neg \square \neg$), PO₄-P in the effluent ($\cdots \blacksquare \cdots$), phosphorous removal efficiency (\square).

521 Finally, also the potential simultaneous phosphorous removal was analized (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 granules were not well-formed. Therefore, in this phase phosphorous was mainly 525 526 removed for growth and assimilation of microorganisms. Subsequently, the granules formation resulted in an improvement of the phosphorous removal to 70%, because the 527 528 higher average diameter of granules may have promoted the development of an anaerobic core where phosporus accumulating organisms (PAOs) may have 529 proliferated. In Phase I (1.80 \pm 0.74 gNaCl⁻·L⁻¹), the phosphorous removal efficiency 530 531 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 removal efficiency dropped to 25%. As well known in the literature [4,11], nitrites can 533 negatively affect the phosphorous uptake activity of PAOs under both anoxic and 534 aerobic conditions. In this work, the obtained results confirmed that the effect of salt 535 536 was detrimental to NOBs, which was reflected in the accumulation of nitrites, as discussed previously. In turn, phosphates uptake dramatically reduced when nitrites 537 concentration was above 1 mg·L⁻¹, as observed in Phase III (5%), in Phase IV (almost 538 539 0%) and until the day 150 in Phase V. Then, when nitrites concentration dropped to zero, the phosporous removal efficiency reprise to increase up to 25% at the end of the 540 experimentation. These observations indicated a probable development of PAOs 541 542 microorganisms in the inner layers of granules. The gradual deterioration of phosphorous removal efficiency was likely caused by a reversible inhibition of PAOs, 543 544 caused by the nitrites accumulation in the saline environment. In order to resume the

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

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

	Phases and time span						
		Phase 0	Phase I	Phase II	Phase III	Phase IV	Phase V
Parameter	Units	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
TSSout	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
ηCOD	%	95 ± 1	94 ± 3	92 ± 4	87 ± 10	83 ± 2	86 ± 1
ηΤΟϹ	%	93 ± 4	95 ± 1	93 ± 5	97 ± 1	99 ± 1	98 ± 2
ηΝ	%	58 ± 27	46 ± 13	30 ± 14	15 ± 3	23 ± 5	29 ± 11
ηΡ	%	59 ± 16	58 ± 6	27 ± 9	6 ± 2	1 ± 1	18 ± 7

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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 high mean dimensions of granules may have promoted the formation of an anaerobic 561 core where PAOs may have grown. The presence of nitrites caused a temporary 562 deterioration of PAOs phosphorous removal efficiency, that increased when nitrites 563 564 were depleted.

565

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