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

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ABSTRACT

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 $COD \cdot m^{-3} \cdot d^{-1}$ and a gradual increase of salinity (from 0.30 to 38 g $NaCl^{-} \cdot L^{-1}$) to promote a biological salt-adaptation. First aggregates (average diameter ≈ 0.4 mm) appeared after 14 days. Extracellular polymeric substances (EPSs) analyses revealed that proteins were mainly higher than polysaccharides, and microorganisms metabolized EPSs as additional carbon source, mostly in feast phase, to face the energy demand for salinity adaptation. No significant worsening of organic matter removal was observed. The initial decrease of nitrification (from 58% to 15%) and the subsequent increase (up to 25%), confirmed the acclimation of AOBs to saline environment, while the accumulation of nitrites suggested NOBs inhibition. The nitrogen removal initially decreased from 58% to 15%, due to the inhibitory effect of salinity, and subsequently increased up to 29% denoting a simultaneous nitrification-denitrification. The dimensions of mature granules (higher than 1 mm) probably involved PAOs growth in the inner anaerobic layers. Nitrites caused a temporary deterioration of phosphorous removal (from 60% to almost zero), that increased up to 25% when nitrites were depleted.

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

1. INTRODUCTION

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

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 nowadays increasingly the focus of research. Currently, activated sludge systems are the main biological processes implemented at full-scale. However, their practical application to treat complex industrial wastewaters is rather limited because the microorganisms are known to be inhibited by toxic and recalcitrant compounds and to 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 subjected to a huge osmotic pressure causing cellular plasmolysis or inhibition of many enzymes [5,6]. Consequently, high salt concentrations affect organic matter, nitrogen

and phosphorus removal. Currently, aerobic granular sludge (AGS) process is one of the most promising technologies for biological wastewater treatment. In this system, 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 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 obtain different redox conditions (anaerobic, anoxic and aerobic) inside the layered 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 industrial saline wastewater [2,4,14–17] has been expressed recently. In all these works, 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 cultivating aerobic granular sludge in a saline environment lead to growth of moderately halophilic genera, such as *Salinicola and Halomonas* with great versatility with respect to salt tolerance. However, few studies have been conducted about granules' stability under salinity conditions [19], therefore some information is still lacking concerning the role and the functionality of extracellular polymeric substances (EPS) in granulation process in a saline environment. EPS are considered as one of the most relevant factors in granules formation [20]. They are made of various compounds (proteins, polysaccharides, humic acids, lipids) [21] with specific biological-physical characteristics, as a jelly-like aspect and consistence, that confer key properties determining microbial aggregation and aerobic granular sludge formation.

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

sequencing 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.

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 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 12 hours. A single cycle included 20 minutes of influent feeding, 333 minutes of aeration, 2 minutes of settling and 5 minutes of effluent discharge. The volume exchange ratio (VER) was fixed at 50% by placing a solenoid valve at half height of the filling height. Air was introduced via a fine bubble aerator placed at the base of the reactor at a flow rate of 3,3 $cm \cdot s^{-1}$ to ensure high shear forces. A programmable logic controller (PLC) Crouzet model Millenium 3 CD20 handled the SBR cycling operations. The reactor was fed with a synthetic saline wastewater prepared with a media composed by NaAc 97.7mM, $MgSO_4 \cdot 7H_2O$ 3.7mM, K_2HPO_4 20mM, KH_2PO_4 10mM, KCl 4.8mM, NH_4Cl 30 mM, according to Beun et al., (2002)[22]. This media 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 to obtain the desired salinity measured as electrical conductivity. The reactor was

seeded with 1.75 l of flocculent activated sludge collected from the municipal wastewater treatment plant of Enna (Sicily, Italy). Some physical properties of this sludge were: sludge volume index after 30 min settling (SVI_{30}) of 111 $mL \cdot gTSS^{-1}$, and mixed liquor total suspended solids concentration next to 2.12 $gMLTSS \cdot L^{-1}$. During the first 29 days (Phase 0), the OLR was gradually increased from 0.4 to 1.6 kg $COD \cdot m^{-3} \cdot d^{-1}$, and the settling time was decreased from 15 min to 2 min to avoid organic and hydraulic loading shocks, respectively, on the inoculum biomass. Subsequently, the 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 $gNaCl^{-} \cdot L^{-1}$), Phase I-15 days (1.80 ± 0.74 $gNaCl^{-} \cdot L^{-1}$), Phase II – 30 days (4.87 ± 0.69 $gNaCl^{-} \cdot L^{-1}$), Phase III – 30 days (11.56 ± 0.31 $gNaCl^{-} \cdot L^{-1}$), Phase IV-30 days (24.31 ± 2.74 $gNaCl^{-} \cdot L^{-1}$), Phase V-30 days (37.79 ± 1.21 $gNaCl^{-} \cdot L^{-1}$).

2.2 Analytical procedures

The influent wastewater and the effluent have been sampled twice per week and analyzed according to the Standard Methods [23]. In particular, the following parameters were measured: total and volatile mixed liquor suspended solids (MLTSS and MLVSS), total suspended solids discharged with the effluent (TSS_{out}) chemical oxygen demand (COD), total organic carbon (TOC), ammonia nitrogen (NH_4-N), anions (nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), chloride (Cl^-)). During all the experimentation, COD was measured by means of chemical titration and, in high salinity conditions, mercury sulphate was added to eliminate chloride interference. The ammonium and all the anions concentrations were measured by means of a ionic 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 (SVI_5) and after 30 min settling (SVI_{30}), hydrophobicity, and extracellular polymeric substances (EPSs) were analyzed once per week to limit the sludge withdrawal.

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 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 measuring the time they took to drop from a fixed height [25]. The hydrophobicity of 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 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 obtained by centrifugation at 5000 rpm for 5 min while the bound EPSs were extracted by a thermal extraction method [21]. The polysaccharides were determined according to the phenol–sulphuric acid method with glucose as standard [26], while the proteins were determined by the Folin method with bovine serum albumin as standard [27]. The 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, while the second sample was taken at the end of the famine phase. Dissolved Oxygen (DO) concentration and pH were monitored during the cycle to identify the end of the feast phase.

3. RESULTS AND DISCUSSIONS

3.1 Granulation process and physical properties of granules

The reactor was seeded with flocculent biomass collected from the wastewater 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 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 mean diameter next to 0.3-0.4 mm (**Figure 2a**) and the granulation rate was next to 44% . In Phase I (1.80 ± 0.74 $gNaCl^{-}\cdot L^{-1}$), the granulation process proceeded and yellow granules (**Figure 1b**) having an average diameter of about 1.1-1.2 mm and an irregular 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 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, some fluffy granules were observed in response to the salt stress, as also noted by Taheri et al. (2012) [28]. In Phase III, the further increase of salinity up to approximately 11.56 ± 0.31 $gNaCl^{-}\cdot L^{-1}$, caused a significant change in the granules appearance, that became translucent (**Figure 1d**). In particular, on day 102, the average 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 $Cl^{-}\cdot L^{-1}$). Although a slight modification of the granules morphology occurred in this period, a further increase in 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 was maintained over 0.6 mm. In Phase IV, despite the further increase of salinity (about

24.31 ± 2.74 $gNaCl^{-}\cdot L^{-1}$), the granules formed during the previous phases were subjected to a maturation process resulting in the increase of their average diameter up to about 1.7 mm (**Figure 1e**). At that time, the granules resulted very compact and structurally stable. The stability of the granules was maintained also in Phase V (37.79 ± 1.21 $gNaCl^{-}\cdot L^{-1}$), 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**). During Phase IV and Phase V, the maturation process resulted in a further increase of 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 the replacement of Ca^{2+} by the abundantly available Na^{+}, in the EPS matrix, caused the deterioration of the granule strength resulting in a weaker and swollen granule structure, in the present study the high salinity resulted in the formation of compact granules in 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 particles, and the high concentration of cations in the bulk that reduced the electric double layers on the surface of granules, favoring the microbial aggregation.

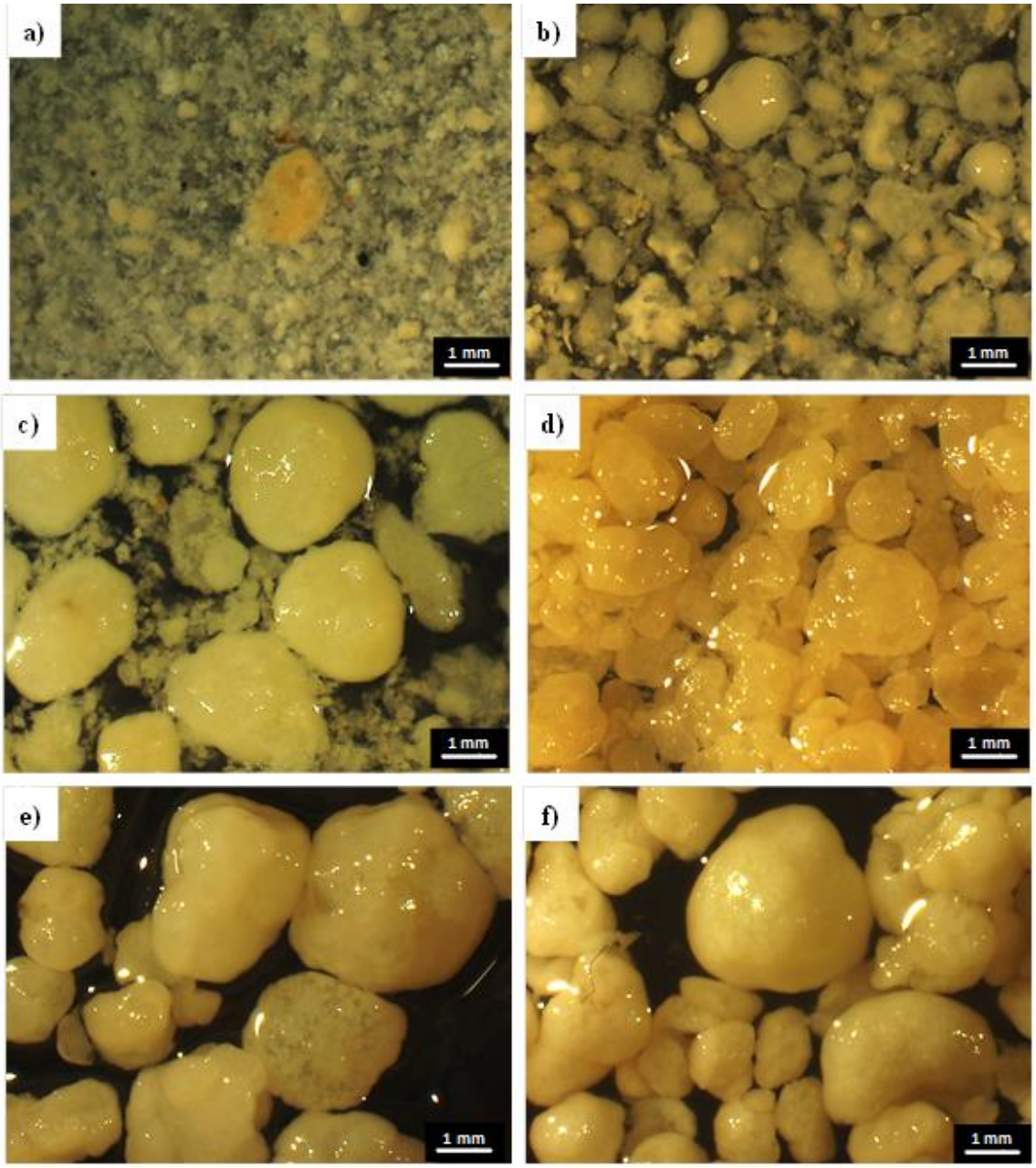

Figure 1: Aerobic granules observed at the stereomicroscope (7X magnification): (a) Phase 0 – day 14, (b) Phase I – day 38, (c) Phase II – day 73, (d) Phase III – day 102, (e) Phase IV – day 131, (f) Phase V – day 157.

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 $g \cdot L^{-1}$ (value of the inoculum)

to 0.85 $g \cdot L^{-1}$. Then, the concentration of biomass in the new operating conditions increased up to 1.3 – 1.4 $g \cdot L^{-1}$. 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 $g \cdot L^{-1}$ and a subsequent washout of TSS_{out} in the effluent with a concentration of 0.33 $g \cdot L^{-1}$.

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 $g \cdot L^{-1}$.

In the same phase, the TSS_{out} in the effluent was almost constant next to 0.15 $g \cdot L^{-1}$ 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 $g \cdot L^{-1}$ 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 $g \cdot L^{-1}$ to 5.4 $g \cdot L^{-1}$ was observed. During this phase, the TSS_{out} concentration in the effluent gradually increased up to 0.54 $g \cdot L^{-1}$ 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.

The settling properties of the aerobic granules were evaluated through the SVI after 5 and 30 minutes. The trends of both the SVI_5 and the SVI_{30} during the five experimental periods are shown in **Figure 2c**. As reported by many authors [31,32], the complete granulation can be identified when the ratio between the SVI_5 and the SVI_{30} is close to one. This condition was observed starting from the day 35 in Phase I, when both the SVI_5 and the SVI_{30} values were close to 55 $mL \cdot g^{-1}$. In Phase II, the SVI_5 and the SVI_{30} values increased to 90 $mL \cdot g^{-1}$ suggesting a slight worsening of the settleability. From the Phase III until the end of the experiment, a gradual decrease of the SVI_5 and the SVI_{30} down to 20 $mL \cdot g^{-1}$ was observed, confirming the achievement of stable and mature aerobic granules.

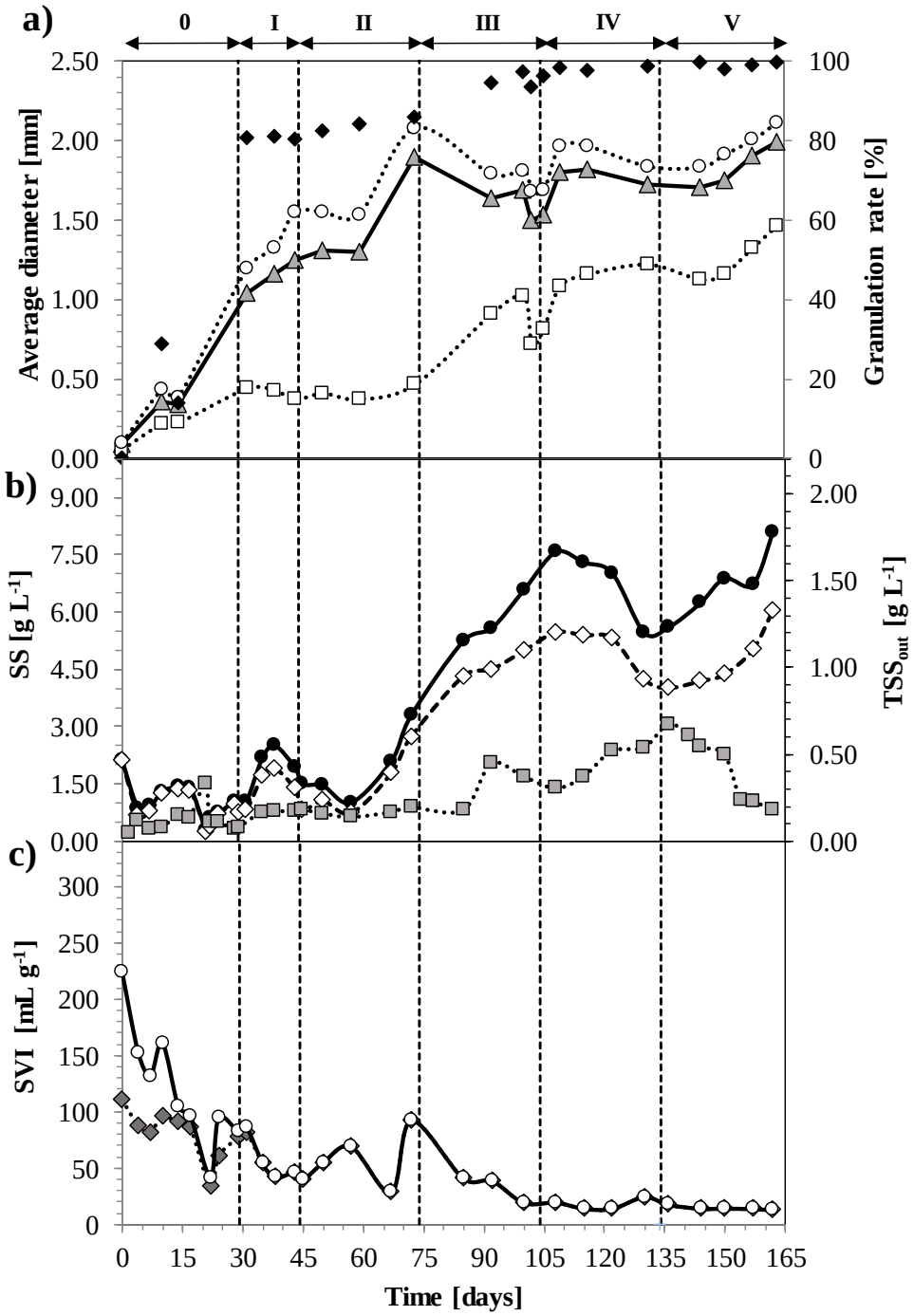

Figure 2: (a) Mean diameter of granules (D50 —△—), particle diameter corresponding to 10% (D10 ···□··) and to 60% (D60 ···○··) cumulative undersize particle size distribution, granulation rate (◆); (b) total suspended solids (—●—) and volatile suspended solids (--◇--) in the reactor, total suspended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

3.2 Extracellular polymeric substances analysis

During the whole experimental period, the EPSs in forms of proteins and polysaccharides were analyzed (**Figure 3**). EPS are metabolic products accumulating at 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 constituted by proteins, polysaccharides, humic acids, and lipids secreted by microorganisms. Proteins are considered to be the main polymeric substances responsible to maintain the granules structure. Furthermore, proteins are mainly present in the inner layer, while polysaccharides concentrate on the outer surface of aerobic granules [34]. **Figure 3a** shows the trend of the proteins content of the granules referred to the unit of MLVSS during the experimentation. In Phase 0, without salt addition, it was registered an increase of protein respect to the inoculum value, because the biomass taken from the conventional activated sludge plant underwent a biological stress due to the new operating conditions (batch feeding, shear forces, hydraulic selection pressure, etc.). Then, when the biomass was acclimating to the new conditions and pseudo 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 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) fulfilling the function of structure of aerobic granules. Subsequently, the following decrease of proteins values confirmed the adaptation of granules to new operational 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 ÷ 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.

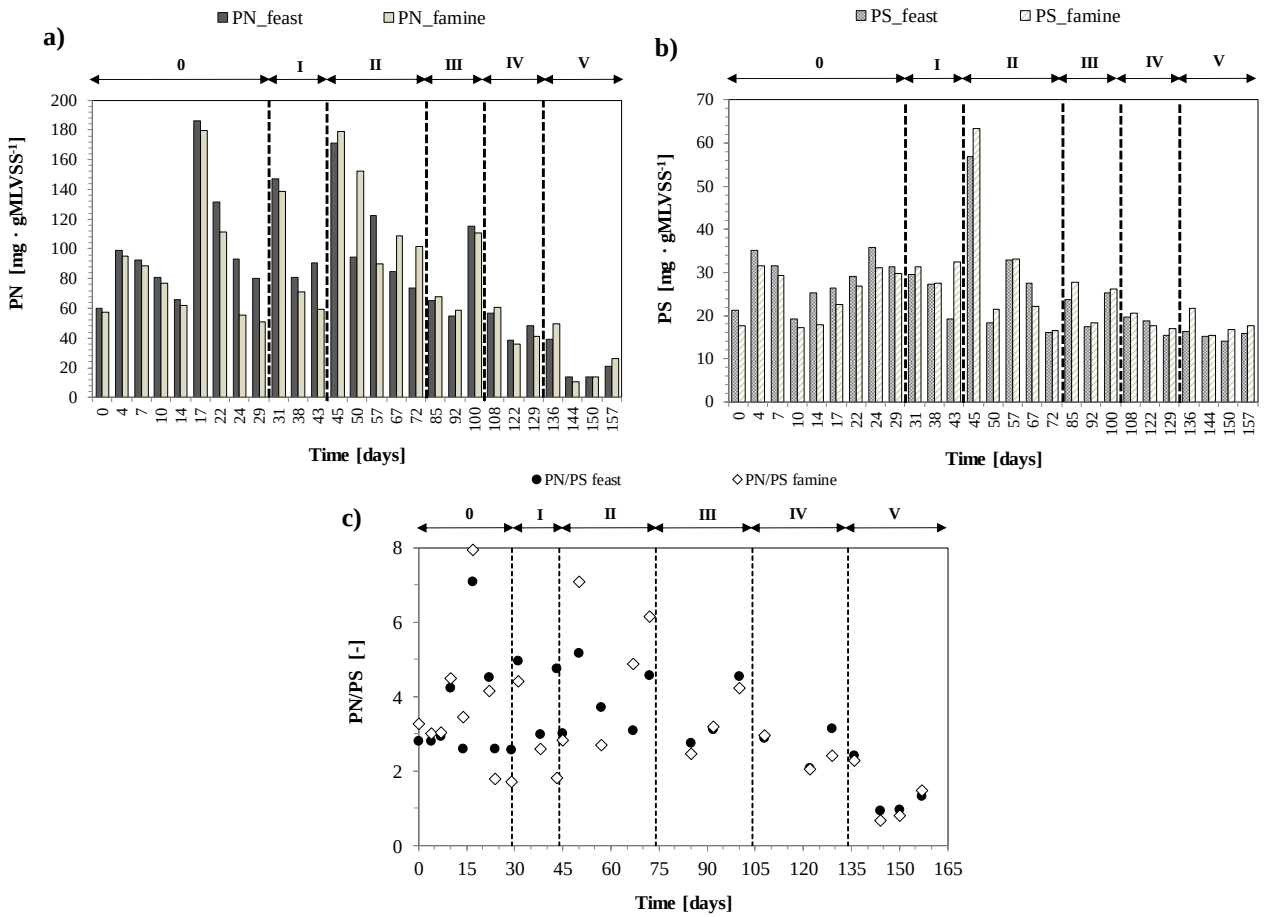

Figure 3: (a) EPS proteins content; (b) EPS polysaccharides content; (c) PN/PS ratio, in feast and famine conditions.

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.

During the Phase 0 with no salt addition, the polysaccharides in feast phase were always 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 polysaccharides) when the substrate was present (feast phase) which was used for growth of microorganisms when the external substrate was depleted (famine phase). However, also in this case, during the following experimental phases, the 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 contrast with what observed by the same authors in a previous study [17], where the biomass was previously adapted to salinity. It should be stressed that, in the present study, the salinity constituted an important environmental variable which affected all the biological and physical processes involved in the granulation phenomenon. At this purpose, a fundamental aspect which cannot be ignored is that the microbial community was probaby changed to adapt to the high salinity environment, as founded by Wan et al. (2014) [35]. So it is possible that halotolerant microorganisms were biologically selected, as also found by [18], ensuring aerobic granules stability under high salinity environment. Moreover, these new microorganisms possessed different metabolic

kinetics, which were not analyzed in this study, compared to common bacterial strains of soft wastewater. As observed by Taheri et al., (2012) [28], the half-saturation 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 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 causes K_S to become higher. Therefore, since in this work the exogenous COD was maintained almost constant for each Phase, it could be assumed that, when the salinity 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 famine phase. This could explain the unusual trend of EPS observed during the feast and famine phases in presence of salinity.

Finally, the evolution of the EPS composition in terms of proteins/polysaccharides ratio (PN/PS) was studied. The PN/PS ratio is considered an important parameter to study the granulation, since it expresses the combined effect of proteins and polysaccharides on 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 polysaccharides vary in a similar way both in feast and in famine conditions. Moreover, the time course of the PN/PS ratio was decreasing and it resulted very similar to that of the proteins, since proteins were the major component of the EPS.

3.3 Hydrophobicity

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

increase of the hydrophobicity [39–41]. The increase of surface hydrophobicity would promote cell-to-cell interaction and further serves as inducing cell force for aggregating and promoting biogranulation [39]. However, high salt concentrations of wastewater directly affects sludge hydrophobicity [29], because Na^+ ions produce a change in the cell surface properties. In particular, the presence of salt involves the replacement of divalent Ca^{2+} ions with monovalent Na^+ ions of the EPS, resulting in a minor reduction of surface electronegativity and therefore in a lower hydrophobicity [4]. **Figure 4a** shows the trend of the granules hydrophobicity during the whole experimental study. In 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 the surface negative charge of bacteria cells and increasing the hydrophobicity. This reduced the zeta potential and the electrostatic repulsions, favoring bridging and microbial aggregation.

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

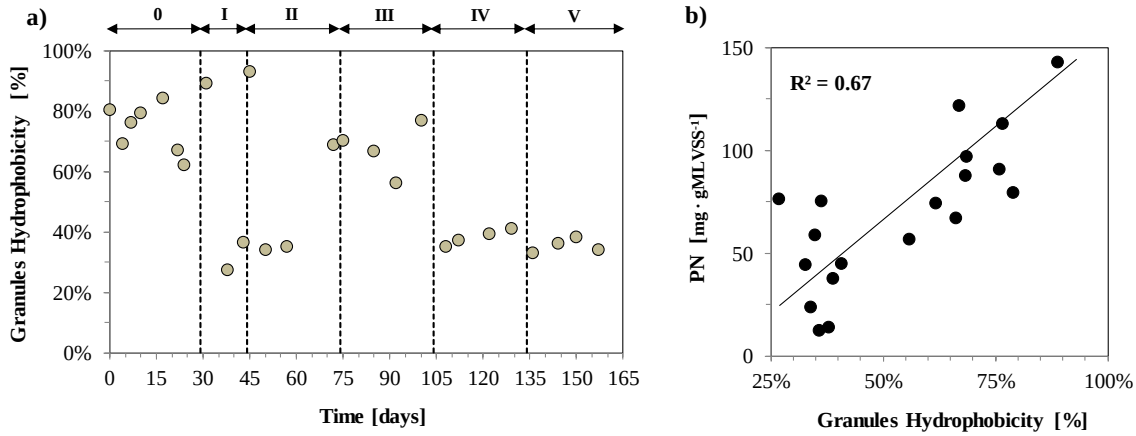

Figure 4: (a) Granules hydrophobicity; (b) correlation between granules hydrophobicity and protein (PN) content. All the represented values are averages between feast phase and famine phase.

3.4 Bench scale plant performances

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

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.

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 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 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 efficiencies of COD and TOC in Phase 0 and in Phase I, resulted quite similar. Subsequently, when the effluent COD grew from Phase II till the end of the experimentation, the effluent TOC always remained quite low, denoting that the 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. Also Wang et al. (2017) [12] observed a similar trend of the effluent COD when the 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 trends of COD and TOC allowed a more precise analysis of organic matter removal of saline wastewater.

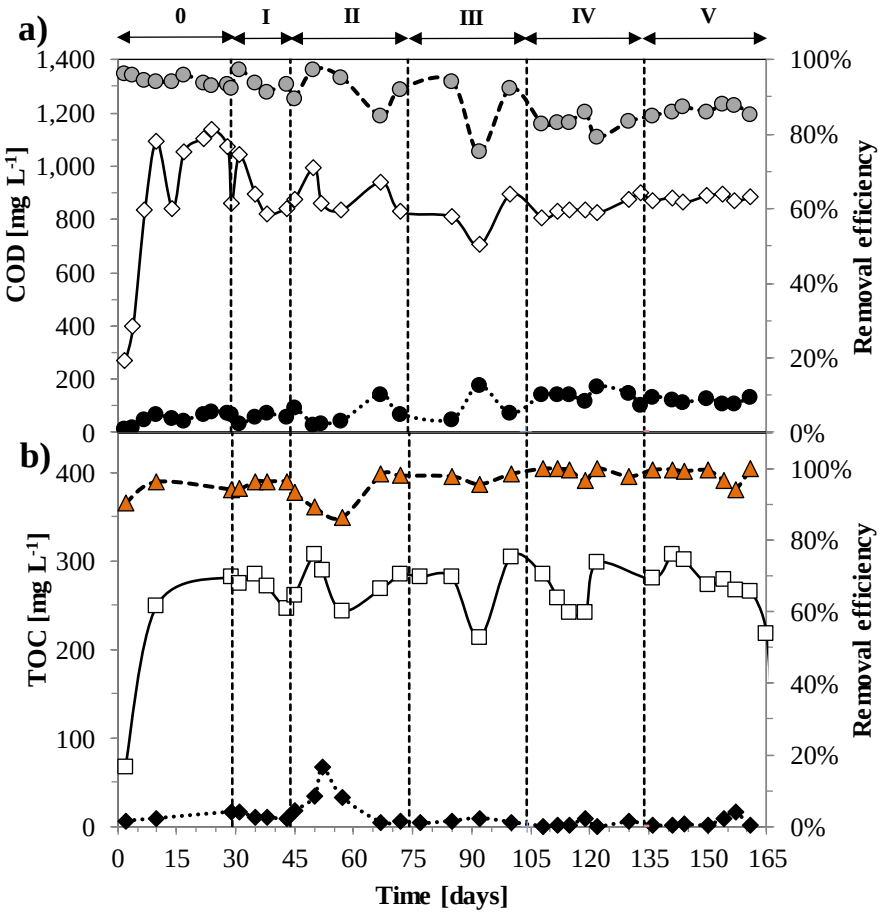

Figure 5: (a) COD in the influent (—◇—), COD in the effluent (···●··), COD removal efficiency (- -○- -); (b) TOC in the influent (—□—), TOC in the effluent (···◆··), TOC removal efficiency (- -▲- -).

3.4.2 Nitrogen and Phosphorous removal

Figure 6a shows how the gradual increase of salinity until Phase III (11.56 ± 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 ± 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

minimum of 5%. During this phase the autotrophic biomass underwent a strong salinity inhibition. Then, in the Phase IV and V the nitrification activity reprised from 5% up to 25%, highlighting an adaptation of nitrifying microorganisms to the high salinity environment, and the nitrogen removal increased from 15% to 29%. Observing the denitrification efficiency, since it is a relative value strictly depending on the nitrification efficiency, it was relatively high between 80-90% from Phase 0 (no salt addition) to Phase II (4.87 ± 0.89 $gNaCl \cdot L^{-1}$), when salt concentration was not too high. 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 denitrification activity was observed (up to 71% in Phase V) that, together with the gradual increase of the nitrification, contributed to increase the total nitrogen removal. This suggested that, not only nitrifying but also denitrifying microorganisms were adapted to the saline environment.

Although in Phase 0 the washout of biomass occurred in the earliest days of the operation, both the absence of salinity and the inoculum seed sludge enriched in nitrifying microorganisms were responsible for the highest nitrification efficiency (**Figure 6b**). Moreover, granules with smaller sizes have a higher specific surface area and thereby a higher specific oxidation capacity, as also observed by Pronk et al., (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 $mg \cdot L^{-1}$, was observed. This was mainly due to the severe washout of the slow growing 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 and the reactor enriched in fast settling biomass, the nitrification efficiency rose to 40%

on day 29. At the beginning of Phase I, on day 31, the nitrification efficiency suddenly collapsed, probably due to an initial inhibitory effect of salinity on the autotrophic bacteria. Subsequently, a gradual reprise of nitrification up to 40% denoted the acclimation of the autotrophic biomass to 1.80 ± 0.74 $gNaCl^{-}\cdot L^{-1}$. In Phase II, a progressive increase of ammonium in the effluent was noted, likely due to the gradual inhibition of the nitrification process when autotrophic microorganisms were exposed to about 4.87 ± 0.69 $gNaCl^{-}\cdot L^{-1}$. In Phase III (11.56 ± 0.31 $gNaCl^{-}\cdot L^{-1}$) it was observed a strong inhibition of nitrifying biomass and the ammonium was mainly removed for assimilation and growth of heterothrophic microorganisms. Based on these observations and on the changes of granules morphology and structure discussed previously, it could 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 (24.31 ± 2.74 $gNaCl^{-}\cdot L^{-1}$) a decrease of ammonium in the effluent was noted and, consequently, the nitrification efficiency increased up to 33%, probably due to the growth and specialization of nitrifying bacteria. Moreover, it was observed a contextual 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 effects of salinity on the bacteria dwelling in the inner layers, providing optimal environmental conditions for the maintenance of the biological processes.

Finally in Phase V (37.79 ± 1.21 $gNaCl^{-}\cdot L^{-1}$), 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.

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 nitrification denitrification (SND) [43] occurred. In the first days of operation, two peaks of nitrates and nitrites concentration of 10 and 5 $mg \cdot L^{-1}$ were observed, respectively. This was mainly due to the small dimensions of granules at that time, that implied a small anoxic layer and a poor denitrification efficiency of about 60%, accordingly. Then, due to the increase of the aerobic granules size and the development 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 concentration produced an initial decrease of the denitrification efficiency, due to a partial inhibition of the denitrifiers microorganisms. After that, the denitrification 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 concentration in the effluent next to 1 $mg \cdot L^{-1}$ was observed. In Phase III, a gradual accumulation of nitrites in the effluent up to 5 $mg \cdot L^{-1}$ was observed, and the 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 this phase. As discussed previously, during Phase III the average size of the granules was reduced and the anoxic layer become thinner, so limiting the denitrification process. Moreover, the nitrite accumulation suggested the inhibition of the autotrophic nitrite oxidizing bacteria (NOB), according to other authors [4,11,15] who noted that NOB are 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 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 effluent rose to 9.6 $mg \cdot L^{-1}$ due to the salt increase (37.79 ± 1.21 $gNaCl^{-} \cdot L^{-1}$) which may 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 $mg \cdot L^{-1}$ 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 acclimation of NOB to the saline environment. Bearing in mind the results above, the high robustness of granular sludge led to a decreased sensitivity of aggregated microorganisms forming granules towards the impact of stress factors such as salinity.

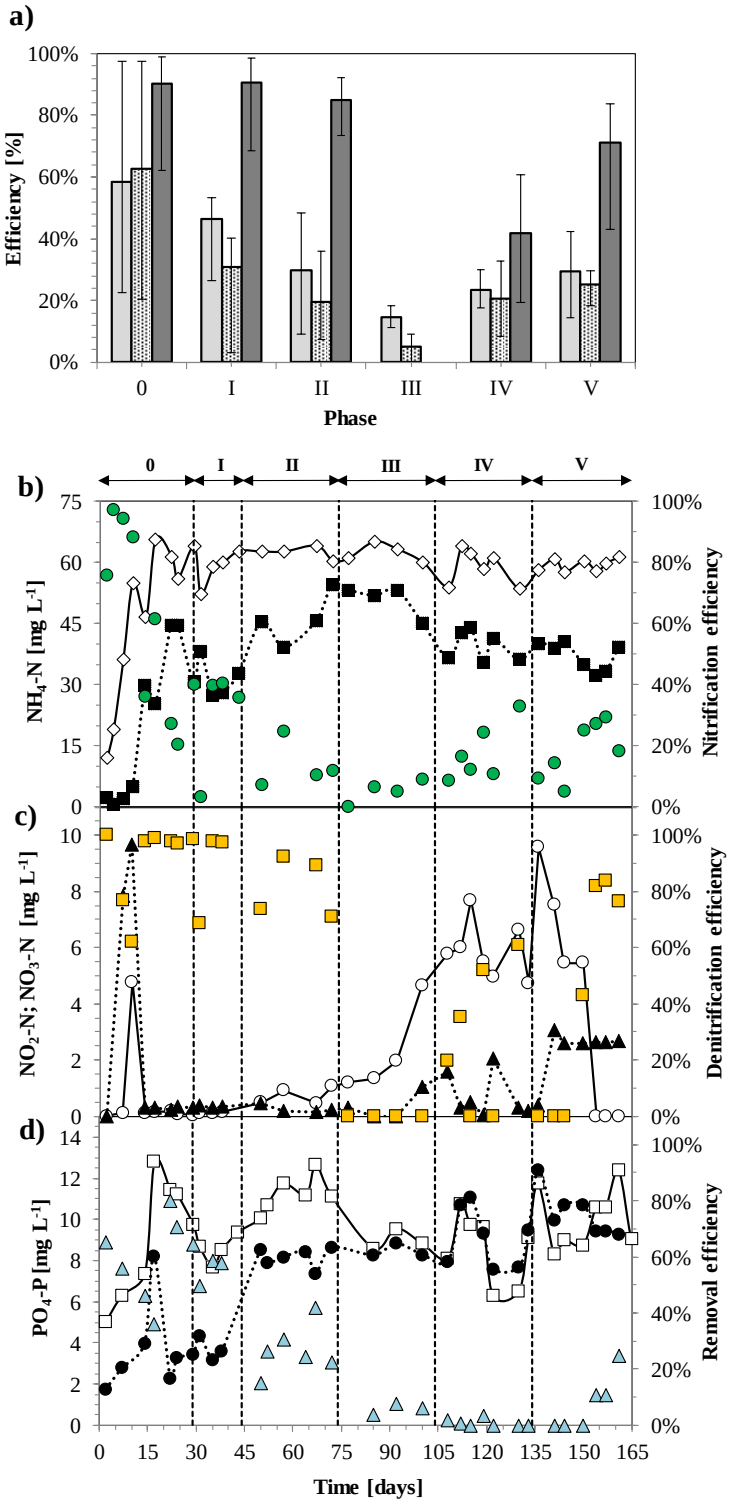

Figure 6. (a) Nitrogen average removal efficiency (□), nitrification average efficiency (▣), denitrification average efficiency (■); (b) NH_4-N in the influent (—◇—), NH_4-N in the effluent (···■··); nitrification efficiency (●); (c) NO_2-N in the effluent (—○—), NO_3-N in the effluent (···▲··), denitrification efficiency (■); (d) PO_4-P in the influent (—□—), PO_4-P in the effluent (···●··), phosphorous removal efficiency (▲).

Finally, also the potential simultaneous phosphorous removal was analized (**Figure 6d**). More specifically, from the beginning of the experimentation until the day 17 (Phase 0), a gradual decrease of the phosphorous removal efficiency was observed. Particularly, it 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 removed for growth and assimilation of microorganisms. Subsequently, the granules formation resulted in an improvement of the phosphorous removal to 70%, because the higher average diameter of granules may have promoted the development of an anaerobic core where phosporus accumulating organisms (PAOs) may have proliferated. In Phase I (1.80 ± 0.74 $gNaCl^{-}\cdot L^{-1}$), the phosphorous removal efficiency slightly decreased to 60%. In Phase II, an increase of phosphorous in the effluent was 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 negatively affect the phosphorous uptake activity of PAOs under both anoxic and aerobic conditions. In this work, the obtained results confirmed that the effect of salt was detrimental to NOBs, which was reflected in the accumulation of nitrites, as discussed previously. In turn, phosphates uptake dramatically reduced when nitrites concentration was above 1 $mg\cdot L^{-1}$, as observed in Phase III (5%), in Phase IV (almost 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 experimentation. These observations indicated a probable development of PAOs microorganisms in the inner layers of granules. The gradual deterioration of phosphorous removal efficiency was likely caused by a reversible inhibition of PAOs, 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 main granules features throughout the whole experimental period.

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

		Phases and time span					
Parameter	**Units**	**Phase 0** **0-29** **(29 days)**	**Phase I** **30-44** **(15 days)**	**Phase II** **45-74** **(30 days)**	**Phase III** **75-104** **(30 days)**	**Phase IV** **105-134** **(30 days)**	**Phase V** **135-164** **(30 days)**
MLTSS	$g \cdot L^{-1}$	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	$g \cdot L^{-1}$	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}	$g \cdot L^{-1}$	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
SVI_5	$mL \cdot g^{-1}$	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	$gNaCl \cdot L^{-1}$	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
ηTOC	%	93 ± 4	95 ± 1	93 ± 5	97 ± 1	99 ± 1	98 ± 2
ηN	%	58 ± 27	46 ± 13	30 ± 14	15 ± 3	23 ± 5	29 ± 11
ηP	%	59 ± 16	58 ± 6	27 ± 9	6 ± 2	1 ± 1	18 ± 7

4. CONCLUSIONS

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

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

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