Comparison between kinetics of autochthonous marine bacteria in activated sludge and granular sludge systems at different salinity and SRTs

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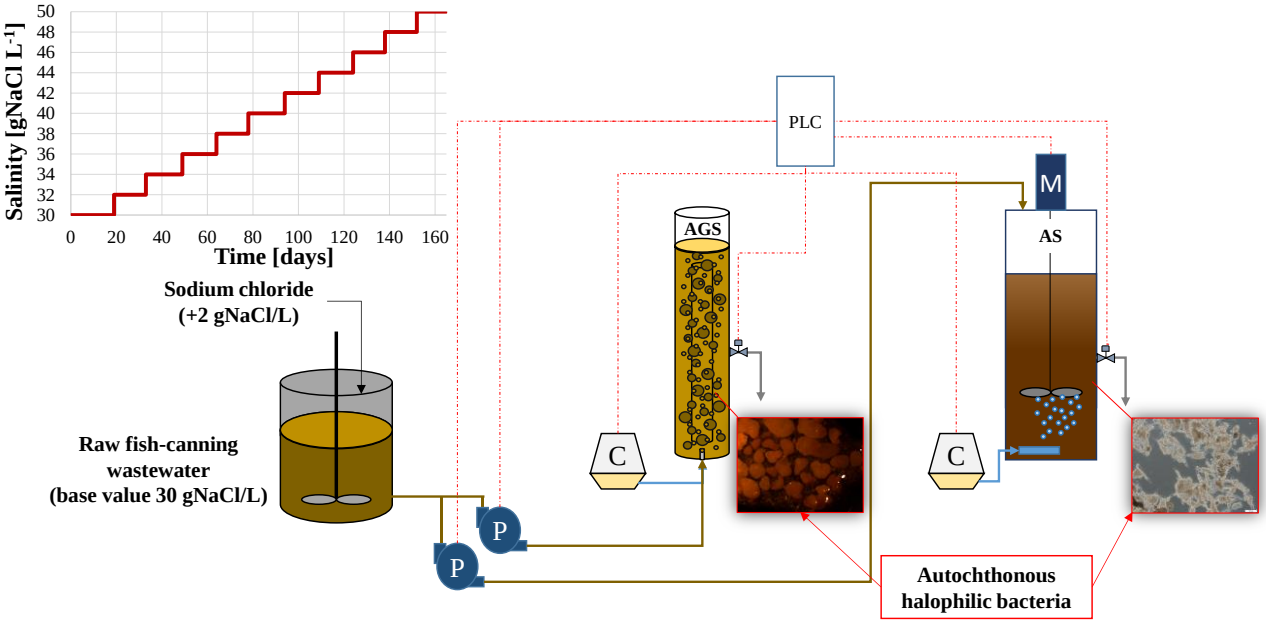
Abstract

Biological nutrient removal performances and kinetics of autochthonous marine biomass in forms of activated sludge and aerobic granular sludge were investigated under different salinity and sludge retention time (SRT). Both the biomasses, cultivated from a fish-canning wastewater, were subjected to stepwise increases in salinity (+2 gNaCl L^{-1}), from 30 gNaCl L^{-1} up to 50 gNaCl L^{-1} with the aim to evaluate the maximum potential in withstanding salinity by the autochthonous marine biomass. Microbial marine species belonging to the genus of Cryomorphaceae and of Rhodobacteraceae were found dominant in both the systems at the maximum salinity tested (50 gNaCl L^{-1}). The organic carbon was removed with a yield of approximately 98%, irrespective of the salinity. Similarly, nitrogen removal occurred via nitritation-denitritation and was not affected by salinity. The ammonium utilization rate and the nitrite utilization rate were approximately of 3.60 $mgNH_4$-N $gVSS^{-1}h^{-1}$ and 10.0 $mgNO_2$-N $gVSS^{-1}h^{-1}$, respectively, indicating a high activity of nitrifying and denitrifying bacteria. The granulation process did not provide significant improvements in the nutrients removal process likely due to the stepwise salinity increase strategy. Biomass activity and performances resulted affected by long SRT (27 days) due to salt accumulation within the activated sludge flocs and granules. In contrast, a lower SRT (14 days) favoured the discharge of the granules and flocs with higher inert content, thereby enhancing the biomass renewing.

The obtained results demonstrated that the use of autochthonous-halophilic bacteria represents a valuable solution for the treatment of high-strength carbon and nitrogen saline wastewater in a wide range of salinity. Besides, the stepwise increase in salinity and the operation at low SRT enabled high metabolic activity and to avoid excessive accumulation of salt within the biomass aggregates, limiting their physical destructuration due to the increase in loosely-bound exopolymers.

Keywords: Activated sludge; aerobic granular sludge; autochthonous-halophilic bacteria; shortcut nitrification; saline wastewater**.**

Graphical abstract



Research Highlights

- Autochthonous marine bacteria were cultivated as granular and activated sludge
- Biomass metabolic kinetics were evaluated at different salinity and SRT
- Salinity did not affect the biomass kinetics within the range of 30-50 gNaCl L^{-1}
- Biomass activity and performances decreased for both system because of a long SRT
- Destructuration of the microbial bioaggragates occurred due to salt accumulation

1. Introduction

Nowadays, several activities, including petroleum, chemical and fish canning, produce large amount of wastewaters featured by high salt (chloride) concentration. In many cases, these wastewaters contain significant concentrations of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS) and total nitrogen (TN) (Chowdhury et al., 2010; Corsino et al., 2016).

Chemical and physical treatments, like membrane separation, ion exchange or electrodialysis have been widely investigated in the past (Fan et al., 2011; Muthukumaran and Baskaran, 2013). Although these methods are highly performing, they lead to secondary pollution (chemicals disposal) and are less cost-effective than biological treatments (energy and chemicals consumption); therefore they are currently limited only to specific applications (He et al., 2017).

Whereas biological treatments are widely recognized as cost-effective, their application in the field of industrial saline wastewater still represent a challenge. Indeed, one of the main drawbacks when treating saline wastewater is related to plasmolysis that involves the breaking of the cellular membrane, leading to the cell death (Lefebvre and Moletta, 2006). This phenomenon occurs when the solute concentration in the cytoplasm is higher than that in the surrounding environment. Similarly, plasmolysis could occur when the aqueous environment is hypertonic compared to the cytoplasm.

Although several studies demonstrated that the acclimation of activated sludge (AS) to salinity can be successfully achieved, the main bottleneck consists in the removal efficiencies of such salt-adapted systems (Campo et al., 2018; Lefebvre and Moletta, 2006; Zhang et al., 2017). To overcome this drawback, many authors suggested the use of aerobic granular sludge (AGS), since bacteria bio-aggregation could help to operate at higher salinity and faster kinetics as well (Wang et al., 2017; Wang et al., 2015). Nevertheless, the research findings suggested that halo-tolerant biomass, even in the form of granular sludge, totally lose its metabolic functionalities over a certain salinity level (> 20 g NaCl L^{-1}) (Jemli et al., 2015), thereby resulting in loss of removal efficiencies.

Recently, Chen et al. (Chen et al., 2018) demonstrated that microbial community diversity and richness, as well as removal performance for nitrogen and organic carbon deteriorated with increasing in salinity from 0 gNaCl L^{-1} to 20 gNaCl L^{-1}. The authors observed that salinity inhibited the dehydrogenase activity of activated sludge, thus decreasing bacterial metabolic activity. For these reasons, many researchers encouraged the use of halophilic bacteria for the biological treatment of high-strength saline wastewater (Gomes et al., 2018; Guo et al., 2016). Recently, several studies on the treatment of saline and hypersaline wastewater by means of halophilic biomass were carried out (Cui et al., 2016; Oren, 2010; Zhuang et al., 2010). Among these, Capodici et al. (2018) successfully cultivated autochthonous activated sludge from a real fish-canning wastewater. The authors assumed the presence of autochthonous active biomass in their study and speculated that it may be belonged to halophilic strains because of their ability to survive in hypersaline saline environment (>30 gNaCl L^{-1}). The removal efficiencies BOD and TSS were higher than 90%. Similarly, more than 95% of the nitrogen was removed via shortcut nitrification-denitrification. Partial nitrification, or nitritation, naturally occurs treating saline wastewater because nitrite oxidizing bacteria (NOB) are more sensitive to the salt concentration than the ammonium oxidizing bacteria (AOB). However Capodici and coauthors tested the effectiveness of the autochthonous biomass only at 30 gNaCl L^{-1} of salinity. Therefore, the ability of these bacteria to survive at higher salinity and its performances were not tested so far. Furthermore, it is possible to speculate that bio-aggregation of these bacteria in aerobic granular sludge (AGS), could represent a further advantage, thereby enhancing the biological performances or enabling the possibility to operate at higher salinity.

Another issue concerning the treatment of saline wastewater is related to the choice of a proper sludge retention time (SRT). Indeed, although at high salinity the biomass yield decreased, thereby limiting the sludge wasting (Ching and Redzwan, 2017), longer SRT could result in excessive "ageing" of the sludge because of accumulation of inert material within the flocs or granules. This in turn affects the biological performances because of the decrease in the biomass active fraction in

the system. Therefore, the choice of a proper SRT allow to avoid the decay of biological performances in the long term.

To the authors' knowledge, no studies reporting about the performances and the kinetics of autochthonous marine biomass at salinity higher than 30 gNaCl L^{-1} nor the effects of SRT exist. Additionally, specific molecular analysis aimed at identifying these bacteria were not performed up to now. In this respect, identification of bacterial communities is indispensable for a better understanding of the biological processes that enable the nutrient removal, especially for the treatment of not conventional wastewater.

The purpose of this study was to validate the approach of cultivating autochthonous marine biomass for the treatment of saline wastewater and to test its maximum potentiality in terms of salinity withstanding. With this aim, this study investigated the biological nutrient removal efficiencies and the metabolic kinetics of autochthonous marine biomass derived from fish-canning wastewater, in forms of activated sludge and granular sludge, in a range of salinity between 30 gNaCl L^{-1} and 50 gNaCl L^{-1} at different SRTs.

2. Materials and Method

2.1 Reactors set-up

Two sequencing batch reactors (SBR), one with aerobic granular sludge (AGS-SBR) and the other with flocculent activated sludge (AS-SBR) were operated. As reported in previous studies (Capodici et al., 2018; Corsino et al., 2018), because the biomass retention capacity of the AGS reactor was double than the AS, with the aim to compare the performances of those systems operating with the same biomass amount, the volume of the AS-SBR was chosen as the double of the AGS-SBR. More precisely, the AGS-SBR was a column-type (100 cm height) with a working volume of 4 L (internal diameter of 8.6 cm) and was characterized by an internal riser 50 cm high with an internal diameter of 5.4 cm. The AS-SBR had an operating volume of 8 L. Both the SBRs were equipped with a feeding pump and a solenoid valve for the effluent discharge placed at the

mid-point of the reactor yielding a volumetric exchange ratio (VER) equal to 50%. The volume of the raw wastewater treated per day was equal to 4 L and 8 L for the AGS-SBR and for the AS-SBR respectively. Therefore, the hydraulic retention time (HRT) was equal to 24 hours in both the reactors.

The AGS-SBR was operated on a 12 h cycle divided into 60 minutes of not-aerated influent upflow feeding, 10 h and 50 min of aeration (650 min), 5 min of settling and 5 min of effluent discharge. Air was introduced via a fine bubble aerator at the base of the reactor at a flow rate of 3 L min^{-1} so that the superficial air velocity was approximately of 2.4 cm sec^{-1}. The AS-SBR cycle included 60 minutes of influent feeding (mixed and not-aerated), 9 h and 30 min (570 min) of aeration, 60 of anoxic mixing followed by 5 minutes of aeration to favour nitrogen stripping, 20 minutes of settling and 5 minutes of effluent discharge. The length of aerated/not-aerated period was set in order to maximize nitrogen removal efficiency. A stirrer device provided the mixing during the non-aerated period and a fine bubble diffuser provided the air supply. A Programmable Logic Controller (PLC) automatically handled the SBRs cycling operations.

The dissolved oxygen (DO) concentration within the bulk was maintained close to the saturation value according to the temperature and salinity.

2.2 Experimental set-up

The AGS-SBR and the AS-SBR were monitored for 165 days. Both the reactors were seeded with autochthonous activated sludge derived from a fish-canning wastewater. For the cultivation of this sludge, a parent reactor working as a conventional SBR was started-up without an activated sludge inoculum. More precisely, this reactor was filled with raw fish-canning wastewater and was operated with a complete sludge retention strategy until the autochthonous activated sludge developed. For further details on the cultivation of the autochthonous biomass, the reader is referred to the literature (Capodici et al., 2018).

The fish-canning wastewater was collected from a local industry that produces canned anchovies (Palermo, Italy). The raw fish-canning wastewater was collected from the canning section of the industry, where the wastewater had a salt concentration of approximately 150 g NaCl L^{-1}. Then, the raw fish-canning wastewater was diluted with tap water 1:5 (v/v) to obtain a salt concentration approximately equal to that of the wastewater at the outlet of the industry (30 g NaCl L^{-1}). After dilution, the COD and the BOD_5 concentration resulted on average in approximately 800 mg L^{-1} respectively, whereas the TN and the NH_4-N were on average equal to 150 mg L^{-1} and 115 mg L^{-1}, respectively (Table 1).

The AGS-SBR and the AS-SBR were seeded with the autochthonous activated sludge derived from the parent SBR reactor with a TSS concentration of approximately 3 gTSS L^{-1}. Subsequently, both the reactors were operated for approximately 3 months until steady conditions were achieved. The salinity was kept constant to 30 gNaCl L^{-1} until full granulation and stable performance (data not discussed) were achieved in the AGS-SBR and AS-SBR systems. At the end of the start-up phase (beginning of this study) the TSS concentration was of approximately 6.3 gTSS L^{-1} and 13.1 gTSS L^{-1} in the AS and AGS reactor, respectively.

Tab. 1: Operating conditions for AGS-SBR and AS-SBR during the experimental phases.

	Phases										
	1	2	3	4	5	6	7	8	9	10	11
Duration [days]	15	15	15	15	15	15	15	15	15	15	15
Salinity [gNaCl L^{-1}]	30	32	34	36	38	40	42	44	46	48	50
COD [mg L^{-1}]	2035 *(±206)*	1989 *(±131)*	2164 *(±147)*	1921 *(±151)*	1857 *(±97)*	2104 *(±86)*	2373 *(±106)*	2098 *(±141)*	2238 *(±153)*	2127 *(±134)*	2094 *(±191)*
BOD_5 [mg L^{-1}]	865 *(±136)*	862 *(±97)*	843 *(±62)*	807 *(±31)*	727 *(±44)*	793 *(±101)*	760 *(±59)*	988 *(±37)*	897 *(±53)*	911 *(±61)*	906 *(±74)*
TN [mg L^{-1}]	147 *(±13)*	138 *(±17)*	145 *(±9)*	140 *(±11)*	134 *(±14)*	136 *(±12)*	136 *(±8)*	175 *(±11)*	173 *(±8)*	186 *(±7)*	147 *(±13)*
SRT [days]	27						14				

When steady state conditions were reached in both the SBRs, salt concentration was gradually increased. In details, although steady state condition is conventionally referred to the SRT, in this study it was assumed that steady state condition were reached when a steady nutrients removal efficiency occurred (fluctuations smaller than 3%). Nevertheless, a minimum duration of 15 days

for each phase was imposed. The experiment was divided into eleven phases, during which salinity was stepwise increased by 2 gNaCl L^{-1} each, by adding a known amount of sodium chloride to the diluted wastewater. In this way, the BOD_5 and total nitrogen concentration in the feed were kept constant during the entire experiment. Salinity was increased up to 50 gNaCl L^{-1} and it was not further increased because of issues related to the oxygen transfer and scaling as a consequence of the high salt concentration.

The SRT was not imposed until Phase 6, corresponding to 40 gNaCl L^{-1} (Table 1) when the sludge wastage flux was set in order to maintain a TSS concentration of approximately 12.0 gTSS L^{-1} and 6.0 gTSS L^{-1} in the AGS-SBR and AS-SBR, respectively. Thus, the SRT was approximately of 27 days in both the SBRs. However, due to the accumulation of inert material within the aerobic granules in the AGS-SBR and to the ageing of the activated sludge in the AS-SBR, since Phase 7 a regular sludge withdrawal, corresponding to a SRT close to 14 days, was performed. More precisely, a selective wastage strategy was performed in the AGS-SBR, consisting in discharging the granules from the bottom of the reactor after the settling phase. In this way, the heaviest granules, likely those with the highest inert matter content, were selectively wasted. In the AS-SBR the sludge was wasted from the liquid bulk during the aeration phase.

In Table 1.1 the main operating conditions and wastewater characteristics are summarized.

2.3 Analytical methods

The chemical-physical parameters (COD, BOD_5, NH_4-N, NO_2-N) were analyzed according to standard methods (APHA, 2005). The TSS and volatile suspended solid (VSS) concentrations were troublesome due to the salt adsorption within the activated sludge flocs. To address this problem, a modified method was used (Capodici et al., 2018). The total nitrogen concentration was determined in Total Nitrogen Measuring Unit TNM-1 (Shimadzu, Japan).

EPS extraction was performed according to the heating method (Le-Clech et al., 2006). For each EPS fraction, the carbohydrates (PS) and the proteins (PN) were determined in accordance with the

phenol–sulfuric acid method (DuBois et al., 1956) and with the Folin method (Lowry et al., 1951), respectively.

The observed yield coefficient (Y_{obs}) was calculated through mass balances between sludge withdrawn and sludge production, dividing by the cumulated BOD_5 removed, according to Eq. 1:

$$Y_{\text{obs}} = \frac{[(X_2 - X_1)V + X_e Q + X_s Q_s]}{(BOD_{\text{in}} - BOD_{\text{out}})Q} \qquad [gVSS\ gBOD^{-1}] \qquad (Eq.1)$$

where X_2 and X_1 are the biomass concentrations (g VSS L^{-1}) at day (n) and (n-1), V is the working volume of the reactor, Q is the influent flow, X_s and X_e are the concentrations of the waste biomass and the effluent (g VSS L^{-1}), Q_s is the volume of waste sludge on a daily base, BOD_{in} and BOD_{out} are the influent and effluent BOD_5 concentration (g L^{-1}), respectively.

The size of the activated sludge flocs and granules was examined by means of a high-speed image analyses sensor (Sympatec Qicpic) that allowed the evaluation of sludge particle size distribution (PSD). The settling properties of the AGS and the AS were evaluated by means of the sludge volume index (SVI). The SVI was calculated as the ratio between the volume occupied by the sludge after a static settling phase and the TSS concentration of the sample. Because of the different nature of the AGS and AS, to compare the achieved results with previous respective studies, the SVI was calculated in a different way. More precisely, for the AGS the SVI was calculated based on the volume of settled sludge after 5 minutes (SVI_5), whereas for the AS the SVI was calculated by considering the volume of settled sludge after 30 minutes (SVI_{30}) (Giesen et al., 2013). The SVI_{30} was calculated also for the AGS in order to evaluate the SVI_5/SVI_{30} ratio.

The dissolved oxygen (DO) concentration in the mixed liquor was measured continuously by means of a WTW IQ Sensor Net System 2020 XT equipped with on line sensor probes. The electrical conductivity (EC) was measured instead of a direct assessment of salinity, because it was more reliable. Therefore, salinity was calculated based on a correlation curve with the EC.

One-way analysis of variance (one-way ANOVA) was used to assess the relationship between salinity changes and biomass kinetics (error level equal to 0.05).

2.3.1 Metabolic kinetic assessment

To evaluate the impact of the salinity increase on nitrogen removal kinetics, ammonium utilization rate (AUR) and nitrite utilization rate (NUR) tests were performed in each experimental phase. Kinetic tests were performed only when steady conditions were reached. These tests were performed in a 1.5 L batch reactor (3 gTSS L^{-1}) at controlled temperature (20°C). Specifically, based on the TSS concentration, a known amount of mixed liquor sample was withdrawn from the respective parent reactors and put in the batch reactor. Ammonium chloride and sodium nitrite were added as ammonia and nitrite sources respectively. Kinetic batch tests were replicated during each experimental phase.

During AUR tests DO was provided via a fine bubble diffuser and it was maintained close to the saturation value (in accordance with the temperature and salinity). NUR tests were performed in not-aerated conditions by using a magnetic stirrer device to mix the sample during the trials. Although anoxic conditions generally occur within the inner layers of granules even in the presence of dissolved oxygen in the bulk, it was decided to perform NUR tests for the AGS under the same conditions of the AS (not-aerated conditions). Sodium acetate was added as external carbon source to enhance the nitrites reduction. In particular, 100 ml of a concentrate solution containing sodium acetate (10 g L^{-1}) was dosed as, to have a concentration within the batch reactor of approximately 500 g L^{-1}.

Moreover, in order to assess the possible presence of NOB, specific experiments were periodically run in a similar way than the AUR tests, by adding sodium nitrite instead of ammonium chloride under aerobic conditions.

Kinetic tests were operated for 2 hours each, during which 10 mL of sample was withdrawn at regular time intervals (10 minutes). Thus, samples were filtered through a 0.45µm membrane for

NH_4-N and NO_2-N analyses. The AUR and NUR were calculated as the slope of the linear regression line of NH_4-N and NO_2-N data. These values were then referred to the volatile suspended solids concentration of the sample. The initial ammonium concentration for each AUR batch test was approximately of 100 mg NH_4-N L^{-1}, whereas the nitrite concentration for the NUR tests was close to 50 mg NO_2-N L^{-1}.

2.3.2 DNA extraction and 16S rRNA amplicon library preparation

Samples (AGS-SBR and AS-SBR) for molecular analysis were collected at Phase 11 (salinity 50 gNaCl L^{-1}) to evaluate the bacterial composition of communities developed at the maximum salinity. DNA was extracted using the FastDNA Spin kit for soil (MP Biomedicals, Santa Ana, CA, USA). Bacterial 16S rRNA amplicon sequencing targeting the V1–V3 variable regions was performed following the procedure described by (Caporaso et al., 2010), using primers adapted from the Human Gut Consortium (Ward et al., 2012). Ten µl of extracted DNA were used as a template and the PCR reaction mix (25 µl) contained dNTPs (400nM of each), MgSO4 (1.5 mM), Platinum® Taq DNA polymerase HF (2 mU), 1. Platinum® High Fidelity buffer (Thermo Fisher Scientific, Waltham, MA, USA), and barcoded library adaptors (400 nM) containing V1–3 specific primers: 27F AGAGTTTGATCCTGGCTCAG and 534R ATTACCGCGGCTGCTGG. PCR settings: initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 20 s, 56°C for 30 s, 72°C for 60 s and final elongation at 72°C for 5 min. All PCR reactions were run in duplicate and then pooled. The amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, Indianapolis, IN, USA). The library DNA concentration was measured with the Quant-iT™ HS DNA Assay (Thermo Fisher Scientific) and quality validated with a Tapestation 2200, using D1 K ScreenTapes (Agilent, Foster City, CA, USA). Based on library concentrations and calculated amplicon sizes, the samples were pooled in equimolar concentrations and diluted to 4 nM.

2.3.3 DNA sequencing16S rRNA amplicon bioinformatic processing

The samples were paired end sequenced (2.301 bp) on a MiSeq (Illumina, San Diego, CA, USA) using a MiSeq Reagent kit v3, 600 cycles (Illumina) following the manufacturer's guidelines for preparing and loading samples on the MiSeq. A 10% Phix control library was added to overcome low complexity issues often observed with amplicon samples (Illumina & Control). Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger et al., 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN:275, and merged using FLASH v. 1.2.7 (Magoč and Salzberg, 2011), with the settings -m 25 -M 200. They were then de-replicated and formatted for use in the UPARSE workflow (Edgar, 2013) and clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090 usearch_global command with -id 0.97. Taxonomic status for each was assigned using the RDP classifier (Wang et al., 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso et al., 2010), using the MiDAS database v.1.20 (McIlroy et al., 2015). The results were analyzed in R (R Core Team 2015) through the Rstudio IDE using the ampvis package v.1.24.0 (Albertsen et al., 2015). Microbial community diversity was estimated by applying richness, Shannon and Simpson indices (Hill family indices) (Hill, 1973). Data analyses were performed with the software PAST (PAleontological STatistics).

3. Results

3.1 Granules and activated sludge characteristics

Physical properties of granular and activated sludge observed at different salinity are reported in Table 2.

Tab. 2: Physical properties of granular and activated sludge at different salinity.

Parameter	Phase	1	2	3	4	5	6	7	8	9	10	11
	SRT	27						14				
	Salinity gNaCl L^{-1}	30	32	34	36	38	40	42	44	46	48	50
Mean size µm	AGS-SBR	2632 *(±215)*	2754 *(±312)*	2901 *(±196)*	3054 *(±307)*	3654 *(±298)*	3821 *(±265)*	2547 *(±138)*	2489 *(±176)*	2601 *(±201)*	2578 *(±217)*	2714 *(±314)*
	AS-SBR	152 *(±28)*	136 *(±35)*	145 *(±31)*	151 *(±17)*	148 *(±15)*	132 *(±28)*	127 *(±19)*	119 *(±16)*	121 *(±23)*	123 *(±17)*	115 *(±21)*
MLVSS g L^{-1}	AGS-SBR	8.8 *(±0.20)*	8.77 *(±0.35)*	7.6 *(±0.31)*	7.17 *(±0.24)*	6.82 *(±0.19)*	5.92 *(±0.31)*	5.64 *(±0.26)*	6.18 *(±0.17)*	5.91 *(±0.12)*	6.2 *(±0.09)*	6.23 *(±0.18)*
	AS-SBR	5.1 *(±0.10)*	4.8 *(±0.24)*	4.8 *(±0.36)*	4.66 *(±0.41)*	4.41 *(±0.24)*	4.32 *(±0.29)*	4.59 *(±0.31)*	4.78 *(±0.19)*	4.7 *(±0.15)*	4.49 *(±0.11)*	4.51 *(±0.23)*
VSS/TSS %	AGS-SBR	66% *(±2)*	63% *(±1)*	60% *(±3)*	60% *(±2)*	59% *(±1)*	53% *(±2)*	54% *(±2)*	58% *(±2)*	66% *(±1)*	64% *(±2)*	65% *(±3)*
	AS-SBR	84% *(±3)*	90% *(±2)*	86% *(±1)*	76% *(±2)*	70% *(±2)*	65% *(±3)*	71% *(±2)*	75% *(±3)*	75% *(±2)*	73% *(±2)*	73% *(±2)*
SVI* mL $gTSS^{-1}$	AGS-SBR	14.9 *(±1.2)*	16.2 *(±1.9)*	16.7 *(±1.3)*	16.9 *(±2.1)*	16.4 *(±1.7)*	18.3 *(±2.4)*	15.6 *(±2.0)*	15.1 *(±1.7)*	15.6 *(±1.5)*	16.1 *(±1.9)*	15.4 *(±0.8)*
	AS-SBR	65.5 *(±3.5)*	69.5 *(±4.2)*	60.7 *(±3.4)*	45.5 *(±2.7)*	45.5 *(±3.1)*	37.5 *(±3.4)*	34.3 *(±1.9)*	35.6 *(±1.7)*	33.1 *(±2.8)*	34.9 *(±4.1)*	33.5 *(±4.6)*
Yobs gVSS $gBOD_5^{-1}$	AGS-SBR	0.165 *(±0.010)*	0.181 *(±0.006)*	0.144 *(±0.012)*	0.160 *(±0.017)*	0.159 *(±0.008)*	0.153 *(±0.022)*	0.169 *(±0.026)*	0.151 *(±0.016)*	0.141 *(±0.031)*	0.118 *(±0.014)*	0.121 *(±0.011)*
	AS-SBR	0.195 *(±0.080)*	0.178 *(±0.033)*	0.138 *(±0.002)*	0.135 *(±0.014)*	0.12 *(±0.013)*	0.113 *(±0.017)*	0.168 *(±0.008)*	0.159 *(±0.016)*	0.164 *(±0.019)*	0.159 *(±0.020)*	0.162 *(±0.006)*

*SVI_5 for AGS and SVI_{30} for AS

Legend: MLVSS: Mixed Liquor Volatile Suspended Solids, VSS/TSS: volatile suspended solids/total suspended solids; SVI: Sludge Volume Index; Yobs: Observed heterotrophic yield coefficient.

The mature granules obtained at the end of cultivation period were characterized by a mean size of approximately 2.6 mm, a SVI after 5 minutes of settling of 14.9 mL $gTSS^{-1}$ and an inert content of approximately 35% (VSS/TSS = 0.65). The mean size of granules rapidly increased during the experiment and granules of approximately 4 mm were obtained at 40 gNaCl L^{-1} of salinity, which was consistent with previous studies that reported the average size of particles increased with salinity (Ji et al., 2018). Until Phase 6, the VSS concentration was decreasing and, accordingly, the inert content of the granules was over 45%, thereby indicating a significant accumulation of inert material. After the SRT was decreased to 14 days (Phase 7 onward) the VSS concentration and the VSS/TSS ratio increased to 65%, thereby suggesting the occurrence of biomass renewing.

The mean size of flocs in the AS-SBR slightly decreased with the increase in salinity from 152 µm to approximately 115 µm at 50 gNaCl L^{-1} of salinity. The VSS concentration in the AS-SBR decreased during the period in which the SRT was maintained at approximately 27 days, reaching a minimum value of 4.32 gVSS L^{-1} at Phase 6, corresponding to an inert content of 35%. After the SRT was decreased, the VSS concentration ranged between 4.5 gVSS L^{-1} and 4.7 gVSS L^{-1} until the end of the experiment. The inert content was close to 27% and it was constant for the rest of the

experiment, thereby indicating that salt accumulation within the flocs did not occurred under lower SRT.

Referring to the SVI values, no significant changes with the increase in salinity were observed in the AGS-SBR. The SVI_5 slightly ranged between 14 mL $gTSS^{-1}$ and 17 mL $gTSS^{-1}$ during the entire experiment, whereas the SVI_5/SVI_{30} value was approximately 1.1. This result was in contrast with the findings of a previous study in which the SVI decreasing with the increase in salinity (from 2 gNaCl L^{-1} to 15 gNaCl L^{-1}) (Wang et al., 2017). This was probably due to the fact that in this study the maturation of granular sludge was achieved before than salinity was increased, whereas in the study by Wang and co-authors, salinity was increased simultaneously with the granulation process.

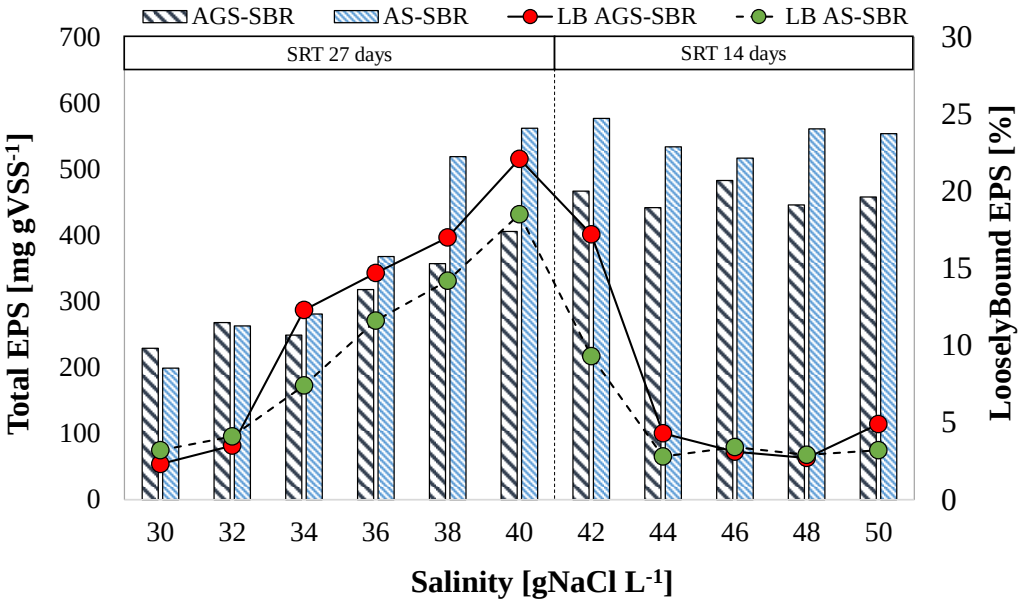
The SVI_{30} decreased with the increase in salinity in the AS-SBR. Starting from an initial value of 85 mL $gTSS^{-1}$, the SVI_{30} constantly decreased to 68 mL $gTSS^{-1}$ at the end of the experiment. This result could be related to the increase of the bulk buoyancy that resulted in the increase of the hydraulic selection pressure. In this way, the reactor started to enrich in faster-settling activated sludge. Similar findings were also achieved in other studies (Chen et al., 2018; Ou et al., 2018a). This was not observed in the AGS-SBR likely because the granules were already hydraulically-selected by the shorter duration of the settling phase.

The observed yield coefficient of the heterotrophic biomass in the AGS-SBR slightly decreased during the entire experiment from 0.168 gVSS g BOD_5^{-1} to 0.121 gVSS g BOD_5^{-1}, suggesting the reduction of new biomass synthesis due to the salinity increase, which was consistent with the literature (Mannina et al., 2016; Wu et al., 2008). The influence of SRT on the observed yield coefficient resulted negligible. Similarly, in the AS-SBR the observed yield coefficient decreased by almost 40%, from 0.195 gVSS g BOD_5^{-1} (Phase 1) to 0.113 gVSS g BOD_5^{-1} at salinity of 40 gNaCl L^{-1}. However, after Phase 7 (42 gNaCl L^{-1}), under SRT of 14 days, the Y_{obs} increased and ranged between approximately 0.159 gVSS g BOD_5^{-1} and 0.168 gVSS g BOD_5^{-1} until the end of the experiment.

The achieved results indicated that the biomass synthesis decreased with the increase in salinity. Nevertheless, a lower SRT enhanced the biomass renewing, as well as the synthesis of new bacterial cells. It is reasonable to explain the biomass activity although the high salinity level due to the dominance of the marine species.

3.2 EPS pattern in activated and granular sludge

The trend of the specific EPS content and that of the loosely-bound fraction in the activated sludge and granular sludge reactors during the experiment are depicted in Fig. 1.



Legend: AGS-SBR: Bound EPS in the AGS reactor; *AS-SBR*: Bound EPS in the AS reactor;
LB AGS-SBR: Loosely Bound EPS in the AGS reactor; *LB AS-SBR*: Loosely Bound EPS in the AS reactor;

Fig.1: Trends of the specific EPS content and percentage of loosely-bound EPS in the activated sludge (AS) and granular sludge (AGS) reactors.

The specific EPS content increased with salinity in both the SBRs, thereby confirming that bacteria produce excess of exopolymers to face the salinity increases (Corsino et al., 2017; Ji et al., 2018). This is likely due to the cell protection role played by EPS against high salinity, as suggested for biofilm communities (Decho, 2013). These exopolymeric substances are an abundant component of

microbial mats developed in hypersaline systems, such as salt ponds, salterns and hypersaline lagoons (Decho and Gutierrez, 2017), where EPS seem to condense with increasing salinity and even form a hydrophobic barrier on the surface of biofilm (Decho, 2013).

Interestingly, the EPS content significantly started to increase in both the reactors after Phase 4, when the salinity was equal to 36 gNaCl L^{-1}. In general, when saline concentration is changing in the bulk, the overproduction of EPS helps to reduce the destruction of microorganisms due to maintaining of the cellular osmotic pressure balance. In halotolerant-activated sludge bioreactors, the response of bacteria to salinity increases, in terms of overproduction of EPS, occurs from salinity levels of approximately 5-10 gNaCl L^{-1} (Campo et al., 2018; Wang et al., 2015). In this study, the response by the halophilic marine bacteria to the salinity increase occurred at salinity over 36 gNaCl L^{-1}. Therefore, based on the result above, it is possible to speculate that the response of marine bacteria to the salinity increase, in terms of overproduction of EPS, started when the gradient of osmotic pressure between the inner and the outer of the bacterial cells exceeded a certain threshold value which is significantly higher than that of halotolerant microorganisms.

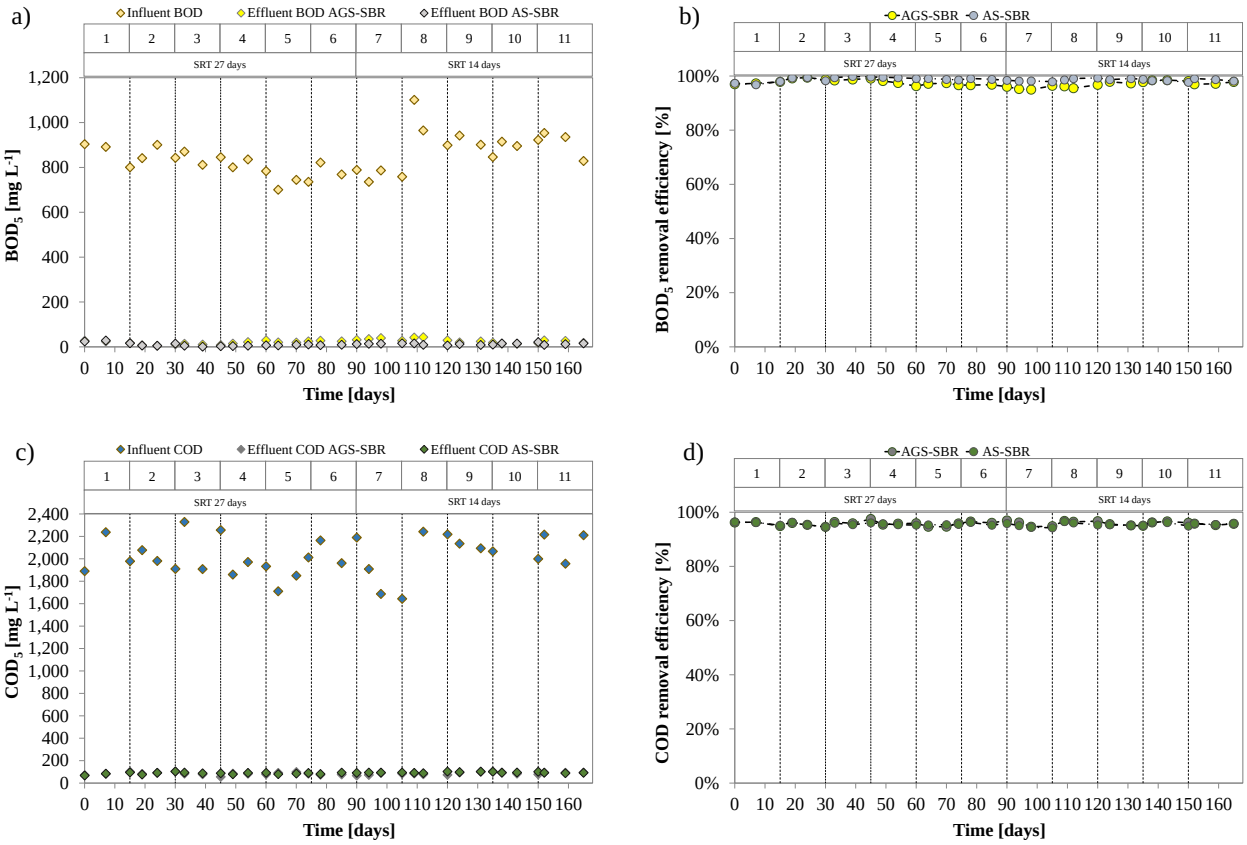
In both the AGS-SBR and the AS-SBR, the EPS were mainly constituted by the tightly-bound fraction (average value >90%). However, it is worth to observe that the loosely bound fraction was increasing when the SRT was not controlled (Fig. 1). Indeed, the loosely bound fraction reached its maximum value at Phase 6 in both the reactors, when the salinity was equal to 40 gNaCl L^{-1}. Specifically, the LB-EPS accounted for approximately 22% and 18% of the total EPS content in the AGS-SBR and AS-SBR, respectively. This result was in good agreement with a previous study (Corsino et al., 2017), in which the authors observed an exponential increase in the LB-EPS content with the increase in salinity under not controlled SRT. After Phase 6, when a shorter SRT was applied, the LB-EPS fraction decreased in both the reactors as far as a stable value of approximately 5% in the AGS-SBR and 3% in the AS-SBR was reached. Previous studies demonstrated that the increase in salinity caused a significant increase in the loosely-bound EPS fraction of halotolerant activated and granular sludge (Corsino et al., 2017; Wang et al., 2015; L. Zhao et al., 2016).

Similarly, other authors observed a significant increase in the loosely-bound EPS of halophilic biomass, in both forms of activated and granular sludge, subjected to drastic salinity increase (Corsino et al., 2018). Ismail et al. (2010) demonstrated that under high salinity the EPSs matrix suffers a deterioration, because of the replacement of calcium ions by sodium ones, thereby resulting in the increase in the loosely-bound fraction. In contrast, the results obtained in this study indicated that by stepwise increasing the salinity and by decreasing the SRT, the amount of loosely-bound EPS decreased. These results likely suggest that the detrimental effect exerted by sodium cations toward the EPS matrix occurs in the long-term and as a response of high salinity increases. Therefore, lower SRT and stepwise salinity increases enable to minimize the EPS matrix deterioration, thereby promoting the formation of sludge with good physical characteristics.

Concerning the EPS composition, the proteins resulted the main EPS constituent. The amount of proteins was more than five times higher than that of carbohydrates and no significant changes were observed in the PN/PS ratio with the increase in salinity and SRT as well. Specifically, the protein concentration increased from 190 mg $gVSS^{-1}$ to approximately 320 mg $gVSS^{-1}$ in the AGS and from 150 mg $gVSS^{-1}$ to approximately 410 mg $gVSS^{-1}$ in the AS during the entire experiment. Similarly, the carbohydrates concentration increased from 40 mg $gVSS^{-1}$ to approximately 65 mg $gVSS^{-1}$ in the AGS and from 30 mg $gVSS^{-1}$ to approximately 82 mg $gVSS^{-1}$ in the AS during the entire experiment. Consequently, the PN/PS ratio was constant to a stable value of approximately 5 during the entire experiment.

3.3 Carbon removal efficiency

Time courses of the BOD_5 and COD in the influent and effluents of the AGS-SBR and AS-SBR, as well as the removal efficiencies in both the reactors are depicted in Fig. 2a and Fig. 2b, respectively.



423

424 **Fig.2:** Influent and effluent concentrations for BOD (a) and COD (c) and the removal efficiencies
425 (b,d) during the eleven experimental phases.

426

427 The BOD_5 in the influent fish canning wastewater ranged approximately between 700 mg L^{-1} and
428 1100 mg L^{-1}. Both the SBRs provided effluents of constant quality in terms of BOD_5, which
429 resulted always below 45 mg L^{-1} and 30 mg L^{-1} in the AGS-SBR and AS-SBR, respectively.
430 Therefore, the carbon removal efficiency was on average equal approximately to 98%, which was
431 much higher than the performance achieved in other studies with salt-adapted microorganisms
432 (Chen et al., 2018; Ou et al., 2018a). A slight decrease was observed in both reactors when the SRT
433 was set equal to 27 days, likely due to the accumulation of salt within the flocs and granules as
434 discussed above. The reduction of SRT since Phase 7 enabled the biomass renewing, resulting in an
435 improvement of carbon removal efficiency. The removal efficiencies in both the reactors were in
436 good agreement with the VSS/TSS ratio values (Table 1), suggesting that when treating saline

wastewater a lower SRT enables to avoid the decrease of the active biomass within the microbial aggregates, thereby preventing the worsening of the biological performances.

A similar trend was observed also concerning the COD. The COD in the influent ranged between 1710 mg L^{-1} and 2423 mg L^{-1}, whereas the COD in the effluent was mostly below 100 mg L^{-1} in both the AGS and the AS systems (Fig. 2c). Overall, both the SBRs provided more than 95% of COD removal. The achieved results indicated that the autochthonous heterotrophic bacteria were extremely active even at high salinity levels. In terms of organic loading rate, both the SBRs were able to remove more than 95% of the influent organic load with an average rate of approximately 2.14 g COD $m^{-3}d^{-1}$. In a previous study, Zhao et al. (Zhao et al., 2016) obtained 80% of COD removal at 35 gNaCl L^{-1} in a SBR with acclimated activated sludge operating with an organic loading rate of approximately 2 g COD $m^{-3}d^{-1}$ (synthetic medium). Similarly, in an salt-adapted AGS system, Ou et al. (Ou et al., 2018b) achieved the removal of approximately 1.06 g COD $m^{-3}d^{-1}$ operating with acetate-based wastewater at 50 gNaCl L^{-1} of salinity. Based on the available literature, salt-adapted microorganisms were able to remove approximately the 50-70% of the influent organic load at salinity higher than 30 gNaCl L^{-1}, showing a decreasing trend with the salinity increase (Ji et al., 2018; Wang et al., 2014). It is worth mentioning that mostly of the cited studies are referred to the treatment of synthetic wastewater. While referring to real wastewater, Val del Rio et al. (Val del Río et al., 2012) obtained the removal approximately of 30% of the influent organic loading rate (4 g COD $m^{-3}d^{-1}$) in a AGS-SBR treating fish-canning wastewater at 30 gNaCl L^{-1} of salinity. Similarly, Figueroa et al. (Figueroa et al., 2008) obtained 90% of COD removal in a AGS-SBR operating with an organic loading rate of 1.5 g COD $m^{-3}d^{-1}$ at 30 gNaCl L^{-1} of salinity after a long acclimation period.

The results achieved in this study demonstrated that the autochthonous marine biomass was able of providing excellent carbon removal performance even at higher salinity than those tested in previous studies (Capodici et al., 2018; Ramos et al., 2015; van den Akker et al., 2015). The dominance of this marine species can explain the biomass activity although the high salinity level. It

is worth mentioning that strategy of stepwise increasing the salinity contributed to avoid sudden increase in the solute concentration around bacterial cells, thereby preventing osmotic stress. Indeed, drastic osmotic shocks would cause plasmolysis because cells lose water through osmosis, thus inhibiting the transport of substrates into the cell and the carbon removal performance (Lang et al., 2005). A gradual adaptation to salinity enables bacteria to face to the higher osmotic pressure by accumulating organic compounds in the cytoplasm, avoiding the loss of the metabolic activity.

The obtained results clearly demonstrated that the use of autochthonous biomass and the stepwise increase in salinity are effective approaches to achieve excellent carbon removal performance for the treatment of wastewater at high salinity.

3.4 Nitrogen removal efficiency

The overall performance of AGS-SBR and AS-SBR systems concerning nitrogen removal with the increased salinity is shown in Fig.4.

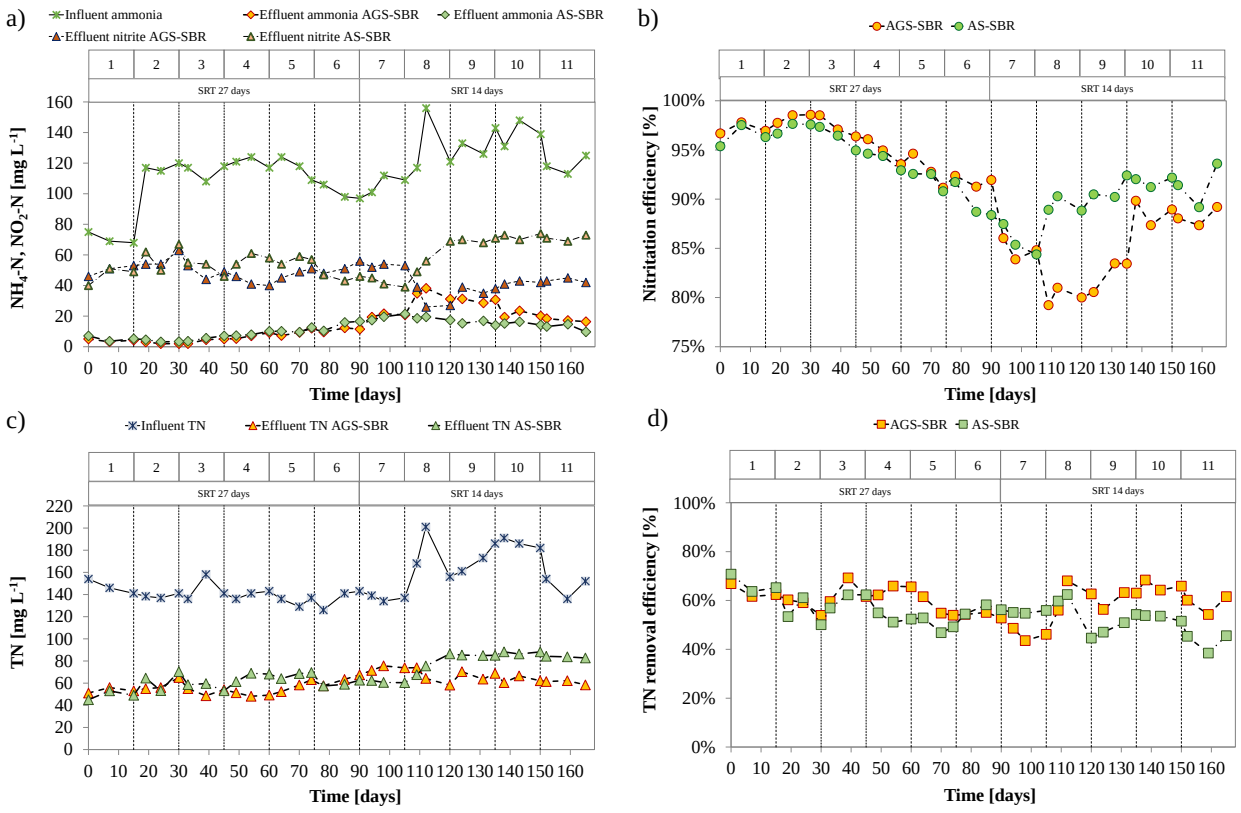


Fig.3: Influent and effluent concentrations of the ammonium and nitrite in the AGS-SBR and AS-SBR (a) and nitritation efficiency (b) at different salinity; influent and effluent concentrations of TN (c) and removal efficiencies (d).

In this study no nitrates were observed in the effluent for the entire experiment, and also kinetic batch tests confirmed that biological activity of NOB bacteria was inhibited in high-saline environment. Besides, also NGS analysis revealed the absence of NOB bacteria in both the SBRs communities. A typical trend of the nitrogen pattern during an operational cycle in the AGS-SBR and AS-SBR (Phase 1) is shown in Fig. S1.

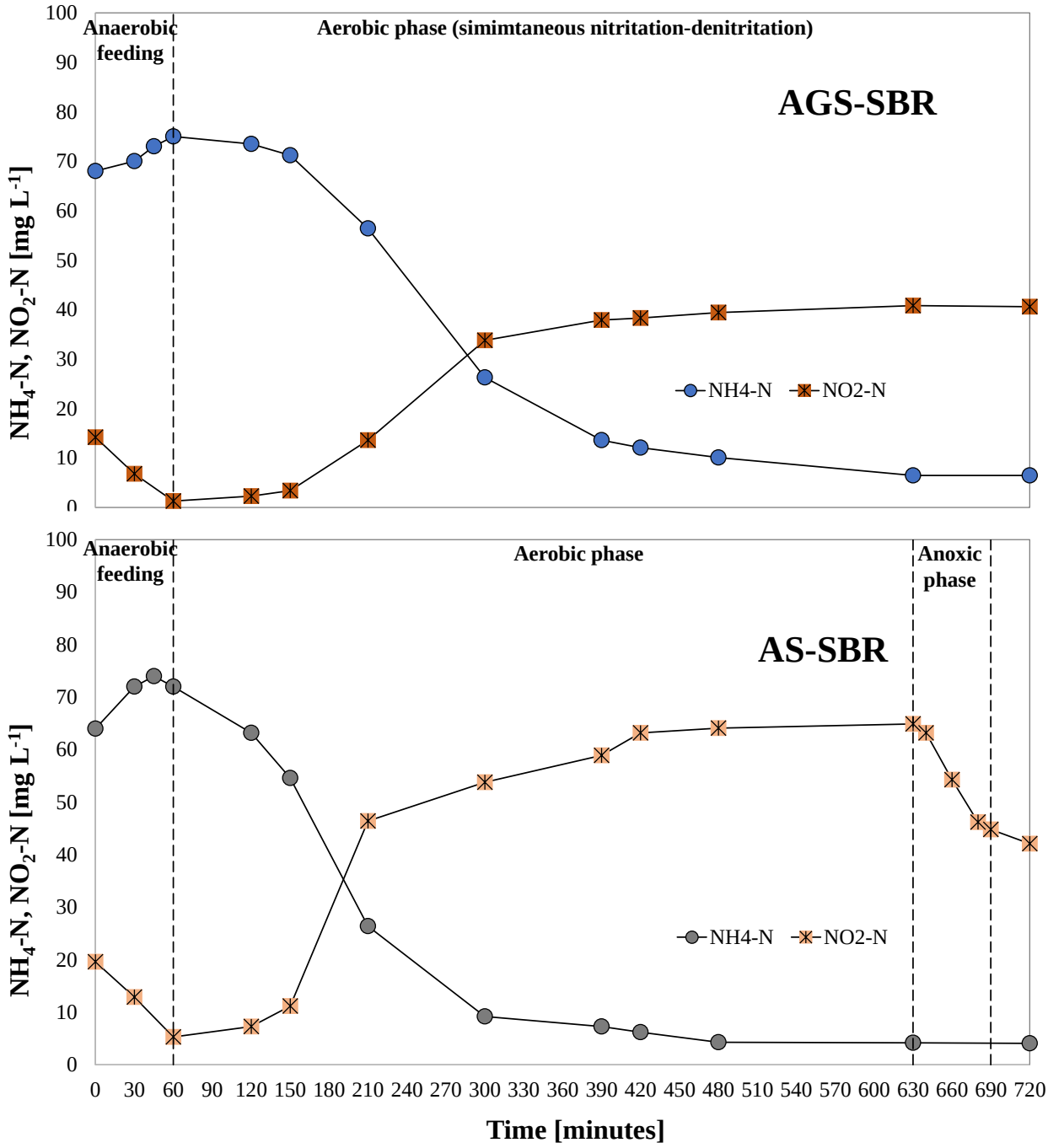
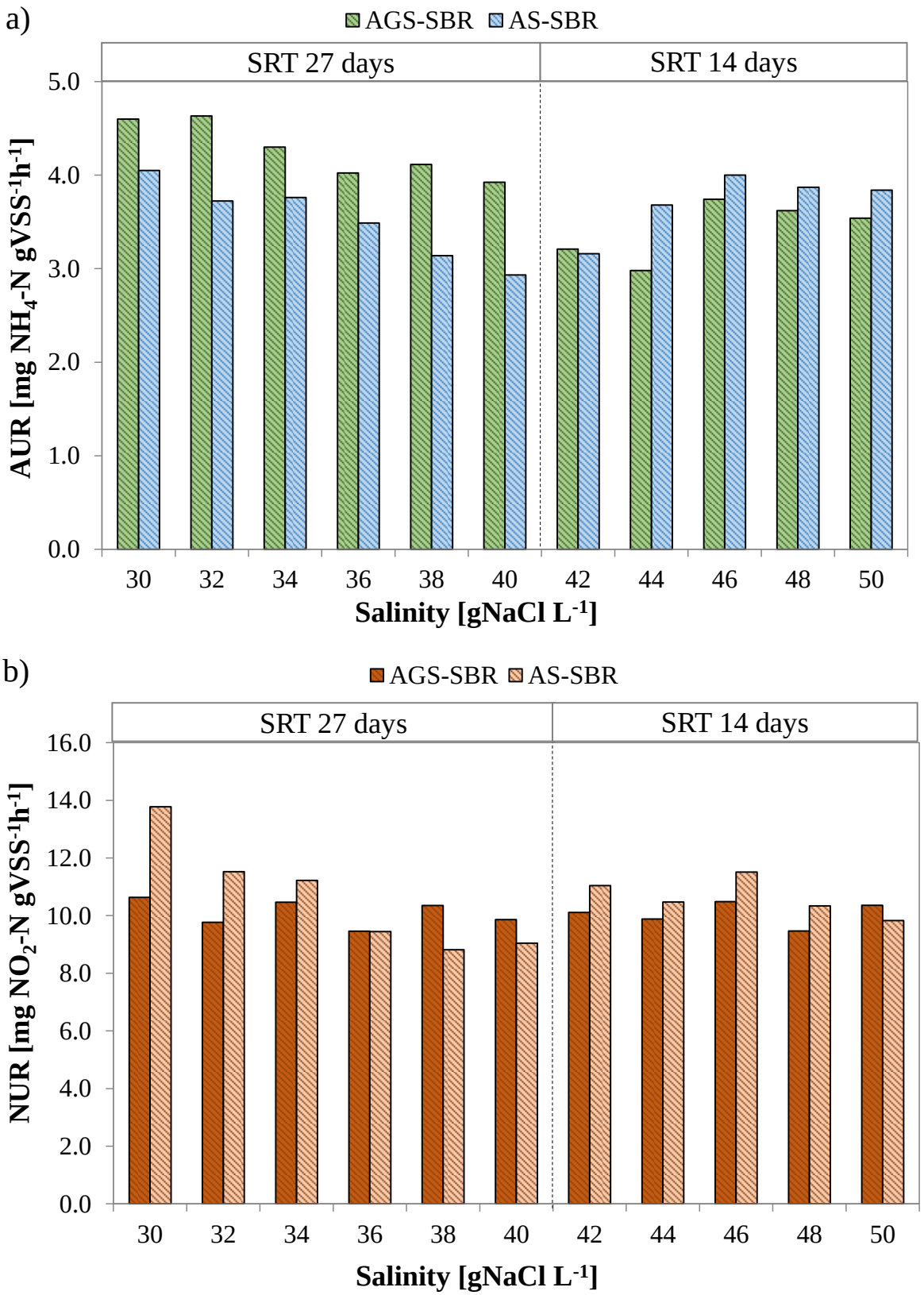


Fig.S1: Trends of ammonium and nitrite during a typical SBR cycle in the AGS and AS systems (Phase 1)

Since the SRT was set equal to 27 days, the effluent ammonium concentration showed a slightly increasing trend in both the SBRs, while maintaining always below 20 mg L^{-1} (Fig. 3a). Accordingly, the nitritation efficiencies decreased from approximately 98% (Phase 1) to 92% and

493 88% in the AGS-SBR and AS-SBR, respectively (Fig. 3b). The achieved results were in good
494 agreement with the sludge ageing previously discussed. Nevertheless, the nitritation performance
495 was approximately close to 90% in both the SBRs, thereby indicating a high activity of the Beta-
496 AOB, mainly belonging to *Nitrosomonas* species, as shown by NGS analysis.
497 When the SRT was decreased at the beginning of Phase 7, the effluent ammonium concentration
498 increased in both the reactors. In particular, the effluent ammonium concentration reached its
499 maximum value at Phase 8 of approximately 36 mg L^{-1} and 23 mg L^{-1} in the AGS-SBR and AS-
500 SBR, respectively. Accordingly, the nitritation efficiency rapidly decreased, reaching the minimum
501 value of 78% in the AGS-SBR and 85% in the AS-SBR. This result could be likely related to the
502 SRT decrease, which caused a wash-out of nitrifies. Nevertheless, both the reactors rapidly adapted
503 to the new operating conditions. Indeed, the effluent ammonium concentration decreased in the
504 subsequent phases, reaching a steady value of approximately 18 mgNH_4-N L^{-1}. At the end of the
505 experiment, the nitritation efficiency was 93% and 88% in the AGS-SBR and AS-SBR,
506 respectively. Based on the achieved results, the salinity did not significantly affect the biological
507 activity of the halophilic AOB strains, which were able to provide high performances during the
508 entire experiment. In contrast, an excessive accumulation of salt and inert material within the
509 granules or flocs worsened the nitritation performances likely because of the decrease in the overall
510 active biomass within the reactors that occurred from Phase 1 to Phase 6 before the SRT was
511 decreased.
512 During the entire experiment, the nitrite concentrations in the effluents of the SBRs ranged between
513 40 mg NO_2-N L^{-1} and 70 mg NO_2-N L^{-1} consistent with the ammonium oxidation performances.
514 The high concentration of nitrites in the effluent suggested that the denitritation process was limited
515 independently of salinity. Nitrites reduction by denitrifies occurred during the anoxic period in the
516 AS-SBR and within the layered structure of the granules in the AGS-SBR, simultaneously with the
517 nitritation process (Corsino et al., 2016).

518 Overall, the total nitrogen concentration in the SBRs effluents ranged between 40 mg TN L^{-1} and 75
519 mg TN L^{-1} (Fig. 3c), resulting in TN removal efficiencies ranging between 60% and 40% in the
520 AGS-SBR and AS-SBR, respectively (Fig. 3d). No significant relation with the increase in salinity
521 was observed. Based on the results above, denitritation was the limiting process for total nitrogen
522 removal. It is reasonable to assume that denitritation was limited by lack of carbon as nitrite
523 concentration in both the effluents did not show any significant relation with salinity. However, a
524 distinction should be made between the AGS-SBR and the AS-SBR. In the latter, because
525 denitritation occurred only during the not-aerated phase, when ammonium was already oxidized to
526 nitrite and the organic carbon was almost completely oxidized. Therefore, this certainly limited the
527 availability of the organic substrate for nitrite reduction. It is reasonable that endogenous
528 denitritation occurred through the use of bacterial internal carbon sources that limited the reaction
529 kinetic (Bernat et al., 2008). In the AGS reactor, in addition to the carbon limitation, it is also
530 reasonable that the high oxygen concentration in the bulk, close to the saturation value, was the
531 limiting factor for the simultaneous nitritation-denitritation process. Indeed, under these operating
532 conditions, it is possible that the oxygen concentrations in the inner layers of the granule was very
533 high, thereby favouring aerobic reactions instead of anoxic.
534
535 *3.5 Nitrogen removal kinetics*
536 Nitritation and denitritation kinetics were evaluated by means of AUR and NUR tests. The results
537 obtained during the experiments are depicted in Fig. 4.



538

539 **Fig. 4:** Values of the ammonium utilization rate (a) and the nitrite utilization rate (b) in each Phase

540 of the experiment.

541

Nitritation and denitritation kinetics showed a high metabolic activity of both nitrifying and denitrifying bacteria although the high salinity level. The achieved results were comparable to those of CAS systems (Metcalf and Eddy, 2015) and were significantly higher than kinetics related to halotolerant biomass (Pronk et al., 2014).

AUR slightly decreased in both SBRs when salinity was increased from 30 gNaCl L^{-1} to 40 gNaCl L^{-1} (Fig. 4a) during the period at 27 days of SRT. More precisely, the AUR in the AGS-SBR decreased from 4.67 $mgNH_4$-N $gVSS^{-1}h^{-1}$ at 30 gNaCl L^{-1} of salinity to 3.97 $mgNH_4$-N $gVSS^{-1}h^{-1}$ at salinity of 40 gNaCl L^{-1}, thereby reducing by approximately 16%. In contrast, the AUR in the AS-SBR decreased from 4.05 $mgNH_4$-N $gVSS^{-1}h^{-1}$ to 2.93 $mgNH_4$-N $gVSS^{-1}h^{-1}$ within the same range of salinity. Thus, the decrease in AUR was of approximately 38% in the AS-SBR, which was 2.4 times more the AGS-SBR. These results were in good agreement with nitritation performances above discussed.

Based on these results, the increase in salinity with a stepwise strategy within a range of 30 gNaCl L^{-1} and 40 gNaCl L^{-1} had a negligible effect on biological activity of AOBs in the AGS (i.e., $p = 0.286$), whereas a significant decrease was noted in the AS, although the one-way analysis of variance indicated a low correlation value (i.e., $p = 0.210$).

After Phase 6, at salinity of 40 gNaCl L^{-1}, the AUR sharply decreased in the AGS-SBR approximately by 22%. At salinity of 42 gNaCl L^{-1} the AUR in the AGS-SBR was approximately 3.20 $mgNH_4$-N $gVSS^{-1}h^{-1}$ and this value was comparable with that in the AS-SBR. Therefore, a sharp decrease in AOBs activity was noted at salinity higher than 40 gNaCl L^{-1}, although this occurred only in the AGS reactor. Such result could be likely due to the accumulation of inert material within the granules and to the limitation to oxygen diffusion. Indeed, at Phase 6, approximately the 78% of aerobic granules were on average bigger that 4 mm, likely because of the inclusions of inert material within the structure of granules, (salt and inert particulate deriving from the raw wastewater). The high salt concentration that decreased the oxygen saturation within the liquid bulk, and the bigger size of granules, increased the resistance to oxygen diffusion within the

inner layers, endangering the ammonia oxidation process. Besides, accumulation of inert inorganic material within the granule caused a decrease in the VSS/TSS ratio that was approximately 53%. Because of the lower VSS/TSS ratio in the AGS, the biologically active fraction of the granule significantly decreased, thereby reducing the ammonium oxidation capacity by the AGS system.

After the SRT was decreased to 14 days, the new operating conditions rapidly reflected on the AOBs activity in the AS-SBR. Indeed, the AUR increased from 3.18 $mgNH_4$-N $gVSS^{-1}h^{-1}$ to approximately 4.0 $mgNH_4$-N $gVSS^{-1}h^{-1}$ at salinity of 46 gNaCl L^{-1}. Thereafter, the AUR ranged between 3.87 $mgNH_4$-N $gVSS^{-1}h^{-1}$ and 3.84 $mgNH_4$-N $gVSS^{-1}h^{-1}$ during the rest of the experiment. Contrarily, the AUR in the AGS-SBR still decreased until Phase 8 at salinity of 44 gNaCl L^{-1}, when it reached its minimum value of approximately 2.44 $mgNH_4$-N $gVSS^{-1}h^{-1}$. Subsequently, a reversal of this decreasing trend occurred. However, the trend reversal point was slightly shifted in time respect to that in the AS-SBR. In the last experimental Phase, at 50 gNaCl L^{-1} of salinity, the AUR reached a value of approximately 3.54 $mgNH_4$-N $gVSS^{-1}h^{-1}$. It is worth mentioning that the shortened in SRT after Phase 6, caused the decrease in the size of granules in the AGS-SBR. This certainly affected the AUR kinetics, because the smaller size of bio-aggregates favoured the increase of oxygen penetration depth, thereby increasing the aerobic zone of the granule which is associated with the capacity for nitritation (Wang et al., 2017).

Overall, the stepwise increase strategy resulted in a decrease in AUR of approximately 2.7% and 0.5% in the AGS-SBR and AS-SBR respectively for each salinity increase. Nonetheless, the stepwise strategy of increasing salinity did not significantly affect the AUR kinetics of the AGS-SBR and AS-SBR systems. The results of one-way analysis of variance (one-way ANOVA) indicated that, considering the entire range of salinity, the salinity increase had a marginal effect on AUR kinetic in the AGS ($p > 0.302$) and the AS reactors ($p > 0.289$).

Based on the results above, the metabolic activity of the AOBs was higher in the AGS than the AS up to a salinity of 40 gNaCl L^{-1}, whereas at higher salinity levels, AUR kinetics were higher in the AS. It is worth mentioning that over a certain salinity, the oxygen diffusion within the liquid bulk

began to be salt-limited (Zannotti and Giovannetti, 2015). Consequently, at high salinity, the ammonia oxidation process could be oxygen limiting. Besides, in the aerobic granules autotrophic bacteria develop in the inner layers (Winkler et al., 2012). Consequently, to provide enough electron donors for the AOBs metabolic functionalities, oxygen has to penetrate deeper within the granule. Therefore, although the compact structure of the granules act as a kind of protecting shield for bacteria, it makes even more difficult the oxygen diffusion within the inner layers. This would explain why over a salinity of 40 gNaCl L^{-1} the metabolic activity of AOBs was higher in the AS-SBR. At lower salinity instead, the oxygen diffusion is not a limiting factor for the ammonia oxidation process. Therefore, within a range of salinity between 30 gNaCl L^{-1} and 40 gNaCl L^{-1} AUR kinetics were higher in the AGS-SBR likely because of the higher active biomass retention in the aerobic granules than the flocculent activated sludge that enabled to enhance a faster oxidation of ammonium. In this respect, the NUR tests confirmed that lower SRT enabled to achieve a significant reprise of metabolic activity of AOBs strains especially in AGS systems.

The values of the NUR at different salinity are shown in Figure 4b. The NUR ranged between 9 $mgNO_2$-N $gVSS^{-1}h^{-1}$ and 10.5 $mgNO_2$-N $gVSS^{-1}h^{-1}$ in the AGS-SBR during the entire experiment, independently of salinity. Conversely, the NUR in the AS-SBR showed a decreasing trend during the period at SRT of 27 days, when salinity was increased from 30 gNaCl L^{-1} to 40 gNaCl L^{-1}. Indeed, the NUR decreased from 13.8 $mgNO_2$-N $gVSS^{-1}h^{-1}$ to approximately 9.0 $mgNO_2$-N $gVSS^{-1}h^{-1}$ at Phase 6 at salinity of 40 gNaCl L^{-1}, thereby decreasing approximately of 40%. After Phase 6, when the SRT was decreased, the NUR in the AS-SBR increased and ranged between 9.8 $mgNO_2$-N $gVSS^{-1}h^{-1}$ and 11.5 $mgNO_2$-N $gVSS^{-1}h^{-1}$ until the end of the experiment.

Based on the results above, denitrifies activity was independent of both salinity and SRT in the AGS-SBR. Although the size of the granules decreased after Period 6, this did not affect the NUR kinetics of the AGS. Indeed, the average size of the granules was larger than 2.5 mm, thereby enabling the achievement of a sufficient anoxic zone within the granule structure that allowed the maintenance of the denitritation capacity.

Conversely, the NUR in the AS-SBR decreased with the increase in salinity until Phase 6 as previously observed for the AUR. However, the decrease of the SRT enabled a recovery of denitrifies metabolism independently of salinity. This result suggested that the biomass ageing in the AS-SBR occurred until Phase 6 caused a significant decrease in the heterotrophic active fraction, whereas a lower SRT allowed the biomass renewing. Indeed, it was calculated that the specific observed heterotrophic yield in the AS-SBR decreased with the increase in salinity from 0.20 gVSS $gBOD^{-1}$ to approximately 0.10 gVSS $gBOD^{-1}$ at salinity of 40 gNaCl L^{-1}. Thereafter, the decrease in the SRT enabled to increase the Y_{obs} to approximately 0.18 gVSS $gBOD^{-1}$, thereby favouring the biomass renewing. These results indicated that the removal rate of nitrites did not show any significant relationship with the increase in salinity ($p > 0.465$), but rather with the SRT.

3.6 Bacterial community analysis

Due to the promising results on biological performance of the autochthonous halophilic-marine biomass in both the SBRs also at 50 gNaCl L^{-1}, the high-throughput sequencing technology was applied on such communities to provide a first insight into their microbial diversity. To the best author's knowledge, although several studies investigated the impact of salinity on microorganisms in activated sludge processes (He et al. 2017), a few data are available about the bacterial diversity of halophilic-marine biomass at salinity higher than 30 gNaCl L^{-1} (Ji et al. 2018).

Both the analyzed microbial communities showed to harbor considerable microbial diversity, with high species richness (Fig. 5a), despite the high salinity experimented in this study. This can be explained by the presence of a high number of rare species, as shown by the rank abundance curves that revealed that these communities were characterized by a strong dominance of a few operational taxonomic units (OTUs) and a long tail of rare OTUs (Fig. 5b). Abundances of the rare OTUs were indeed 93.3% and 93% of the total OTUs in AGS-SBR and AS-SBR communities, respectively. Abundant OTUs were defined as those comprising 1% or more of the community, and rare OTUs comprised < 1% (Campbell et al., 2011).

As previously suggested (Besemer, 2016) for other microbial communities, the presence of high amounts of rare taxa, many of which not actively metabolizing, provides the genetic capability to respond to changes in environmental conditions.

Although the taxon richness resulted to be higher in the AGS-SBR sample (Fig. 5a), the halophilic-marine biomass showed similar taxon composition in both the SBRs. On phylum level (Fig. 5c), samples were dominated by OTUs affiliated to Bacteroidetes (60% in AS-SBR and 64% in AGS-SBR). Proteobacteria were also present at high percentages (31.91 % in AS-SBR and 30.82% in AGS-SBR), while both the phyla Planctomycetes (1.57% in AS-SBR and 0.30% in AGS-SBR) and Firmicutes (1.29% in AS-SBR and 1.34% in AGS-SBR) contributed less to the dominant portion of the communities. Results are in agreement with previous studies reaveling Bacteroitedes as one of the most abundant phylum composing marine microbial communities, due to their important role played in the organic degradation in such environments (Díez-Vives et al., 2012). Although a direct comparison with data available in literature is difficult, due to the lack of information on microbial diversity of halophilic-marine biomass, it should be reasonable compare results obtained here with the outputs of studies on the effect of salinity on microbial composition in activated processes (He et al. 2017, Chen et al. 2018, Zhang et al. 2016). Our results seemed to be in agreement with different studies that revealed the dominance of Proteobacteria, Bacteroidetes and Firmicutes in activated sludges grown at high salinity conditions (He et al. 2017, Chen et al. 2018, Zhang et al. 2016) and that highlighted the high tolerance to salt stress of Bacteroidetes (Chen et al. 2018).

From Fig. 5d and Fig. 5e, the community composition on class and family level was further analyzed. Bacteroitedes were dominated by members of the Flavobacteria (49.20% in AS-SBR and for 55.37% in AGS-SBR) and included members of the Bacteroidia (3.84% in AS-SBR and 4.53% in AGS-SBR). Among the Flavobacteria, the family Cryomorphaceae predominated, with the highest contribution from members of the genus *Owenweeksia* (38 in AS-SBR and 45% in AGS-SBR). The family Flavobacteriaceae, mainly the genus *Vitellibacter* (7.25% in AS-SBR and 4.40%

671 in AGS-SBR), was also abundant. For the Bacteroidia, *Marinifilum* represented the most abundant
672 genus (2.09% in AS-SBR and 2.69% in AGS-SBR).
673 Within the Proteobacteria, members of Alpha (14.59% in AS-SBR and 6.72% in AGS-SBR),
674 Delta-Proteobacteria (4.53 in AS-SBR and 10.76 in AGS-SBR) and Gamma-Proteobacteria (8.53 in
675 AS-SBR and 6.17 in AGS-SBR) were also prominent. The microbial composition found in the
676 analyzed samples, can explain the performance of the autochthonous halophilic-marine biomass in
677 both the SBRs at 50 gNaCl L^{-1}. The dominance of Cryomorphaceae in both the samples, indeed,
678 agrees with the ecology of this family, that comprises primarily marine genera (McBride, 2014a).
679 Moreover, the dominant genus *Owenweeksia* contains only a single species isolated from sand-
680 filtered seawater, collected at Port Shelter in Hong Kong (Lau, 2005) and the genera *Vitellibacter*
681 and *Marinifilum* comprised mainly marine species isolated from seawater (Ruvira et al. 2013,
682 Thevarajoo et al. 2016, Xu et al. 2016). In addition, the presence of high percentages of members
683 belonging to Proteobacteria are generally in agreement with studies evaluating the salt tolerance of
684 microbial communities in activated and granular sludge processes (He et al. 2017, Ji et al. 2018).
685

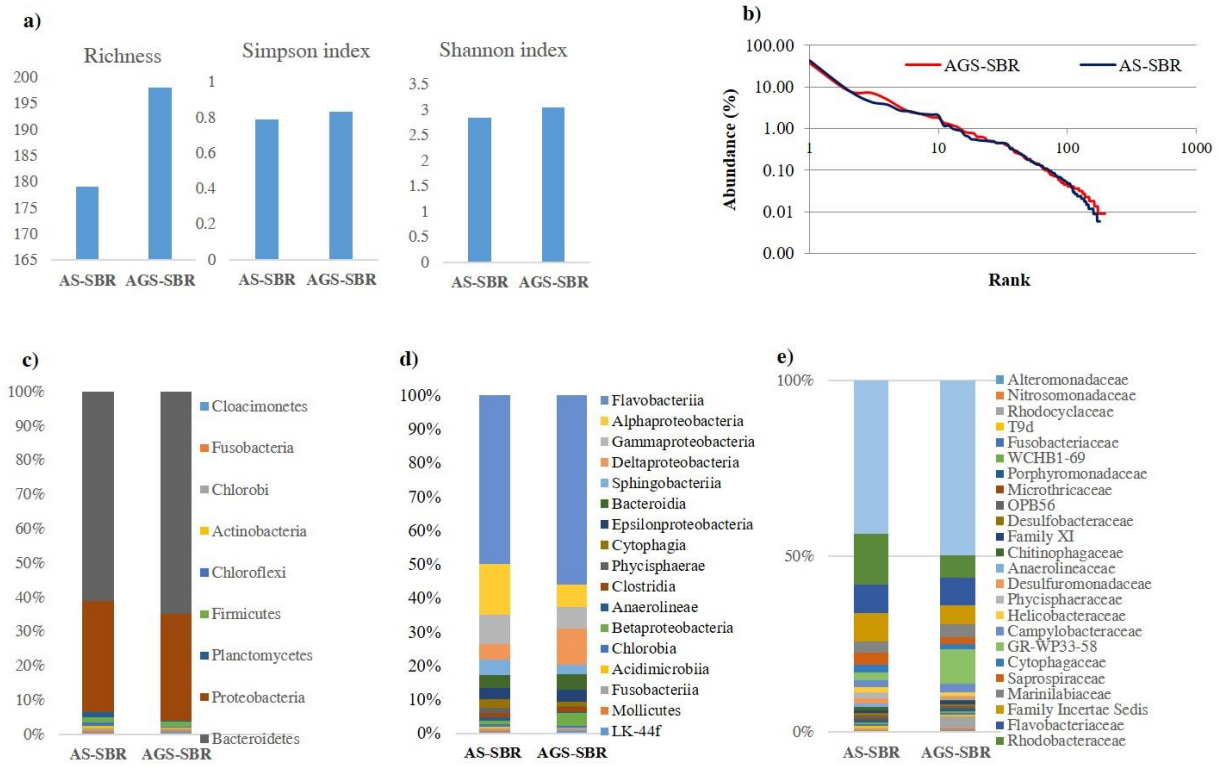


Fig.5: Microbial diversity and composition of the halophilic communities grown in the SBRs (AGS-SBR and AS-SBR) developed at the highest salinity experimented (50 gNaCl L^{-1}), obtained from NGA data. Microbial diversity, estimated by applying Richness, Simpson and Shannon indices (a). Rank–abundance curves of communities for determining population relative abundances. Curves are displayed on a log–log scale for clarity (b). Relative abundances (%) of the most representative bacterial taxa in microbial communities at phylum (c), class (d) and family (e) level. Data are reported as operational taxonomic units (OTUs).

4. Discussion

4.1 Autochthonous or allochthonous biomass?

One of the aim of this study was to compare the performance of the autochthonous biomass with that allochthonous acclimated to salinity. The results discussed above indicated that the autochthonous halophilic-marine bacteria enabled very high nutrient removal efficiencies compared to halotolerant biomasses (He et al., 2017; Zhang et al., 2017). In terms of nutrient removal performance, both the SBRs enables more than 95% of the influent organic load (2.25 g COD $m^{-3}d^{-}$

^{1}), which resulted higher than that obtained in other studies with acclimated biomass operating at lower salinity and treating synthetic wastewater (Ji et al., 2018; Ou et al., 2018a; Wang et al., 2014). While referring to nitrogen removal, complete nitritation was successfully achieved within the entire range of salinity investigated. In contrast, in previous literature, nitrogen removal is considered the main concern related to the treatment of saline wastewater. In the majority of the available studies in the literature, complete nitrification is generally achieved up to 20 gNaCl L^{-1} (Pronk et al., 2014), whereas stable nitritation was achieved up to a salinity of approximately 45 gNaCl L^{-1}, although it rapidly collapsed at higher salt concentration (García-Ruiz et al., 2018). Moreover, in terms of physical structure of the bio-aggregates, it was observed that detrimental effects on the EPS matrix occurred as a result of long SRT, whereas it was not affected by the increase in salinity as reported in previous studies with acclimated biomasses (Corsino et al., 2017; Wang et al., 2016).

The results above confirmed that the use of autochthonous-halophilic bacteria represents a valuable solution for the treatment high-strength carbon and nitrogen saline wastewater. The results also indicated that both kinetics and performances were significantly higher than those achievable with acclimated halotolerant microorganisms, even at higher salinity (Lefebvre and Moletta, 2006; Wang et al., 2017). Results reported in literature suggest that although the acclimatization of conventional activated sludge to the salinity is possible (Lefebvre and Moletta, 2006; van den Akker et al., 2015; Wang et al., 2015), the achievement of high ammonium oxidation rates and nitrogen removal efficiencies is limited within a range of salinity between 5 gNaCl L^{-1} and 33 gNaCl L^{-1} (Pronk et al., 2014; Wang et al., 2017). Moreover, the autochthonous biomass had a better biodiversity compared to the acclimated biomass as proven by the considerable microbial diversity that gained a higher nutrient removal performances and process stability at high salinity levels.

It is worth mentioning that a comparison between the results above with others referred to the autochthonous-halophilic biomass under similar operating conditions was not carried out because of the lack of knowledge in the literature. Besides, the achieved results suggested that halophilic

biomass could tolerate higher salinity levels than those tested in this study. However, it has to be mentioned that under higher salinity levels several technical drawbacks could occur, like salt deposits on equipment and devices, as well as scaling on the air diffuser that create management difficulties independently on the effects of the salinity on the bacterial metabolism.

The result discussed above demonstrated that the start-up of a plant designed for the treatment of a saline wastewater with an inoculum of autochthonous biomass is more appropriated than using allochthonous bacteria. Indeed, the cultivation of the autochthonous biomass offers benefits in terms of higher metabolic kinetics, higher microbial diversity, stable process operation, thereby enabling an effluent of superior quality, while saving energy and footprint. Therefore, both from a management and process point of view, the system's start-up without any inoculum, thus allowing developing the autochthonous biomass, represent a valuable operating strategy to achieve highly performing systems.

4.2 Granular or activated sludge?

Overall, biological performances were comparable in both the activated and the granular sludge reactors, thereby indicating that, when a stepwise increase strategy of the salinity is performed, the granulation process did not provide significant improvements in the nutrient removal processes. Previous studies demonstrated that the aerobic granulation of halophilic sludge offers an advantage in case of drastic salinity fluctuations (Corsino et al., 2018). Indeed, because of the bigger and denser structure the aerobic granules are able to withstand drastic and moderate salinity fluctuations in the short and long-term better than activated sludge. Nonetheless, if a stepwise strategy of salinity increases is performed, the halophilic activated sludge shows a similar response to salinity increases compared to halophilic granular sludge. The bacterial community analysis did not highlight significant differences between the AGS-SBR and AS-SBR. Indeed, although the taxon richness resulted to be higher in the AGS-SBR sample, the halophilic-marine biomass showed a very similar taxon composition in both the SBRs.

Biological performances resulted mostly affected by the SRT and by the decreasing solubility of the oxygen due to the salinity in both the systems. Indeed, the oxygen transfer and diffusion processes, as well as the biomass ageing significantly affected both the carbon and the nitrogen removal kinetics and performances more than salinity. This was more noticeable in the AGS-SBR because of the higher size and density of the microbial aggregates that represented an additional barrier to the oxygen diffusion and favoured the buildup of inert material.

Nevertheless, although the kinetic and the biological performances were comparable within the entire range of salinity investigated in this study, it is worth mentioning that, because of the higher biomass retention capacity, AGS systems could be able to enable almost 50% of volume saving.

5. Conclusions

Nutrient removal performances and kinetics of autochthonous marine bacteria in forms of activated and granular sludge were assessed at different salinity levels, ranging from 30 gNaCl L^{-1} to 50 gNaCl L^{-1} in two SBRs. The achieved results demonstrated that the cultivation of the autochthonous biomass offers benefits in terms of higher metabolic kinetics, higher microbial diversity and stable process operation than allochthonous acclimated biomass, thereby enabling an effluent of superior quality. At the maximum salinity level tested, the microbial community was dominated in both the systems by marine species belonged to the genera *Owenweeksia* (Cryomorphaceae), *Vitellibacter* (Flavobacteriaceae) and *Marinifilum* (Bacteroidia). Both the SBRs provided a high-quality effluent, enabling performances approximately of 98% and 90% in terms of carbon removal and nitritation efficiency, respectively. Denitritation process was limited independently of salinity, resulting in TN removal efficiency lower than 70%. Nevertheless, the biomass activity and performances slightly decreased as a result of a long SRT (27 days) because of salt accumulation within the activated sludge flocs and granules. Although the high salinity, AUR and NUR of approximately 3.60 $mgNH_4\text{-}N\ gVSS^{-1}h^{-1}$ and 10.0 $mgNO_2\text{-}N\ gVSS^{-1}h^{-1}$, respectively, indicated a high activity of nitrifying and denitrifying bacteria. The obtained results also indicated that a lower SRT (14 days),

favoured the discharge of the granules and flocs with higher inert content, thereby enhancing the biomass renewing and a high metabolic activity. The operating strategy of stepwise increasing the salinity and decreasing the SRT is crucial for a dual proposal when treating saline wastewater: first to avoid an excessive buildup of salt within the biomass aggregates and second to avoid their physical destructuration. Indeed, both the strategies enabled to achieve sludge with good physical properties, because of the decrease in the LB-EPS fraction. Lastly, if a stepwise strategy of salinity increases is performed, the halophilic activated sludge shows a similar response to salinity increases compared to halophilic granular sludge.

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Figure caption

Fig.1: Trends of the specific EPS content and percentage of loosely-bound EPS in the activated sludge (AS) and granular sludge (AGS) reactors.

Fig.2: Influent and effluent concentrations for BOD (a) and COD (c) and the removal efficiencies (b,d) during the eleven experimental phases.

Fig.3: Influent and effluent concentrations of the ammonium and nitrite in the AGS-SBR and AS-SBR (a) and nitritation efficiency (b) at different salinity; influent and effluent concentrations of TN (c) and removal efficiencies (d).

Fig. 4: Values of the ammonium utilization rate (a) and the nitrite utilization rate (b) in each Phase of the experiment.

Fig.5: Microbial diversity and composition of the halophilic communities grown in the SBRs (AGS-SBR and AS-SBR) developed at the highest salinity experimented (50 gNaCl L^{-1}), obtained from NGA data. Microbial diversity, estimated by applying Richness, Simpson and Shannon indices (a). Rank–abundance curves of communities for determining population relative abundances. Curves are displayed on a log–log scale for clarity (b). Relative abundances (%) of the most representative bacterial taxa in microbial communities at phylum (c), class (d) and family (e) level. Data are reported as operational taxonomic units (OTUs).

Fig.S1: Trends of ammonium and nitrite during a typical SBR cycle in the AGS and AS systems (Phase 1)

Table caption

Tab. 1: Operating conditions for AGS-SBR and AS-SBR during the experimental phases.

Tab. 2: Physical properties of granular and activated sludge at different salinity.