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Stable nutrient removal from wastewater with fluctuating seawater content ensured by an adaptable aerobic granular sludge microbiome

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ABSTRACT

Seawater intrusion in coastal regions can alter the wastewater composition, threatening the microbial communities in wastewater treatment processes. An aerobic granular sludge (AGS) system was challenged by fluctuations in wastewater salinity levels promoted by seawater intrusion events for 286 days, divided into two stages. During stage I, the seawater content in wastewater increased stepwise, and over stage II the seawater content in wastewater oscillated throughout the day. Most of the time, the AGS effectively removed COD during the anaerobic phase, regardless of the wastewater salt content. Ammonium removal was slightly unstable (ca. 75 ± 19 %) during stage I, with nitrite release in the effluent. Over stage II, the ammonium content in the wastewater was fully removed. The nitrite content in the effluent decreased, and nitrate became the main nitrogen form released. Phosphate removal was quite unstable at the beginning, improving over time with complete removal achieved during stage II (ca. 98.4 ± 1.1 %). Taxa involved in nitrogen and phosphorous removal were identified in the AGS microbiome at both stages but with superior abundance in the latter stage. A diverse core microbiome, mainly composed of extracellular polymeric substances-producing bacteria (*Thauera*, *Flavobacterium*, *Paracoccus*) and denitrifying bacteria (*Thiotrix*, *Azoarcus*, *Aequorivita*) was identified in stage II. The AGS system efficiently managed daily oscillating seawater levels in wastewater, corroborated by the effective removal performance that seemed to be sustained by an adaptable AGS microbiome.

Introduction

Wastewater treatment plants (WWTPs) play a crucial environmental role, treating the wastewater produced by humans during their daily activities and returning it into the water cycle. WWTPs currently face multiple challenges such as the high salinity levels in wastewater, which not only undermine the efficiency of biological wastewater treatment processes but also cause deterioration of the WWTP infrastructures (Guo et al., 2023). Approximately 5 % of worldwide effluents are saline or hypersaline, and this trend is likely to continue and even increase (Cui et al., 2016). Salt's presence in wastewater can arise from several sources, namely the discharge of industrial saline effluents (e.g., petroleum refineries, leather processing, fish canning), the use of seawater (e.g., flushing purposes) to address the freshwater shortage in some coastal areas (Alam et al., 2022), among others. Also, the prevalence of climatic changes and sea level rise have increased the frequency of seawater intrusion events, directly augmenting the salt levels in wastewater (Bosserelle et al., 2022).

Biological processes are typically the most applied approaches in full-scale WWTPs due to their cost-effectiveness and lower environmental impact. Within the biological processes, aerobic granular sludge (AGS) has emerged as a robust technology now being applied worldwide for treating domestic and industrial wastewater (https://nereda.royalhaskoningdhv.com/). The merit of this technology is attributed to its ability to withstand several pollutants, high settling velocity, and its great tolerance to harsh scenarios in which saline environments are included (Ely et al., 2022; Yue et al., 2023; Amorim et al., 2016). The AGS consists of round-shape microbial aggregates structured in a three-dimensional matrix allowing the distribution of microorganisms in aerobic, anoxic, and anaerobic layers. Due to this layered structure, the simultaneous removal of pollutants from wastewater can occur in a single reactor (Hamza et al., 2022; de Kreuk et al., 2005).

The AGS technology has been frequently applied for treating urban wastewater with low or moderate salinity levels that do not vary much over time (Amorim et al., 2016; Oliveira et al., 2021). However, high salinity levels can exert an inhibitory effect on the functionality of

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microbial communities responsible for wastewater treatment, considering that the increase of the osmotic pressure in the mixed liquor sludge can impair the microorganism's metabolism and ultimately lead to cell death (Cortés-Lorenzo et al., 2012). Bassin et al. (2011) showed that the presence of salts in the form of NaCl in an AGS system resulted in nitrite accumulation that affected the polyphosphate-accumulating organisms (PAO) activity, disrupting the metabolic pathway linked to phosphate removal. To prevent the latter outcome, it is crucial to gradually adapt freshwater-based microbial communities to increasing salinity levels. Studies have shown that an acclimation period to increasing salinity levels allows microorganisms to better thrive in saline conditions and likely maintain nutrient removal performances (Zhang et al., 2019; Campo et al., 2018). Currently, research exploring the influence of salinity in AGS systems has risen, particularly those assessing the removal performance and stability during the gradual adaptation to varying salt levels (Wang et al., 2017; Quartaroli et al., 2019; Pronk et al., 2014). A clear example was the study by Corsino et al. (2016), who assessed the ability of the AGS technology to treat fish-canning wastewater with salinity levels up to 50 g NaCl/L. In that scenario, simultaneous nitrification–denitrification and chemical oxygen removal (COD) removal occurred, but nitrification deteriorated above 50 g NaCl/L. In another study carried out by Pronk et al. (2014), a stable granular structure and efficient ammonium removal at salinity levels up to 20 g NaCl/L were reported, though nitrite oxidation and phosphorus removal were severely inhibited. Similarly, He et al. (2020) demonstrated effective phosphorus removal at 10 g NaCl/L, but the process was drastically inhibited at 20 g NaCl/L, while nitrogen removal remained unaffected. Contrastingly, Quartaroli et al. (2019) reported moderate ammonium removal rates (ca. 64 %) at salinity levels exceeding 30 g NaCl/L, without nitrite accumulation at 40 g NaCl/L. Hou et al. (2019) showed that daily NaCl fluctuations improved the biomass settling ability, which contributed to higher COD removal efficiencies. Tang et al. (2022) observed rapid granulation and efficient settleability of salt-tolerant aerobic granular sludge exposed to wastewater with an organic loading rate of 3.6 kg COD/m^3 and a salinity content of 30 g NaCl/L. The findings from the above studies outstand the resilience of AGS technology to withstand hypersaline environments. However, most studies on this topic use NaCl as the sole salt source in the wastewater, which may not fully reflect the complexity of real wastewater. In fact, studies on the influence of seawater, a complex chemical matrix with diverse inorganic salts, on nutrient removal and microbiome dynamics remain scarce, despite their importance for understanding what occurs in real scenarios. Similarly, research on the effect of daily salinity oscillations in wastewater due to seawater intrusion, an ever-growing phenomenon, in AGS systems, is limited.

This study aims to explore the effectiveness of an AGS system for the treatment of domestic wastewater whose salinity oscillates throughout the day, mimicking a real scenario of seawater intrusion, by evaluating the effect of such complex saline source on the reactor treatment performance whilst exploring the AGS microbiome dynamics, aiming at the identification of patterns in the microbial community underpinning the reactor functionality. To our knowledge, this is the first study that explores daily salinity oscillations in the feeding regime of an AGS reactor using seawater as the salt source instead of NaCl.

Methods

AGS reactor operation

A laboratory-scale sequencing batch reactor (SBR) with 110 cm of height, 6.5 cm of internal diameter, and a working volume of 2.4 L was used. The seed sludge consisted of AGS from a full-scale WWTP with a Nereda® system (Frielas, Lisbon, Portugal). The reactor was operated in eight successive treatment cycles of 3-hours per day. Each treatment cycle comprised four sequential phases, namely anaerobic filling (60 min), aeration (112 min), settling (3 min), and effluent withdrawal (5 min). Aeration was provided by an air diffuser placed at the bottom of the reactor at 4 L/min. In each cycle, a volumetric exchange ratio of 40 % was applied. The pumps for feeding and effluent withdrawal as well as the airflow meter were controlled through an automatic timer (Siemens Logo! 230 RC). The reactor was operated at room temperature (25 ± 3 °C).

The reactor was fed with a synthetic wastewater that resulted from the mixture of two concentrated media (A and B) prepared according to de Kreuk et al. (2005) and salty water. This latter was prepared by dissolving Red Sea salts (Red Sea ®) in water. In each treatment cycle, 89 mL of each concentrated media was mixed with 772 mL of salty water. The concentrations of ammonium (NH_4^+), phosphate (PO_4^{3-}), and COD in the wastewater were 47, 18, and 331 mg/L, respectively. The organic loading rate and nitrogen loading rate were 1.01 kg COD/m^3/d and 0.14 kg N-NH_4^+/m^3/d. The reactor operation lasted for 286 days and was divided into two stages. In stage I (from day-0 to day-130), the wastewater salinity was progressively increased, as follows: 1.5 g/L from day-0 to -19 (slightly saline); 5 g/L from day-20 to -31 (moderately saline), 10 g/L (highly saline) from day-31 to -88, and 15 g/L from day-89 to -130 (highly saline). In stage II (from day-131 to day-286), the seawater intrusion phenomena were mimicked by varying the wastewater salinity concentrations along the day, alternating four cycles of high salinity (7.5 g/L) with four cycles of very high salinity levels (22.5 g/L). The classification of salt concentrations was made based on the saline water classification reported elsewhere (The Chemistry of Processes in the Hydrosphere, 2007).

An unexpected phenomenon of overgrowth of filamentous bacteria occurred shortly after the beginning of stage I. Some control strategies were applied to avoid this overgrowth, as follows: (I) reduction of COD concentration in the wastewater by half (166 mg/L) from day-41 to day-89; (II) daily nitrogen (N_2) supply at the bottom of the reactor for 23 days (day-62 to day-85), during the last 10 min of the feeding phase; (III) re-seed of the reactor with fresh AGS biomass from Frielas' WWTP on day-74 to restore microbial balance by enriching the biomass with healthier bacterial populations, particularly those involved in COD and nutrient removal. After the re-seeding process, the AGS biomass concentration in terms of mixed liquor total suspended solids (MLTSS) was 9.86 g/L and had a sludge volume index at 5 min (SVI_5) of 0.05 L/g TSS.

Analytical methods

Wastewater, influent (after anaerobic feeding), and effluent liquid samples were collected twice a week. Samples were filtered through syringe nylon membrane filters (0.45 μm pore-size) to remove the suspended solids and subsequently used for analysis. The ammonium, nitrite, nitrate, and phosphate concentrations were determined using photometric test kits (Spectroquant®, Merck Millipore). The COD concentration was determined using a photometric test kit (Hach® Lange) suitable for samples with chloride levels up to 20 g/L. The levels of pH and salinity were monitored using appropriate probes.

Total and volatile suspended solids (TSS and VSS, respectively), MLTSS, and SVI_5 were assessed along the operation according to standard methods (APHA, 1998). Bed height was measured daily by directly observing the height of the biomass inside the reactor column.

AGS microbiome analysis

Extraction, purification, and quantification of genomic DNA

Throughout the reactor operation, biomass samples were collected monthly during the aeration phase to ensure that representative samples of the bacterial population within the reactor were taken. After removing most of the reactor's medium from the collection tube, the granular biomass was frozen until further use. Subsequently, the granules were aseptically crushed, and the resulting biomass suspension was used for the extraction of genomic DNA using DNeasy UltraClean Microbial Kit (Qiagen ®). The genomic DNA was purified with the GRS PCR

& Gel band purification kit (Grisp ®) and further quantified through fluorimetry using Qubit (Thermo Fisher Scientific). The purified genomic DNA was stored at −20 °C until sequencing.

DNA sequencing and data analysis

Genomic DNA sequencing was accomplished through Illumina Next-Generation sequencing technology performed at GATC-Eurofins. The procedure included DNA amplification, library preparation, sequencing of the V3-V4 hypervariable region of the 16 s rRNA, and bioinformatic data analysis. The taxonomic abundance, community and clustering profiling, and multiple linear regression were performed on the MicrobiomeAnalyst web-based platform (https://www.microbiomeanalyst.ca/). Bioinformatic data submitted was filtered to remove features that could affect data modeling and subsequently normalized by total sum scaling. Neither data rarefaction nor transformation were performed.

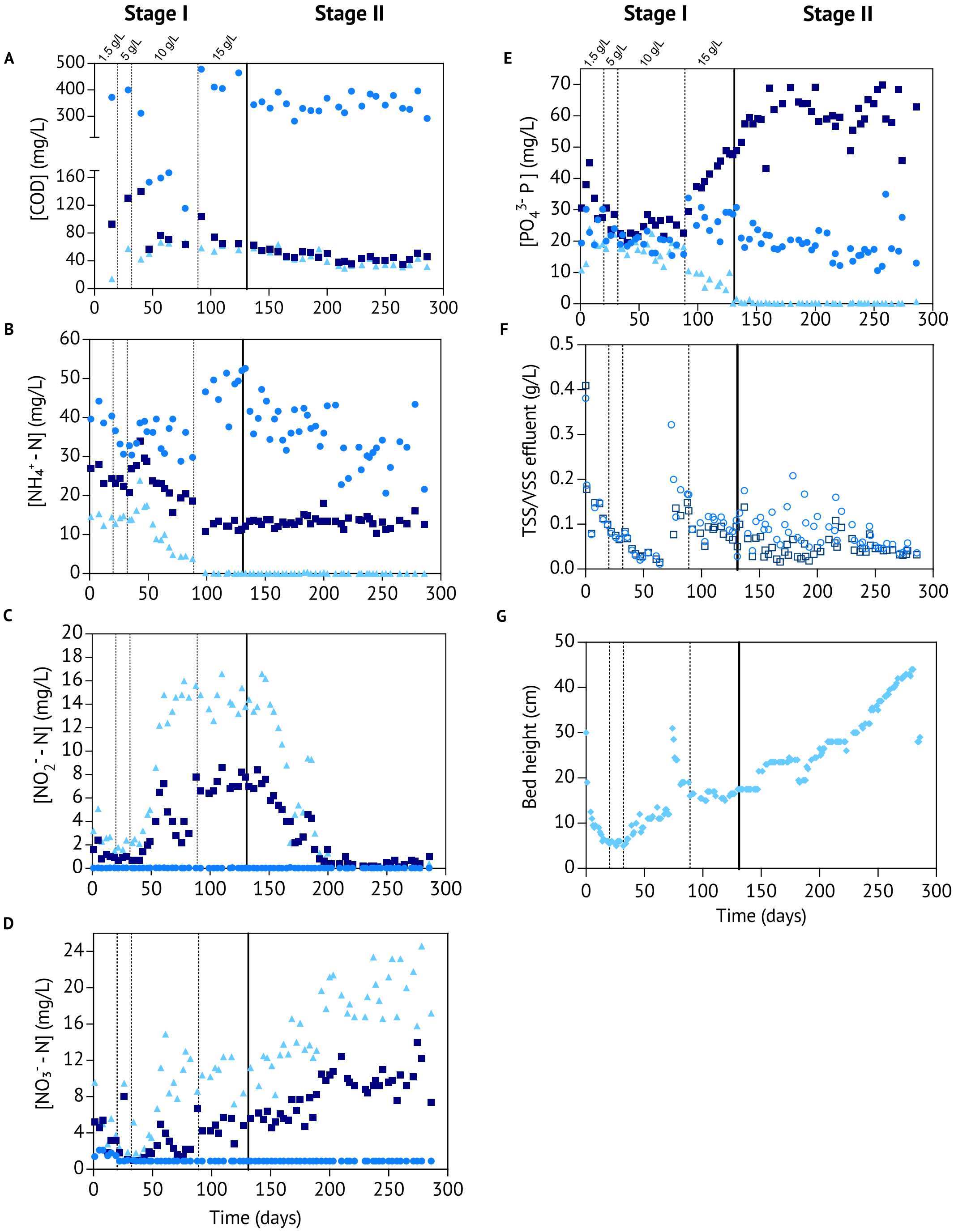

Fig. 1. Concentrations of COD **(A)**, ammonium **(B)**, nitrite **(C)**, nitrate **(D)**, and phosphate **(E)** along the AGS reactor operation. Concentrations in the wastewater (●), inside the reactor by the end of the feeding phase (■), and in the effluent (▲) are presented. The TSS (○) and VSS (□) **(F)** concentrations in the effluent, and the biomass bed height (◆) **(G)** over reactor operation.

Results

Reactor removal performance

The removal of COD, nitrogen, and phosphorus during reactor operation was assessed (Fig. 1**)**. From the beginning of operation until day-41, the COD concentration in the wastewater was quite stable (361.3 ± 44.9 mg O_2/L, on average), with COD levels in the reactor bulk liquid increasing by the end of the anaerobic feeding but decreasing to levels of ca. 38.2 mg O_2/L during the aerated phase (Fig. 1**A**). From day-41 to day-89, when the seawater content in the wastewater was 10 g/L, the COD concentrations in the wastewater were reduced by half, and COD levels after the anaerobic feeding mostly stabilized, and no major COD consumption occurred during the aerobic phase of the treatment. After the AGS re-inoculation on day-74, the COD removal capacity decreased from 61 to 43 %, but a sudden improvement occurred, with 88 % of the COD removed on day-92. Indeed, from day-110 onwards, when the reactor was fed with wastewater containing 15 g/L of seawater salts, the COD levels in the reactor bulk liquid after feeding and in the effluent were very similar (an average difference of 5.7 ± 2.9 mg O_2/L).

During the daily fluctuations of seawater content in wastewater (stage II), the overall COD removal was 87.8 ± 3.0 %, like those observed by the end of stage I. The COD removal was highly stable during this stage, and oscillations of the seawater content in the wastewater over the day did not affect the COD removal, with COD levels in the effluent always below the acceptable discharge limit of 125 mg O_2/L (maximum of 63.6 mg O_2/L on day-158).

In terms of ammonium, from the beginning of stage I to day-54, the average levels at the effluent were ca. 15.3 ± 2.9 mg NH_4^+-N/L. After this day onwards, with the increase of the seawater level in the wastewater to 10 g/L, a gradual decrease of the ammonium content in the effluent was noted, until reaching values near zero on day-92, which were kept like that until the end of stage I, when the seawater concentration in the wastewater was 15 g/L **(**Fig. 1**B)**. During stage I, about 75.1 ± 19.1 % (on average) of the ammonium in wastewater was removed, while in stage II, the AGS system achieved 100 % of ammonium removal.

Regarding nitrite, levels in the bulk liquid after anaerobic feeding and in the effluent increased throughout stage I (Fig. 1**C**). In fact, from day-57 to the end of stage I, when seawater levels increased from 10 to 15 g/L in wastewater, a rising tendency was observed, with nitrite levels in the effluent increasing up to 16.6 mg NO_2^--N/L. From the beginning of stage II, during which the seawater content in wastewater oscillates over the day, the nitrite content inside the reactor after feeding and in the effluent gradually decreased and, from day-193 onwards, nitrite was no longer detected in the effluent.

Nitrate levels detected in the effluent remained low during the stepwise increase in seawater content from 1.5 to 10 g/L, which occurred from the beginning of stage I to day-57 (Fig. 1**D**). As the AGS biomass capacity to remove ammonium increased from day-57 to the end of stage II, the nitrate levels in the effluent also increased, accounting for the main end-product of nitrification.

At the beginning of stage I, phosphate removal was very low (Fig. 1**E**). By the middle of stage I (day-64 onwards), when seawater content in the wastewater reached 10 g/L, the phosphorus metabolism improved, and phosphate release at the end of the anaerobic feeding phase started to occur. By the end of stage I, complete removal of phosphate from wastewater with 15 g/L seawater salts started to occur, continuing like that along stage II.

During the beginning of stage I until day-74, when the seawater content gradually increased from 1.5 to 10 g/L, both TSS and VSS effluent concentrations experienced an accentuated decrease, from 0.38 to 0.01 g TSS/L, and from 0.41 to 0.02 g VSS/L, respectively (Fig. 1**F**). Throughout this period, there was an overgrowth of filamentous bacteria, and to prevent this, the excess of suspended filamentous biomass was weekly removed, resulting in the decrease of solids content in effluent. On day-74, the reactor was re-seeded with fresh AGS biomass, leading to an increase of TSS and VSS effluent concentrations in the following days, which then stabilized until the end of stage I. During stage II, characterized by the daily seawater oscillations in wastewater, the TSS effluent concentration varied, discharging about 0.10 ± 0.04 g TSS/L (on average), while the VSS concentration remained relatively constant (0.05 ± 0.03 g VSS/L). From day-232 until the end of stage II, both TSS and VSS effluent concentrations were kept constant.

From the beginning of operation to day-36, when seawater content increased from 1.5 to 10 g/L, the biomass bed height decreased but then started to increase. The highest bed height (ca. 31 cm) was reached on day-74, coinciding with the re-seed of fresh AGS biomass (Fig. 1**G**). Some biomass was washed out in the following days, leading to a decrease in the biomass bed height until day-103, which remained like that until the end of stage I. During stage II, the bed height increased gradually over time, reaching the highest value of 44 cm on day-280.

AGS microbiome under the influence of fluctuating seawater exposure

AGS taxonomic composition and dynamics

Pseudomonadota was the most dominant phylum in the biomass during the whole reactor operation, with relative abundances higher than 59.4 % on all sampling days (Fig. 2**A**). After the re-inoculation of fresh AGS on day-74, the relative abundance of Pseudomonadota decreased by about 10 %. Nevertheless, this phylum recovered its predominance straight after and, on the two last sampling days of stage I, it was the most dominant phylum (ranging from 88.3 % to 90.1 %). In contrast, during stage II, Pseudomonadota gradually decreased its relative abundance which varied between 61 % and 87 %. Until day-74, the second most represented phylum in the AGS community was Bacteroidota but after the re-inoculation (day-74a), its relative abundance started to decrease until reaching 6.5 % on day-111, when seawater level in wastewater was 15 g/L. Over stage II, the abundance of Bacteroidota tended to increase, achieving ca. 34.6 % by the end of operation. The remaining phyla in the AGS microbiome showed relative abundances lower than 3 % along operation, except for Acidobacteria (day-0).

At the class level, the most abundant ones during reactor operation were Alphaproteobacteria, Betaproteobacteria, Flavobacteriia, and Gammaproteobacteria which together composed at least 85.3 ± 11.4 % (on average) of the total community on each day (Fig. 2**B**). Other bacterial classes were also identified in the AGS community including Chitinophagia, Holophagae, Saprospira, and Cytophagia, which presented relative abundances below 8 % across most sampling days, and Actinobacteria, Bacteroidia, Clamydiia, Clostridia, Epsilonproteobacteria, Ignavibacteria, Oligloflexia, Phycisphaerae, Rhodothermia, and Spirochaetia, which were detected at even lower relative abundances (<3%).

The β-diversity of the bacterial community at the genus level was performed to assess the dissimilarity between the AGS microbiome composition over time. The principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity matrix revealed that the AGS microbiome composition varied over the reactor operation. The analysis revealed the presence of two clusters (represented by red and blue ellipses), with samples mostly distributed along the x-axis (Fig. 2**C**). In this analysis, the proximity of the plotted samples reflects the degree of similarity in their taxonomic composition. Samples from days-0, -89, and -111 (stage I) grouped closely within the same cluster, but samples from days-0 and -89 were much closer to each other than to the sample of day-111. The remaining samples of stage I (days-18, -32, -67, -74(a), and -74(b)) shared similarities in their composition according to their proximity in the PCoA and they were much closer to samples of stage II than to the other stage I samples. Moreover, the sample collected on day-89, 15 days after the AGS re-seed, exhibited high dissimilarity from the sample collected immediately after the bioaugmentation on day-74(a). The last three samples from stage II (days-215, -246, and -277) were relatively close to each other, suggesting the existence of a high

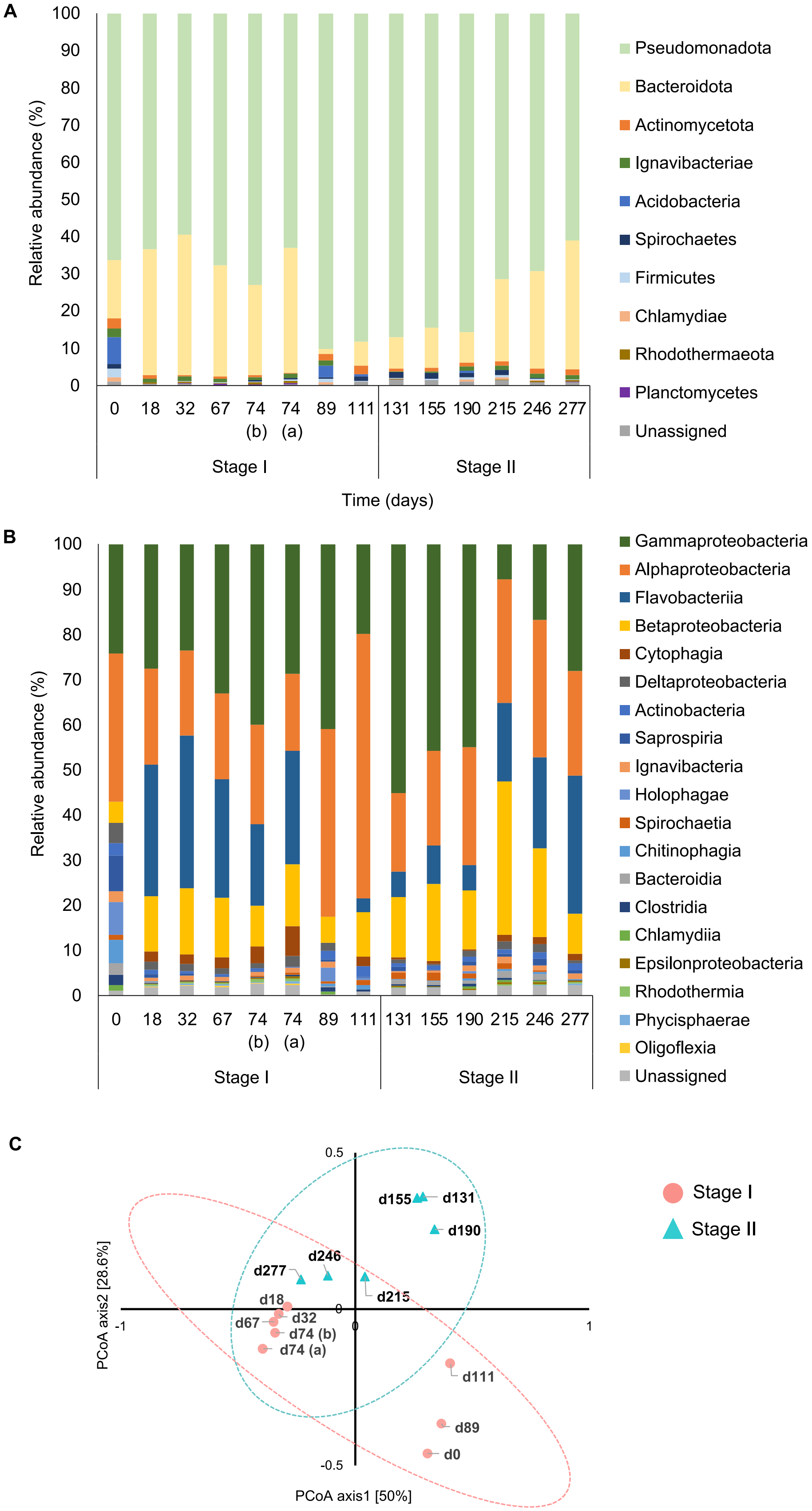

Fig. 2. Relative abundances of bacterial phyla **(A)** and classes **(B)** in the AGS biomass along reactor operation. **(C)** Two-dimensional PCoA plot of beta-diversity based on the Bray-Curtis index for dissimilarity. Samples of each sampling day of stage I (●), and stage II (▲) are presented. PERMANOVA was used for p-value determination, with F = 3.53, $R^2 = 0.23$; *p-value*: 0.03.

similarity in their genera composition. Meanwhile, these three latter samples are closer to samples of stage I than to the ones of stage II. For samples from days-131, -155, and -190, they grouped closely within the blue cluster. Nonetheless, they were relatively distant from the remaining samples of the same stage, and even more distant from those of stage I. Regarding PERMANOVA analysis, a *p-value* of 0.03 ($\alpha = 0.05$) was calculated, indicating a statistically significant difference in dissimilarity among the genera composition of the analyzed samples.

A heat map presenting the biomass core microbiome during the daily oscillations in seawater content (stage II) is shown in Fig. 3**A**. A total of 23 bacterial genera were persistently identified throughout stage II, comprising members of the top 10 dominant bacterial genera on that stage namely: *Aequorivita*, *Azoarcus*, *Arenibacter*, *Mariniflexile*, *Flavobacterium*, *Neomegalonema*, *Pseudorhodobacter*, *Thauera*, *Thiolamprovum*, and *Thiothrix*.

Most of the bacterial genera in the core kept their relative abundances over time, except *Aequorivita*, *Mariniflexile*, and *Thiolamprovum*, whose relative abundances increased over stage II, and the *Thiothrix* genus, whose relative abundance decreased. For the *Aequorivita* genus, with the gradual increase in its abundance over this stage, by the end, it became the most abundant genus in the microbiome, with a relative abundance of 20.3 %. Four bacterial genera namely *Aequorivita, Pseudorhodobacter, Thauera*, and *Thiothrix* stood out with higher relative abundances. The remaining genera had relative abundances below 2 % on every sampling day, including *Alkaliphilus*, *Brevundimonas*, *Frigoribacterium*, *Hyphomonas*, *Ignavibacterium*, *Leadbetterella*, *Lentimicrobium*, *Pseudoarcobacter*, *Rhodobacter*, *Saccharomonospora*, and *Sandaracinus*.

A multiple linear regression analysis was performed using a linear model ($p < 0.05$) to assess the significance of the differences at the genus level between the two operational stages. Ten genera showed significantly higher relative abundances in stage II compared to stage I, including *Arenibacter*, *Brevundimonas*, *Flavobacterium*, *Lentimicrobium*, *Pseudoruegeria*, *Rhizobium*, *Saccharomonospora*, *Thauera*, *Thiothrix,* and *Azoarcus*. Except for the *Pseudoruegeria*, all the latter genera also belonged to the stage II core microbiome.

To ascertain the microbial composition established due to exposure to daily seawater level oscillations in the wastewater (stage II), a depth analysis of the top 10 dominant genera on the last sampling days was performed (Fig. 3**B)**. The relative abundance of *Thauera* and *Aequorivita* genera fluctuated from days-215 and -277, with the *Thauera* abundance decreasing from 25.9 % to 4.2 %, losing its position to the *Aequorivita*, which increased its relative abundance from 10.5 % to 20.3 %. However, on day-246, these two genera had similar relative abundances (ca. 11 %). The *Pseudorhodobacter, Arenibacter,* and *Paracoccus* genera kept their relative abundance across the last three sampling days. Contrarily, the relative abundances of *Thiothrix*, *Thiolamprovum, Defluviimonas*, and *Mariniflexile* genera increased over time, achieving their highest relative abundances on day-277. Apart from *Nitrosomonas* and *Defluviimonas* genera, the other eight genera identified on these three sampling days also belong to the core microbiome (Fig. 3**A**).

Discussion

Efficiency of the AGS system to manage seawater intrusion in wastewater

The nutrient removal capacity remained stable in the AGS reactor exposed to seawater intrusion events, demonstrating the robustness and adaptability of AGS systems in managing salt stress scenarios, which are of increasing concern for WWTPs located near coastal areas.

As for COD removal, neither the stepwise seawater content increase in the wastewater (stage I) nor the daily seawater oscillations (stage II) adversely affected the AGS biomass capacity to remove COD, whose levels in the effluent never exceeded the limit values for the discharge of treated wastewater (125 mg O_2/L), established by the European Commission (Council Directive 91/271/EEC). Nevertheless, at the beginning of the operation, the aerobic COD consumption potentiated the growth of filamentous bacteria on the granules' surface. When filamentous bacteria become dominant in the AGS biomass, nutrient and COD removal efficiencies often decrease because the oxygen transfer can be reduced up to 50 %, limiting oxygen supply to the aerobic

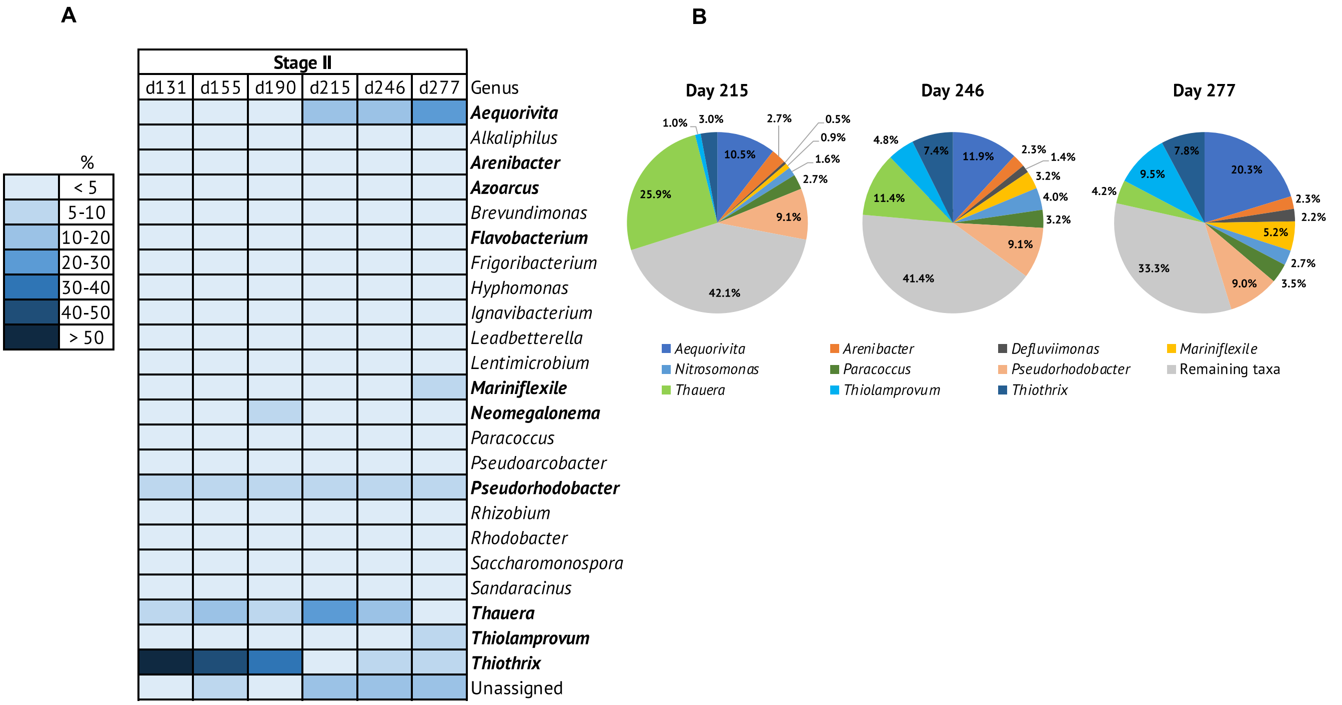

Fig. 3. Heatmap based on the relative abundances of bacterial genera that persisted in biomass over stage II **(A)**. Color changes the intensity from light to dark blue according to the increasing relative abundances. Genera included in the top 10 of most abundant of stage II are highlighted in bold. Top 10 dominant bacterial genera on the last sampling day (day-277) and their relative abundance on days-215 and −246 of stage II **(B)**. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

microorganisms within the granules (Liu et al., 2018). The supply of N_2 for 23 days and the re-inoculation with fresh AGS allowed for the suppression of the filamentous growth on the AGS biomass. The addition of fresh granules was essential to restore the microbial balance within the reactor by increasing the proportion of bacterial populations involved in the reactor's performance, relative to the proportion of filamentous bacteria. Afterward, the system promptly recovered its COD removal capacity during the anaerobic phase. In a study by Carrera et al. (2021), the treatment of industrial saline wastewater with ca. 10.7 g NaCl/L and a COD content of 370 mg/L in a pilot-scale reactor, achieved COD removal efficiencies between 60 to 80 %. Herein, even when the salinity level of the wastewater was 22.5 g/L (more than double that of the latter study), the COD removal efficiency was always higher than 82 %. Previous studies also reported that the COD removal process was unaffected when AGS systems handled seawater-based wastewater (Corsino et al., 2019; Sarvajith and Nancharaiah, 2020; Huang et al., 2019). Even though AGS biomass can be affected by high salinity in wastewater, our results showed that COD removal remained highly efficient at seawater levels up to 22.5 g/L. High salt content often leads to poor sludge-settling properties and even triggers drastic biomass losses, eventually ending in low COD removal rates which were not observed in the present study. In a previous study by Li et al. (2019), no significant impact was observed when AGS was challenged with wastewater containing 10 g NaCl/L (94 % of COD removal). However, increasing NaCl concentration to 20 and 40 g/L deteriorated the COD removal capacities which declined to 72 % and 54 %, respectively. Under those conditions, excess levels of salt can hinder oxygen diffusion into the granules, affecting the metabolism of microorganisms responsible for COD removal (He et al., 2020).

During the early days of operation, the ammonium removal was not complete, probably due to the inhibitory effect that salinity triggered on ammonia-oxidizing bacteria (AOB) activity, which required an acclimation process. It is noteworthy that the acclimation of the allochthonous community in biological systems through a progressive salinity increase is necessary to mitigate the negative impact of salt on nitrogen or phosphorus removal. However, this acclimation period often demands time for the gradual development of a halotolerant population (Pronk et al., 2014; He et al., 2020). Bassin et al. (2011) also verified that an acclimation period of AGS to gradually increasing salinity levels was needed to enhance salt tolerance in AOB. From day-50 onwards, when the seawater content in the wastewater was 10 g/L, a gradual improvement in the ammonium removal performance was observed, with complete removal achieved 42 days later. Nonetheless, the filamentous bacteria overgrowth during that period may have also contributed to the impairment of the overall nitrification process, due to the competition for the available oxygen among AOB, nitrite-oxidizing bacteria (NOB), and filamentous bacteria. After the enrichment of the AGS population over filamentous bacteria, the ammonium removal efficiency was unaffected by either the high seawater content (up to 15 g/L seawater salts) during stage I or the oscillating seawater concentrations in the wastewater that over the day fluctuated from 7.5 g/L to 22.5 g/L. Also, the ammonium content in the effluent was always negligible during stage II, which may indicate that the fluctuations in wastewater salinity did not affect the established AOB population which was able to thrive and reach an efficient removal performance. Consistent with this, Quartaroli et al., (2019) found that increasing the wastewater salinity from 5 to 40 g NaCl/L did not disrupt the ammonium removal efficiency in an AGS system but further salinity increases up to 55 g NaCl/L affected the system performance.

After the AGS recovered the capacity to oxidize ammonium in stage I, the nitrite removal performance started to occur, but not completely, as nitrite and nitrate were the main end nitrification products in the effluent. This implied that the progressive salinity increase, temporarily inhibited the NOB activity, requiring time for NOB to adapt. Indeed, the nitrite levels in the effluent just started to decrease during stage II where the seawater content in wastewater oscillated over the day, with complete nitrite conversion into nitrate only occurring from day-193 onwards. This late response of NOBs to fully restore their biological function was probably caused by their lower tolerance and higher sensitivity to salt compared to AOB (Cui et al., 2016). The deterioration of the NOB activity with the increasing salt content in wastewater was also reported by Bassin et al. (2011), when AGS was exposed to hypersaline conditions (ca. 33 g NaCl/L), with nitrite concentrations in the effluent reaching up to 20 mg/L. The inhibition of nitrite conversion is not surprising, as nitrifiers are in the outermost layer of the granules and are therefore more exposed to stressors. In fact, AOB and NOB seemed to be the first bacterial groups affected by the osmotic pressure resulting from the high salt content in the bulk liquid (Winkler et al., 2013).

Regarding phosphate removal, from the operation beginning to day-64 (stage I), when salinity increased from 1.5 to 10 g/L, both phosphate release during anaerobic feeding and phosphate uptake during the aerated period were compromised. The stepwise seawater increase coupled with the excessive growth of filamentous bacteria over this period were possibly the principal factors disturbing the PAO's ability to remove phosphate. Furthermore, the nitrite accumulation in the bulk reactor liquid occurring at the same time triggered the deterioration of phosphate removal, as the inhibition of the PAO activity is often linked to nitrite accumulation (Bassin et al., 2011; Pronk et al., 2014). When the nitrite content in the effluent was negligible, anaerobic phosphate release and aerobic phosphate uptake occurred efficiently. Similarly, complete phosphate removal from the wastewater likely occurred during the daily oscillations of seawater content in wastewater.

AGS microbiome adaptation to seawater intrusion

Over reactor operation, the AGS bacteriome was highly diverse and dynamic. Pseudomonodota and Bacteroidota, the most representative phyla in the AGS microbiome over the whole reactor operation, were also reported as dominant in the AGS biomass of a reactor treating saline wastewater with 12 g NaCl/L (Ely et al., 2022).

Alphaproteobacteria and Gammaproteobacteria were the two most abundant classes followed by Flavobacteriia and Betaproteobacteria. The dominance of these classes in biomass was previously reported in a study using AGS to treat fish-canning wastewater with salinity concentrations up to 6 g/L (Paulo et al., 2021). Also, Alphaproteobacteria, Betaproteobacteria, and Flavobacteriia classes were detected in high abundance in a study using AGS to treat hypersaline wastewater with 30 g NaCl/L (Niu et al., 2023). Their predominance in the AGS microbiome may be due to the ability of bacteria from Alphaproteobacteria and Betaproteobacteria classes to remove organic and nitrogen compounds, especially at high salinity levels, as these classes can proliferate in such conditions (Chen et al., 2018).

The AGS bacteriome was dynamic during reactor operation. Nevertheless, the PCoA analysis showed that the biomass microbiome shared similarities in its genera composition. At the beginning of the operation, the AGS community composition changed, and later, with the AGS re-inoculation, the community composition reverted to one like that at the beginning. The supply of fresh mature granules ensured the re-establishment of essential bacterial groups, corroborated by the improvement of the biological removal performance, especially evident for phosphate removal. Another shift in the microbiome composition was driven by the seawater content oscillation throughout each day, as the community was adapting to the new salt stress scenario imposed on the reactor, which was also confirmed by the wide genera diversity found in the microbiome.

A core microbiome was identified in the AGS microbiome exposed to daily seawater oscillations (stage II). It comprised a wide variety of bacterial groups related to biological removal processes, that may have guaranteed the stability and effectiveness of the AGS system. Eight of the top 10 most abundant taxa in the biomass by the end of operation also belonged to the core microbiome (Fig. 3**B**). That is the case of the *Aequorivita* genus, which was the most abundant in the AGS microbiome by the end of stage II. Indeed, this genus was previously detected in the AGS microbial community, showing its capacity to survive and grow in extremely saline environments (Yue et al., 2023). It has been also identified as a denitrifying bacterium (DNB), which can play an essential role in maintaining the stability of the AGS community while ensuring efficient denitrification (Zeng et al., 2018). *Arenibacter* was another halotolerant taxon identified in the core microbiome of AGS biomass. This taxon is mostly found in marine microbial communities, but it has also been identified in the microbiome of the biomass from biological systems treating salts-containing wastewater (Zerva et al., 2021). The biological functions of *Arenibacter* can include the synthesis of a wide range of carbohydrates but also the degradation of hydrocarbons and polycyclic aromatic hydrocarbons (Li et al., 2017). These features likely allow members of this genus to adapt well to constantly changing environments or persist in stress scenarios. Despite its low relative abundance on the core microbiome (<5%), *Arenibacter* abundance remained constant during the whole stage II, proving that this genus was well-established in the community and may have helped the system to withstand the salt stress conditions, and eventually, contribute to the stability of the reactor operation. *Mariniflexile* is another marine bacterial genus frequently detected in saline environments and that was identified as a member of the core microbiome. As a bacterium adapted to marine environments, it is not surprising that *Mariniflexile* easily proliferated in the AGS biomass treating saline wastewater. Interestingly, in a previous study, this taxon was also identified in the core microbiome of AGS treating fish-canning wastewater (Paulo et al., 2021).

Moreover, out of the 23 identified bacterial genera identified in the AGS core microbiome, 12 taxa are recognized for their denitrifying capacity namely: *Aequorivita*, *Azoarcus*, *Ignavibacterium*, *Flavobacterium*, *Lentimicrobium*, *Paracoccus*, *Pseudoarcobacter*, *Pseudorhodobacter*, *Thauera*, *Thiothrix*, *Rhodobacter* and *Rhizobium* (Zeng et al., 2018; Trubitsyn et al., 2014; Wang et al., 2021; Szabó et al., 2017; Ding et al., 2023; Tao et al., 2023; Wang et al., 2021; Zhou et al., 2019). The high DNB diversity detected in the AGS microbiome can be attributed to their spatial distribution within granules. DNBs are in the inner region of the granule, in an anoxic/anaerobic microenvironment, which can confer higher protection from the salt stress experienced by bacterial groups located in the outer layer of the granules (Nancharaiah and G. Kiran Kumar Reddy., 2018).

The *Flavobacterium* genus has been identified as a PAO but can also act as a denitrifier and putative extracellular polymeric substance (EPS)-producer (Pishgar et al., 2021; Świątczak and Cydzik-Kwiatkowska, 2018). Over most sampling days of the stepwise seawater increase stage (stage I), it was detected at extremely low abundances, although an increase in its abundance was noted from day-111 onwards (ca. 3.1 %). This coincided with the period when a notable improvement in phosphate removal was also observed, suggesting that the persistence of *Flavobacterium* within the bacteriome during stage II is in line with the excellent phosphate removal performance observed. Despite the sensitivity of PAO to salinity has been extensively reported, our results showed that the salt-acclimation period together with the AGS re-inoculation may have been essential for the establishment of a well-adapted PAO community that enabled to complete restoring this biological function. A similar trend was reported by de Graaff et al. (2020), who stated that biological phosphorus removal can efficiently occur with wastewater containing seawater salts up to 35 g/L and the impact due to NaCl-based wastewater at the same salinity concentration is markedly distinct. The observed increase in the relative abundance of the *Thiothrix* genus from stage I to stage II may have also contributed to the improvement of phosphate removal, considering that certain species within the *Thiothrix* genus (i.e., *Thiothrix caldifontis)* are recognized as putative PAO (Rubio-Rincón et al., 2017). The *Thiothrix* genus is often found in WWTPs and some of their members can grow in up to 10 g NaCl/L and survive in up to 30 g NaCl/L (Ravin et al., 2021), which justifies the persistence of this taxon in the AGS community. In this regard, the presence of *Flavobacterium* and possibly *Thiothrix* may have ensured phosphorus biological removal despite the challenges inherent to the seawater content fluctuations in wastewater.

Concerning nitrification, the slow-growing *Nitrosomonas* genus, mainly responsible for the second step of the nitrification process, was also detected on the three last sampling days of stage II. Notably, NOBs are regarded as highly sensitive bacteria, and any stress condition often leads to nitrification failure (Jin et al., 2010). The presence of *Nitrosomonas* on those days is consistent with complete nitrification, which started to occur in the middle of stage II. This proves that the fluctuating in the salinity level of the wastewater over the day did not compromise the resilience of the AGS system, and even favored nutrient removal processes, demonstrating the resilience of certain bacterial communities to keep their metabolic activity such as the ones responsible for nitrogen and phosphorus removal. On the AGS core microbiome subjected to daily seawater content oscillation in wastewater (stage II), it was also possible to identify four well-known bacterial genera related to EPS production, such as *Thauera*, *Paracoccus*, *Flavobacterium*, and *Neomegalonema* (Paulo et al., 2021; Szabó et al., 2017; Suhonen et al., 2023). The EPS secreted by AGS plays an essential role in the aggregation of microorganisms, granulation, and stability of the AGS, however, their synthesis can also be stimulated by external stimuli. In stressful scenarios such as saline wastewater, an overproduction of EPS is expected to occur which can act as a key polymer to ensure bacterial granule integrity while protecting bacteria against unfavorable conditions of the external environment (Costa et al., 2018; Wang et al., 2021). Literature reports that exposure to salt can lead to changes in the quantity and quality of the EPS produced by AGS biomass (Oliveira et al., 2021; Corsino et al., 2017). The presence of genera related to EPS production during this stage may have contributed to the granular integrity and ultimately promoted the occurrence of biological treatment processes, such as COD and nutrient removal performances that were not impaired by the daily oscillating seawater content in the wastewater over stage II. In this scenario, the EPS producers may have protected bacterial cells from osmotic shocks by providing a physical barrier and helping to maintain the stability of the biofilm, which allows bacteria to survive within it. Ultimately, the core microbiome within AGS biomass showed the thriving of several distinct genera (both halophilic and non-halophilic) under salt stress events, highlighting the resilience and adaptive abilities of the AGS populations when exposed to diverse environmental challenges. It is worth noting that the salt acclimation process during stage I might have contributed to a smoother adaptation of the AGS microbiome to the salt stress, maintaining the functional diversity, which is important for avoiding total system failure. If the AGS biomass was exposed to seawater content fluctuations without any previous acclimation, this might have caused instability in the reactor's performance.

Conclusions

By challenging the AGS to seawater intrusion in wastewater, the following main conclusions can be drawn:

- Regardless of the wastewater seawater content and feeding mode (gradual increase or fluctuating levels), the COD effluent levels consistently met the permissible discharge limits. Filamentous bacteria overgrowth on the granules' surfaces temporally impaired the nitrogen and phosphate removal, but these processes were later enhanced and fully restored after re-inoculation with fresh biomass.
- Stable and effective COD, nitrogen, and phosphate removal performances were achieved even when the seawater content in wastewater fluctuated over the day.
- The AGS bacteriome was versatile and easily adapted to the fluctuating salinity content in wastewater, outstanding the AGS microbial flexibility and robustness.

- Both halophilic and non-halophilic bacteria were present within the AGS microbiome.
- The presence of a wide diversity of EPS-producing bacteria within the AGS microbiome may have protected other bacteria from the osmotic shocks triggered by daily seawater content oscillations.
- The AGS system's capacity to withstand variable seawater levels over a day corroborated the potential of this technology to be used in WWTPs, especially those closer to the coastline.

CRediT authorship contribution statement

Catarina Miranda: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Paula M.L. Castro:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Catarina L. Amorim:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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