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Microbiome adaptation of aerobic granular sludge allows process resilience during the treatment of seawater-based wastewater with pharmaceuticals

Catarina Miranda [a], Alexandra S. Maia [b,c], Maria Elizabeth Tiritan [d,e], Paula M.L. Castro [a], Catarina L. Amorim [a,*]

[a] Universidade Católica Portuguesa, CBQF–Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, 4169-005 Porto, Portugal
[b] Associate Laboratory i4HB–Institute for Health and Bioeconomy, University Institute of Health Sciences - CESPU, 4585-116 Gandra, Portugal
[c] UCIBIO - Applied Molecular Biosciences Unit, Translational Toxicology Research Laboratory, University Institute of Health Sciences (1H-TOXRUN, IUCS-CESPU), 4585-116 Gandra, Portugal
[d] Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia do Porto, Portugal
[e] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Portugal

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A B S T R A C T

Coastal wastewater treatment plants often face multiple stressors (e.g., pharmaceuticals and oscillating seawater levels) simultaneously, and their combined effects on biological treatment systems are still largely underestimated. In this study, an aerobic granular sludge (AGS) reactor was challenged over a four-month period with wastewater that had daily fluctuations in seawater content (7.5 to 22.5 g/L) and occasionally contained two pharmaceuticals, venlafaxine (VNF) and tramadol (TRA), and their metabolites (*O*-desmethylvenlafaxine and *O*-desmethyltramadol), a combination that closely mimics real-world conditions. Over time, pharmaceuticals removal improved, especially for VNF and TRA. For VNF, monitored using an enantiomer-discriminating method, non-enantioselective removal was observed, indicating that the removal most probably occurred through adsorption. Despite the pharmaceuticals' presence in wastewater, the chemical oxygen demand removal was efficient (89 ± 3 %), and ammonium removal was complete, with nitrate as the main nitrification end-product. During the period of sporadic pharmaceuticals presence in wastewater, extracellular polymeric substances (EPS) content within AGS increased up to 196 ± 5 mg/g TSS, possibly contributing to the improvement of pharmaceuticals' adsorption and AGS functional stability. The AGS core microbiome comprised several functional taxa sustaining the system performance under stress exposure. The AGS bacteriome steadily adapted to the changes in wastewater composition, presenting distinct bacterial signatures in each stage. *Paracoccus*, an EPS-producing genus, was enriched during pharmaceuticals load, which may have been crucial for the system's stability. The adaptable and versatile microbiome of the AGS under multiple wastewater stressors contributed to the system's resilience, expanding its applicability for wastewater treatment in vulnerable areas.

1. Introduction

Pharmaceuticals, including antibiotics, analgesics, β-blockers, hormones, and antidepressants, are extensively consumed worldwide to improve human health [1]. The increasing inflow of pharmaceuticals in wastewater due to high consumption, alongside the limited capacity of wastewater treatment plants (WWTPs) to eliminate them and their metabolites [2], has led to their release and accumulation in aquatic environmental compartments at concentrations ranging from ng/L to μg/L [3]. The persistence of pharmaceuticals has brought significant concerns for human and environmental health over the past decades, as many of these compounds can pose serious threats to non-target organisms when present in aquatic ecosystems. In the case of chiral pharmaceuticals, the concern is even higher, as their enantiomers may differ in environmental fate and biological effects, including toxicity [4].

Venlafaxine (VNF), a serotonin-norepinephrine reuptake inhibitor is a chiral pharmaceutical administrated as a racemate and has been used as a first-line pharmaceutical for the treatment of depression and anxiety [5]. VNF can be biologically converted into *O*-desmethylvenlafaxine (*O*-DVNF), its main chiral active metabolite. Therefore, apart from VNF, which was detected at levels up to 260 ng/L and 220 ng/L in influents and effluents of European municipal WWTPs, its metabolite was

* Corresponding author.
E-mail address: camorim@ucp.pt (C.L. Amorim).

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detected at even higher concentrations of 640 and 610 ng/L, respectively [6]. Recently, the metabolite *O*-DVNF was detected at levels 3 times higher than VNF in surface waters with an enantiomeric fraction (EF) far from 0.5 in many sampling points [7]. Furthermore, VNF and *O*-DVNF were recently added to the 4th Watch List of Priority Substances under the EU Water Framework Directive, as they pose significant risks to aquatic environments (Decision 2022/1307/EC) [8]. Consequently, these substances require strict monitoring in wastewater streams before their discharge into the environment. Tramadol (TRA) is a chiral synthetic opioid analgesic commonly prescribed to alleviate acute and chronic pain [9]. TRA presents low biodegradability, and because of this, it has been regularly detected in several water sources, including WWTP effluents (88–416 ng/L) [10], surface waters (3–382 ng/L) [11], and, at extremely low levels, in seawater (< 0.1–1.0 ng/L) [12]. The active chiral metabolite of TRA, *O*-desmethyltramadol (*O*-DTRA), has also been detected in effluents of WWTPs at concentrations ranging from 50 to 245 ng/L [10] and in surface waters with a higher proportion of one enantiomer [7]. Studies have shown that exposure to TRA, even at trace levels, affected the behavior and development of some fish species [13–15]. With the increasing consumption patterns of TRA and VNF, along with the widespread presence of these pharmaceuticals and their metabolites in various aquatic compartments, it is imperative to understand how these compounds behave in WWTPs.

Biological treatment systems have been widely applied in WWTPs, mainly due to their cost-effectiveness, easier maintenance, and lower production of by-products compared to physicochemical methods [16]. However, many pharmaceuticals are resistant to biodegradation and, even at trace levels, can threaten the microbial communities involved in treatment processes [17,18]. Additionally, nearly 90 % of chiral pharmaceuticals are marketed as racemates (including VNF and TRA) [19]. Although enantiomers share identical thermodynamic properties, their metabolization and transformation in biological processes may differ.

Aerobic Granular Sludge (AGS) has revolutionized the biological treatment in WWTPs, emerging as one of the most promising technologies for treating municipal and industrial wastewater. Over two decades, the Nereda® AGS technology has been implemented in over 20 countries across six continents (https://nereda.royalhaskoningdhv.com/). The reason for this success has been attributed to its attractive features, including fast biomass settling rates, high adsorption ability, effective sludge retention, and layered microbial structure that ensures simultaneous organic carbon and nutrient removal [20,21]. In comparison to conventional activated sludge systems, the implementation of the AGS technology significantly reduces land footprint by up to 75 % and lowers energy costs by up to 50 %, making it a more competitive and sustainable alternative [21]. Additionally, the AGS has in its composition a high content of extracellular polymeric substances (EPS), which not only ensure the self-aggregation of microbial cells but also confer protection and structural stability to granules, especially in stressful conditions (e.g., nutrient limitation, high salinity levels, presence of recalcitrant pollutants) [22,23]. Moreover, the EPS matrix also provides binding sites to trap organic and inorganic pollutants in wastewater [24].

AGS has been proven as an efficient technology for managing wastewater containing recalcitrant pollutants, including pharmaceuticals, because of its robustness in keeping the main biological removal processes in the presence of such stressors [17,18,25–28]. Nevertheless, most of the studies have been performed in freshwater [17,18], while studies examining the influence of additional stressors, such as salts, on the removal performance are rare [25,26]. Salts are often present in wastewater. However, in WWTPs that are close to the coast, the wastewater salinity levels can oscillate over the day because of the intrusion of seawater driven by the tides [29]. Indeed, the presence of high and oscillating salt levels in wastewater can affect the metabolism of microorganisms that are unable to thrive under varying osmotic pressures, leading to the collapse of their metabolic activities [30]. Notwithstanding, the effect of the simultaneous presence of oscillating seawater salinity concentrations and pharmaceuticals in wastewater is a combination not yet explored in previous research on AGS systems. To bridge this gap, an AGS reactor was operated for a four-month period for the treatment of seawater-based wastewater that sporadically contained a mixture of two chiral pharmaceuticals (VNF and TRA) and their respective metabolites (*O*-DVNF and *O*-DTRA), all frequently detected in influents and effluents of WWTPs. The fate of the pharmaceuticals in the AGS system exposed to oscillating seawater salinity levels was assessed over two months of intermittent pharmaceuticals feeding, a scenario that closely mimics real-world conditions. In this way, this study aims to evaluate the effect of the simultaneous presence of multiple stressors (pharmaceuticals and seawater salt content oscillations) on the main AGS biological removing processes (organic carbon and nitrogen removal) and to ascertain the system's ability and main mechanisms for pharmaceuticals removal. In addition, the production and compositional variability of EPS was assessed due to its critical role in system stability. The evolution of the AGS microbiome was followed over the operation and linked to the variations observed in the system performance and EPS production.

To our knowledge, this is the first study reporting the efficiency of an AGS system in managing the simultaneous presence of chiral pharmaceuticals and their metabolites and fluctuating seawater salinity content in wastewater. This study provides valuable insights into the effects of wastewater stressors on the AGS microbiome and its influence on the overall system performance, offering practical guidance on the technology's effectiveness in addressing real-world wastewater treatment challenges.

2. Material and methods

2.1. Chemical reagents

Tramadol hydrochloride (TRA) and *O*-desmethylvenlafaxine (*O*-DVNF) were acquired from Merck and Venlafaxine hydrochloride (VNF) from TCI. *O*-desmethyltramadol hydrochloride (*O*-DTRA) was purchased from LGC GmbH. All standards had a purity of >98 %. The stock standard solutions of each pharmaceutical were prepared in methanol or ultra-pure water according to their solubilities to obtain a final concentration of 1 mg/mL and stored at −20 °C in amber glass flasks.

2.2. AGS reactor set-up

A 2.4 L laboratory-scale column reactor with a height of 110 cm and an inner diameter of 6.5 cm was used. The operation of the sequencing batch reactor (SBR) consisted of eight successive treatment cycles of 3 h. Each treatment cycle consisted of four sequential phases: anaerobic feeding (60 min), during which wastewater was introduced at the bottom of the reactor; aeration (112 min), with air sparged at the bottom at an airflow rate of 4 L/min; settling (3 min); and effluent withdrawal (5 min). Approximately 39.5 % of the reactor liquid was discharged in each cycle, resulting in a hydraulic retention time of 7.6 h. The pumps for feeding and effluent discharge were automatically controlled using a Siemens Logo! 230 RC timer. Neither pH nor dissolved oxygen were controlled. The reactor was operated at room temperature, around 25 ± 3 °C. The biomass used to seed the reactor was collected from a laboratory-scale AGS reactor exposed to daily oscillations in seawater salt content for 9 months [31].

2.3. Wastewater composition and operating strategy

The reactor operation lasted for 133 days, and it comprised three main stages during which the stressors in wastewater varied as presented in Table 1. The wastewater used to feed the reactor intends to closely mimic urban wastewater from a coastal WWTP which often experiences seawater intrusion mainly due to tides. For that, two concentrated media (A and B) were prepared, according to the

Table 1
Operating conditions of the AGS-SBR.

Stage	Length of operation	Duration time (days)	Wastewater stressors	
			Daily seawater oscillations (7.5–22.5 g/L)	Mixture of VNF, TRA and their metabolites (8–16 μg/L)
I	0–16	16	✓	X
II[a]	17– 89	72	✓	✓
III	90–133	43	✓	X

[a] Each pharmaceutical/metabolite was added to the wastewater for a period of 48 h, on days 17, 45, 59, 73, 80, and 87.

composition described by de Kreuk et al. [32]. Medium A consisted of CH_3COONa (0.49 g/L), $MgSO_4.7H_2O$ (0.08 g/L), and KCl (0.03 g/L), while medium B was composed of Na_2HPO_4 (0.06 g/L), KH_2PO_4 (0.03 g/L), and NH_4Cl (0.18 g/L). Chemical oxygen demand (COD) and ammonium content in the feeding wastewater were 331 and 47 mg/L, respectively. In each treatment cycle, approximately 89 mL of each media was diluted with 772 mL of saline water, which was prepared using seawater salts purchased from Red Sea®. Seawater intrusion was simulated in the feeding regime by promoting daily oscillations of seawater salinity content in wastewater from moderate (7.5 g/L; low tide) to very high (22.5 g/L; high tide) levels, alternating every two treatment cycles. The salinity concentrations for each tidal phase were chosen based on the findings of the study by Pólvora et al. [33]. During stage II, the wastewater sporadically contained a mixture of two pharmaceuticals (TRA, VNF), and their main metabolites (*O*-DTRA and *O*-DVNF) to simulate the simultaneous presence of different pharmaceuticals and metabolites commonly observed in real wastewater. Target compounds were chosen based on their massive prescription, consumption, and prevalence in the environment. As such, each target compound was individually added to medium A to achieve a final concentration in wastewater that varied from 8 to 16 μg/L. The pharmaceuticals concentration is intended to replicate the variability often observed in real-world wastewater conditions; however, the concentrations were slightly higher than those usually reported in wastewater [34–36] to explore the resilience of the AGS system in such challenging conditions.

2.4. Analytical methods

2.4.1. Physicochemical analyses

Wastewater, influent (after anaerobic feeding), and effluent samples were collected twice a week to monitor the COD and nitrogen removal. Liquid samples were filtered through 0.45 μm nylon syringe membrane filters (Chromafil® PET filters), and the filtrate was subsequently used for analysis. Ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) concentrations were measured using Spectroquant® photometric test kits (Merck), while COD was measured using a Hach® Lange test kit suitable for samples with chloride concentrations up to 20 g/L. The bed height of the settled biomass was measured after 3 min of settling by directly observing the biomass height (in cm) inside the reactor column. Total and volatile suspended solids (TSS and VSS, respectively) concentrations in the effluent were determined according to the Standard Methods for the examination of Water and Wastewater [37]. The electrical conductivity and pH were monitored on each sampling day using conductivity and pH meters.

2.4.2. Quantification of the target pharmaceuticals and metabolites

During stage II, wastewater samples were collected on the first day of each shock load, and effluent samples were taken daily at the same treatment cycle About 250 mL of each collected sample were filtered through 0.45 μm glass microfiber filters (Whatman™), acidified to pH 2.0 with 10 % (*v*/*v*) sulfuric acid, and then underwent solid phase extraction (SPE) using Oasis® Mixed-mode cation exchange sorbent cartridges (150 mg, 6 mL, Waters™) for sample pre-concentration, performed on a 12-position vacuum manifold system (Supelco™ Visiprep). Additional information on the SPE procedure can be found in the supplementary material (SM1). The quantification of each target pharmaceutical or metabolite was performed in an UltiMate 3000 Dionex-ultra-high performance liquid chromatography (UHPLC, Thermo Scientific) coupled to an ultra-high resolution quadruple time-of-flight (UHR-QqTOF) mass spectrometer (Impact II, Bruker) equipment. The chromatographic separation was conducted using a Chiral Stationary Phase column, namely Astec Chirobiotic™ V, 5 μm (150 mm length × 2.1 mm internal diameter, and 5 m particle size) (Sigma Aldrich). The mobile phase consisted of ethanol/10 mM aqueous ammonium acetate buffer (92.5/7.5, *v*/*v*), adjusted to pH 6.8 [38]. Elution of the chiral compounds was performed isocratically at a flow rate of 0.32 mL/min, with a run time of 30 min, and an injection volume of 5 μL. The Bruker Compass Data Analysis software (version 5.0) was used to process the acquired data. Method validation and quality control procedures are described in supplementary material (SM2). The results obtained for linearity and range, quantification and detection limits, and matrix effect are summarized in Table SM1. The recovery, accuracy, and intra- and inter-batch precision are presented in Table SM2.

2.5. Data analysis

Pharmaceuticals, COD, and ammonium removal efficiency from the wastewater were calculated based on the following equation:

$$\textit{Removal efficiency}\ (\%) = \frac{(C_w - C_{effl})}{C_w} \text{x } 100$$

where C_w and C_{effl} are the concentrations of each compound in wastewater and effluent, respectively.

The enantiomeric fraction (EF) was determined using the equation:

$$EF = \frac{C\ (S)}{(C\ (S) + C\ (R)\ )}$$

where C(*S*) and C(*R*) are the concentrations of (*S*) and (*R*) enantiomers of the chiral pharmaceuticals in the effluent.

2.6. Biochemical characterization of EPS produced by AGS

To evaluate the changes in the EPS production and composition over reactor operation, a total of ten AGS biomass samples were periodically harvested from the reactor during stages II and III. To ensure sampling representativeness, mixed liquor samples were collected during the aeration phase of the treatment cycle and subsequently sieved (< 1 mm) to retain only mature AGS. These granules were rinsed twice with deionized water and further used for EPS extraction, which was performed according to Felz et al. [39]. Shortly, 1 % (*w*/*w*) of AGS underwent alkaline extraction in 0.5 % sodium carbonate aqueous solution. The mixture was heated at 80 °C with constant mixing at 400 rpm for 35 min. Subsequently, the solution was centrifuged, and the resulting supernatant was precipitated with 1 M hydrochloric acid to achieve a pH of 2.2. EPS, in the form of a gel-like pellet, were obtained after centrifugation. The biochemical composition of EPS was characterized in terms of their main components: proteins and polysaccharides. To this end, each EPS sample was dissolved in a 1 M NaOH solution and mixed until reaching pH 8.5. The resulting liquid solution was then used to determine the EPS content in proteins and polysaccharides, following the modified Lowry [40] and anthrone sulfuric [41] colorimetric-based methods, respectively.

2.7. AGS microbiome composition analysis by Next-Generation Sequencing technology

During stages II and III, fourteen AGS samples were collected during the aeration phase of the treatment cycle to ensure sample representativeness. The AGS was crushed in sterile conditions, and genomic DNA was extracted using the DNeasy PowerSoil ProKit (Qiagen®), following the manufacturer's instructions. Genomic DNA purification was performed using GRS PCR & Gel band purification kit (Grisp®), and the concentration of the purified DNA was subsequently estimated with the Qubit fluorometer (Thermo Fisher Scientific). The purified genomic DNA was further stored at −20 °C until sequencing.

Illumina Next-Generation Sequencing technology of the fourteen genomic DNA samples was performed at GATC-Eurofins. The process comprised a multi-step procedure, that included DNA amplification, library preparation, sequencing, and bioinformatic data analysis. Paired-end gene sequencing of the V3-V4 region of the 16S rRNA gene was carried out on the Illumina Miseq sequencing platform using two specific primers, namely 357 F (5'-TACGGGAGGCAGCAG-3′) and 800 R (5'-CCAGGGTATCTAATCC-3′).

The MicrobiomeAnalyst web-based platform (https://www.microbiomeanalyst.ca/) was used to analyze the AGS taxonomic abundance, community profiling (alpha- and beta-diversity, and core microbiome), and perform sample comparisons. The raw sequence data of the AGS samples was deposited in the NCBI database with the BioProject accession number PRJNA1201194 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1201194).

2.8. Statistical analysis

Statistical analysis was conducted using the SPSS software (SPSS Inc., New York, USA, version 29). The Shapiro-Wilk was used to verify the normality of the data distribution, and Levene's test to evaluate the variance homogeneity ($p > 0.05$). Differences in the concentrations of each EPS component were assessed using a One-way ANOVA followed by Tukey's post hoc for multiple mean comparisons, with statistical significance defined as $p < 0.05$.

3. Results and discussion

3.1. AGS reactor performance

3.1.1. Fate of target pharmaceuticals and metabolites in the AGS reactor

During stage II, apart from daily oscillations of the seawater content in wastewater, like in stage I, the AGS biomass was intermittently exposed to a mixture of TRA, *O*-DTRA, VNF, and *O*-DVNF in wastewater. No previous acclimatization of the AGS biomass to the target compounds was performed. The concentrations of the different pharmaceuticals and metabolites in wastewater and effluent during the six intermittent shock loads are shown in Fig. 1.

TRA concentration at the effluent on the first dosing day of each

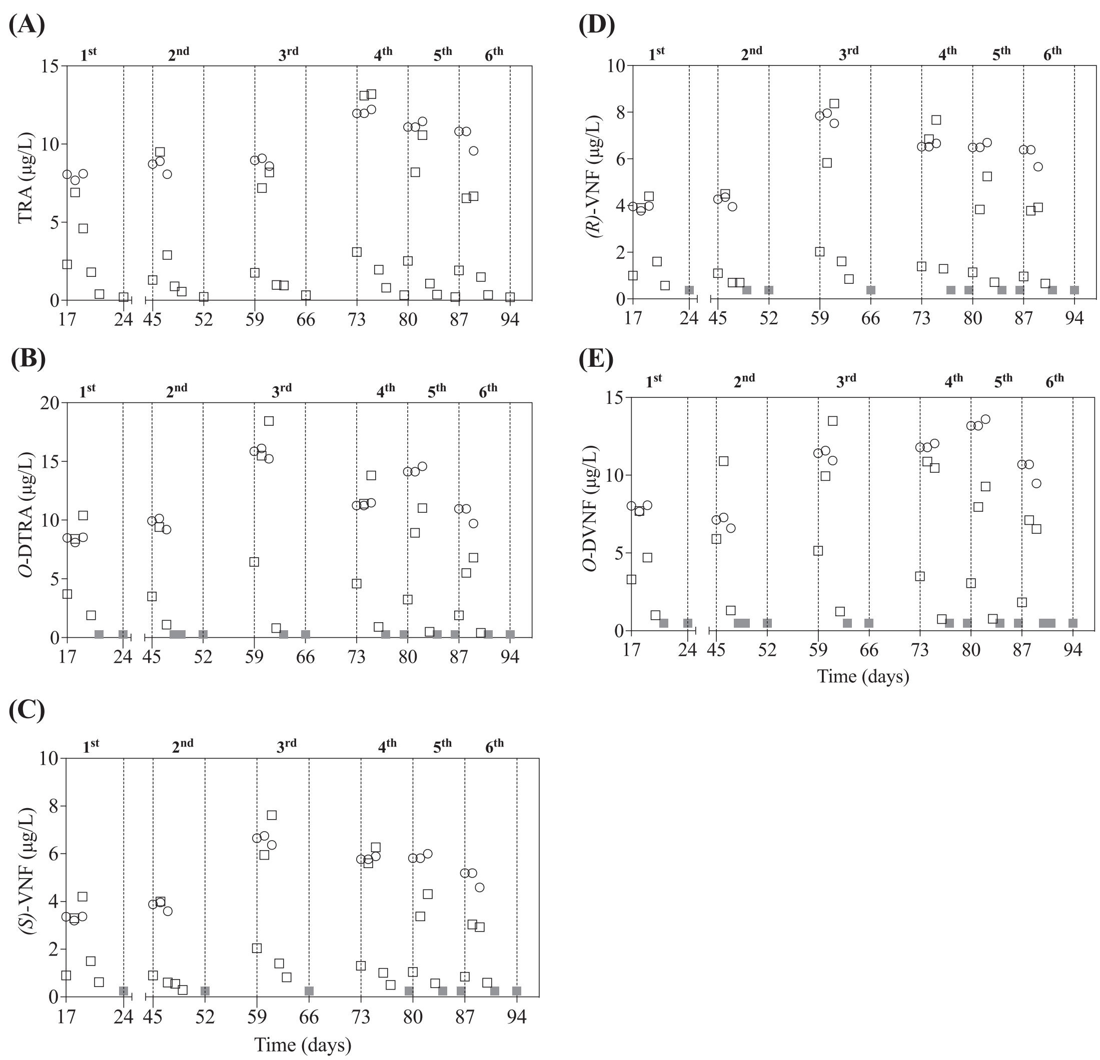

Fig. 1. Concentrations of TRA **(A)**, *O*-DTRA **(B)**, (*S*)-VNF **(C)**, (*R*)-VNF **(D)** and, *O*-DVNF **(E)** in wastewater (○), and effluent (□) during each pharmaceutical shock load. Effluent analyte levels that were detected below the method quantification limit (MQL: 0.25 μg/L for *O*-DTRA, 0.25 μg/L for (*S*)-VNF; 0.372 μg/L for (*R*)-VNF, and 0.5 μg/L for *O*-DVNF) are presented by light gray squares (■).

shock load was relatively lower compared to that in wastewater, varying from 1.3 to 3.1 μg TRA/L, indicating that the system was able to remove ca. 85 to 74 % on the first dosing day of 2nd and 4th shock loads, respectively (Fig. 1A). Biodegradation was unlikely to occur in the period of the treatment cycle as the AGS community was not enriched with specific degrading cultures nor acclimated to TRA over long-term periods, as reported before [42]. Therefore, the adsorption of TRA onto AGS biomass seems to be the main plausible mechanism for TRA removal. During some shock loads, especially on the 2nd and 3rd days, effluent concentrations of TRA were found to be higher than those in wastewater. This suggests that TRA accumulated in the reactor bulk liquid during previous treatment cycles, leading to a substantial increase in their effluent concentrations. After ceasing the load of wastewater with the mixture of pharmaceuticals, TRA concentrations in the effluent steadily decreased, and after 7 days, concentrations were markedly reduced, reaching trace effluent levels (0.2 ± 0.1 μg TRA/L, on average for all shock loads). Overall, TRA removal was achieved across all shock loads, although not completely, consistent with the previous findings on TRA removal in activated sludge systems [10].

Conversely, the removal of *O*-DTRA was lower than that of its parent compound (Fig. 1B). During the 1st to 4th shock loads, the system removed over half of the *O*-DTRA dosed in the wastewater, with the lowest removal efficiency observed on the first dosing day of the 1st shock load (56 %). However, from day-80 onwards, *O*-DTRA concentrations at the effluent gradually decreased, thus improving the removal efficiencies observed on the first dosing day of the 5th (77 %) and 6th shock loads (83 %). In adsorption processes, the removal efficiency relies on the physicochemical characteristics of the target compounds, for instance, the octanol/water partition coefficient (log K_{ow}), a parameter that defines the adsorption of pharmaceuticals residing in the water phase onto sludge [43]. Molecules with log $K_{ow} < 2.5$ have low adsorption potential, $2.5 < \log K_{ow} < 4$ have medium adsorption potential, and log $K_{ow} > 4$ present high adsorption potential [44]. Although both compounds are considered to have low adsorption potential based on their log K_{ow} values, the *O*-DTRA has a lower log K_{ow} (1.72) compared to TRA (2.45), suggesting that TRA is more likely to adsorb onto AGS than *O*-DTRA.

The analytical method enabled the monitoring of the behavior of *(S)* and *(R)* enantiomers of VNF on the AGS system (Fig. 1C, D) and thus to estimate the EF. For all shock loads, the concentration of VNF enantiomers in the effluent was very similar, with average EF values varying from 0.47 ± 0.01 to 0.49 ± 0.02 on the 6th and 3rd shock loads, respectively. The EF values were close to 0.5 in all shock loads, revealing that VNF was removed in a non-enantioselective form (no preferential removal of *(S)*-VNF over *(R)*-VNF) which is a typical behavior of an abiotic removal mechanism, in which adsorption is included [45]. As observed for other target compounds, the adsorption of both VNF enantiomers was more pronounced in the last shock loads, reaching removal efficiencies of up to 84 % for (*S*)-VNF and 85 % for (*R*)-VNF on the first dosing day of the 6th shock load (Fig. 1C, D). VNF in the bulk reactor liquid was in a partially positively charged form, as the pH was ca. 7.66 ± 0.18, below its pKa (8.9). As such, adsorption may have occurred through electrostatic interactions between the positively charged groups of VNF, and the negatively charged groups of the EPS present in the outer surface of AGS [24]. Previous studies have also reported the adsorption of several types of emerging contaminants onto AGS, both in saline and non-saline wastewater [17,18,25–27]. Amorim et al. [27], reported the capacity of AGS for the removal of several chiral pharmaceuticals, including VNF. Higher removal efficiencies for most of the pharmaceuticals were achieved; however, the pharmaceuticals were the sole stressor in wastewater, and as such the influence of the salinity was not considered. The fact is that salt sources in wastewater may compete with other compounds for binding sites, which may potentially hinder the adsorption capacity of AGS for pharmaceuticals, as previously speculated by Oliveira et al. [26].

Removing the *O*-demethylated metabolites of TRA and VNF proved to be more challenging for the AGS biomass than the removal of their parent compounds. Nevertheless, the AGS system progressively improved its capacity to adsorb *O*-DVNF over time, with effluent concentrations decreasing from 3.1 μg/L to 1.8 μg/L, which corresponded to *O*-DVNF removal efficiencies of 77 % and 83 %, reached on the first dosing day of the 5th and 6th shock loads, respectively (Fig. 1E). WWTPs often do not fully eliminate *O*-DVNF, which has been detected at concentrations as high as 1600 ng/L in effluents from a Swedish WWTP [46]. In a study by Rúa-Gómez et al. [47], removal rates of 56 % for VNF and 41 % for *O*-DVNF were reported in a German WWTP operating with activated sludge, with biodegradation proposed as the main removal mechanism. In this study, adsorption prevailed over biodegradation, as indicated by the concentration profiles and by the EF, in the specific case of VNF.

3.1.2. *Reactor treatment performance*

The reactor performance in terms of the main biological removing processes and AGS settling properties is presented in Fig. 2. During stage I, in the absence of the target pharmaceuticals in wastewater, a high and stable COD removal efficiency was observed (88.9 ± 4.7 %), with average COD levels in the effluent of 39.4 ± 9.1 mg O_2/L (Fig. 2A). In general, the intermittent feeding with pharmaceuticals (stage II) did not compromise the COD removal, achieving low effluent levels of 37.5 ± 8.8 mg O_2/L (on average). When pharmaceuticals were no longer present in wastewater (stage III), the AGS system kept an efficient COD removal capacity, confirmed by the low average levels in the effluent (37.8 ± 11.2 mg O_2/L). No major differences were observed in the overall COD removal performance along operation, whose content in the effluent (maximum 60.8 mg O_2/L on day-98) was always far below the emission limit value established by the European Commission of 125 mg O_2/L (council directive 91/271/EEC). Moreover, from days-18 to −49, COD was predominantly consumed during the anaerobic feeding phase of the treatment cycle. Nonetheless, a progressive increase of COD levels after anaerobic feeding was noted from day-54 to the end of stage II, with COD levels increasing up to 110.4 mg O_2/L on day-84. This was probably explained by a slight inhibition exerted by the pharmaceuticals on the metabolic processes involved in COD uptake during the anaerobic phase of the cycle. Later, in stage III, COD levels after the anaerobic feeding phase returned to levels achieved in stage I, underscoring the AGS system's resilience under the presence of pharmaceuticals. Previous studies also reported a similar trend in COD removal by AGS when exposed to fluoxetine (0.93 mg/L) or tetracycline (300 μg/L) in wastewater, as minimal COD levels were consistently found in the effluent [17,28]. Likewise, COD removal performance was not impaired when an AGS reactor was fed with increasing sulfamethoxazole levels (500–750 μg/L), with COD removal efficiencies similar to those found in this study [48]. Contrarily, the COD removal efficiency was impacted in an AGS reactor continuously exposed to a mixture of pharmaceuticals (diclofenac, naproxen, trimethoprim, and carbamazepine) dosed at concentrations of up to 7 mg/L each for 180 days; however, a stable COD removal was achieved 100 days later [49]. A similar result was achieved in the study by Amorim et al. [27], who reported a decrease in COD removal performance when an AGS reactor was fed with a mixture of eight chiral pharmaceuticals (each dosed at 1.3 μg/L). The authors indicated that the presence of pharmaceuticals had a higher impact on COD uptake during the anaerobic phase of the cycle, consistent with the findings of this study. However, both AGS systems were able to recover after ceasing the feeding with pharmaceuticals.

Concerning nitrogen removal (Fig. 2B), ammonium (NH_4^+-N) content in wastewater was completely consumed during the aerobic phase, with average effluent levels of 0.06 ± 0.07 mg NH_4^+-N for all reactor stages. As such, ammonium removal performance was stable and effective, reaching 100 % removal efficiencies over time, revealing that the nitrification was not impaired by the pressure of pharmaceuticals or high salinity levels, showing the robustness of the ammonia-oxidizing bacteria (AOB) to cope with the applied stressors. This is in line with

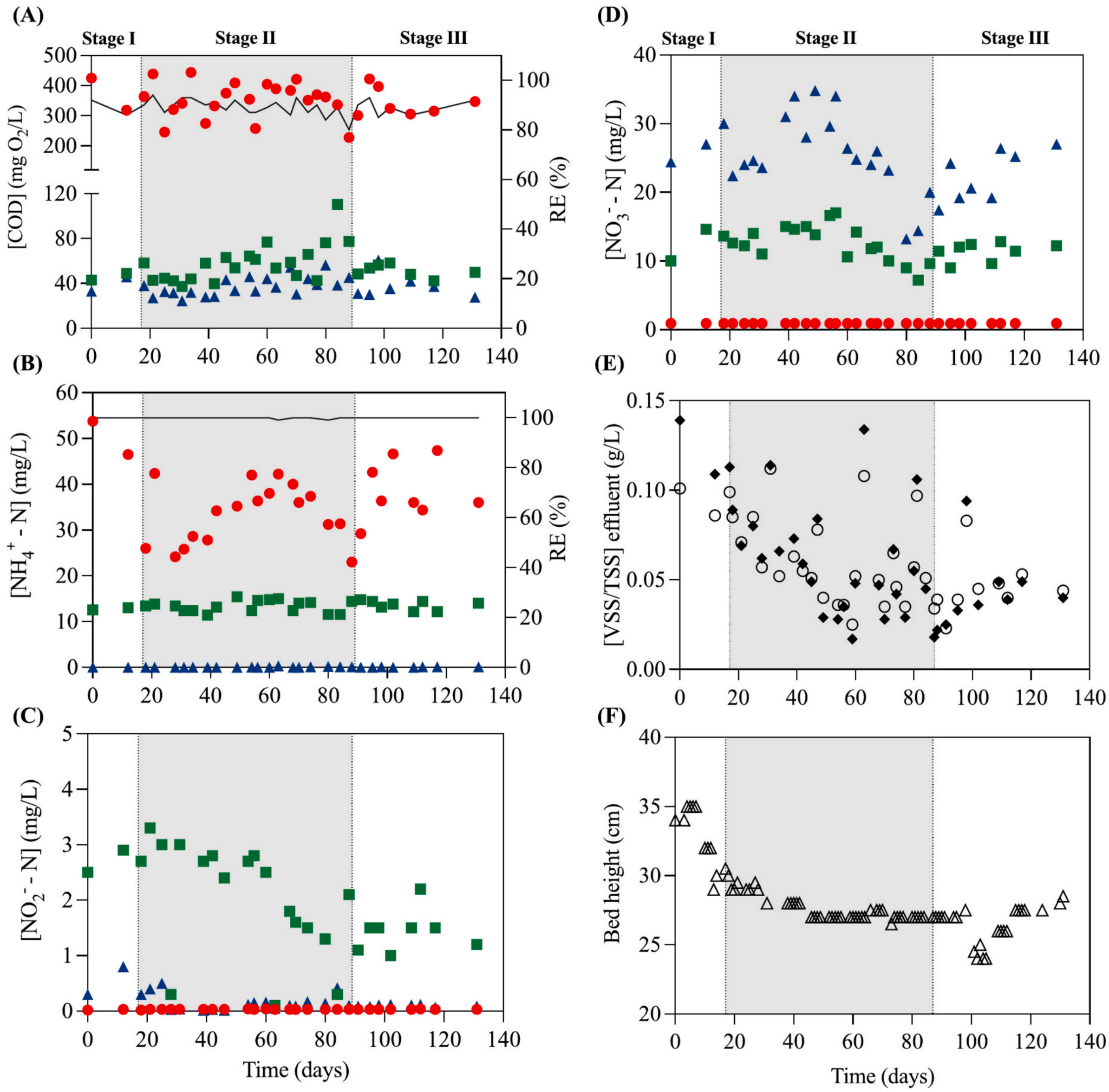

Fig. 2. COD **(A)**, NH_4^+-N **(B)**, NO_2^--N **(C)**, NO_3^--N **(D)** concentrations along AGS reactor operation. COD and NH_4^+-N removal efficiencies (RE %) are plotted on the right y-axis. Concentrations (mg/L) in wastewater (●), in the reactor bulk liquid by the end of the anaerobic phase (■), and in the effluent (▲) are shown. The TSS (◆) and VSS (○) concentrations in the effluent **(E)**, and the biomass bed height (△) over reactor operation **(F)**. The gray-shaded area covers the period during which the wastewater contained a mixture of pharmaceuticals (stage II).

the findings of the study by Zhang et al. [50], who reported efficient ammonium removal in an AGS system subjected to increasing doses of tetracycline (up to 500 μg/L). Similarly, effective ammonium removal was reported in an AGS reactor dosed with 0.93 mg/L of the antidepressant fluoxetine [17]. Other studies have shown contrasting results, with ammonium removal performance severely affected in granular systems exposed to a mixture of antibiotics [51] or the simultaneous presence of pollutants and salt [26]. Also, Shi et al. [52] reported a temporary inhibition of COD and ammonium removal in an AGS system exposed to fluoroquinolone antibiotics (900 μg/L), which may be attributed to the detrimental effects of antibiotics on the metabolic activity of bacteria.

As for nitrite (NO_2^--N), levels after the anaerobic feeding phase and at the effluent were consistently low in all reactor stages (Fig. 2C). These findings proved that the first and second steps of the nitrification effectively occurred, possibly supported by the AOB and nitrite-oxidizing bacteria (NOB) communities present in the AGS biomass whose metabolic functions were not affected by the pharmaceuticals nor by the salinity oscillations in wastewater. This aligns with existing studies showing an efficient NOB activity in AGS-based systems exposed to pharmaceutical stress [17,48].

Most of the time, nitrate (NO_3^--N) content in the reactor after the anaerobic feeding was stable, achieving ca. 12.25 ± 2.36 mg NO_3^--N/L (on average) in all stages (Fig. 2D). An increasing trend of the effluent nitrate concentration started to be observed on day-39, with values over 31 mg NO_3^--N/L, probably due to a slight inhibition of the denitrification process due to the exposure to pharmaceuticals. However, from day-59 to the end of stage II, effluent concentrations of nitrate and nitrite decreased, indicating a gradual recovery of the denitrification process. Nevertheless, nitrate was the main end-product of nitrification during the whole operation. Indeed, a similar accumulation pattern was observed in AGS systems treating wastewater with pharmaceuticals namely: a mixture of fluoroquinolones [18], sulfamethoxazole [48], or diclofenac [53].

The AGS reactor profile in terms of VSS and TSS at the effluent is shown in Fig. 2E. During stage II, TSS and VSS levels at the effluent gradually decreased, with the lowest solid concentrations achieved on day-59 (0.017 g TSS/L and 0.025 g VSS/L). In stage III, when the pharmaceuticals were no longer present in wastewater, the solids content kept relatively constant with levels around ca. 0.04 g/L for TSS and VSS. The variations in the biomass bed height over reactor operation are presented in Fig. 2F. From day-18 to the end of stage II, bed height values ranged from 27 to 30.5 cm. Even though subtle variations in biomass bed height were observed over this stage, that could be due to the periodical collection of AGS samples for monitoring the AGS microbial composition and EPS biochemical composition. Overall, the presence of the stressors in wastewater had no major effects on the biomass bed weight or TSS/VSS concentrations. This result is consistent with a previous study by Castellano-Hinojosa et al. [54], who exposed a continuous-flow AGS-based system to very low concentrations (1.5 to 60 ng/L) of distinct anticancer drugs, with results exhibiting no significant effect on effluent suspended solids. In contrast, the long-term feeding with ofloxacin, norfloxacin, and ciprofloxacin (at mg/L levels) in an AGS system led to an increase in the biomass effluent solid content, demonstrating that these antibiotics compromised the granular integrity [18]. Apparently, in the current study, the selected pharmaceutical concentrations may have been too low to affect the structure and integrity of the granules, or it could be because the pharmaceuticals used are not antibiotics, which typically exert selective pressure on microbial communities and can compromise granule integrity.

3.1.3. EPS production and biochemical composition

Evaluation of the EPS produced by AGS over operation and their content in polysaccharides (PS) and proteins (PN) is shown in Fig. 3, with the sum of PS and PN representing the total EPS production. An increase in the total EPS concentration became noticeable on day-66 (172.9 mg/g TSS), with the highest content achieved later, on day-80 (196.4 mg/g TSS). Moreover, the total EPS concentration on day-80 was significantly higher ($p < 0.05$) than the one obtained immediately before the 1st shock load (day-17), when the wastewater was free of pharmaceuticals. These results suggest a late response in the EPS production due to the presence of pharmaceuticals in wastewater, requiring approximately 49 days for the AGS biomass to considerably increase its PN and PS content in EPS. Similarly, Oliveira et al. [26], who explored the effect of 2-fluorophenol (20 mg/L) and moderate salinity content (6.5 g NaCl/L) in an AGS reactor, observed that the two stressors triggered EPS production, but the response did not occur instantaneously after first exposure, it took time to occur. In terms of PS, concentrations differed significantly ($p < 0.05$) among samples during stage II, ranging from 10.9 ± 0.4 to 20.9 ± 0.7 mg/g TSS, reported on day-59 and day-80, respectively.

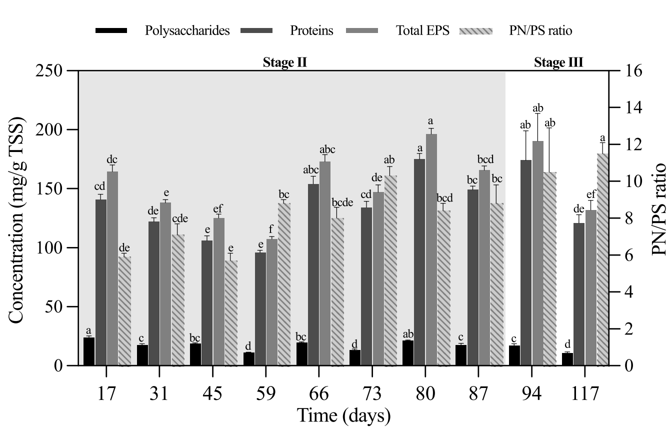

Fig. 3. EPS production by AGS. The polysaccharide (PS) and protein (PN) concentrations, total EPS content, and PN/PS ratio are shown. Results are shown as means ± SD ($n = 3$). Columns showing different letters within the same group are significantly different from each other according to Tukey's test at $p < 0.05$. The gray-shaded area covers the period during which the wastewater contained a mixture of pharmaceuticals (stage II).

PN was the most dominant component in all EPS samples, varying from 96.4 ± 1.9 to 175.4 ± 4.8 mg/g TSS, observed on day-59 and day-80 of stage II, respectively. When pharmaceuticals were no longer present in wastewater (stage III), PN concentrations increased up to 173.5 ± 25.1 mg/g TSS (on day-94), whereas PS decreased to levels of 10.6 ± 1.05 mg/g TSS (on day-117), yielding the highest PN/PS ratio during this stage.

The PN/PS ratio, which is related to aggregation and stability of AGS [55], increased over time, almost doubling from 5.9 to 11.5 in 100 days. Moreover, the relative hydrophobicity of EPS can also be estimated using the PN/PS ratio which, herein, increased during the feeding with pharmaceuticals, suggesting a greater hydrophobicity of the produced EPS, and perhaps more available binding sites for the adsorption of the pharmaceuticals, like that reported by Zhang et al. [56]. This is consistent with the gradual improvement of the pharmaceuticals' removal observed over the shock loads in stage II (Fig. 2).

Previous studies have also reported an overproduction of EPS, especially of the PN component, when AGS-based systems were challenged with different pharmaceuticals [50,52,57]. Salinity itself can also trigger EPS production. In fact, a direct relationship between these variables was previously established by Corsino et al. [58], who demonstrated that increasing NaCl concentrations in wastewater led to a rise in main EPS components, with PN showing the most significant increase. Under saline conditions, PN in EPS can attenuate the effects of the elevated osmotic pressure between bacteria and the bulk liquid, thereby creating a protective environment against salt stress [58]. In this study, the combination of the multiple stressors in wastewater may synergistically have triggered the EPS production, thereby contributing to the excellent tolerance of AGS to such stressors. This possibly occurred because the EPS produced on the outer surface of the granules may have offered special microbial protection against the stressors in wastewater. The adsorption of the target pharmaceuticals on the functional charged groups of EPS (e.g., carboxyl, phosphate, hydroxyl, sulfhydryl) present on the surface of the granules might have prevented their cellular diffusion and thus protected the functional bacterial groups from the pharmaceuticals' action [24]. In this sense, the EPS produced by AGS was crucial in maintaining the optimal equilibrium of the system operation by sheltering the functional bacterial groups, which, in turn, ensured the simultaneous removal of COD and ammonium in the presence of the target pharmaceuticals.

3.2. AGS microbiome dynamics under pharmaceuticals and seawater oscillations scenarios

3.2.1. Alpha and beta diversity

The alpha and beta diversity were estimated to follow the changes in the AGS microbiome over stages II and III (Fig. 4). The Chao1 index estimates the species richness by measuring the number of species in a community (Fig. 4A). At the beginning of stage II (day-17), species richness was low, gradually increasing until day-59 and slightly decreasing from this day onwards. This suggests that the pharmaceuticals initially selected a highly rich community, but by the middle of this stage, community richness decreased and stabilized as only certain species could sustain their populations under prolonged exposure to stressors, which may be linked to the toxicity of the pharmaceuticals, that probably affected the survival of such bacterial populations. After ceasing the supply of pharmaceuticals (stage III), the species richness decreased as fewer species were able to persist without the selective pressure of pharmaceuticals. The Shannon index, which measures the species diversity within a community, showed that diversity was low at the beginning of stage II (day-17), but increased straight after the beginning of the pharmaceutical shock loads, possibly because this stress promoted a greater diversity in the bacterial community (Fig. 4B). The Simpson index, accounting for both species richness and evenness,

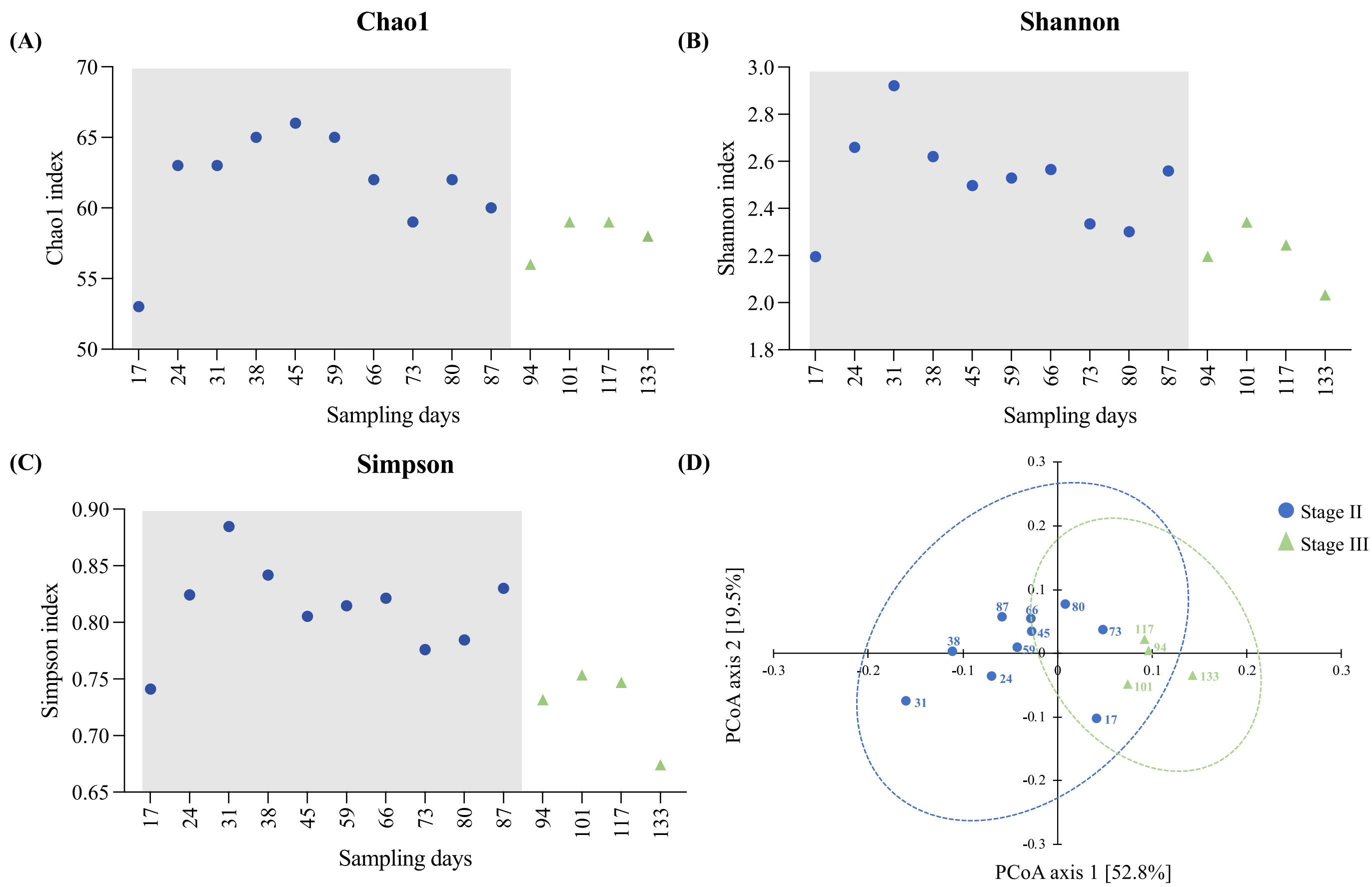

Fig. 4. Alpha diversity of the AGS bacterial community, at the genus level, for stages II and III explained by Chao1 **(A)**, Shannon **(B)**, and Simpson **(C)** indexes. Two-dimensional PCoA plot of AGS bacterial genera beta-diversity based on Bray-Curtis index for dissimilarity (PERMANOVA; F = 6.07, R^2 = 0.34, $p < 0.002$) **(D)**. Sampling days of stage II (●), and stage III (▲) are presented. The gray-shaded area covers the period of stage II during which the wastewater contained a mixture of pharmaceuticals.

shared a very similar pattern to the Shannon index, and thus, a less diverse bacterial community was observed in stage III, when the wastewater was free of pharmaceuticals (Fig. 4C). These results demonstrated that when AGS biomass was exposed to pharmaceuticals, their presence in wastewater might have created selective conditions where certain species could thrive alongside others. During stage II, the pharmaceuticals may have inhibited the predominance of some species in AGS that would otherwise outcompete others, allowing less competitive species to thrive, thus increasing the overall diversity. Moreover, the increase in EPS concentration during this stage might also have supported the survival of a greater number of species. In stage III, in the absence of pharmaceuticals, dominant species could reassert themselves, leading to reduced diversity. Zhang et al. [50], also reported an increased microbial diversity in an AGS system exposed to tetracycline up to 200 μg/L, concomitant to higher EPS production; however, further increase of the tetracycline concentration to 500 μg/L led to a decrease in EPS production, as well as in microbial community diversity and richness.

The beta diversity of the bacterial community was performed to compare biomass sample composition at the genus level. A principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity metric revealed a temporal variation in the AGS microbiome composition, with samples of each stage being mostly separated along the x-axis, indicating that there is a significant shift in the community structure between stages II and III (Fig. 4D). Biomass samples from stage II exhibited notable dispersion in the PCoA, implying high dissimilarity among them. AGS samples from days-24, −31, and− 38 are also somewhat distant from the remaining samples of stage II, showing that AGS community composition evolved during this stage. Conversely, samples from the middle to the end of stage II shared great proximity among them, and thus, the variations in their community composition are expected to be low, suggesting that these samples present a distinct bacterial population signature. Initially, the AGS bacterial community appears to be responding to a new selective pressure, which ended up in the middle of stage II in the development of a new microbial niche with a stable composition. A new shift in bacterial communities occurred in stage III, when pharmaceuticals were no longer present in wastewater, demonstrating the restructuring of the community. As such, samples of stage III (days-94, −101, −117, and − 133) have a high degree of similarity among them but are relatively far from samples of stage II. Interestingly, the AGS sample from day-17 (taken before the 1st pharmaceutical shock) and samples from days-94, −101, and − 117 are relatively close to each other, suggesting that by the end of the operation, the AGS microbiome may have reverted to a similar composition to that encountered before the AGS exposure to pharmaceuticals.

3.2.2. Evolution of the AGS microbiome composition and dynamics

The AGS bacteriome composition was assessed to unveil the key bacterial genera in each operational stage (Fig. 5). In general, the microbial community composition at the genus level changed over AGS operation, indicating a progressive adaptation of the microorganisms in response to the variations in wastewater composition. Independently of the wastewater stressors, *Thiolamprovum* was the most dominant genus on all sampling days. However, its average relative abundance was variable over the operation, ranging from 37.8 ± 5.2 % to 49.3 ± 3.8 %, observed in stages II and III, respectively. *Nitrosomonas*, a well-known

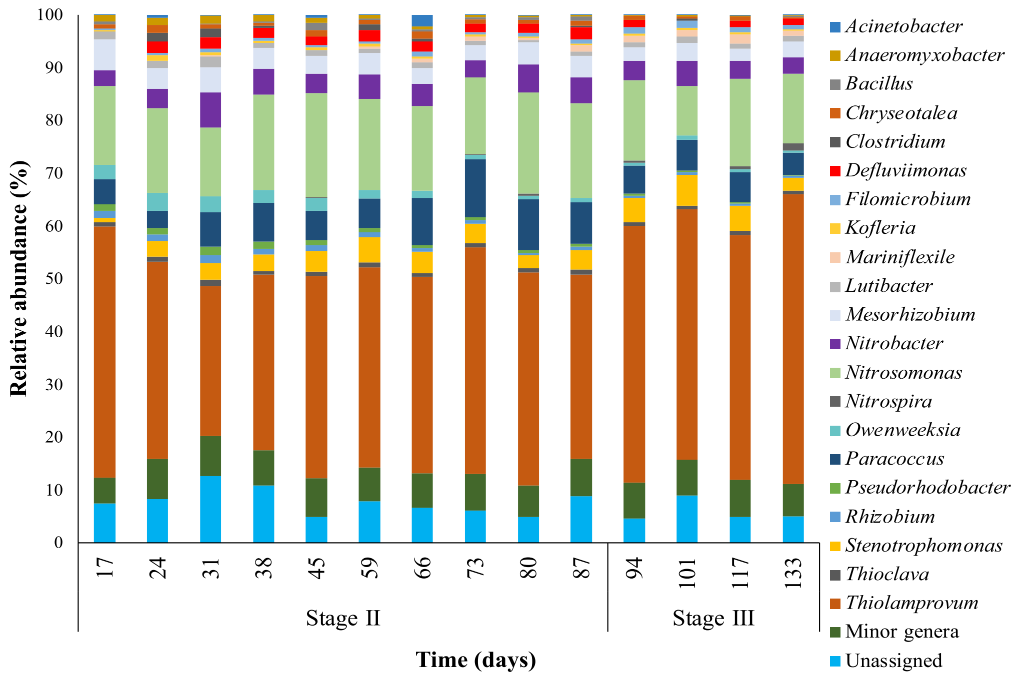

Fig. 5. AGS relative abundances at genus level on different sampling days of stages II and III. The group "Minor genera" includes all bacterial genera with relative abundances <1 % on all sampling days whereas "Unassigned" includes all taxa not assigned to a specific genus.

AOB, was the second most abundant genus identified in all biomass samples. Nonetheless, the average relative abundance of *Nitrosomonas* was slightly higher in stage II (16.7 ± 2.1 %) than in stage III (13.6 ± 3.1 %). *Nitrobacter*, a NOB genus, was also detected on all sampling days (varying from 3.1 % to 6.6 %), albeit at considerably lower abundances compared to *Nitrosomonas*. *Nitrospira* was the second NOB genus identified in the AGS microbiome, although not consistently. Compared to other nitrifying bacteria, *Nitrospira* had very low average relative abundances (0.2 ± 0.4 %) throughout the operation. Reactor performance indicated that the presence of multiple stressors in wastewater did not compromise the efficiency of the nitrification, as ammonium was entirely removed from the wastewater, and nitrite levels in the effluent remained consistently low (Fig. 2B, C). This is supported by the presence of AOB (*Nitrosomonas*) and NOB (*Nitrospira* and *Nitrobacter*) in the AGS bacteriome during the operation. Among NOB, *Nitrospira* was the most affected genus during stage II, although it was able to recover and proliferate after ceasing the feeding with pharmaceuticals. Indeed, NOB are more sensitive to adverse environments than AOB [59], which may explain the pattern observed in this study. Notably, the persistence of those nitrifying bacterial genera in the AGS bacteriome underscores the extraordinary resilience of the AGS system in sustaining these taxa, irrespective of the pressures imposed by the wastewater.

In addition, other bacterial genera with roles in biological removing processes were identified at variable relative abundances over the operation, including *Acinetobacter* (ranging from 0.03 % to 2.3 %), *Stenotrophomonas* (ranging from 0.8 % to 5.9 %) and *Paracoccus* (ranging from 3.2 % to 11.0 %). Members of these genera comprise heterotrophic microorganisms that have been reported to play crucial roles in EPS production, denitrification, and degradation of several organic carbons [57,60–62]. Accordingly, the presence of these genera in the bacteriome may corroborate the high COD removal efficiencies attained during the operation while contributing to the resilience of the AGS system under wastewater stressors. After ceasing the feeding with pharmaceuticals in stage III, some bacterial taxa were identified at their highest relative abundances, such as *Thiolamprovum* (55 %), *Stenotrophomonas* (5.9 %), *Mariniflexile* (1.7 %), *Filomicrobium* (1.4 %), and *Nitrospira* (1.4 %). Among these genera, *Nitrospira* was also identified in the biomass of AGS treating hypersaline pharmaceutical wastewater; however, as in this study, its abundance was affected by the wastewater composition [57].

Additionally, a linear discriminant analysis effect size (LEfSE) was performed to assess the statistically significant differences in the AGS microbiome (at genus level) between stages II and III (Fig. 6A). Through the LEfSE, it is possible to identify the discriminative biomarkers that characterize each stage by ranking them according to their LDA scores, thus providing a bacterial fingerprint of each operational stage. When the AGS was exposed to pharmaceuticals and their metabolites (stage II), *Owenweeksia, Anaeromyxobacter, Pseudorhodobacter, Rhizobium, Thioclava, Bradymonas, Bdellovibrio, and Oceanospirillum* genera were significantly more abundant in the AGS microbiome. These findings revealed that the simultaneous salt and pharmaceuticals presence selectively enriched the biomass in specific bacterial genera. Perhaps, members of these genera might have a competitive advantage mechanism related to their metabolic ability, adaptation to stress, and/or interaction within the bacterial community allowing them to survive and thrive under this stress scenario. That was the case of *Owenweeksia*, the biomarker with the highest LDA score. Members of *Owenweeksia* have been regarded as active decomposers of organic matter in marine environments [63]. *Anaeromyxobacter* genus has been recognized for its effective mechanisms for bioremediation [64], while *Rhizobium* has been identified as a denitrifier genus with the simultaneous ability to produce EPS [65]. *Bdellovibrio* was also identified in the microbiome of an AGS system treating wastewater containing several antibiotics [66]. Members of the genus *Bdellovibrio* have been reported as predatory bacteria able to attack a wide variety of gram-negative bacteria [67]. This possibly included some gram-negative bacteria that could proliferate under pharmaceutical stress, thus creating an abundant prey population for *Bdellovibrio*, leading to their enrichment during stage II. Despite its competitive advantage over other bacterial populations, *Bdellovibrio* had extremely low abundance in the bacteriome, as it was included in the minor genera population (Fig. 5).

Contrarily, in the absence of pharmaceuticals (stage III), *Thiolamprovum* became one of the six biomarkers of this stage along with the *Mariniflexile*, *Prochlorococcus*, *Candidatus Combothrix*, *Ilumatobacter*, and *Methylocaldum* genera. Among these biomarkers, *Thiolamprovum* and *Mariniflexile* genera were previously identified in AGS treating saline wastewater amended with endocrine disruptors [25]. Most of the

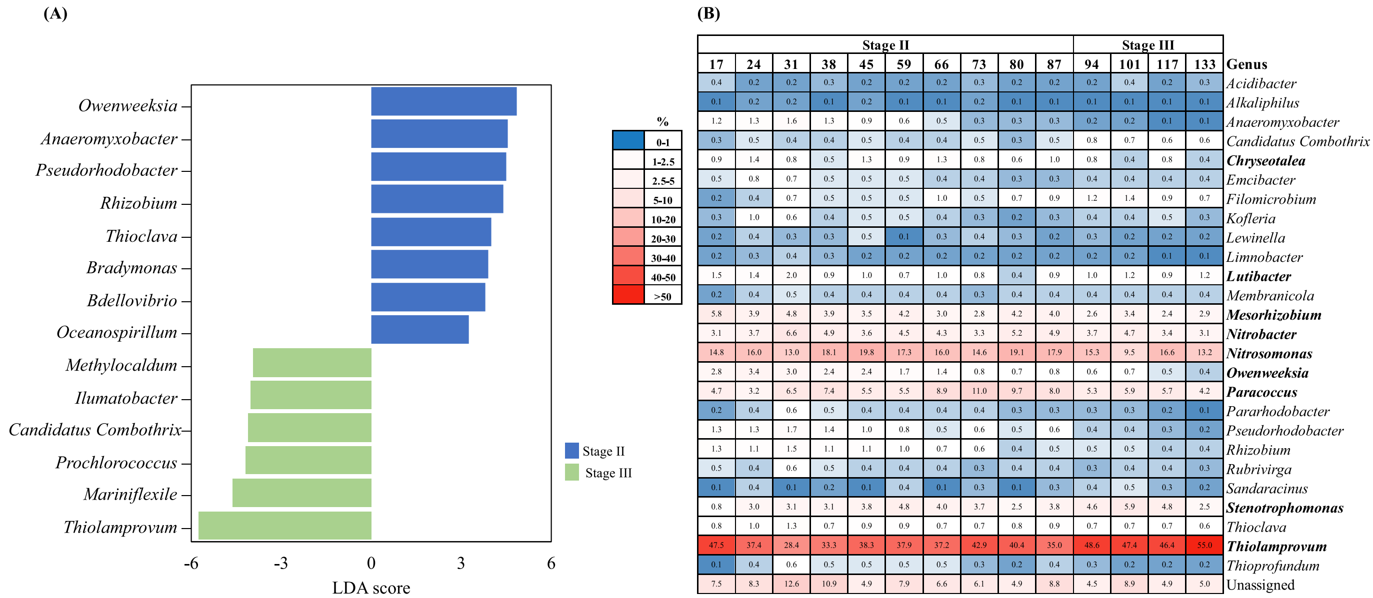

Fig. 6. LEfSE of the AGS microbiome at the genus level, presenting a LDA score > 2.0 and a p <0.05 (B). The most significant genera were listed in descending order according to their LDA scores (represented in the x-axis). Heatmap including the persistent bacterial genera in AGS biomass over stages II and III (C). The top 10 most abundant genera along operation are marked in bold. Color intensity changes from blue to red according to their increasing relative abundances. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

biomarkers were isolated from marine environments [68–70], which supports their enrichment in the bacteriome when the daily oscillations in seawater content were the sole selective pressure in wastewater. Accordingly, a restructuration of the AGS microbial community occurred, since the absence of pharmaceuticals in wastewater eliminated the selective pressure that previously favored other bacteria capable of tolerating such stressors. Thus, a new bacterial population signature was established.

The heap map showing the variations in the abundance of the persistent genera within the AGS core microbiome is shown in Fig. 6B. The AGS core microbiome was vast, with a total of 26 bacterial genera identified, 9 of them belonging to the top 10 most dominant taxa of the bacteriome (marked in bold in the heat map). Most of the genera were detected in the core microbiome at similar relative abundances, irrespective of the changes in wastewater composition. Several bacterial genera of the core microbiome were closely related to system performance functions, namely nitrification (*Nitrosomonas* and *Nitrobacter*), denitrification (*Pseudorhodobacter*, *Paracoccus, Mesorhizobium, Stenotrophomonas*, *Rhizobium* and *Lutibacter*), and EPS production (*Paracoccus*, *Rhizobium, Stenotrophomonas*, *Mesorhizobium*) [65,71–75]. Both ammonium and nitrite oxidizers' microorganisms coexisted in the core microbiome, which aligns with the stable and effective nitrification observed throughout the operation. According to Li et al. [76], the enrichment of slow-growing nitrifying bacteria resulted in AGS with a strong structure and efficient settling capacity, which, in turn, allowed its high stability. A greater diversity of denitrifying bacteria (DNB) genera was found in the core microbiome compared to AOB and NOB. The excellent tolerance of DNB to stressors may be attributed to their location in the inner layer of the granules, where they are more protected from the detrimental conditions experienced on the surface of the granules [77]. Nitrogen performance indicated incomplete denitrification, with nitrate always predominating in the effluent, possibly due to the presence of pharmaceuticals. Even though effluent nitrate concentrations decreased in stage III, considerable levels persisted in the end of the treatment cycle. It should be mentioned that the high levels of dissolved oxygen in the reactor's bulk liquid may have further suppressed denitrifying activity, which occurs in the anoxic core of the granules. A contrasting pattern was observed at the microbiome level, where most denitrifying bacterial genera had higher relative abundances in stage II than in stage III. This could be attributed to the competitive advantage of DNB under pharmaceutical exposure, enabling them to outcompete more sensitive bacterial groups, thus increasing their abundances. This was the case of the *Paracoccus* genus, as higher relative abundances began to be observed from day 66 (8.9 %), reaching its highest abundance on day 73 (11.0 %). However, after ceasing the feeding with pharmaceuticals, its abundance gradually declined to 4.2 %, like the level observed when the wastewater was free of pharmaceuticals (4.7 % on day 17). Members of the *Paracoccus* genus exhibit a crucial role in biofilm formation and stability, mostly due to their capacity to synthesize EPS [78]. Indeed, the organic carbon present in the wastewater can be used by bacteria as a source to produce EPS, which is crucial to ensure granular stability and integrity as well as to offer protection against adverse external environments [75]. Results demonstrated that AGS exposure to pharmaceuticals induced changes in the composition of EPS with the PN concentrations significantly increasing ($p < 0.05$) compared with those reported at the beginning of stage II (Fig. 3). Corsino et al. [79] have reported that the increase of the PN content in EPS reinforces the structural stability of the AGS due to an increase in the hydrophobic properties of the cell surface, which consequently triggers the microorganism aggregation in the EPS matrix. As a result, the granules became denser and stronger enough to withstand the external stressors. Moreover, PN has been regarded as the main EPS component capable of interacting with distinct classes of pharmaceuticals [24].

The higher EPS production (from day-66 onwards) was consistent with the enrichment of the *Paracoccus* and *Acinetobacter* in the microbiome (Fig. 5). These two genera have been reported to be involved in the biosynthesis and secretion of EPS [60,78], which may explain their contribution to the AGS stability and the effective adsorption of the target pharmaceuticals and metabolites onto AGS. Therefore, the pressure exerted by the pharmaceuticals stimulated the synthesis of EPS by specific bacterial populations, and this adaptative response was crucial to alleviate the toxicity of such compounds within the biomass. This aligns with the study by Jiang et al. [57], who reported the enrichment of the *Paracoccus* genus in an AGS system treating diluted pharmaceutical wastewater containing 25 g NaCl/L. As discussed previously, the adsorption onto AGS is proposed here as the main removal mechanism, which may have occurred through the interaction of pharmaceuticals and the binding sites of EPS, thus avoiding the diffusion of pharmaceuticals into the inner layers of AGS. In this sense, the presence of EPS-producing genera in the AGS microbiome may have been essential for creating a thick biofilm layer on the outer surface of AGS, sheltering the functional bacterial groups inhabiting the outer (AOB, NOB) and inner (DNB) layers of the AGS, thereby preserving the biological removal processes.

After the withdrawal of pharmaceuticals from wastewater (stage III), the decrease in the total EPS content was accompanied by the reduction in the abundance of EPS-producing genera in the AGS microbiome. Conversely, other halotolerant taxa were able to reestablish their abundance in the bacteriome and emerged as biomarkers of stage III (Fig. 6A). This was evident for *Thiolamprovum*, whose abundance was initially affected by the pharmaceuticals (28.4 %, on day-31) but progressively increased during stage II (42.9 %, on day-73). However, when daily salinity oscillations became the sole stressor in wastewater, this genus successfully thrived and reached its highest relative abundance (55.0 %, day-133). Consistent with the findings of the current study, the *Thiolamprovum* genus was previously identified in the core microbiomes of AGS exposed to wastewater with high salinity levels [25,31], which may explain its role in enhancing the system's resilience to salt stress.

4. Conclusion

This study assessed the robustness of an AGS system to withstand the short-term loads of two pharmaceuticals and their metabolites (VNF, TRA, *O*-DVNF, and *O*-DTRA), along with daily salinity oscillations in wastewater. Pharmaceuticals were largely removed from the wastewater, with adsorption proposed as the main removal mechanism. Despite the exposure to wastewater stressors, COD and ammonium removal remained effective, with the effluent always meeting the discharge limits. The feeding with pharmaceuticals stimulated EPS production, which has promoted pharmaceuticals' adsorption and avoided their diffusion within AGS. Shifts in the composition and structure of the microbiome revealed its gradual adaptation to the imposed stress conditions. Nevertheless, a core microbiome was maintained and comprised several bacterial genera (e.g., AOB, NOB, DNB, EPS producers) responsible for sustaining the system's performance and stability over operation.

In this study, synthetic wastewater with a controlled composition was used to ensure scientific validity and to develop a comprehensive understanding on the AGS behavior when exposed to combined stressors. Achieving the required level of control would not be feasible with real wastewater due to its unpredictable and fluctuating composition. However, further validation using real wastewater would be valuable to explore AGS behavior in real-world conditions.

CRediT authorship contribution statement

Catarina Miranda: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alexandra S. Maia:** Writing – review & editing, Validation, Methodology, Investigation. **Maria Elizabeth Tiritan:** Writing – review & editing, Validation, Supervision, Methodology. **Paula M.L. Castro:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Catarina L. Amorim:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2025.107592.

Data availability

Data will be made available on request.

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