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Adaptative biological response of aerobic granular sludge to events of single or combined wastewater related stressors

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ABSTRACT

Wastewater comprises various stressors and their individual and combined immediate effects on aerobic granular sludge (AGS) are still underexplored. In this study, the AGS was exposed for 24 hours to wastewater with varying salt concentrations (up to 30 g NaCl L^{-1}) alongside pharmaceuticals (diclofenac, DCF or carbamazepine, CBZ). Differences in extracellular polymeric substances (EPS) production and composition were observed between single and combined stressor exposures. The removal of pharmaceuticals was influenced by the wastewater salinity level and the type of pharmaceutical, with a positive correlation found between the EPS polysaccharides content and the removal efficiency at salinity levels up to 10 g NaCl L^{-1} . The combination of salinity and pharmaceuticals in wastewater also impacted the AGS bacteriome composition, with the bacteriome of the AGS not-exposed to stressors showing greater similarity to that of AGS exposed to DCF than to that exposed to CBZ, at each wastewater salinity level. Functional profiling suggested that short-term exposure to stressors slightly increased the relative abundance of mismatch repair, cell motility, and homologous recombination functions in the AGS microbiome. Summing up, the stressors impact on AGS bacteriome structure and EPS production varies depending on the pharmaceutical and whether it is combined with salt or not. This study unveiled the immediate AGS response to both single and combined stressors exposure, but a more thoughtful characterization of the bacteriome composition over the early adaptation period to stressors is needed to understand the community succession and to identify key microbial groups.

1. Introduction

Wastewater treatment plants (WWTP) receive complex aqueous matrices containing a wide range of chemical and biological pollutants. Concerning chemical pollutants, wastewater often contains several organic and inorganic compounds, such as toxic/recalcitrant organic compounds, ammonium and inorganic salts.

The occurrence of different chemical pollutants in WWTP constitute potential stress factors for the microbial communities involved in the biological treatment process, which can lead to changes in their diversity and functions, ultimately negatively impacting treatment process efficiency (Amorim et al., 2018; Chen et al., 2018; Udaiyappan et al., 2017; Pronk et al., 2014). Among organic pollutants, pharmaceuticals, due to their extensive use in a variety of fields such as medicine, livestock, aquaculture, and people's daily life constitute a great environmental concern and a challenge for wastewater treatment due to their complexity and recalcitrancy (Adeoye et al., 2024). Carbamazepine (CBZ) and diclofenac (DCF) are two persistent and recalcitrant pharmaceutical pollutants commonly found in wastewaters (Adeoye et al., 2024). In wastewaters from different sources, pharmaceuticals often co-exist with other stress factors such as high salinity. For instance, the effluents of the pharmaceutical industries, in addition to containing pharmaceutical compounds, may also contain large amounts of dissolved inorganic salts, e.g. chlorides at concentrations up to 25 g L^{1} , which increase the wastewater salinity (Gadipelly et al., 2014; Monteagudo et al., 2014). High concentrations of salts are also present in urban wastewaters combined with other pollutants including pharmaceuticals, not only due to discharges of saline industrial effluents, but also to discharges from snow melting operations, toilet flushing with seawater, and seawater intrusion into coastal WWTPs, among others

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(Cahoon and Hanke, 2019; Osman et al., 2017).

Current biological treatments used in WWTPs do not fully remove pharmaceuticals, which persist in effluents discharged into the environment. Moreover, these treatments are often disturbed by fluctuations in wastewater composition, particularly when stress factors are present, leading to temporary process disruptions (Amorim et al., 2018; Oliveira et al., 2021). Aerobic granular sludge (AGS) is a recent biological technology that has been successfully applied for the treatment of urban and industrial wastewater (Bengtsson et al., 2018; Paulo et al., 2021a). In fact, the worldwide count of full-scale installations using the AGS technology has been increasing, with over 100 projects currently in operation (https://nereda.royalhaskoningdhv.com/en/projects). The AGS is a special case of biofilm composed of microorganisms immobilized within a self-produced matrix of extracellular polymeric substances (EPS), forming compact spherical sludge aggregates without the need for the addition of carrier materials. The secreted EPS play a key role in the aggregation process of microorganisms but can also contribute for the removal of pollutants from wastewater through sorption onto EPS functional groups (Gao et al., 2011). In addition, EPS protect microorganisms from harsh conditions in wastewater (e.g., the presence of stressors), as the increased production of EPS is a natural bacterial response to stress (Xia et al., 2018). Nevertheless, the biochemical composition of EPS depends on the microorganisms present in the AGS, and the environmental conditions, further determining the response of AGS to recalcitrant pollutants, and consequently the efficiency of the AGS wastewater treatment (More et al., 2014; Wu et al., 2019). Most of studies have examined the effect of single stressors on EPS production (Corsino et al., 2017; Dong et al., 2017; Wei et al., 2015), while changes in EPS due to multiple pollutants, especially at the early stages of exposure when a system disruption frequently occurs, remain underexplored (Amorim et al., 2018; Chen et al., 2018; Pronk et al., 2014). Moreover, the existing research tends to focus on the long-term recovery or stabilization of AGS systems, while the immediate effects on the AGS microbiome and EPS composition, critical for understanding the dynamics of system behavior and optimization of rapid response strategies, remain underexplored.

The aim of this study was to address the gaps mentioned in the literature by assessing the initial adaptative response of the AGS biomass when exposed to single and combined stress factors (pharmaceuticals and salinity), in terms of EPS production and microbiome composition and functions. For this, the AGS was exposed over 24 hours to wastewater with different salinity levels (up to 30 g NaCl L^{-1}) containing either CBZ or diclofenac DCF. The applied pharmaceuticals concentrations was higher than that usually detected in wastewater to replicate a demanding and extreme condition. This will allow for a more thorough understanding of the dynamic microbial processes responding to the sudden shock loading event of combined stressors, mimicking a real-world wastewater treatment scenario, which is critical for developing optimization and control strategies to be applied after possible process disturbances.

2. Material and methods

2.1. Chemicals

Diclofenac (DCF) and carbamazepine (CBZ) of > 98 % of purity were purchased from Sigma-Aldrich (Steinhein, Germany). Stock solutions of the individual pharmaceuticals were prepared at a concentration of 3 g L^{-1} using water as solvent and stored at −20 °C.

2.2. Short-term batch assays

AGS collected from a Nereda® plant at the Frielas urban WWTP, Lisbon, Portugal, was used as inoculum. The AGS was washed twice with sterilized water and sieved to remove fine particles. Aerobic batch experiments were conducted by inoculating the AGS (1 g of wet granules) in 75 mL of synthetic wastewater. Synthetic wastewater was composed by: CH_3COONa 5.17 g L^{-1}, NH_4Cl 1.89 g L^{-1}, K_2HPO_4 0.97 g L^{-1}, KH_2PO_4 0.29 g L^{-1}, $MgSO_4.7H_2O$ 0.89 g L^{-1}, KCl 0.35 g L^{-1}, and 10 mL L^{-1} of a trace element solution (De Kreuk et al., 2005) which results in a wastewater with a COD, ammonia and phosphate content of 330, 46 and 18 mg L^{-1}, respectively. Wastewater containing different levels of salt in the form of NaCl (0, 5, 10, 20 and 30 g L^{-1}) was used. Each tested pharmaceutical (DCF or CBZ) was added to the wastewater to achieve a final concentration of 8 mg L^{-1}. The selection of these pharmaceuticals was based on their recalcitrancy during wastewater treatment. Also, the applied concentrations were higher than those often detected in wastewater streams to simulate an extreme environment imposing a high degree of toxicity to the biomass in the short-term experiment, as followed in other studies (Hai et al., 2018; Sathishkumar et al., 2020). Flasks in which the AGS was exposed to wastewater with different salinity contents but without any pharmaceutical were also included in the experiment as well as flasks with non-saline wastewater and pharmaceutical but without AGS (abiotic control). The flasks were incubated for 24 hours, protected from light, at 25 °C with constant shaking (150 rpm). All the assays were performed in triplicate under sterile conditions.

2.3. Pharmaceuticals concentration in the aqueous phase over time

To analyze the concentration of pharmaceuticals in the aqueous phase, samples were collected at the end of 1, 3, 5, 8 and 24 hours of incubation, centrifuged at 14000 rpm (Hettich Zentrifugen model Universal 320 R, Germany) for 10 min at 4 °C, and the supernatant was collected. The CBZ and DCF concentrations were detected and quantified by high-performance liquid chromatography (HPLC) using a System Gold 126 (Beckman Coulter, Fullerton, CA, USA) equipped a Diode Array Detector. The HPLC separation was performed in a reversed phase 250–4 HPLC - Cartridge LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany), operated in isocratic mode at room temperature, at a flow rate of 1.0 mL min^{-1} and an injection volume of 20 μL. The mobile phase was composed of 75 % of acetonitrile, and 25 % of 0.1 % triethylamine aqueous solution acidified to pH 2.14 with trifluoroacetic acid. The detection wavelength was set to 286 nm for CBZ and 270 nm for DCF.

The AGS removal efficiency for the tested pharmaceuticals was calculated for each time point using the following equation:

$$\text{Removal efficiency (\%) at time } x = \frac{C_i - C_x}{C_i} \times 100, \qquad (1)$$

where C_i and C_x are the concentration of each pharmaceutical in the bulk liquid at the beginning and after x hours of incubation in mg L^{-1}, respectively.

2.4. EPS extraction and biochemical analysis

After the short-term exposure experiments (24 hours), AGS samples were collected by sieving for EPS analysis. The EPS were extracted using the heat method according to previous research (McSwain et al., 2005). Briefly, the AGS samples were washed twice with deionized water, resuspended in 0.05 % of NaCl and grounded using a mortar. The resulting suspension was soaked in an water bath at 80 °C for 30 min, and then centrifuged at 8000 rpm at 4 °C for 20 min. The supernatant was collected as EPS extract. The EPS content in polysaccharides was estimated by the anthrone-sulfuric acid method, using glucose as standard (Frølund et al., 1996). The EPS content in proteins and humic acids was estimated using a modified Lowry method with bovine serum albumin as standard (Frølund et al., 1995).

2.5. AGS microbiome analysis

At the end of the batch assay, the granules were collected by sieving and then crushed. Genomic DNA was extracted using the Power-Soil® DNA Isolation Kit (Qiagen, Hilden, Gr), according to the manufacturer's instructions. A total of 9 samples were selected for Illumina sequencing, three from AGS exposed to each pharmaceutical (CBZ or DCF) and three from AGS not exposed to pharmaceuticals, all at NaCl concentrations of 0, 10 and 30 g L^{-1} in the wastewater. Sequencing of the V3-V4 region of the 16S rRNA gene was performed with the Illumina MiSeq sequencing platform at Eurofins Genomics (Germany) with the primer pair (from 5'-3') 357 F - TACGGGAGGCAGCAG (Turner et al., 1999) and 800 R - CCAGGGTATCTAATCC (Kisand et al., 2002). Raw sequences were demultiplexed according to their index sequences, primers were clipped (no mismatches were allowed) and the sequences were merged using the software FLASH (Magoč and Salzberg, 2011). After being assembled, reads with sizes significantly different from the target region were discarded as well as reads containing "N" bases. Merged reads were clipped to 285 bp to remove low quality bases. Chimeric reads were identified and removed based on the de-novo algorithm of UCHIME (Edgar et al., 2011) as implemented in the VSEARCH package (Rognes et al., 2016). The remaining set of high-quality reads was processed using minimum entropy decomposition to partition the marker gene reads into Operational Taxonomic Units (OTUs) (Eren et al., 2015, 2013).

To assign taxonomic information to each OTU, DC-MEGABLAST alignments of cluster representative sequences to the sequence database [NCBI_nt (Release 2019–10–10)] were performed (Altschul et al., 1990). The taxonomic assignment for each OTU was then transferred from the set of best-matching reference sequences (lowest common taxonomic unit of all best hits). Further processing of OTUs and taxonomic assignments was performed using the QIIME software package (version 1.9.1 (Caporaso et al., 2010). Abundances of OTUs were normalized using lineage-specific copy numbers of the 16S rRNA gene to improve estimates (Angly et al., 2014). All sequences obtained are available at the NCBI platform under the accession number PRJNA648344.

Rarefaction curves were constructed using R software (R version 4.0.0) with *rarecurve* function from *vegan* package (Oksanen et al., 2020). The alpha diversity indices were calculated with functions *diversity*, *specnumber* and with *diversity/log(specnumber)*, respectively, from the *vegan* package.

2.6. In silico metagenome analysis

Functional profile inference was performed using Piphillin software (Iwai al., 2016) based on the OTU sequences and the OTU abundance table obtained from the 16S rRNA gene sequencing data. The results obtained were matched against the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) database of phylogenetically referenced prokaryotic genomes using an identity cut-off of 97 % to obtain a list of KEGG orthologs (KO) and their abundance for each sample.

2.7. Statistical analysis

Statistical analysis was performed using R software (R version 4.0.0, 2020–04–24). Normality distribution of data was tested with *shapiro.test* function in *stats* package and homogeneity of variances was tested using *leveneTest* function in *cars* package (Fox et al., 2021).

Differences in EPS content and composition among salinity levels for each pharmaceutical were tested using one-way and two-way ANOVA (function *aov*) combined with post hoc Tukey's honest significant differences method (function *TukeyHSD*) whenever ANOVA assumptions were met.

Correlation analyses between the removal efficiency of pharmaceuticals and the EPS composition were performed and plotted with *ggscater* function from *ggpubr* package (Kassambara, 2020) using Pearson correlation. Salinity levels were divided in two groups: low salinity, from 0 to 10 g NaCl L^{-1} , and high salinity, from 20 to 30 g NaCl L^{-1} .

The variation in OTU composition among samples was analysed by assessing differences in a dissimilarity matrix built with Bray-Curtis measure (function *vegdist* from package *vegan* in R) after transforming the OTU abundance table into a relative abundance table. Information in the Bray-Curtis dissimilarity matrix of the OTU abundance table was graphically assessed with a principal coordinate analysis (PCoA) using *cmdscale* function (from package *vegan* in R) to create the PCoA ordination. The significance of the observed patterns in the PCoA was analysed by assessing differences in sample scores across PCoA axis 1 (PCoA1) and PCoA axis 2 (PCoA2). The normality test revealed normality for both axis in PCoA2 (Shapiro-Wilk: p value > 0.1). Therefore, patterns were assessed with one-way ANOVA (function *aov*) combined with post hoc Tukey's honest significant differences method (function *TukeyHSD*). In addition, a Bray-Curtis dissimilarity matrix was used to perform an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram with Primer v6 software (Anderson et al., 2008).

3. Results

3.1. Effect of salinity and pharmaceuticals on EPS production and composition

After 24 hours, the granules in all flasks kept their integrity and their EPS content after exposure to the different stress conditions is depicted in Fig. 1a. Overall, the total EPS content was significantly influenced by the salinity level (p value <0.05, two-way ANOVA). Nevertheless, for each wastewater salinity level, the total EPS content differed among pharmaceutical types (p value <0.05, two-way ANOVA for the interaction of factors pharmaceutical x salinity).

Comparing the isolated effect of each pharmaceutical in EPS production among different wastewater salinity levels, distinct trends were observed. In wastewater without salt, higher concentrations of EPS were observed when AGS was exposed to DCF (104.09 ± 2.59 mg g^{-1} TSS) in comparison to the respective controls (92.06 ± 0.98 mg g^{-1} TSS, $p < 0.05$), while the opposite was observed for AGS exposed to CBZ (84.04 ± 1.22 mg g^{-1} TSS, $p < 0.05$). In wastewater with 5 and 10 g NaCl L^{-1}, no significant differences were observed between EPS production in AGS exposed to DCF and those non-exposed to pharmaceuticals, but a significantly lower concentration of EPS was produced in wastewater containing CBZ (83.45 ± 0.83 mg g^{-1} TSS and 85.03 ± 0.92 mg g^{-1} TSS, respectively). At 20 and 30 g NaCl L^{-1}, significantly lower production of EPS was obtained for AGS exposed to DCF (100.55 ± 0.75 mg g^{-1} TSS and 83.97 ± 0.42 mg g^{-1} TSS, respectively) comparing to AGS not exposed to pharmaceuticals (114.65 ± 1.67 mg g^{-1} TSS and 102.51 ± 1.65 mg g^{-1} TSS, respectively). At 20 g NaCl L^{-1}, a significantly lower secretion of EPS was obtained for AGS exposed to CBZ (96.49 ± 0.58 mg g^{-1} TSS) comparing to AGS exposed to DCF or non-exposed to pharmaceuticals. For AGS exposed to 30 g NaCl L^{-1}, the highest values of EPS concentrations were obtained in wastewater containing CBZ (139.11 ± 1.60 mg g^{-1} TSS), while the lowest values were observed in wastewater containing DCF (83.97 ± 0.42 mg g^{-1} TSS).

For AGS free from pharmaceutical exposure, the total EPS production in the absence of salt (92.06 ± 0.98 mg g^{-1} TSS) was similar to that obtained with added salt at 5 and 10 g NaCl L^{-1} (96.04 ± 0.90 mg g^{-1} TSS, 96.11 ± 0.26 mg g^{-1} TSS, respectively) (Fig. 1a), but increased significantly at 20 g NaCl L^{-1} and 30 g NaCl L^{-1} (114.65 ± 1.67 mg g^{-1} TSS, 102.51 ± 1.65 mg g^{-1} TSS, respectively). When DCF was present, the total EPS was, on average, significantly higher in the absence of salts (104.09 ± 2.59 mg g^{-1} TSS), registering the lowest EPS production of 83.97 ± 0.42 mg g^{-1} TSS in wastewater containing 30 g NaCl L^{-1}. On the other hand, when exposed to CBZ, the EPS production in the absence

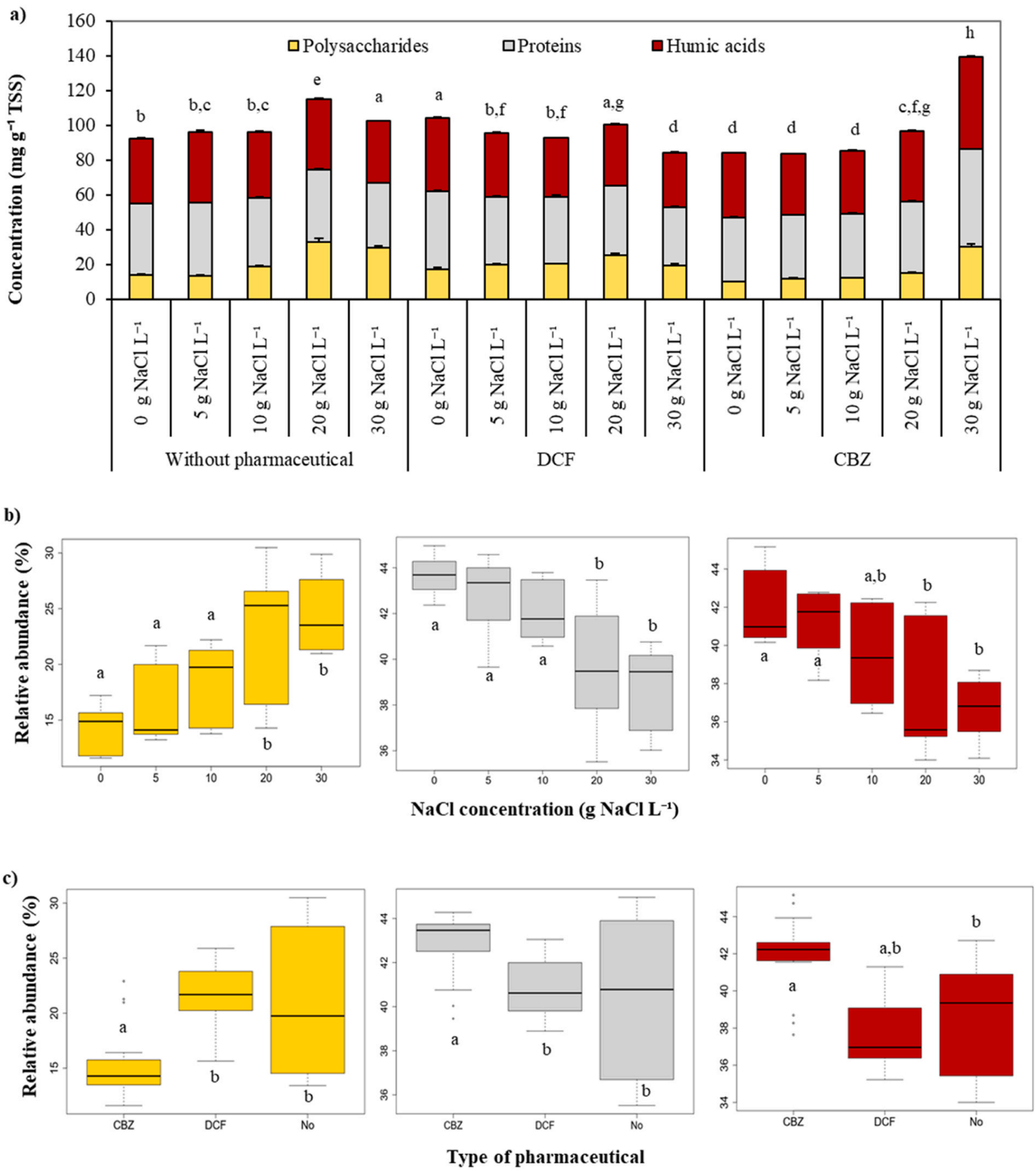

Fig. 1. Total EPS (mg g^{-1} TSS) produced by AGS and its composition (a) in polysaccharides (yellow), proteins (grey) and humic acids (red) after short-term exposure to wastewater with different NaCl concentrations combined, or not, with the pharmaceuticals diclofenac (DCF) and carbamazepine (CBZ). The error bars indicate the standard deviation of the mean of triplicates. Columns that do not share a letter differed significantly in total EPS concentration (Tukey's test at $p < 0.05$). The boxplots represent the distribution of the relative abundance of polysaccharides (yellow), proteins (grey) and humic acids (red) according to the salinity level (b) and the type of pharmaceutical (c) present in the wastewater. Means that do not share a letter in boxes differed significantly according to Tukey's test at $p < 0.05$.

of salt (84.04 ± 1.22 mg g^{-1} TSS) closely resembled that observed with salt at 5 and 10 g NaCl L^{-1} (83.45 ± 0.83 mg g^{-1} TSS and 85.03 ± 0.92 mg g^{-1} TSS, respectively). As the salt content increased to 30 g NaCl L^{-1}, there was a significant increase in EPS production, reaching 139.11 ± 1.6 mg g^{-1} TSS, marking the highest level of EPS produced across all tested conditions (Fig. 1a).

The EPS produced by AGS were mainly composed of proteins and humic acids in all stress conditions applied (Fig. 1a). The highest relative abundance of proteins and humic acids were registered for wastewater with 0 and 5 g NaCl L^{-1} and CBZ (43.70 ± 0.37 % proteins and 44.60 ± 0.42 humic acids at 0 g NaCl L^{-1}; 43.85 ± 0.37 % proteins and 42.10 ± 0.42 % humic acids at 5 g NaCl L^{-1}). The concentrations of proteins and humic acids in EPS varied within a small range, with the lowest content in proteins (36.47 ± 0.35 %) and humic acids (34.97 ± 0.69 %) registered after exposure to wastewater with 30 g L^{-1} and 20 g NaCl L^{-1}, respectively.

As depicted in Fig. 1b, the relative abundance of polysaccharides in EPS composition consistently increased with higher salinity levels, being significantly higher ($p < 0.05$) for salinity levels of 20 and 30 g NaCl

L^{-1} . An opposite trend was observed for proteins and humic acids.

Considering the effect of pharmaceuticals, the average relative abundance of polysaccharides in EPS composition was significantly lower ($p < 0.05$) for AGS exposed to CBZ than for non-exposed AGS, neither for AGS exposed to DCF (Fig. 1c). The opposite was observed for proteins, with significantly higher relative abundance registered in EPS from AGS exposed to CBZ ($p < 0.05$; Fig. 1c). Regarding humic acids, their relative abundance was significantly higher in AGS exposed to CBZ compared to AGS free from pharmaceuticals exposure ($p < 0.05$). No significant differences were observed between AGS samples exposed to CBZ and DCF regarding humic acids content (Fig. 1c).

3.2. The fate of DCF and CBZ over time

The concentration of DCF and CBZ were followed over time (Figs. 2a and 2b). Removal of DCF and CBZ was observed in all conditions, being higher at all time points when flasks contained AGS in comparison with abiotic controls without AGS (Figs. 2a and 2b). In fact, except for AGS exposed to wastewater with CBZ and 30 g NaCl L^{-1} (ANOVA combined with Tukey's HDS test: $p < 0.05$), significant differences between the overall removal efficiencies and each abiotic control were observed (Figs. 2c and 2d).

Overall, the removal efficiency of DCF was higher than that of CBZ at each salinity level (Figs. 2c and 2d). The highest removal percentages were registered for AGS exposed to wastewater containing DCF and 5 g NaCl L^{-1} . In such conditions, the maximum pharmaceutical removal capacity was practically achieved after 8 hours with ca. 31.2 ± 3.6 % of DCF removed (Fig. 2b). From this point onwards, only a minor increase in DCF removal occurred, reaching 36.6 ± 1.5 % by the end of 24 hours. For the remaining wastewater salinity levels, the removal efficiency of DCF was lower, ranging from 13.3 ± 4.2–20.0 ± 3.6 % after 8 hours, and increasing to values between 22.1 ± 0.6 and 34.5 ± 2.3 % by the end of 24 hours. Nevertheless, salts presence in the wastewater, at concentrations ranging from 5 to 20 g NaCl L^{-1} , significantly enhanced the removal efficiency of DCF (Tukey's HDS test: $p < 0.05$). Contrarily to what was observed for DCF, when AGS was exposed to wastewater with CBZ, the maximum pharmaceutical removal capacity was practically achieved at 8 hours to all salinity levels, remaining relatively unchanged thereafter (Fig. 2a). For this pharmaceutical, the highest removal percentages were observed at 5 and 10 g NaCl L^{-1} , reaching a maximum of 21.6 ± 1.94 % and 24.3 ± 2.1 %, respectively. The increase of wastewater salinity to 5 or 10 g NaCl L^{-1} , significantly enhanced the pharmaceutical removal capacity in comparison to that observed in non-saline wastewater (Tukey's HDS test: $p < 0.05$). When increasing the wastewater salinity to 20 g NaCl L^{-1} , the removal capacity for CBZ decreased to ca. 13 %, reaching a similar removal efficiency to that

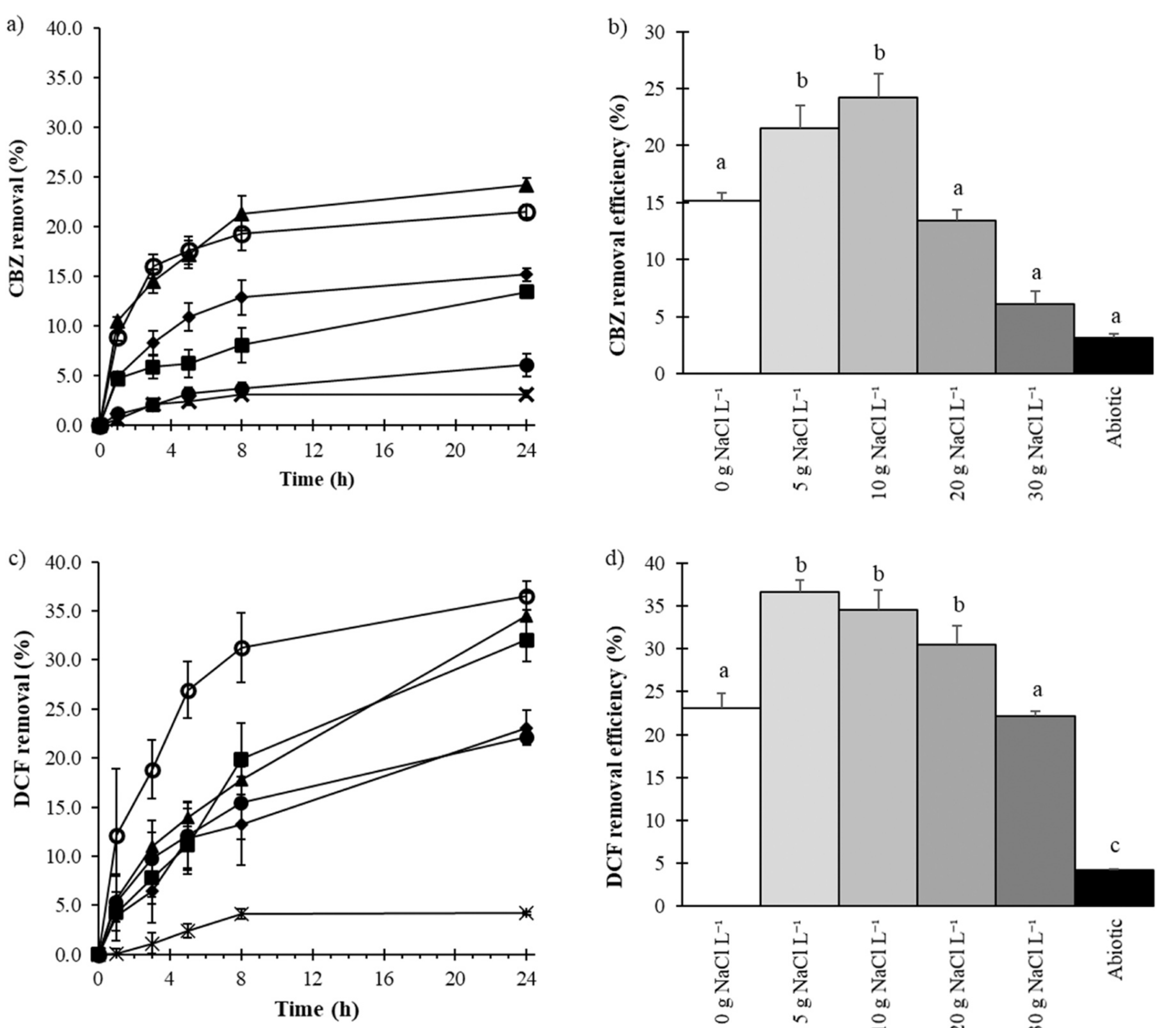

Fig. 2. Removal efficiency (%) of CBZ (a) and DCF (c) over time at different salinity levels (□- 0, □- 5, ▲ - 10, ■ - 20, □ - 30 g NaCl L^{-1}), in the abiotic control (×), and the overall removal efficiency (%) of CBZ (b) and DCF (d) by the end of 24 hours. The columns and data points represent the mean of triplicates and the error bars indicate the standard deviation of the mean. Different letters in columns indicate significant differences for each pharmaceutical removal at the different wastewater salinity levels (ANOVA combined with Tukey's HDS test: p value < 0.05).

observed in non-saline wastewater (Tukey's HDS test: $p > 0.05$).

The removal efficiency of pharmaceuticals depends on the salinity level and on the type of pharmaceutical in the wastewater (two-way ANOVA: $p < 0.05$). In fact, the relationship between the removal efficiency and the wastewater salinity level was dependent on the type of pharmaceutical present since the *p* value of the interaction between both factors was significant (two-way ANOVA: $p < 0.05$).

Even though wastewater salinity levels up to 10 g NaCl L^{-1} improved the overall AGS removal capacity towards DCF and CBZ in comparison to those observed in non-saline wastewater, after 24 hours of exposure more than one half of the initial amount of these pollutants were still present in the wastewater.

Considering the short-term nature of the experiment and the recalcitrancy of CBZ and DCF, the pharmaceuticals removal occurred most probably by adsorption to AGS and not by degradation. The EPS's functional groups at granules surfaces represent possible sorption sites for organic pollutants (More et al., 2014). Therefore, a correlation analysis between the removal efficiency of each pharmaceutical and the EPS composition (Fig. S1) was performed. For each pharmaceutical, a separate analysis was performed for AGS exposed to low salinity (up to 10 g NaCl L^{-1}) and to high salinity (from 20 to 30 g NaCl L^{-1}) wastewater levels.

For AGS exposed to DCF, there were significant correlations ($p < 0.05$) for low salinity wastewater levels, with the polysaccharides relative abundance positively correlated with the removal efficiency (R=0.87) and the opposite for both proteins (R=-0.74) and humic acids (R=-0.71). Therefore, up to 10 g NaCl L^{-1} in wastewater, the increase in the relative abundance of polysaccharides and the decrease in proteins and humic acids contents of the EPS (Fig. 1b) led to an increase of the DCF removal efficiency.

Regarding AGS exposed to wastewater with CBZ, there was a significant correlation ($p < 0.05$) between the relative abundance of polysaccharides and the removal efficiency, which was positive for low levels of wastewater salinity (R=0.91) and negative for high levels of wastewater salinity (R=-0.97). No significant correlations were observed between the relative abundance of proteins and the removal efficiency at low levels of wastewater salinity, although at high salinity levels these two parameters were positively correlated ($p < 0.05$, R=0.95). Regarding humic acids, there was a significant correlation for both ranges of salinity ($p < 0.05$), which was negative for low levels of salinity (R=-0.85) and positive for high levels of salinity (R=0.95). Therefore, up to 10 g NaCl L^{-1} in wastewater, alongside the increase of polysaccharides and the decrease of humic acids relative abundances (Fig. 1b), there was a higher removal of CBZ. From 20–30 g NaCl L^{-1} in wastewater, the concomitant increase of polysaccharides and decrease in proteins and humic acids (Fig. 1b) led to a decrease in the CBZ removal efficiencies.

3.3. Combined effect of salt and pharmaceuticals on bacteriome dynamics

To study the immediate effect of salt and pharmaceuticals on the AGS microbiome composition, the biomass at the end of the exposure was analyzed by high-throughput sequencing analysis. A total of 302,320 high-quality reads were obtained and clustered into 396 OTUs. After removing the unclassified OTUs (3 OTUs, 0.9 % of the total number of high-quality reads) and the ones that assigned to microalgae taxa (6 OTUS, 4.2 %), a total of 287,093 reads remained, which corresponded to 387 OTUs. The rarefaction curve for each sample reached saturation, indicating that the OTUs detected in each sample were representative of the bacterial communities (Fig. S2).

The bacterial communities of the AGS exposed to different conditions were compared by estimating their alpha diversity indexes (Table 1).

From a total of 13 different bacterial phyla detected, 9–10 were detected in each sample, except for the AGS microbiome after exposure to non-saline wastewater with CBZ in which 11 different phyla were detected (Fig. 3a). Independently of the stress conditions, the most abundant phylum was *Proteobacteria*, with relative abundances ranging from 49.6 % to 66.4 %, followed by *Bacteroidetes* (from 16.0 % to 29.6 %). Within the 90 bacterial families detected, the most abundant ones (> 2.0 %) are presented in Fig. 3b. The families that had at least 5 % of relative abundance in each AGS microbiome were *Rhodocyclaceae,* mostly represented by the *Rhodocyclus* genus, *Chitinophagaceae,* mostly represented by *Ferruginibacter and Terrimonas genera, Candidatus Competibacteraceae* represented by *Plasticicumulans genus,* an unidentified family of the order *Cytophagales* represented by *Chryseolinea* genus and *Flavobacteriaceae,* mostly represented by *Chryseobacterium and Flavobacterium* genera. Nevertheless, the relative abundances of the five families mentioned varied in each sample according to exposure conditions: for AGS exposed to saline wastewater only, or to saline wastewater containing DCF, the relative abundances of *Rhodocyclaceae* and *Candidatus Competibacteraceae* tended to increase with salinity, a trend that was not observed for the AGS exposed to saline wastewater with CBZ. In fact, when AGS was exposed to CBZ, the highest relative abundance of *Rhodocyclaceae* was observed in non-saline wastewater (21.6 % of the total abundance for that sample); opposingly, *Candidatus Competibacteraceae* had a higher relative abundance in AGS exposed to saline wastewater containing either 10 or 30 g NaCl L^{-1} (8.8 and 8.5 %, respectively); the relative abundance of *Chitinophagaceae* decreased as the salinity level of the wastewater increased, for AGS free from pharmaceutical exposure, as well as for AGS exposed to DCF. In AGS exposed to CBZ, the relative abundance of *Chitinophagaceae* was higher in saline wastewater with 10 g NaCl L^{-1}. Concerning the unidentified family of the order Cytophagales, a higher relative abundance was observed in AGS exposed to wastewater with 30 g NaCl L^{-1}, compared to those exposed to lower wastewater salinity levels. Overall, a higher relative abundance of Cytophagales was observed in AGS exposed to CBZ (ranging from 7.9 % to 9.9 %) than in those exposed to DCF, or to salt free from pharmaceuticals (ranging from 4.3 % to 6.7 %). On the contrary, *Flavobacteriaceae* had higher relative abundance in AGS exposed non-saline wastewater (ranging from 6.7 % to 7.8 %) than in the ones exposed to saline wastewater (ranging from 1.5 % to 7.4 %).

Although with less than 2.0 % of relative abundances, the genera *Phenylobacterium, Paracoccus, Acidovorax, Zoogloea, and Thauera* were detected in all biomass samples.

To visualize differences in AGS bacteriome composition after exposure to the different stress conditions, a PCoA plot was constructed based on the OTUs relative abundance (Fig. 4a). The distribution of the AGS samples across the plot suggests distinct impacts of the different stress conditions on the microbiome composition. Samples distribution across axis PCoA1 seems to match the salinity gradient: AGS samples exposed to non-saline wastewater are on the right side of the plot, while those exposed to saline wastewater with 30 g NaCl L^{-1} are in the left side of the plot. Analysis of sample scores across PCoA1 revealed that there are

Table 1
Alpha-diversity indices estimated for the AGS bacterial community after exposure to wastewater containing different pharmaceuticals and different salts concentrations.

	NaCl concentration in wastewater (g NaCl L^{-1})	H	R	E
Wastewater without pharmaceuticals	0	4.63	212	0.865
	10	4.50	205	0.845
	30	4.45	200	0.840
Wastewater with DCF	0	4.66	229	0.857
	10	4.45	201	0.838
	30	4.43	212	0.827
Wastewater with CBZ	0	4.10	194	0.779
	10	4.44	195	0.842
	30	4.31	201	0.813

H: Shannon diversity; R: richness; P: Pielou's evenness; DCF – diclofenac; CBZ – carbamazepine.

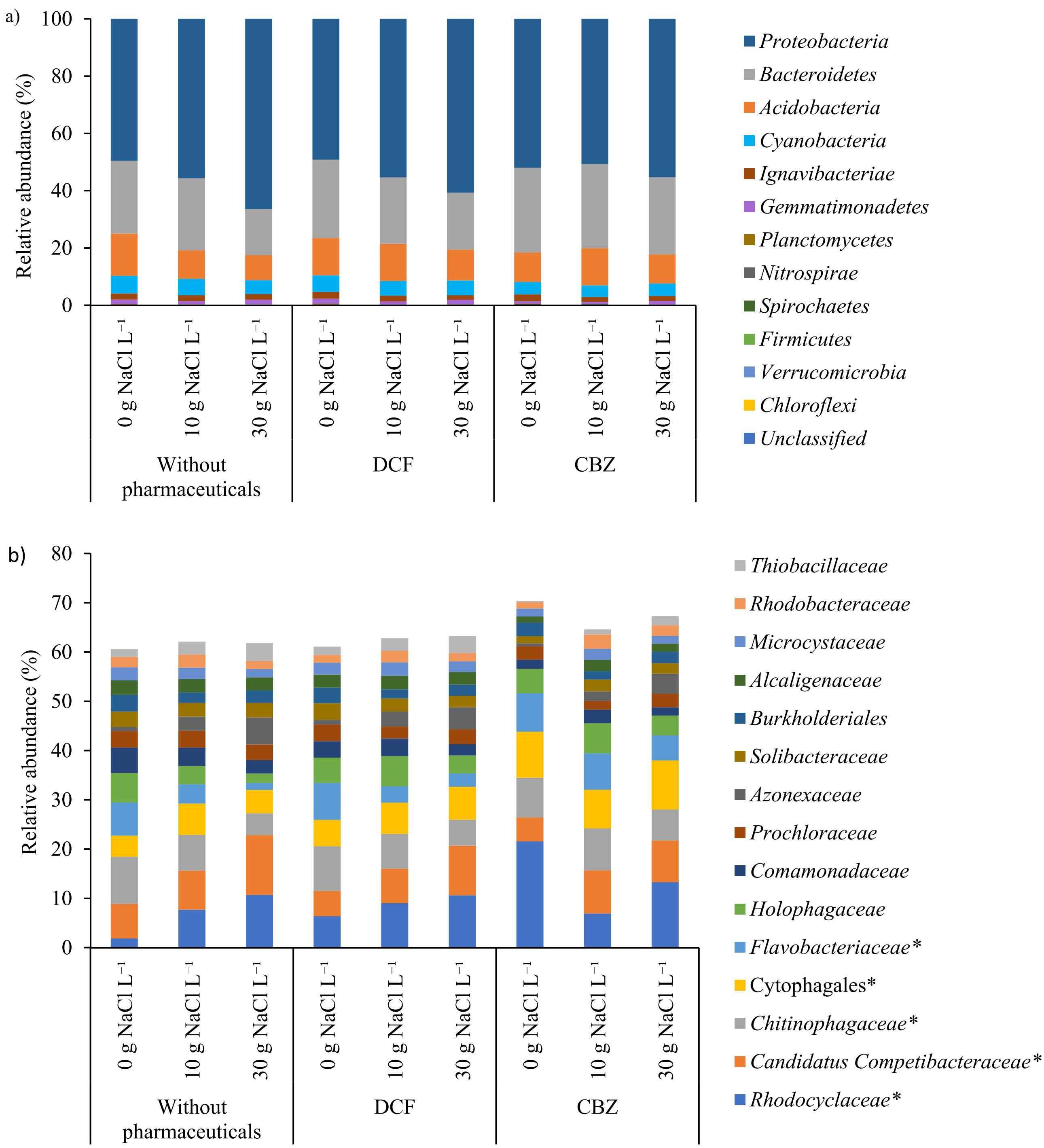

Fig. 3. Taxonomic composition of the AGS bacteriome after exposure to the different stress events. The relative abundance of bacterial communities at the phylum level (a). The relative abundance of the most abundant bacterial families (> 2.00 %) (b). The families that had an average relative abundance for all samples of more than 5 % are identified with * . For the unidentified family of the order Cytophagales (yellow), the name of the order was kept in the legend.

significant differences in terms of OTU composition depending on the wastewater salinity level to which they were exposed (one-way ANOVA, p value< 0.05), between 0 and 30 g NaCl L^{-1} (Tukey's HSD test, p value $<$ 0.05) but no significant differences were observed across samples distributed along PCoA2 axis (one-way ANOVA, p value $>$ 0.05).

In the dendrogram that represents the relatedness of the bacterial communities from the different AGS samples (Fig. 4b), the ones exposed to saline wastewater grouped separately from those exposed to non-saline wastewater. Moreover, AGS samples exposed to wastewater containing 10 g NaCl L^{-1} grouped in the same cluster with more than 80 % of similarity. At each salinity level, bacterial communities not exposed to either pharmaceutical had higher similarity to those exposed to DCF, than to those exposed to CBZ (Fig. 4b). In addition, the similarity between communities from AGS samples not exposed to pharmaceuticals, to those exposed to DCF, was higher in the presence of salt than in its absence. The bacterial community of AGS exposed to wastewater only containing CBZ did not group with any of the other AGS samples (Fig. 4b) showing a more dissimilar bacterial community structure (< 70 %).

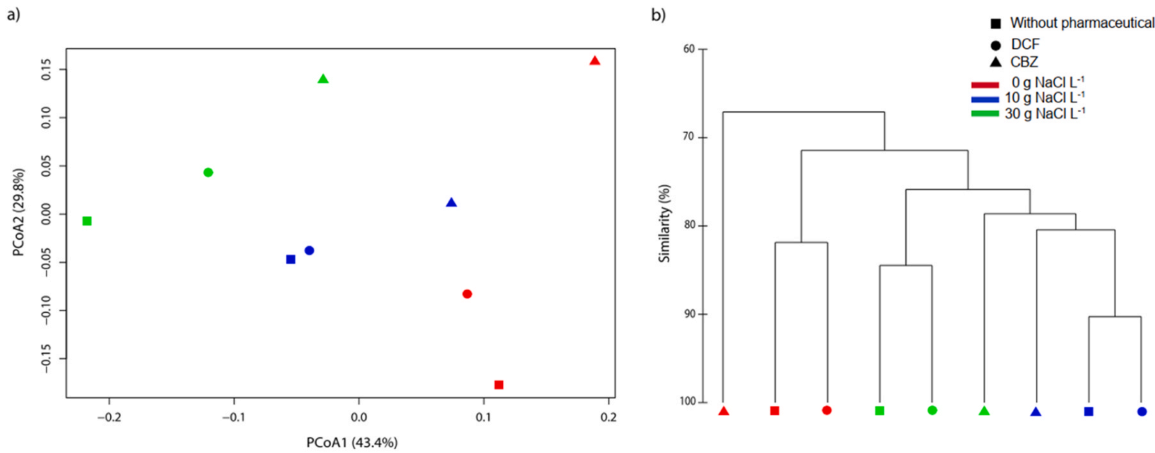

Fig. 4. Principal coordinates analysis (PCoA) plot based on the OTUs abundance of the AGS microbiome after exposure to different stress events (wastewater with diclofenac (DCF) or carbamazepine (CBZ) or without pharmaceuticals at different salinity levels) (a). PCoA1 and PCoA2 axes are plotted with the corresponding percentages of explained variation. The UPGMA dendogram representing the relatedness of bacterial communities from the different AGS samples (b). In both figures distances between samples were calculated with Bray-Curtis dissimilary measure on a relative abundance OTU table.

3.4. *In silico functional analysis*

The functional potential of the AGS microbiomes after exposure to different stress conditions was predicted to investigate how exposure to different levels of salinity and to different pharmaceuticals may affect the biofilm metabolism. Overall, a total of 5132 functional orthologs were predicted for all biomass samples. In the AGS exposed to saline wastewater or to saline wastewater with DCF, a lower number of orthologs were inferred at 10 g NaCl L^{-1} than at 0 and 30 g NaCl L^{-1} (Fig. S3). In AGS samples exposed to wastewater containing CBZ, higher numbers of inferred orthologs were observed as the NaCl concentration of the wastewater increased (Fig. S3).

Inferred pathways with an average relative abundance higher than 0.5 % are indicated in the heatmap of Fig. 5. Inferred functions related with ATP binding cassette (ABC) transporters, two-component system and quorum sensing are within the most abundant (> 2 %), presenting slightly different relative abundances depending on the stress conditions. In the case of ABC transporters and quorum sensing functions, higher relative abundances were observed in the AGS microbiome not exposed to stressors, whilst for functions related with two-component system the opposite was observed.

Differences were found for other stress response related functions (with relative abundance > 0.5 %) such as cell motility and homologous recombination. Genes related with these functions had slightly higher relative abundances in AGS exposed to stressors than in AGS non-exposed to any stressor. Also, the predicted relative abundance of genes for mismatch repair was higher for AGS exposed to wastewater with NaCl and pharmaceuticals than for AGS not exposed to any stressor.

4. Discussion

Exposure of AGS to shock loading events of 24 hours of wastewater containing simultaneously salt and pharmaceuticals affected the EPS production. Moreover, different combinations of stress factors induced a different impact on EPS amount and composition.

Considering the isolated impact of NaCl on EPS secretion, significantly higher EPS concentrations were observed when AGS was exposed to wastewater containing 20 and 30 g NaCl L^{-1}. Wu et al., (2019) observed that higher salinity levels (up to 50 g NaCl L^{-1}) triggered higher EPS secretion by AGS after 6 hours of exposure to the saline treatments. In our study, further increasing salt content to 30 g NaCl L^{-1} led to a slight reduction in EPS production both by AGS free from

KEGG Pathways		0 g NaCl L-1			10 g NaCl L-1			30 g NaCl L-1		
		Without pharmaceutical	DCF	CBZ	Without pharmaceutical	DCF	CBZ	Without pharmaceutical	DCF	CBZ
Carbohydrate metabolism	Glycolysis / Gluconeogenesis	0.77	0.83	0.93	0.85	0.87	0.85	0.88	0.89	0.91
	Citrate cycle (TCA cycle)	0.61	0.67	0.78	0.70	0.71	0.70	0.72	0.73	0.76
	Amino sugar and nucleotide sugar metabolism	0.77	0.80	0.84	0.81	0.82	0.81	0.84	0.85	0.85
	Pyruvate metabolism	1.13	1.18	1.29	1.22	1.22	1.20	1.23	1.23	1.27
	Glyoxylate and dicarboxylate metabolism	1.23	1.21	1.22	1.22	1.22	1.25	1.21	1.21	1.20
	Propanoate metabolism	0.93	0.94	0.95	0.94	0.94	0.93	0.95	0.94	0.96
	Butanoate metabolism	0.92	0.93	0.92	0.93	0.93	0.90	0.93	0.93	0.94
Energy metabolism	Oxidative phosphorylation [PATH:ko00190]	1.38	1.47	1.66	1.51	1.54	1.53	1.56	1.55	1.61
	Carbon fixation pathways in prokaryotes [PATH:ko00720]	0.90	0.97	1.11	1.01	1.03	1.01	1.04	1.04	1.08
	Methane metabolism [PATH:ko00680]	0.79	0.79	0.83	0.78	0.78	0.84	0.77	0.78	0.76
	Nitrogen metabolism [PATH:ko00910]	0.54	0.54	0.55	0.53	0.53	0.53	0.54	0.53	0.52
	Sulfur metabolism [PATH:ko00920]	0.70	0.68	0.64	0.66	0.65	0.68	0.64	0.64	0.62
Lipid metabolism	Fatty acid biosynthesis [PATH:ko00061]	0.54	0.51	0.49	0.51	0.50	0.52	0.49	0.50	0.49
	Fatty acid degradation [PATH:ko00071]	0.58	0.53	0.45	0.52	0.51	0.49	0.51	0.50	0.49
Nucleotide metabolism	Purine metabolism [PATH:ko00230]	1.60	1.62	1.66	1.63	1.63	1.63	1.64	1.64	1.64
	Pyrimidine metabolism [PATH:ko00240]	1.06	1.08	1.12	1.09	1.10	1.10	1.10	1.10	1.11
Amino acid metabolism	Alanine, aspartate and glutamate metabolism [PATH:ko00250]	0.66	0.65	0.66	0.65	0.66	0.68	0.66	0.66	0.65
	Glycine, serine and threonine metabolism [PATH:ko00260]	0.85	0.82	0.79	0.81	0.81	0.83	0.79	0.81	0.80
	Cysteine and methionine metabolism [PATH:ko00270]	0.76	0.75	0.75	0.75	0.76	0.75	0.75	0.75	0.75
	Valine, leucine and isoleucine degradation [PATH:ko00280]	0.84	0.77	0.65	0.75	0.75	0.73	0.73	0.73	0.71
	Phenylalanine, tyrosine and tryptophan biosynthesis [PATH:ko00400]	0.52	0.53	0.55	0.54	0.54	0.55	0.55	0.55	0.55
Metabolism of cofactors and vitamins	Folate biosynthesis [PATH:ko00790]	0.57	0.60	0.67	0.62	0.63	0.62	0.64	0.63	0.65
	Porphyrin and chlorophyll metabolism [PATH:ko00860]	0.85	0.88	0.96	0.88	0.89	0.91	0.90	0.90	0.92
Translation	Ribosome [PATH:ko03010]	1.43	1.46	1.54	1.48	1.49	1.50	1.50	1.50	1.51
	Aminoacyl-tRNA biosynthesis [PATH:ko00970]	1.79	1.83	1.96	1.87	1.87	1.90	1.89	1.90	1.91
Replication and repair	Mismatch repair [PATH:ko03430]	0.46	0.50	0.55	0.51	0.52	0.51	0.52	0.52	0.54
	Homologous recombination [PATH:ko03440]	0.54	0.61	0.72	0.64	0.65	0.63	0.67	0.66	0.69
Membrane transport	ABC transporters [PATH:ko02010]	3.18	3.09	2.92	3.15	3.10	3.08	2.99	3.02	3.03
	Bacterial secretion system [PATH:ko03070]	0.74	0.72	0.66	0.68	0.69	0.71	0.69	0.68	0.66
Signal transduction	Two-component system [PATH:ko02020]	2.48	2.68	2.91	2.72	2.74	2.71	2.80	2.74	2.84
Cellular community	Quorum sensing [PATH:ko02024]	2.44	2.20	1.72	2.19	2.13	2.04	1.94	1.98	1.95
Cell motility	Bacterial chemotaxis [PATH:ko02030]	0.65	0.70	0.76	0.70	0.71	0.72	0.74	0.72	0.75
	Flagellar assembly [PATH:ko02040]	0.73	0.81	0.90	0.83	0.84	0.83	0.87	0.83	0.89

> 3.0%; 2.5-3.0%; 2.0-2.5%; 1.5-2.0%; 1-1.5%; 0.5-1%; < 0.5%

Fig. 5. Heatmap of the inferred KEGG pathways present in the AGS microbiomes after exposure to the different stress conditions. Only pathways that had an average relative abundance for the related KEGG orthologs higher than 0.50 % are presented.

pharmaceuticals exposure, and AGS exposed to DCF. This decrease may be related to differences in the composition and/or metabolic activity of the AGS microbial community. In these scenarios, the impact of osmotic pressure led to a slight reduction in microbial functions related with ABC transporters and quorum sensing (e.g. biofilm formation) despite the increase in oxidative phosphorylation. This could indicate that at 30 g NaCl L^{-1}, in the conditions established in this study, although cells needed to produce more ATP to meet energy demands related with stress regulation (increased oxidative phosphorylation inferred functions), energy was used towards other metabolic pathways rather than membrane transportation or EPS production, such as mismatch repair, that had a slightly higher abundance of inferred functional genes.

In the presence of DCF, the EPS production at 5 and 10 g NaCl L^{-1} was slightly lower than that obtained in non-saline wastewater. Again, at moderate salinity levels (5 and 10 g NaCl L^{-1}) and in the presence of DCF, microorganisms may have prioritized other metabolic processes over EPS production to counterbalance the osmotic pressure, a trend that was no longer observed when the wastewater salinity was 20 g NaCl L^{-1}. This suggests that, depending on the type of stressors and on their concentration in wastewater, microorganisms adjusted their metabolism to better cope with the stressful condition they were facing. Studies on the effect of drugs and salts on AGS reactors operation reported an increase in EPS secretion at higher salinity levels (Corsino et al., 2015; Wu et al., 2019) and when antibiotics and anti-inflammatory pharmaceuticals were present in the wastewater (Muñoz-Palazon et al., 2021; Wan et al., 2018). The opposing tendencies observed in the present study may also be related to the short-term nature of the present assay, as the 24 hours period may be insufficient for microbial communities to reach maximum EPS production.

The composition of the EPS produced also changed with increasing salinity. Although the EPS were mainly composed of proteins and humic acids in all samples, the polysaccharides relative abundance in EPS increased with salinity while the proteins and humic acids contents decreased. Meng and colleagues Meng and colleagues (2019) also described that alginate-like exopolysaccharides production by AGS was higher at moderate salinity levels (around 1 % NaCl). Moreover, salinity is described by Wu et al. (2019) as a wastewater stressor that has an impact on EPS composition, namely increasing the polysaccharides fraction. In fact, previously it was reported that when exposed to stressors, a protective mechanism of AGS that is often activated is the production and accumulation of polysaccharides, which form a network in the outer layer of the sludge biomass thus allowing it to resist the harsh external environment (Dong et al., 2017). Nevertheless, further studies are needed to understand the mechanisms by which salinity specifically influences the production of polysaccharides.

In what concerns the relative abundance of the three main EPS components in AGS samples non-exposed to pharmaceuticals or exposed to DCF, no significant differences were observed. The opposite was verified for AGS exposed to CBZ, with significantly higher relative abundances of proteins and humic acids, and significantly lower relative abundance of polysaccharides. The microbial community analysis revealed that the microbiome of AGS exposed to CBZ had lower overall diversity and richness and a distinct structure compared to that of AGS exposed to wastewater without pharmaceuticals or with DCF. The microbial composition of the AGS influences the EPS composition as reviewed by Flemming and Wingender (2010).

The pharmaceuticals concentration in the aqueous phase decreased over time although more than half of the initial content of pharmaceuticals remained in the aqueous phase by the end of 24 hours. Considering the recalcitrancy of the pharmaceuticals along with the short-term nature of the assay, the observed decrease in concentration is most likely due to sorption onto biomass. The fact that a higher removal rate occurred during the first 8 hours, slowing down thereafter, corroborates the adsorption hypothesis. After the first 8 hours of exposure, the saturation of the EPS binding sites probably occurred, reducing sorption, and achieving an equilibrium. Pharmaceuticals adsorption to AGS was previously described as the most probable mechanism of pharmaceuticals removal in AGS reactors exposed to DCF and other pharmaceuticals (Amorim et al., 2014; Bessa et al., 2021). Possibly, the EPS's functional groups at granules surface may act as potential sorption sites for organic pollutants (More et al., 2014).

Therefore, the removal efficiency of pharmaceuticals by AGS at different wastewater salinity conditions was analyzed as well as the correlation between pharmaceuticals removal and EPS composition. Overall, higher removal efficiencies were obtained for DCF than for CBZ. Nevertheless, removal efficiencies increased for both DCF and CBZ in wastewater containing up to 20 and 10 g NaCl L^{-1}, respectively. This increase was accompanied by an increase of the polysaccharides concentration in EPS as described by Meng et al., (2019), that observed a higher alginate-like exopolysaccharides production by the AGS at moderate salinity levels (around 1 % NaCl). Moreover, Wu et al., (2019) also reported that the salinity level has an impact on the EPS composition, increasing polysaccharides concentrations and promoting the presence of different types of polysaccharides, which in turn affected pollutants adsorption capacity. At the highest wastewater salinity level tested (30 g NaCl L^{-1}), lower pharmaceuticals removal efficiencies were observed. Salt may have competed with the pharmaceuticals for binding sites in the EPS, resulting in lower removal efficiency of the pharmaceuticals as hypothesized in Oliveira et al., (2021). A positive significant correlation between the relative abundance of polysaccharides and the removal efficiency of both pharmaceuticals was observed in the present study for wastewater salinity levels up to 10 g NaCl L^{-1}. For the removal efficiency of CBZ there was also a significant positive correlation with proteins and humic acids relative abundances for wastewater salinity levels of 20 and 30 g NaCl L^{-1}. The tyrosine and tryptophan portions of protein and humic acid were described to interact with pollutants forming a complex through hydrogen bonds and van der Waals forces, contributing to removal from the aqueous phase (Li et al., 2021).

The co-existence of stressors also had an impact on the AGS microbiome. Nevertheless, after exposure to different stress conditions, the microbiomes of the different AGS samples shared a total of 95 OTUs gathering the majority (80.2 %) of the obtained reads. This may be due to the common origin of the inoculum used in each stress condition and/or to the short-time exposure applied in this experiment. In addition, bacteria aggregation in the form of granules, or the EPS produced due to the activation of protection mechanisms, could have contributed to protect cells from environmental stressors and pollutants present in the surrounding environment. The dense physical structure of the AGS limits mass transfer and protects bacteria, especially those located in the inner layers of the granule, from shock loading with pollutants (Rosa-Masegosa et al., 2021). Given the methodology here applied, microbiome data may encompass not only the active microbial population but also some dead and dormant microorganisms. This did not hinder the possibility to observe changes in the relative abundance of bacterial groups due to exposure to the different stress conditions, but it can reduce the variability observed among samples. Nevertheless, after exposure to different stressors combinations, it was possible to observe a joint effect of salinity and pharmaceuticals on the AGS microbiomes: at each salinity level tested, the bacterial community structure from AGS free from pharmaceutical exposure, had higher similarity to those exposed to DCF than to those exposed to CBZ. Additionally, AGS exposed to CBZ had overall lower community diversity and richness. This might be due to the recalcitrant properties of CBZ (Muñoz-Palazon et al., 2021). Moreover, pharmaceuticals with low water solubility, like CBZ, are known to inhibit bacterial growth the most (Ilhan et al., 2022).

The AGS microbiomes were dominated by members of 5 bacterial families namely *Rhodocyclaceae, Chitinophagaceae, Candidatus Competibacteraceae*, an unidentified family of the order Cytophagales and *Flavobacteriaceae*. Nevertheless, the relative abundance of each taxon varied depending on the stress applied, with more pronounced differences among AGS samples exposed to different wastewater salinity levels. Results suggest that exposure to different stressors mainly affected the relative abundance of each taxon, without leading to the suppression or proliferation of new dominant taxa, consistent with the fact that a common initial inoculum was used across all conditions tested. Bacteria belonging to the *Rhodocyclaceae* family are frequently detected in AGS biomass and identified as having a role in EPS production, denitrification, and removal of refractory pollutants (Xia et al., 2018). In the present study, *Rhodocyclus* was the most abundant bacterial genus within this family known for its contribution for EPS secretion (as reviewed in Hamiruddin et al., 2021).

In agreement with what was observed in the present study, *Rhodocyclaceae* members were previously described to dominate the AGS bacterial community exposed to high wastewater salinity levels (Zhang et al., 2018a). Another abundant bacterial family in this study was *Candidatus Competibacteraceae*, which was especially abundant in AGS samples that were exposed to higher salinity levels. In this family are included glycogen-accumulating organisms (GAOs) that use aerobically stored glycogen to enable anaerobic carbon uptake, which is subsequently stored as polyhydroxyalkanoates. This is the case of the members of the *Plasticicumulans* genus detected for this family. GAOs competes with PAOs for glycogen, compromising phosphate removal in the AGS systems (Oehmen et al., 2006). In this study, AGS samples exposed to wastewater containing pharmaceuticals and high salinity content showed higher abundance of members of the *Rhodocyclaceae* family (with PAO members) than *Candidatus Competibacteraceae* (with GAO members). A high salinity environment with recalcitrant compounds seemed to not immediately compromise the PAOs' community. Inversely to what was observed for the two previous bacterial families, the relative abundances of members of the *Flavobacteriaceae* and *Chitinophagaceae* (organic matter decomposers) were lower in AGS samples that were exposed to higher salinity, as previously reported by Zhang et al. (2018a). Bacteria belonging to the *Chitinophagaceae* family are known to have chitinolytic activity which may help to inhibit the growth of filamentous bacteria and improve granulation (Aqeel et al., 2016). *Ferruginibacter* and *Terrimonas*, the most abundant genera for this family in this study, are known to degrade organic matter and excrete EPS allowing for better sludge aggregation (Zhang et al., 2022). *Flavobacteriaceae* members, here represented mostly by *Chryseobacterium* and *Flavobacterium*, are described to be involved in EPS production (Zhang et al., 2019), being frequently detected in AGS and often described as part of the AGS core microbiome together with *Rhodocyclaceae* (Xia et al., 2018). Sun et al. (2017) completely separated the core and shell of AGS to understand the microbial distribution in each layer, finding in the inner core members of the *Rhodocyclaceae* family whilst *Flavobacteriaceae* were dominant in the outer layer.

Members of an unidentified family of Cytophagales also had higher abundance in AGS samples exposed to higher salinity and in AGS samples exposed to CBZ. The *Chryseolinea* genus was the one identified for this family, and their members are known for their capacity to degrade small organic molecules as well as large polymers such as proteins and polysaccharides (Zhang et al., 2022). The Cytophagales order was found to be enriched in AGS treating hyper-saline pharmaceutical wastewater (Jiang et al., 2021).

Although with lower abundance, other bacterial groups potentially involved in EPS secretion were detected in all biomass samples besides those above mentioned, such as *Phenylobacterium, Paracoccus, Acidovorax, Zoogloea,* and *Thauera* (Paulo et al., 2021b; Zhang et al., 2019; Zhang et al., 2018b). Overall, EPS secretion-related bacterial genera were not compromised by the exposure of AGS to combined wastewater stressors (high salinity and pharmaceuticals). Nevertheless, the relative abundance of these genera varies among samples exposed to different stress conditions which could explain the observed differences in total EPS production and composition.

The *in silico* functional inference analysis revealed that, independently of the stressful scenario that the AGS was exposed to, the most abundant inferred functions were ABC transporters, two-component system and quorum sensing. Nevertheless, the relative abundance of such inferred functions slightly varied depending on the stress applied: if those of ABC transporters and quorum sensing functions were higher in AGS microbiomes non-exposed to stressors, the ones relative to oxidative phosphorylation and two component systems function were higher in microbiomes exposed to stressors. The ABC transporters are important for the cytomembrane permeability of bacterial cells, regulating not only to toxics excretion but also the sugar transport (Ou et al., 2018; Zhao et al., 2021). After 24 hours, the presence of stressors in wastewater seemed to decrease the abundance of inferred functions related to ABC transporters. A similar trend was reported by Liu et al., (2022) through metagenomics analysis, that described that an increase of salinity led to the decrease of gene expression related to ABC transporters, weakening this pathway for the osmotic pressure balance in activated sludge. Quorum sensing is a cell density-dependent signalling system that enables microorganisms to communicate with each other, allowing for the regulation of different physiological processes such as biofilm formation, motility, among others (Lv et al., 2024). Quorum sensing plays an important role in the AGS formation and stability by accelerating the synthesis of the EPS, a common bacterial response to stressful situations (Wang et al., 2017). A correlation between EPS production and the abundance of quorum sensing inferred functions was observed for the conditions tested as described above when explaining the EPS secretion results, except for samples exposed to CBZ at 30 g NaCl L^{-1}.

The relative abundance of genes related with cell motility (bacterial chemotaxis and flagellar assembly) and replication and repair (mismatch repair and homologous recombination) were predicted to slightly increase with wastewater salt concentration in AGS samples non-exposed to pharmaceuticals and on the ones exposed to both stressors (salts and pharmaceuticals), despite having higher abundance in the AGS microbiomes that were exposed to CBZ. Therefore, exposure to salinity and pharmaceuticals, especially to CBZ, which is known to be more recalcitrant than DCF, even for short time periods, may cause DNA damage in AGS resulting in higher abundance of genes codifying bacterial DNA repair functions. In addition, these stresses may induce cell motility that can itself be a trigger for biofilm formation and for other mechanisms of adaptation to unfavourable conditions (Rossi et al., 2018).

Given the complexity of the composition of the WWTP influents it is of utmost relevance to study the combined immediate effect of different types of pollutants in the AGS microbial composition and metabolic related functions, to better understand the triggered cellular adaptative responses and which conditions will affect the AGS process performance, towards an improved use of this technology. It is important to notice that the AGS is composed by microorganism with different growth rates and a 24 hour period of exposure to stressors is short for bacterial community adaptation, especially for slow-growing organisms. Nevertheless, by exploring the early-stage response of the bacterial community to the stressors it is possible to ascertain on how microorganisms begin to adjust their metabolism to the new environmental conditions.

5. Conclusion

This study helped to unveil the immediate dynamic microbial processes responding to the sudden exposure of AGS to combined stressors as frequently experienced in full-scale WWTP.

The total EPS content secreted by the AGS was significantly influenced by the wastewater salinity level. Whenever salt and pharmaceuticals co-exist in the wastewater, the impact of salinity on the EPS production was dependent on the type of pharmaceutical present but also on the salt content. Removal efficiency was consistently higher for DCF than for CBZ, even when salinity levels rose to 20 g NaCl L^{-1} , and a positive correlation was found between EPS polysaccharide content and removal efficiency at salinity levels up to 10 g NaCl L^{-1} . Despite the short exposure period, the salinity level, pharmaceutical type, and their combination induced significant shifts in the structure and functional diversity of the AGS microbiome. These impacts were primarily observed in the abundance of specific bacterial groups under different stress conditions, rather than as broad shifts in the microbial composition.

Functional inference analysis of the microbiome revealed that the presence of stressors that are not completely removed from wastewater induced the increase of the abundance of functions related with stress resistance, possibly enabling the AGS community to adapt to the adverse environment. Understanding the early stage response of the AGS microbiome to combined stressors is critical for developing optimization and control strategies to prevent or reverse process disturbances in WWTP.

CRediT authorship contribution statement

Marta Alves: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Catarina L Amorim:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Paula M.L. Castro:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Isabel Henriques:** Writing – review & editing, Methodology, 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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psep.2024.12.080.

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