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Bioaugmentation of Aerobic Granular Sludge with Dye-Decolorizing Yeast for Textile Industrial Wastewater

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Abstract: A sequencing batch reactor (SBR) inoculated with activated sludge and bioaugmented with a dye-decolorizing yeast strain—*Yarrowia lipolytica* (HOMOGST27AB) was assembled to form yeast-bioaugmented aerobic granular sludge (AGS). The bioaugmented AGS-SBR was operated for the treatment of synthetic saline wastewater (12 g L^{-1}) intermittently fed with a reactive textile dye (Navy Everzol ED) at 25, 15, and 7.5 mg L^{-1}. Dye degradation did not occur, although some dye adsorbed to the granules. AGS-SBR performance in removing carbon and nitrogen was good and was not affected by the dye addition. Bioaugmentation with the yeast *Y. lipolytica* (HOMOGST27AB) occurred with success, proved by sequencing samples from granules throughout the reactor operation. The AGS core microbiome gathered essentially microorganisms from the *Proteobacteria* and *Bacteroidetes* phyla. The microbial profile showed a dynamic microbiome established at Phase I of the operation, with a high decrease in the abundance of *Ignavibacterium* from the initial biomass to the granules formed and an increase in *Actinobacteria*, *Cytophagia*, *Flavobacteria*, and *Alphaproteobacteria* in the remaining phases of the bioreactor operation.

Keywords: aerobic granular sludge; yeast; bioaugmentation; textile dye; decolorization; synthetic saline wastewater

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1. Introduction

Water is the most valuable natural resource for life on earth. Industrial wastewaters generated by the textile industries are one of the most important sources of global water pollution. These industries generate high amounts of dyed polluted effluents that are discharged into water bodies, leading to adverse ecological and human health effects [1]. These wastewaters are often charged with azo dyes that have intense color, together with surfactants, detergents, salts, solvents, and other compounds that are difficult to degrade because of their recalcitrancy. The amount of these compounds in textile wastewater is difficult to generalize because they differ in composition, depending on many different factors such as the type of process, chemicals, dyes, fabrics, and machinery used. However, dye effluents are usually high in color, pH, suspended solids (SS), chemical oxygen demand (COD), biochemical oxygen demand (BOD), metals, temperature, and salts [2]. Azo dyes are composed of one or more azo groups (R-N=N-R') that could be microbiologically reduced to amines, leading to dye decolorization under anaerobic/aerobic conditions [3]. Alternating anaerobic/aerobic conditions can be more effective for the treatment of dye-containing effluents [4].

Activated sludge systems are commonly used for biological wastewater treatment but need large surface areas for system implementation that, in some regions, are a limited resource [5]. Aerobic granular sludge (AGS) is an innovative technology for wastewater treatment with many advantages compared with conventional activated sludge systems.

The lower initial investment cost, either in terms of energy consumption or less space needed due to the higher compactness of the granules formed with excellent settling properties, is one of the main advantages of AGS [5]. The high tolerance to chemical toxicity, good effluent clarification, and high biomass retention contribute to the increasing interest in AGS technology, since the amount of wastewater that can be treated in the bioreactor increases [5–8]. Moreover, the simultaneous removal of carbon and nutrients with high shock load resistance, all in a single reactor, contributes to the ecological benefits of this technology [6,9]. AGS comprises suspended microorganisms that bend together to form spherical aggregates which form clusters [10]. These structures are formed by an extracellular polymeric substance (EPS) matrix produced by the microorganisms involved, not needing an external carrier [8,11]. EPS is composed of proteins, polysaccharides, humic compounds, and nucleic acids [12]. EPS leads to the creation of a layered structure that protects microorganisms from direct contact with the external environment and from the toxicity of the wastewater contaminants [12]. Additionally, some studies showed that AGS technology is efficient in degrading toxic contaminants such as fluorophenol and endocrine disruptors, among others [6,13].

Despite all the benefits that AGS technology promises, the microorganisms present in the bioreactors may not be able to degrade specific pollutants. Single strains or microbial consortia have been used to bioaugment AGS systems for degrading contaminants such as herbicides and organofluorines, among others [14–16]. A few studies regarding the use of bacterial strains with dye-decolorizing capacity using AGS in SBR with synthetic wastewater were investigated with good results. Maqbool et al. (2020) tested *Pseudomonas aeruginosa* strain ZM130 for the remediation of a reactive black-5 dyed wastewater with more than 80% color removal [17]; Franca et al. (2020) tested a mix of bacterial strains in an SBR fed with a synthetic textile wastewater containing the dye Acid Red 14 with 91% color removal [18]. In addition, results from the studies of Lourenço et al. (2015) demonstrated the potential application of non-specific bioaugmented AGS technology for dyed wastewater, with color removal of 75–80% [19]. In addition, some yeasts are also being used for bioaugmentation with promising results. Louhasakul et al. (2020) tested *Yarrowia lipolytica* for bioaugmentation and biovalorization of oily industrial wastes [20], and Wen et al. (2022) used a high-efficiency salt-tolerant yeast, *Meyerozyma guilliermondii* W2, to biofortify a pilot-scale wastewater treatment plant to treat high-salinity organic wastewater [21]. However, to the best of our knowledge, there are no previous reports on the use of yeast strains to carry out bioaugmentation in AGS systems.

In the present study, AGS granulation was promoted simultaneously with bioaugmentation with a dye-decolorizing yeast tolerant to salinity. The objective of this study was to access the ability of the yeast-bioaugmented AGS to remove an azo dye commonly used in the textile industry in saline environments. The reactor performance for main nutrient removal processes and the composition of the microbial community were evaluated during reactor operation.

2. Materials and Methods

2.1. Chemicals and Materials

Textile dye Navy Everzol ED (NE), kindly provided by the company AQUITEX, SA (Porto, Portugal), was prepared by dissolving the dried powder in distilled water at the desired concentration. Stock, standard, and working solutions of dye were filtered on syringe nylon membrane filters (0.22 µm pore-size) to sterilize. Acetonitrile, potassium chloride, and potassium phosphate monobasic (phosphate buffer) for HPLC mobile phases were purchased from Merck (Darmstadt, Germany). HPLC grade solvents were filtered with 0.45 µm Glass microfiber filters (Whatman™). Ultrapure water was supplied by a Milli-Q Gradient A-10 (Millipore) system (18.2 MΩcm, organic carbon $\leq$ 4 µg L^{-1}). SBR influent media were prepared with analytical-grade chemicals (Sigma–Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany). Sodium acetate was purchased from

Merck (Darmstadt, Germany). All other chemicals used in this study were analytical grade (Sigma–Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany).

2.2. Cultivation Conditions

A yeast strain, *Yarrowia lipolytica* (HOMOGST27AB), previously isolated, with decolorizing capacity for Navy Everzol ED dye was selected for reactor bioaugmentation [22]. The inoculum at 2% was prepared in Normal Decolorization Medium (NDM) [22] and incubated in an orbital shaker at 25 °C/100 rpm to a final concentration of 1.6×10^6 CFU mL^{-1}. After centrifugation and washing with SBR medium [10], the recovered pellet was reconstituted in 10 mL of SBR medium and introduced into the reactor through a syringe during the aeration phase on day 3 of the reactor operation. Pure cultures of *Y. lipolytica* were maintained in a Normal Solid Decolorization Medium (NSDM).

2.3. AGS Sequencing Batch Reactor (SBR) Set-Up

A lab-scale SBR with 110 cm of height, 6.5 cm of internal diameter, and 2.325 L of working volume was inoculated with the activated sludge from the aeration tank of an industrial wastewater treatment plant from a local brewing industry (Porto, Portugal) and bioaugmented with *Y. lipolytica* (HOMOGST27AB) inoculum prepared as described above. The bioreactor was operated in 3 h cycles during the bioaugmented granulation process and then in 6 h cycles (after day 65), at room temperature (25 ± 2 °C). The experiment was carried out without pH and oxygen control. The system worked in 4 stages: 60 min of inlet anaerobic feeding, aeration (112 and 292 min for the 3 and 6 h-cycles, respectively), gradual decrease from 30 to 3 min of settling, and 5 min of effluent withdrawal. The inlet feeding was introduced from the bottom of the bioreactor; bottom aeration was sparged at an airflow rate of 4 L min^{-1} and the withdrawn liquid in each cycle was approximately 40%. Automatic timers were used to control the pumps during cycles. The SBR influent wastewater was composed of two solutions, as described by De Kreuk et al. (2005) [10]. Briefly, solution A was composed of $C_2H_3NaO_2$ 63 mM, $MgSO_4.7H_2O$ 3.6 mM, and KCl 4.7 mM, and solution B was composed of NH_4Cl 35.4 mM, Na_2HPO_4 4.2 mM, KH_2PO_4 2.1 mM, and 10 mL L^{-1} of trace element solution [10]. NaCl (12 g L^{-1}) was added to solution B throughout the experimental period to mimic the salinity of a textile industrial wastewater [23]. In each cycle, a total of 89 mL of solution A and B together with 772 mL of tap water was introduced from the bottom of the reactor to fulfill the working volume. The dye NE was introduced weekly (1 cycle/week) into the reactor from the top in phase II at 25 mg L^{-1}, phase III at 15 mg L^{-1}, and phase IV at 7.5 mg L^{-1}. Phase I (granulation) and phase V occurred without dye (Table 1).

Table 1. Operating conditions tested in the SBR.

Phase	Length of Operation (Days)	Days of Operation	Cycle Time (h)	Anaerobic Feeding (min)	Aeration (min)	Inlet Acetate Concentration (mM)	Inlet Dye Concentration (mg L^{-1})
I	0–89	90	3 (0–64 d) 6 (65–89 d)	60 60	85–112 292	5.9	-
II [a]	90–145	55	6	60	292	5.9	25
III [a]	146–243	97	6	60	292	5.9	15
IV [a]	244–271	27	6	60	292	5.9	7.5
V	272–289	17	6	60	292	5.9	-

[a] Dye Navy Everzol ED applied 1 cycle/week.

2.4. Analytical Methods

Samples from the bioreactor were regularly collected (out of the dye addition days) from the inlet, influent, and effluent and filtered through nylon membrane syringe filters (0.45 µm pore-size) (Chromafil® PET filters, Macherey-Nagel, Düren, Germany) to remove biomass.

Quantification of nitrite (NO_2^--N), nitrate (NO_3^--N), phosphate (PO_4^{3-}-P), and ammonium (NH_4^+-N) was performed with photometric test kits (Spectroquant®, Merck Millipore, Burlington, MA, USA), according to the manufacturer's instructions. Total organic carbon (TOC) was measured using a Total Organic Carbon Analyzer (Shimadzu, Kyoto, Japan). Total suspended solids (TSS), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge retention time (SRT), and sludge volume index after 5 min of settling (SVI5) were determined according to Standard Methods (APHA, 1998). The AGS bed volume was determined at the end of the settling period using a graduated scale placed on the reactor column.

Quantification of the Navy Everzol ED dye was carried out by chromatography based on the method of Mata et al. (2015) [24,25] with some modifications: analysis was conducted on HPLC Agilent 1260 Infinity II (Santa Clara, CA, USA) with a Kromasil® C18 column, 250 × 4.6 mm i.d. 5 µm particle size, and 100 Å pore size with a guard column (Kromasil® 3.0 × 4.6 mm). The mobile phase used was composed of sodium phosphate buffer (0.7 mL min^{-1}—25 mM, pH 5.5) and acetonitrile running on a 30 min linear gradient from 100:0 to 50:50 (v/v), followed by a 5 min linear gradient up to 15:85 (v/v) and ending with a step back to 100:0 for another 5 min. Each sample was analyzed in duplicate and stored at −20 °C until analysis.

The adsorption capacity of the aerobic granular sludge was estimated using Equation (1):

$$\text{Adsorption capacity} = (V\ (Ci - Cf))/W \quad (1)$$

where V is the working volume of the SBR, W is the dry weight of granules inside SBR, and Ci and Cf are the initial and the final dye concentrations in each cycle, respectively.

2.5. Recovery of Y. lipolytica from AGS and Identification

To ascertain if the dye-decolorizing yeast was incorporated into the granules, isolation of *Y. lipolytica* from the reactor AGS was carried out by plating crushed granules onto agar (NSDM). Colonies that had the size, color, and morphology that corresponded to *Y. lipolytica* were selected. DNA extraction was performed using a PowerSoil® DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA), according to manufacturer instructions. The internal transcribed spacer ITS was amplified with universal primers ITS-4 (5′ TCCTCCGCTTATTGATATGC-3′) and ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′). PCR was performed using 25 pmol of each primer, 25 µL of NZYTaq 2x Green Master Mix, 1 µL of DNA, and 22 µL of nuclease-free H_2O. Amplification occurred for 35 cycles, with 3 min denaturation at 95 °C, followed by 30 s annealing at 50 °C for ITS and 2 min extension at 72 °C and with a final extension at 72 °C for 10 min. The quality of DNA was evaluated by electrophoresis on 0.8% agarose gel. Sequencing was performed at Eurofins genomics (Leipzig, Germany), using ITS-5 primer. To confirm the phylogenetic affiliation, similarity searches were performed using the BLAST program from the National Centre of Biotechnology Information website: http://www.ncbi.nlm.nih.gov/ (accessed on 9 July 2021).

2.6. Microbial Community Analysis of AGS

AGS samples from the bioreactor were regularly collected throughout the operation during the aeration period of the cycle to obtain representative samples of the microbial population. Under aseptic conditions and using a sterile potter and pestle, the biomass sample was crushed and the resulting suspension was stored at −20 °C until use. Some samples were selected representing the different phases of the bioreactor (days 0, 86, 142, 177, 240, and 266) for further analysis.

DNA extraction was performed using PowerSoil® DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA), according to manufacturer instructions. DNA extracted was quantified using a Qubit fluorometer (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. DNA bioreactor samples were used to perform next-generation sequencing (NGS) analysis through Eurofins (Konstanz, Germany). This platform was used for DNA amplification, preparation of library sequencing, and bioinformatic analysis.

Two primers covering the V3-V4 hypervariable region (357F—TACGGGAGGCAGCAG and 800R—CCAGGGTATCTAATCC) were used, and paired-end sequencing based on the 16S rRNA gene was conducted (Illumina MiSeq platform). Microbiome analysis comprehends demultiplexing, clipping of primer sequences, merging, quality filtering, and microbiome profiling. To assign taxonomic information of the OTUs, DC-MEGABLAST alignments of cluster representative sequences to the sequence database were performed. Reference sequences with a minimum of 70% and 80% of identity and representative sequence, respectively, were selected, and then OTUs and taxonomic assignments were performed with the QIIME software package (version 1.9.1, http://qiime.org/, accessed on 27 September 2022). The raw sequence data were deposited in Sequence Read Archive (SRA) from NCBI database, under the accession number BioProject ID PRJNA885052 (http://www.ncbi.nlm.nih.gov/, accessed on 27 September 2022).

2.7. EPS Extraction

An experimental assay was performed at the end of the reactor operation to verify if the dye was linked to the EPS present in the granules or to the biomass. EPS was extracted by adding sodium carbonate (Na_2CO_3) and heating at 80 °C continuously mixing according to Felz et al. (2016) [26].

2.8. Image Analysis

AGS representative samples were collected during the aeration phase throughout bioreactor operation, and images under a magnifying glass coupled to a digital camera (C-5060WZ, Olympus, Tokyo, Japan) were randomly captured to observe the AGS morphology.

2.9. AGS Ability for Dye Removal in Batch Conditions

AGS ability for removing Navy Everzol ED dye was evaluated in culture flasks. The experiments were conducted in 100 mL sterile flasks containing 50 mL of SBR influent medium with [NE] = 7.5 mg L^{-1} and 5 mL of AGS from the bioreactor and heat-inactivated AGS (adsorption control). Cultures were incubated in an orbital shaker at 25 °C at 100 rpm. Experiments were performed in duplicate under sterile conditions and protected from light. Abiotic control was also established.

3. Results and Discussion

A bioreactor was inoculated with activated sludge from an industrial wastewater treatment plant and a dye-decolorizing yeast *Yarrowia lipolytica* (Phase I) to form bioaugmented granular sludge, under salty conditions (12 g L^{-1}). In the subsequent phases (II to IV), a textile dye (Navy Everzol ED) was inserted at different concentrations (25, 15, and 7.5 mg L^{-1}) to evaluate whether the decolorization of the dye would occur. Dye feeding was ceased in phase V. The reactor performance in the removal of carbon and nutrients, granular biomass properties, and microbial community were evaluated.

3.1. Reactor Performance—Removal of C and N

Figure 1 shows the profile of the carbon, ammonium, nitrate, and nitrite during AGS-SBR operation. The reactor performance was evaluated throughout the operation.

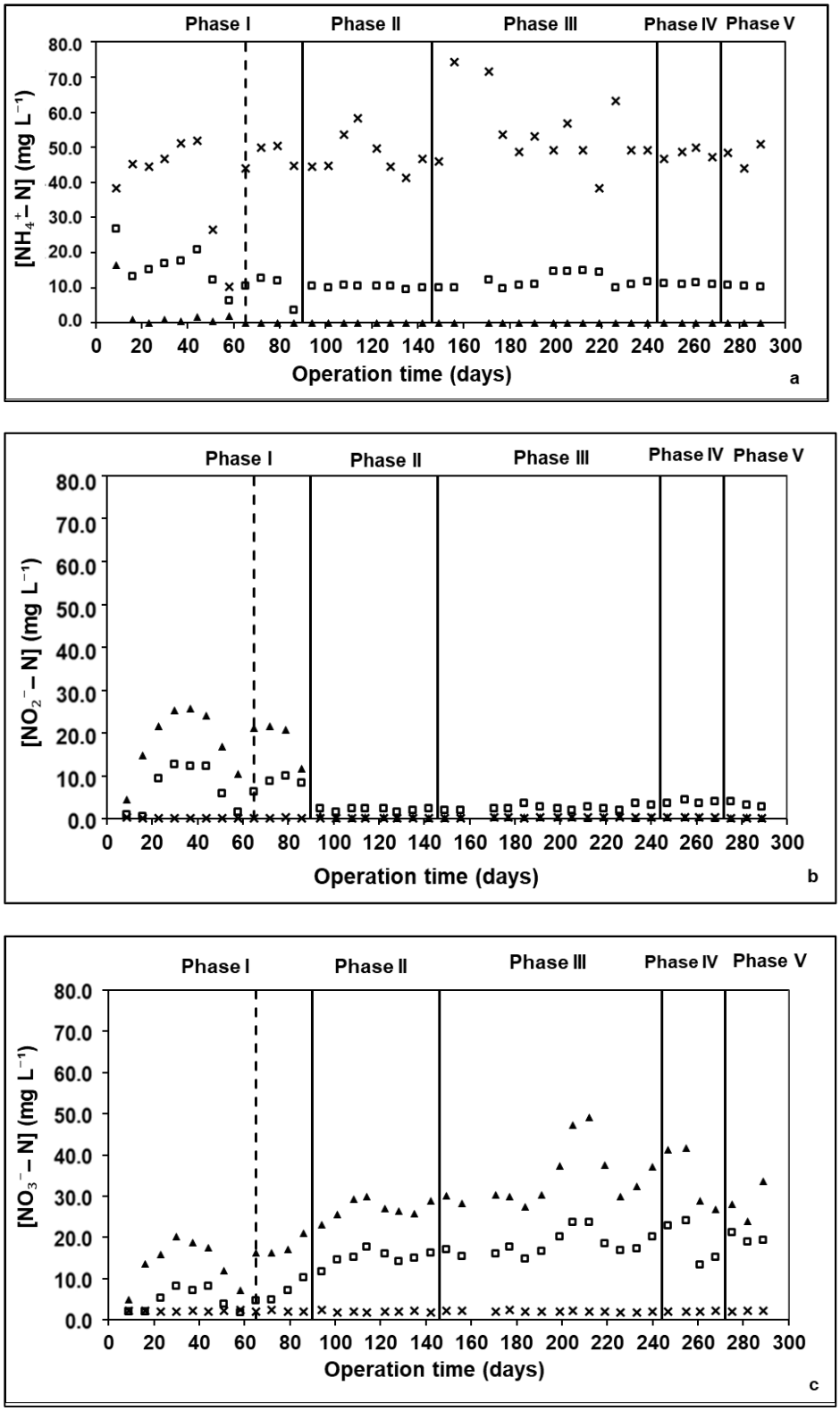

Figure 1. NH_4^+-N (**a**), NO_2^--N (**b**), and NO_3^--N (**c**) concentrations during AGS-SBR operation. Concentrations in reactor influent (×), after anaerobic feeding (□), and effluent (▲) are shown. The dashed vertical line indicates the beginning of 6 h cycles.

The bioreactor exhibited efficient carbon removal, with values of about 40 mg L^{-1} in the effluent (Figure S1 Supplementary Material), stable throughout the reactor operation (~75% removal). Although the carbon supplied was practically all consumed in the anaero-

bic phase, some amount of carbon was still present in the effluent (about 40 mg L^{-1}). This could be due to the presence of non-biodegradable organic matter. In the anaerobic phase of the bioreactor, microorganisms such PAOs (Polyphosphate-accumulating organisms) and GAOs (Glycogen-accumulating organisms) are of extreme importance for carbon removal. The selection of PAOs and GAOs is favored due to the absence of oxygen as a terminal electron acceptor. These organisms, commonly present in AGS-SBR, are intracellular accumulators of storage polymers from volatile fatty acids and use acetate from the influent medium as carbon and energy source for the aerobic phase [27]. In our bioreactor, removal of phosphate was not efficient (data not shown). Several authors mentioned that the presence of salt (NaCl) is difficult for the overall performance of the bioreactor, especially the effective removal of phosphate [28,29]. High salinity may interfere directly or indirectly with cell division, inhibiting some microorganisms' growth and affecting the performance of the AGS-SBR [29]. Nevertheless, the high content of salt did not affect the efficiency of ammonium removal or the nitrification process. Figure 1a shows that the ammonium provided to the SBR was consumed throughout all phases of the bioreactor operation. Somehow, the AGS structure could have protected the AOBs (Ammonia-oxidizing bacteria) and other nitrifying bacteria from the salt and the dye. All the ammonium (NH_4^-) provided was oxidized into nitrite (NO_2^-) and then into nitrate (NO_3^-) by these bacteria (Figure 1b,c). The addition of the dye Navy Everzol ED in Phase II (day 94) did not affect the performance of the SBR. Previous studies with dyes had reported similar results in terms of the stability of carbon removal [4,30]. The AGS-SBR bioaugmentation with *Y. lipolytica* was efficient and did not alter the performance of the SBR. Results from Li et al. (2015) showed a similar carbon removal (nearly 80%) in a submerged membrane bioreactor with activated sludge bioaugmented by a yeast *Candida tropicallis* TL-F1 for degradation and detoxification of Acid Red B [30]. Sarvajith et al. (2018) stated that the removal of carbon, nitrogen, and phosphorus was not impacted by azo dye loading in an AGS reactor operated for 80 days under microaerophilic conditions [31]. Another study reported the efficient removal of chemical oxygen demand and detoxification in a lab-scale activated sludge-based membrane bioreactor bioaugmented with a halotolerant yeast (*Pichia occidentalis* G1) [4]. In addition, Assadi et al. (2018) stated that the presence of high nitrate concentrations had a very small effect on the decolorization performance of biomass [32]. Furthermore, their results also showed that the reactor could maintain its performance in the presence of the dye compounds.

3.2. AGS Settling Properties

During the experimental operation, the bed volume of the AGS and the solid content of the bioreactor effluent were regularly measured (Figure 2). During phase I, the bed volume decreased due to the process of granulation. Phase II started with the addition of 25 mg L^{-1} of dye, which seemed to not cause high stress to the AGS since the bed volume did not decrease much. In fact, in Phase III, bed volume reached its maximum value and the solid content released in the effluent was the lowest of that found during SBR operation, which meant that the AGS was operating in good conditions. Bed volume in phases IV and V was maintained but the TSS value increased, probably due to the renovation of granular biomass.

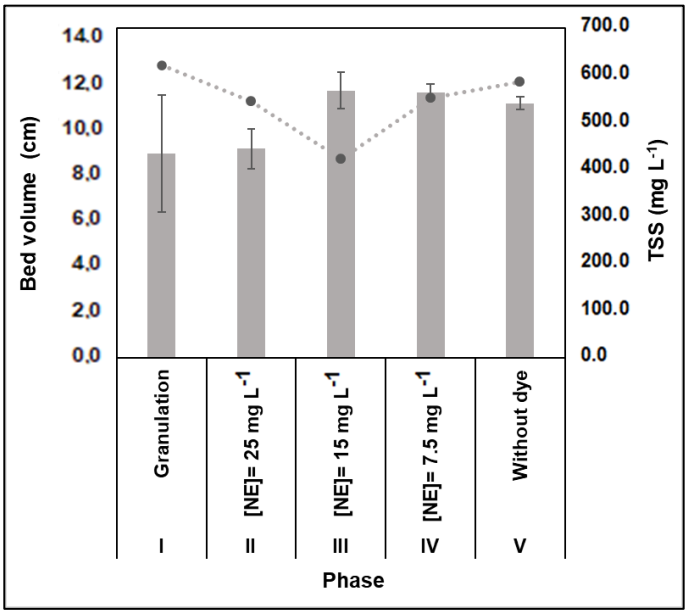

Figure 2. Bed volume and effluent TSS throughout the experimental operation. The average bed volume is represented by columns in each phase and the average of TSS in the effluent is represented by the dotted line.

Table 2 summarizes the AGS properties and its physical characteristics during bioreactor operation. MLSS slightly decreases with the addition of the dye in Phase II. In phases III and IV, in which the dye concentration decreased, it was noticed that MLSS tended to increase, reaching its maximum in Phase V, without the dye addition. Accordingly, the same tendency was observed for MLVSS during the bioreactor operation. The ratio between MLVSS and MLSS that represented the biomass activity remained stable during the reactor operation.

Table 2. Aerobic granular sludge properties along bioreactor operation.

Phase	MLSS (g L^{-1})	MLVSS (g L^{-1})	MLVSS/ MLSS	SVI_5 (mL g^{-1}_{sst})	SRT (Days)
I	9.7	8.82	0.91	20.5	117.4
II	9.0	8.26	0.92	16.7	90.2
III	10.9	9.9	0.91	17.5	97.4
IV	12.3	11.2	0.90	19.3	142.2
V	20.7	18.9	0.91	14.5	231.3

Notes: MLSS, mixed liquor suspended solids; MLVSS, mixed liquor volatile suspended solids; SRT, sludge retention time; SVI_5, sludge volume index after 5 min of settling.

SRT decreased in Phase II and then gradually increased along the operation. SVI_5 decreased in Phase II but slightly increased in Phase III and IV, decreasing again in Phase V, indicating more dense and compact granules at the end of operation. Values of SRT and SVI_5 show the increase of AGS compressibility and settleability.

3.3. Dye Removal

From Phase II to Phase IV of bioreactor operation, Navy Everzol ED dye was added to one cycle per week at the respective concentration, during the feeding phase, just prior to the start of aeration. Figure 3 shows the average of the dye concentration during the reactor cycles of two consecutive days after dye addition. Namely, influent samples collected 5 min after the start of the aeration, a sample of the effluent of the same 6 h cycle, and the subsequent effluents of Day 1 cycles (3 cycles) (mix effluent sample). On Day 2, the influent, effluent, and mix effluent samples were collected.

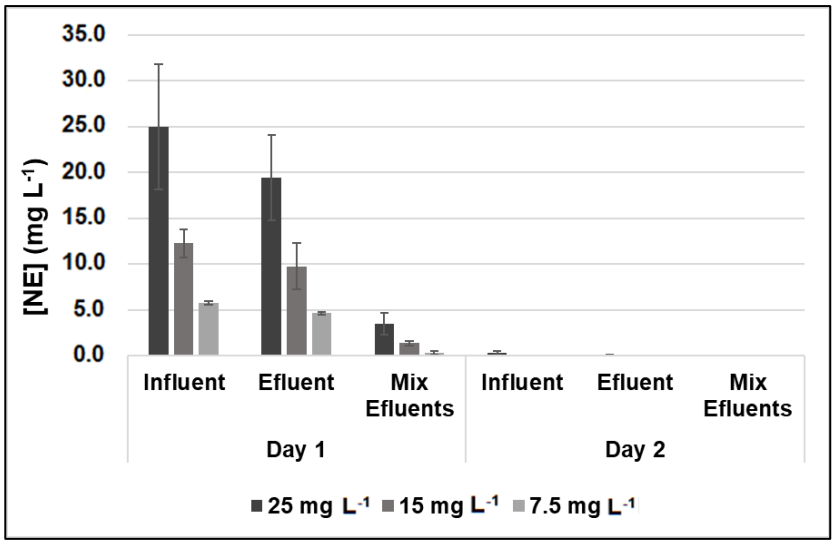

Figure 3. Decrease of dye concentration in the reactor during 2 days of cycles. (Complementary data is shown in Figure S2 in Supplementary Material).

Results showed that in Phase II, the dye concentration decreased in the effluent, resulting in the removal of 51.6% of the total dye fed during this period (Table 3). In Phase III, the added dye concentration decreased to 15 mg L^{-1} and the removal was 54.1%. In Phase IV, the dye feed decreased to 7.5 g L^{-1}, and the dye removal was 60.6%. During each cycle, some dye was retained in the reactor by adsorption of the dye to the aerobic granular biomass (Table 3).

Table 3. Summary of the SBR performance for Navy Everzol ED dye (NE) removal and adsorption.

Phase	[NE] Mass Balance (mg)		% of Removal	Adsorption (mg g^{-1} VSS)
	[NE] in the Inlet	[NE] in the Effluent		
I	-	-	-	-
II	464.4	226.6	51,6 ± 8.9	3.9 ± 1.1
III	284.9	131.7	54.1 ± 7.0	1.86 ± 0.25
IV	53.5	21.1	60.6 ± 4.1	0.98 ± 0.08
V	-	-	-	-

Adsorption capacities of 3.9, 1.86, and 0.98 mgg^{-1} VSS were estimated for phases II, III, and IV, respectively. No metabolites released were detected in the effluent, corroborating the hypothesis of dye adsorption to the aerobic granules.

There was adsorption of the dye to the granules, which remain colored until the end of the reactor operation (no desorption observed). Figure 4 shows the evolution of the granules' color before and after the dye addition. On day 90, before the addition of the dye, granules were yellowish/beige with irregular form, and after feeding with the dye Navy Everzol ED, the granules began to show some blue dots of dye adsorption all over their surface (Figure 4b–d).

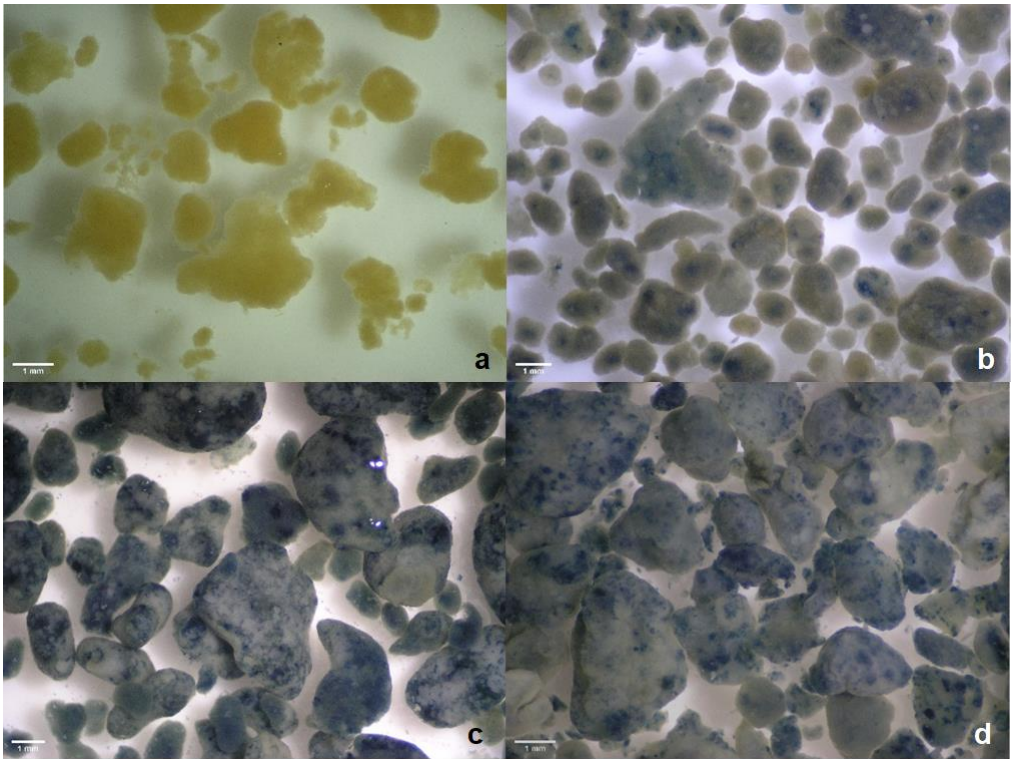

Figure 4. Examples of the evolution of granules' color throughout the reactor operation under a magnifying glass: (**a**) day 90 (before dye); (**b**) day 100; (**c**) day 156; (**d**) day 268.

To evaluate if the dye was linked to the biomass or the EPS from the granules, some assays involving EPS extraction were performed. After the EPS extraction, the supernatant that contained the EPS remained light yellowish and the pellet remained blue, which suggests that the dye did not adsorb to the EPS but to the biomass (Supplementary Material Figure S3). There are several reports on the capacity of AGS for the biosorption of several contaminants, including dyes [33]. In a previous study, it was observed that the yeast strain *Y. lipolytica* (HOMOGST27AB) used for the bioaugmentation partially adsorbs the Navy Everzol ED dye [22]. These observations indicated that the dye could be adsorbed to the biomass of AGS, namely to the *Y. lipolytica* (HOMOGST27AB). Some similar studies stated that some microorganisms, inclusively from granular sludge, have the ability to adsorb large molecules of organic pollutants, such as dye molecules [34]. Meehan et al. (2006) tested the decolorization of Remazol Black-B using a thermotolerant yeast (*Kluyveromyces marxianus* IMB3) and concluded that the decolorization occurred due to biosorption to the yeast cells and not due to a metabolic reaction [35]. Wang et al. (2020) studied a marine bacterium (*Aliiglaciecola lipolytica*) that first adsorbed the azo dye Congo Red onto cells by the secreted EPS (46%), and then the rest of the decolorization was achieved by the action of an azoreductase and laccase [36,37]. In addition, immobilized dead cells from *Candida tropicalis* were efficient as a dye biosorbent from textile wastewater [38].

3.4. Ability for Dye Removal in Batch Conditions

The ability for dye removal of bioaugmented AGS in batch conditions was investigated. The color of the dye in the supernatant was totally removed both in live (91%) and heat-inactivated AGS (88%). The granules were blue both in live and heat-inactivated AGS due to the dye adsorption (Supplementary Material Figures S4 and S5). This result confirmed that the AGS biomass was able to remove dye by adsorption and no desorption occurred. It is known that biosorption is common to several yeasts, dead or alive [39]. Aksu and Dönmez (2003) studied and compared the biosorption capacities of several yeasts to remove Remazol Blue reactive dye and concluded that among nine yeasts, *Candida lipolytica* was

the one that exhibited the highest dye uptake capacity [40]. In addition, a thermotolerant yeast (*Kluyveromyces marxianus* IMB3) was efficient in adsorbing Remazol Black-B, and immobilized dead cells of *Candida tropicalis* were efficient in removing dyes from textile wastewater [35,38].

3.5. Recovery of the Bioaugmented Yeast along Reactor Operation

During reactor operation, several samples of the bioaugmented AGS were collected to evaluate the presence of the dye-decolorizing yeast on the granules. Plating successive dilutions of crushed AGS allowed the recovery of the bioaugmented yeast throughout reactor operation. The identity of the yeast was confirmed by sequencing of the ITS region. These results showed that the yeast *Y. lipolytica* was incorporated into the granules until the end of the reactor operation. To the best of our knowledge, this is the first study reporting the successful formation of aerobic granules bioaugmented with a yeast strain.

In the present study, the dye removal was not complete, even with the dye-decolorizing yeast bioaugmentation. Although the bioaugmentation was successfully achieved, possibly the amount of yeast was not sufficient for complete dye degradation to occur; it is possible that they faced fierce competition from the other microorganisms present in the reactor. Another explanation could be the time of contact that is needed for the yeast to decolorize the dye. According to previous studies, *Y. lipolytica* (HOMOGST27AB) needs 48–72 h to decolorize reactive dyes [23], so possibly this was a limiting step for the dye decolorization in a 6 h cycle bioreactor. Another explanation could be the location of the yeast in the granule—in the inner layers, more protected from external toxics, the dye may be inaccessible for the yeasts to degrade [29].

3.6. Microbial Community of AGS

A total of 318 OTUs were detected in the AGS microbiome. The bacterial composition of the granules at the class level along with the identification of the respective phylum are presented in Figure 5. Bacteria belonging to the Phylum *Proteobacteria* were the most abundant in all samples (47.9%), followed by the *Bacteroidetes* (27.2%); for the activated sludge used to inoculate the bioreactor (day 0), in which it was the dominant Phylum, was the *Ignavibacteriae* (25.4%). This phylum was abundant in the activated sludge inoculum, however, through the granulation *Ignavibacteriae* decreased to a residual number (<1%). *Nitrospirae* and *Acidobacteria* had a similar behavior, being present at 6.1% and 3.4% in the biomass inoculum, respectively, but significantly decreasing in the following phases (<1%). On the other hand, *Acidobacteria* were not present in the initial granules but slightly increased along the subsequent phases. Another phylum that increased during bioreactor operation was *Actinobacteria*, reaching 25.4% in phase IV. Other phyla, such as *Gemmatimonadetes*, showed a relative abundance lower than 2%. Considering class level, overall, *Betaproteobacteria* (19.2%), *Alphaproteobacteria* (18.0%), *Flavobacteriia* (16.9%), and *Actinobacteria* (14.6%) were the most abundant (Figure 5). The most abundant class in the seed sludge was *Ignavibacteria* (25.5%), followed by *Betaproteobacteria* (24.3%). In the subsequent samplings (including Phase I-b), the most abundant classes were *Flavobacteriia*, *Alphaproteobacteria, Betaproteobacteria*, *Actinobacteria*, and *Gammaproteobacteria*. The abundance of *Actinobacteria* increased from 3.2% in phase I-b to 25.4% in Phase IV. The same tendency was observed for *Cytophagia* (1.8% to 13.3%). The inverse tendency was observed for *Flavobacteriia* that decreased from 30.1% in Phase I-b to 11.2% in Phase IV, *Alphaproteobacteria* (from 27.9% to 15.7%) and *Gammaproteobacteria* (11.4% to 3.5%). The relative abundance of *Betaproteobacteria* varied from 12.5% to 25.0% along the phases. The remaining classes had a relative abundance lower than 2.0%.

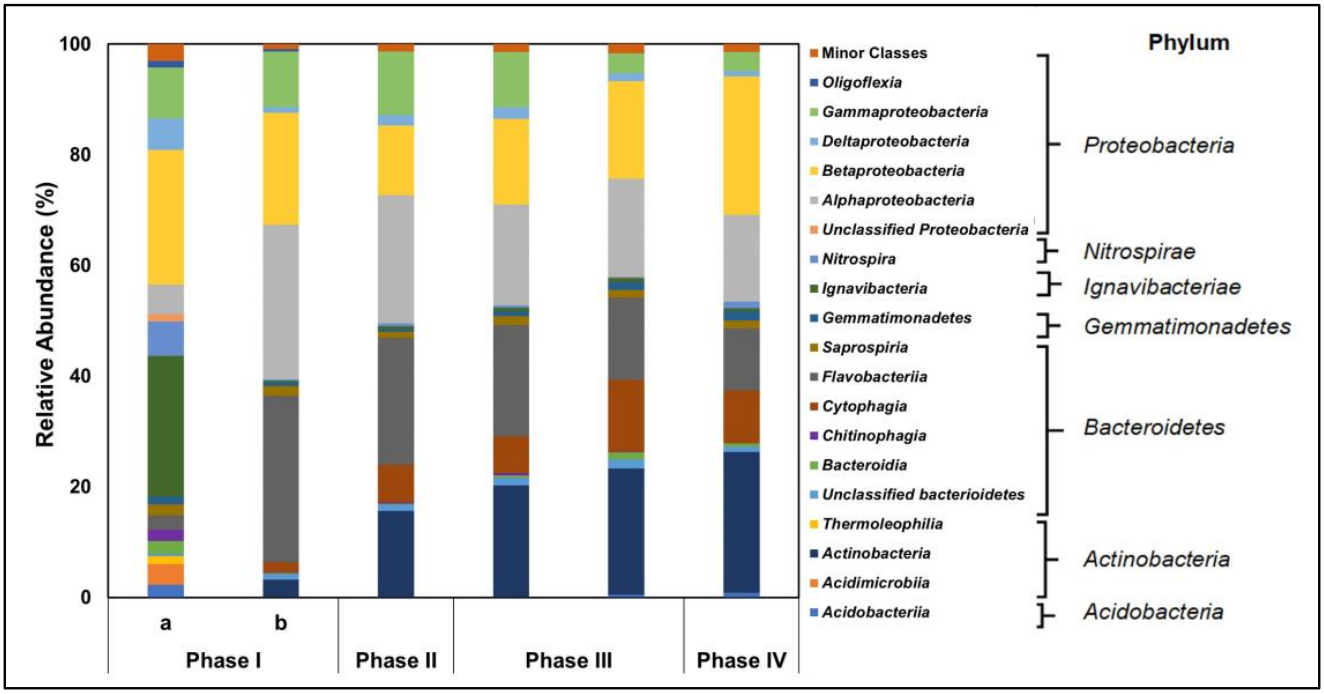

Figure 5. Relative abundance of bacterial groups at class level with their respective phyla. Phase I-IV represents the different phases of the reactor according to Table 1; Phase I-a shows the relative abundance of bacteria of the seed sludge and Phase I-b shows the relative abundance of bacteria in the final of granulation.

Figure 6 represents the heatmap with the most abundant genus of bacteria present in the AGS biomass. When the genus was unknown, the immediately preceding category was represented. *Proteobacteria* was the most abundant phylum of the AGS biomass, comprising seven of the most abundant genera belonging to this phylum (*Mesorhizobium*, Unknown (2), *Azoarcus*, *Thauera*, *Minicystis*, and *Acinetobacter*), though *Ignavibacterium* was the most relative abundant phylum in the seed inoculum.

Organisms from the *Proteobacteria* phylum are generally dominant in AGS and were shown to play a crucial role in the granulation process [41]. These Gram-negative organisms have lipopolysaccharides with cohesive properties on their outer surface that facilitate attachment to the suspended sludge particles and formation of granules [42]. *Proteobacteria* also increase EPS production, promoting the adhesion of floc sludge to become granules [41]. Moreover, the removal of carbon, nitrogen, and phosphorus was shown to increase due to the presence of the organisms from this phylum, playing a significant role in the removal of other pollutants [43]. *Mesorhizobium* and *Thauera* were the most abundant organisms from the *Proteobacteria* phylum found in the AGS-SBR. *Mesorhizobium* is a known nitrogen-fixing bacteria and *Thauera* is an important flocculent aerobic denitrification microorganism that contributes to the degradation of organic matter in wastewater treatment plants and also improves EPS secretion in AGS [43,44].

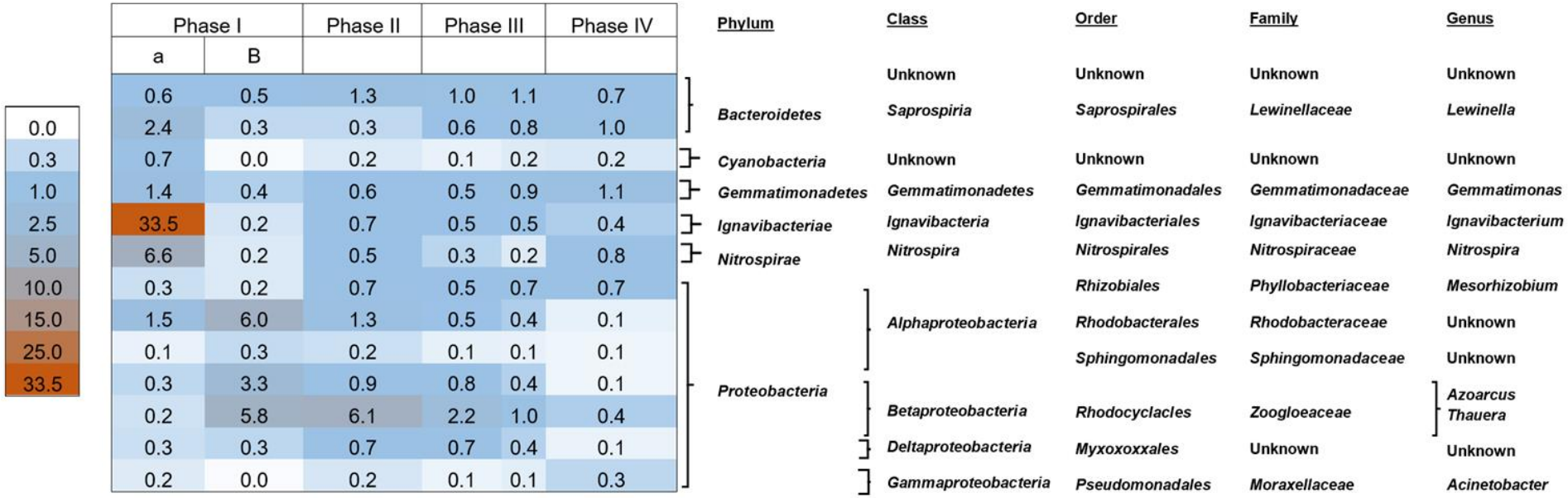

Figure 6. Heatmap representing the most abundant bacterial genus in AGS biomass (% relative abundance). When the genus was unknown, the immediately preceding category was represented.

Bacteroidetes were the second-most abundant Phylum in the AGS-SBR. These Gram-negative, mostly aerobic non-spore-forming bacteria (ex. *Lewinella*) are widely distributed in the environment and are commonly found in wastewater treatment plants [43]. They are one of the most important organisms responsible for the nitrification process of the AGS and the production of EPS and granule formation. On the other hand, an excess of these organisms could damage the stability of granules' structure [41]. In less abundance in the AGS-SBR, we found organisms from *Nitrospirae*, *Ignavibacteria*, *Gemmatimonadetes*, and *Cyanobacteria* phyla. *Nitrospirae* comprises nitrobacteria such as *Nitrospira*, which are common ammonia-oxidizing bacteria that are present in wastewater treatment plants [43]. *Gemmatimonadetes* are known to be a polyphosphate-accumulating microorganism, playing a role in removing phosphate from wastewater and also in the production of EPS [41]. *Ignavibacteria*, the most abundant organisms in the activated sludge provided, are a strictly anaerobic heterotrophic bacterium with the capacity to decompose organic matter in activated sludge [45]. *Cyanobacteria* are photoautotrophic organisms that are commonly present in wastewater treatment plants and have been reported to be useful for the treatment of wastewaters with some chemicals such as antibiotics, pesticides, and detergents since they can metabolize nitrogen, phosphorus, carbon, and sulfur compounds [46]. In the present study, *Cyanobacteria* were present in very low quantity, decreasing from the granulation to the subsequent phases, and therefore possibly not contributing much to the performance of the reactor.

4. Conclusions

In this study, the granulation of activated sludge bioaugmented with the dye-decolorizing yeast *Yarrowia lipolytica* (HOMOGST27AB) in synthetic saline wastewater was successful. AGS-SBR performance in terms of carbon and nitrogen removal from simulated saline wastewater was not affected by the presence of dye Navy Everzol ED. Dye degradation was not achieved by the microbial community present in the AGS-SBR, possibly because the time of contact with the dye was not enough for the decolorization to occur or the proportion of yeast in the AGS was not adequate for dye degradation. Even so, the dye was adsorbed in the granules. Molecular analysis showed the dynamic diversity of AGS-SBR microbial community profile where *Proteobacteria* and also *Bacteroidetes* were predominant. Further studies should be conducted to fully understand the dye adsorption mechanisms by the yeast augmented AGS.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11061654/s1, Figure S1. TOC during AGS-SBR operation. Figure S2. Navy Everzol ED (NE) concentrations during AGS-SBR operation; Figure S3. Example of the EPS extraction assays; Figure S4. Batch liquid cultures containing aerobic granules in SBR medium with Navy Everzol ED dye ([NE] = 7.5 mg L^{-1}); Figure S5. Batch liquid cultures containing heat-inactivated aerobic granules in SBR medium with Navy Everzol ED dye ([NE] = 7.5 mg L^{-1}).

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Data Availability Statement: The raw sequence data of 16S rRNA gene (conducted on Illumina MiSeq platform) of the microbial community from aerobic granular sludge was deposited in Sequence Read Archive (SRA) from NCBI database [47].

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