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Impact of Bioaugmentation on the Bioremediation of Saline-Produced Waters Supplemented with Anaerobic Digestate

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Abstract: Bioremediation of produced waters has been widely investigated in the last decades. More recently, microalgae-based treatments have been developed to produce biomass. The objective of this study was to determine, at lab scale, the remediation efficiency of three origin of microorganisms: a consortium of three halotolerant and halophilic microalgae and their associated bacteria, bacteria from liquid digestate, and aromatic-degrading bacteria selected to perform bioaugmentation. The medium was composed of artificial oil-produced water and seawater, and contained nutrients from liquid digestate. In order to identify what plays a role in nitrogen, chemical oxygen demand, and aromatics compounds elimination, and to determine the effectiveness of bioaugmentation to treat this mix of waters, 16S rRNA analyses were performed. Combination of microorganisms from different origins with the selected aromatic-degrading bacteria were also realized, to determine the effectiveness of bioaugmentation to treat these waters. Each population of microorganisms achieved similar percentage of removal during the biological treatment, with 43–76%, 59–77%, and 86–93% of elimination for ammonium, chemical oxygen demand, and aromatic compounds (with 50% of volatilization), respectively, after 7 days, and up to with 100%, 77%, and 99% after 23 days, demonstrating that in the case of this produced water, bioaugmentation with the specialized aromatic-degrading bacteria had no significant impact on the treatment. Regarding in detail the populations present and active during the tests, those from genus *Marinobacter* always appeared among the most active microorganisms, with some strains of this genus being known to degrade aromatic compounds.

Keywords: ammonium; aromatic compounds; aromatic-degrading bacteria; chemical oxygen demand; digestate bacteria; microalgae; microbial diversity; photosynthetic production; RNA analysis

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1. Introduction

Within the fossil energy industry, produced waters (PWs) generated during oil and gas production contain inorganic salts with total dissolved solids varying from 100 mg·L^{-1} to 300 g·L^{-1} [1,2] and organic molecules such as aliphatic hydrocarbons, polycyclic aromatics hydrocarbons (PAHs), phenolic compounds, benzene, toluene, ethylbenzene, xylene (BTEX), and organic acids [3]. Due to these compounds, PWs could be a serious source of pollution and they need to be treated before discharge to prevent environmental issues.

Over the last few decades, microalgae have gained interest for their ability to fix carbon dioxide (CO_2) by photosynthesis and to produce biomass. They are considered as a promising feedstock for several renewable energy production processes and also for the production of valuable molecules in pharmaceutic, cosmetic, and food sectors [4–7]. Microalgae cultivation

needs large amounts of waters, and the use of seawater and wastewater will be an essential strategy for the conservation of water resources.

In order to bring the nutrients needed for efficient microalgae production, a complementation of PWs is necessary as they contained low concentration of nitrogen and phosphorous and frequently need to be diluted due to their high salinities. Liquid digestate, a nutrient-rich effluent from anaerobic digestion processes, is a good candidate to fill this role. With highly variable compositions depending on the feedstocks used for the anaerobic digestion process, liquid digestates can be highly turbid (up to 51,000 NTU) and can contain up to 3.5 $g \cdot L^{-1}$ of total nitrogen and 380 $mg \cdot L^{-1}$ of total phosphorus [8]. Considering the composition and the high turbidity, liquid digestate has to be pretreated (membrane separation [8]) and/or diluted before being use as culture medium, in order to prevent the inhibitory effect of ammonium (NH_4^+) present in high concentrations (>200 $mg \cdot L^{-1}$) and to reduce turbidity to give microalgae access to light [9].

With this idea of using PWs to produce biomass, several studies investigated the bioremediation efficiency of microalgae or bacteria/microalgae consortia to treat the wastewaters in parallel with biomass production. Concerning PWs, several studies have been conducted. By screening different strains growing in saline PWs, Godfrey [10] showed that microalgae were able to remove up to 84 and 72% of nitrogen and phosphorous from PW, respectively. Similar screening experiments conducted by Abdulquadir et al. [11] and Lutzu and Turgut [12] showed a nitrogen assimilation around to 55–57%, and phosphorous removal ranging from 60 to 89%. Parsy et al. [13] and Concas et al. [14] reported a total nitrogen elimination when using PWs. Concerning chemical oxygen demand (COD), percentage of removal is more dispersed, with removal ranging from 23 to 90% depending on the PW composition and the proportion used [11,13,15,16]. More specifically, Marques et al. [17] investigated the removal of PAHs using *Nannochloropsis oculata* marine microalgae. While microalgae grew less with increasing PW load, more than 85% of the PAHs were eliminated after 14 days' treatment, with a better removal of low-molecular PAHs (2–3 aromatic rings). With a different approach, Cinq-Mars et al. [18] and Babatsouli et al. [19], in addition to measuring bioremediation, performed metagenomics identification to estimate population diversity and gained a better comprehension of the treatment of wastewater.

The objective of this study was to determine the remediation efficiency of each microbial community present in a medium composed of artificial-produced water, liquid digestate, and artificial seawater, in order to identify what roles each community play in nitrogen, COD, and aromatics compounds elimination. We also wanted to determine the effectiveness of bioaugmentation for the treatment of these wastewaters. The novelty of this research lies in following the activity of three distinct microbial communities (a consortium of three halotolerant and halophilic microalgae and their associated bacteria, bacteria from liquid digestate, and selected aromatic-degrading bacteria) and the mixes of these communities. Diversity of each mixture was monitored with DNA and RNA analyses to determine the microorganisms present initially (16S rDNA analyses) and the main active genera of microorganisms in each biological treatment (16S rRNA analyses at days 2, 4, and 7). In addition, bioremediation in term of ammonium, COD, and aromatic compound was identified for each biological treatment in this complex medium using real liquid digestate as nutrient source.

2. Material and Methods

2.1. Artificial-Produced Water Supplemented with Liquid Digestate as Culture Medium

Artificial-produced water supplemented with liquid digestate was used as culture media to grow microalgae and bacteria. Compositions of artificial-produced water (aPW) was based on the composition of aPW used by Parsy et al. [20], except that total dissolved solids (TDS) was adjust to 150 $g \cdot L^{-1}$ instead of 30 $g \cdot L^{-1}$ with Instant Ocean salts. Organic phase was also simplified and contained (expressed in $mgC \cdot L^{-1}$): acetate: 25.22; ethanol: 14.04; phenol: 16.12; benzene: 10.66; toluene: 6.59; ethylbenzene: 9.80; o-xylene: 1.41; m-xylene: 3.76; naphthalene: 0.94; phenanthrene: 2.24. Synthetic water was made using

chemical products purchased from Sigma-Aldrich. Liquid digestate came from an industrial biogas plant using wastes from agriculture, urban, and food industry (France). Liquid digestate had a turbidity of 825 NTU, a pH of 8.4, total dissolved solids of 9.0 $g \cdot L^{-1}$, and contained 2.66 $g \cdot L^{-1}$ of NH_4^+, 2.52 $gO_2 \cdot L^{-1}$ of COD, 94 $mg \cdot L^{-1}$ of phosphate, 1.91 $gC \cdot L^{-1}$ of inorganic carbon, and 175 $mgC \cdot L^{-1}$ of volatile fatty acids. Liquid digestate was frozen at −20 °C for storage.

2.2. Strains and Cultivation for Inoculum Preparation

Three halotolerant microalgae were selected for this study. They were previously identified through 18S rDNA sequencing for microalgae [21,22] and are referred to as strains *Dunaliella salina* CA113, *Nannochloropsis oceanica* CA101, and *Tetraselmis suecica* CA106. Strains were purchased from Greensea (Mèze, France).

Each microalgae strain was cultivated individually in glass bottles (500 mL of working volume) containing sterile *f* medium [23] at 30 $g \cdot L^{-1}$ of salinity in a simulated seawater (Instant Ocean salts, Aquarium Systems, France). Mixing was ensured by air bubbling, while the pH was maintained at 8.0 ± 0.5 thanks to regular injections of CO_2. Light was provided with 14/10 h light/dark periods, by 3 white LED lamps (CorePro LEDtube, 400–750 nm wavelength, 23 W, 2700 lm, 6500 K, Philips, Netherlands). Photosynthetically active radiation (PAR) was adjusted from 50 to 150 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$, during cellular growth to avoid photoinhibition or photolimitation. Growth was monitored by spectrophotometry (Thermoscientific Evolution 201 UV–visible spectrophotometer, USA). At the end of each batch, cultivated cells were withdrawn and reinoculated with an initial optical density at 680 nm (OD_{680}) of 0.3.

The bacteria and archaea present in the liquid digestate were also studied. No cultures of these microorganisms were performed. During the tests, these cells were brought directly by liquid digestate used as nutrient source. Liquid digestate was frozen at −20 °C for storage. It was thawed a few hours before the experiments started. A large diversity of active microorganisms was brought in despite the freezing/thawing of the liquid digestate before the experiments started.

In addition, three halotolerant bacteria, known to degrade phenol, BTEX, or PAH [24–26], were also investigated to perform bioaugmentation: *Marinobacter hydrocarbonoclasticus* SP.17^T (DSM 8798), *Vibrio cyclotrophicus* P-2P44^T (DSM 14264), and *Halomonas organivorans* G-16.1^T (DSM 16226). Strains were purchased from the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH. Each strain was cultivated individually in glass test tubes (15 mL working volume) containing sterile Marine Broth 2216 (BD Difco, UK). Growth was monitored by spectrophotometry (Camspec M107 Spectrophotometer, Spectronic Camspec Ltd, UK). At the end of each batch, cultivated cells were withdrawn and reinoculated with an initial optical density at 600 nm (OD_{600}) of 0.1.

2.3. Growth Test and Monitoring

Seven conditions were tested, by selecting the microorganisms added to the culture medium. These conditions are presented in Figure 1.

For better comprehension, each condition is symbolized by letters in all figures: abiotic (O); microalgae and associated bacteria (Mb); bacteria from liquid digestate (Bd); aromatics-degrading bacteria (Ba); microalgae and associated bacteria + bacteria from liquid digestate (Mb + Bd); bacteria from liquid digestate + aromatics-degrading bacteria (Bd + Ba); microalgae and associated bacteria + bacteria from liquid digestate + aromatics-degrading bacteria (Mb + Bd + Ba). Each condition contained microalgae suspension, liquid digestate, and aromatic-degrading bacteria suspension. However, digestate and suspensions were sterile-filtered (syringe filter with hydrophilic polyethersulfone membrane; 0.2 µm, Pall Corporation, USA) depending on the condition tested. For instance, microalgae condition (Mb) contained the microalgae suspension (unfiltered) and permeates of liquid digestate and aromatic-degrading bacteria suspension. In this way, all tests started under similar

physicochemical conditions. For abiotic condition, all volumes added in the tubes were sterile-filtered.

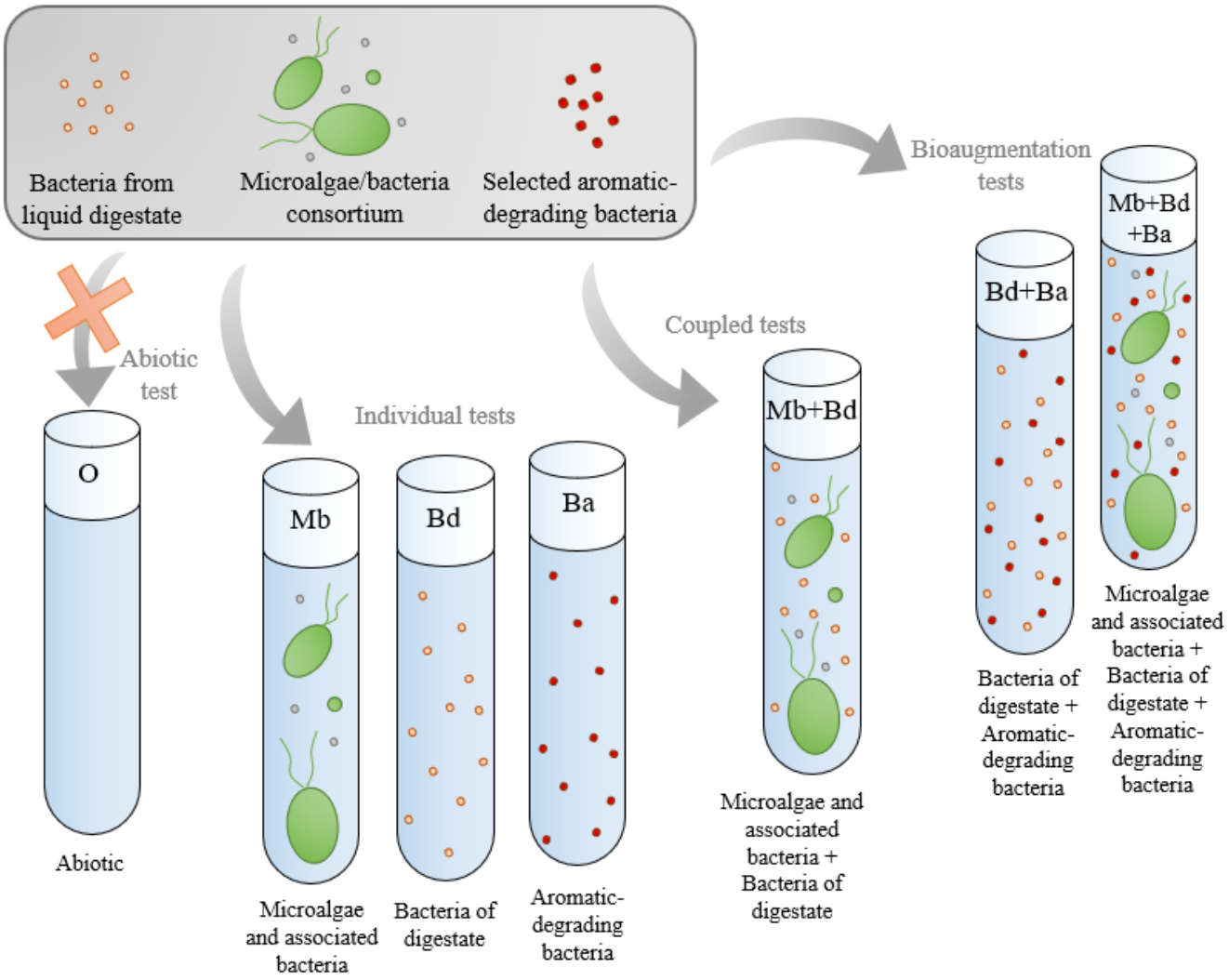

Figure 1. Schematic representation of the experimental plan.

Tests were performed in glass test tubes (working volume 80 mL). Each condition was performed in triplicate. Medium was composed of 31%v/v of aPW, 5%v/v of liquid digestate, and artificial seawater (to complete to 100%), with a final salinity of 70 $g \cdot L^{-1}$. 5% v/v of liquid digestate was used as a source of nutrient, in accordance with previous results [13]. Medium was inoculated with microalgae consortium and selected bacteria or their permeates, with volumes and concentrations carefully selected to have initial concentration for each selected microalgae of $1.2 \cdot 10^5$ cells$\cdot mL^{-1}$, and initial concentration for each selected bacteria corresponding to 0.033 of OD_{600}.

Initial pH was 8.2 ± 0.1. Tubes were incubated for 23 days at room temperature. Reactor mixing was ensured by sterile air bubbling, while the pH was maintained at 8.2 ± 0.3 thanks to regular injections of pure CO_2. Light was provided with 14/10 h light/dark periods, by one white LED lamp (CorePro LEDtube, 400–750 nm wavelength, 23 W, 2700 lm, 6500 K, Philips, Netherlands). Photosynthetically active radiation (PAR) was adjusted to 50 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$.

To monitor the growth of microorganisms in each condition, samples were collected every 2 days to monitor the OD_{600} by spectrophotometry (Thermoscientific Evolution 201 UV–visible spectrophotometer, USA). For conditions including microalgae cells (Mb, Mb + Bd, Mb + Bd + Ba), the evolution of the microalgae consortia population was followed

by cell counting, using Malassez cell counting chamber (Herka, France) to distinguish microalgae species. Mobile strains (*D. salina* and *T. suecica*) were immobilized during cell counting by mixing them with commercial Lugol's iodine solution in a 50:50 proportion 10 min before counting (Remel Gram iodine solution, Thermo Scientific, USA). Mixing with Lugol's solution was also helpful to distinguish *D. salina* and *T. suecica* cells. pH was also measured by using a pH-meter ProfiLine pH 1970i (Xylem Analytics) to adjust the frequency of CO_2 injections used to regulate pH. For the abiotic condition, pH was monitored only at the beginning and at the end of the test to avoid contamination of the medium.

2.4. Microbial Diversity Monitoring

For each condition, initial microbial populations were monitored thanks to DNA extraction, PCR amplification of the 16S rDNA, and MiSeq sequencing. Then, to identify the active microbial species, culture samples were taken at day 2, 4, and 7 and were subjected to RNA extraction, reverse transcription of the 16S rRNA, PCR amplification of the 16S rcDNA, and MiSeq sequencing.

2.4.1. Sampling, Nucleic Acid Extraction, and RNA Reverse Transcription

For initial microbial population diversity, 1 mL of suspensions at day 0 were centrifugated in 1.5 mL tubes at 13,400 g. DNA extractions were performed on pellet using DNeasy PowerSoil kit (Qiagen, Germany) following the manufacturer's instructions. The success of the extractions was verified by electrophoresis with 1% agarose gel containing ethidium bromide. DNA samples were stored at −20 °C until further use.

For active microbial population diversity, 1 mL of suspensions were centrifugated in 1.5 mL tubes at 13,400 g at days 2, 4, and 7. Then, 1 mL of RNAprotect Bacteria Reagent (Qiagen, USA), was added. Samples were stored at −80 °C for less than four weeks before use. RNA extractions were performed using a Fast RNA Pro Soil Direct kit (Qiagen, USA), according to the manufacturer's instructions. All samples were treated with TURBO DNA-free Kit AM1907 (Invitrogen, USA), following the manufacturer's instructions, to remove DNA traces. The absence of DNA in the RNA extracts was checked by PCR followed by 1% agarose gel electrophoresis. Reverse transcriptions of RNA extracts were then performed using Reverse Transcriptase M-MLV (Invitrogen, USA), following the manufacturer's instructions. cDNA samples were stored at −20 °C until further use.

2.4.2. PCR Amplification

PCR of the V4–V5 region of the 16S rRNA gene (from cDNA and DNA) was performed using AmpliTaq Gold™ 360 Master Mix (Applied Biosystems, USA) with the following reaction mix: AmpliTaq 1X, 515F primer 0.2 µM (5′ GTGYCAGCMGCCGCGGTA [27]), 928R primer 0.2 µM (5′ ACTYAAAKGAATTGRCGGGG [27]), and cDNA or DNA 1 µL. The amplification was performed with an initial denaturation step at 95 °C for 10 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 58 °C, and 40 s at 72 °C. The amplification reaction ended with a 7 min extension step at 72 °C. The amplification was confirmed by analysis of the reaction mix by 1% agarose gel electrophoresis.

2.4.3. Sequencing and Bioinformatics Analyses

Amplicons were sequenced by the Plateforme Génome Transcriptome de Bordeaux (Université de Bordeaux, INRAE, France) using Illumina MiSeq 250 bp paired-end technology. Bioinformatics processing of the data was performed using the method described by Escudié et al., (2018) [28] on the Galaxy FROGS pipeline [29]. After a preprocessing step (merging, denoising, and dereplications of the reads), the sequences were clustered into operational taxonomic units (OTUs) with an aggregation distance of three bases. OTUs containing less than 0.0005% of the total sequences were deleted, as were chimeric OTUs. Taxonomic assignments were performed using the Silva database v.128 [30]. The data were deposited in GenBank under accession number PRJNA924123. For OTUs with no taxo-

nomic classification or incomplete classification, sequences were individually processed in BLASTn (NCBI). The micro-organism with the lower E-value was taken into account to identify these sequences.

2.5. Dissolved Compounds Monitoring

At day 0, 7, 14, and 23, samples were taken and filtered (syringe filter with hydrophilic polyethersulfone membrane (0.2 µm), Pall Corporation, USA). Permeates were used to monitor dissolved nutrients and organic/inorganic carbon. COD and NH_4^+ concentrations were evaluated by spectrophotometric methods with LCK1014, 1414 kits (for COD), and LCK303 kit (for NH_4^+) (Hach Company, USA). As aromatic compounds absorb light at UV wavelength, absorbance spectra were also realized between 240 and 300 nm. The absorbance peaks were then integrated to determine relative concentrations of aromatic compounds (from aPW) [31–34]. For NH_4^+, COD, and aromatic compounds, the relative removal percentages were then calculated in each sample as the difference between the concentrations on day 0 and days 7, 14, and 23.

To determine statistical differences between NH_4^+, COD, and aromatic compounds remediation, one-way analyses of variance were performed using "Rstudio" software. Then, Tukey's tests were performed to evaluate differences in remediation efficiency between each condition. For statistical tests, a confidence level of 95% (significance α level of 0.05) was considered. Thus, p-values < 0.05 were deemed to be statistically significant.

3. Results and Discussion

3.1. Microbial Growth

In order to study the bioremediation of aPW, liquid digestate, and seawater, seven conditions were tested with various microbial populations (Figure 1; O: abiotic; Mb: microalgae and associated bacteria; Bd: bacteria from liquid digestate; Ba: aromatics-degrading bacteria; Mb + Bd: microalgae and associated bacteria + bacteria from liquid digestate; Bd + Ba: bacteria from liquid digestate + aromatics-degrading bacteria; Mb + Bd + Ba: microalgae and associated bacteria + bacteria from liquid digestate + aromatics-degrading bacteria). Growth for each condition is shown in Figure 2. For abiotic condition (O), the media stayed clear and OD_{600} remained constant at 0.037 ± 0.008. This condition is not shown in Figure 2.

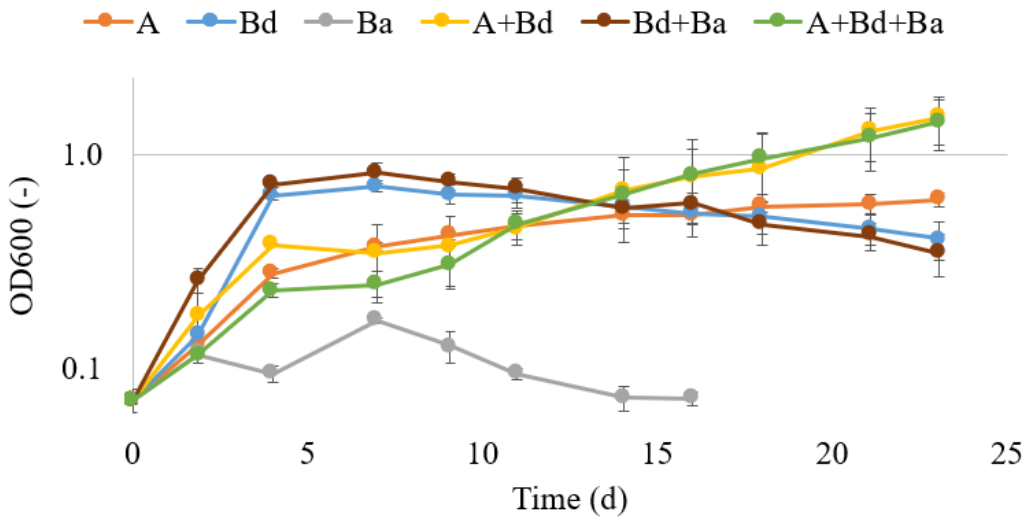

Figure 2. Evolution of optical density at 600 nm for each condition. Mb: microalgae and associated bacteria; Bd: bacteria from liquid digestate; Ba: aromatics-degrading bacteria; Mb + Bd: microalgae and associated bacteria + bacteria from liquid digestate; Bd + Ba: bacteria from liquid digestate + aromatics-degrading bacteria; Mb + Bd + Ba: microalgae and associated bacteria + bacteria from liquid digestate + aromatics-degrading bacteria. Values correspond to mean ± standard deviation, n = 3.

Growth in all conditions with only bacteria (Bd, Ba, Bd + Ba) finished in 4–7 days before reaching a plateau and declining, probably due to the lack of nutrients to support bacterial growth. The growth of aromatic-degrading bacteria (Ba) was lower than the other conditions with bacteria (Bd, Bd + Ba). It could be hypothesized that the three aromatic-degrading bacteria were less efficient in adapting to the new culture medium at a salinity of 70 $g \cdot L^{-1}$ than the large diversity of bacteria from liquid digestate (conditions Bd and Bd + Ba), resulting in lower growth. Concerning microalgae, *N. oceanica* and *T. suecica* were the species with the higher growth. *D. salina* growth was not observed. As microalgae growth is slower, OD_{600} of tests including microalgae (Mb, Mb + Bd, Mb + Bd + Ba) did not reach a plateau. However, microalgae concentrations stopped increasing at day 16 ($2.7 \pm 1.8 \cdot 10^6$ cells$\cdot mL^{-1}$ for *T. suecica*, $8.4 \pm 5.6 \cdot 10^5$ cells$\cdot mL^{-1}$ for *N. oceanica*), and the slow increase of OD_{600} was probably due to slow bacterial development that occurs in parallel due to the organic matter released by microalgae. As algal growth was finished, tests were not continued after 23 days. Growth rate of microalgae consortia was 0.2 d^{-1} after 9 days of culture, being similar to growth rates monitored on individual microalgae in effluent containing *f* medium and 80% of aPW with similar composition [20].

3.2. Microbial Diversity

3.2.1. Initial Populations

Microbial diversity was monitored in each individual condition (Mb, Bd, and Ba) at day 0, immediately after the start of the cultures, thanks to the V4–V5 region sequencing of the 16S rRNA gene. The most abundant detected genera (with more than 5% of the total number of read) in each condition at the beginning of the experiments are shown in Figure 3. It is important to note that samples could be cross-contaminated, as permeates of liquid digestate, microalgae, and aromatic-degrading bacteria suspensions were used in the corresponding conditions, and 0.45 µm filters do not retain free DNA from lysed cells. Contaminations could be detected in small proportions.

A total of 193 OTU representing 70 genera were identified. Considering only the genera with more than 5% of the total number of read, 16 genera were represented. More precisely, liquid digestate microbial population (Figure 3, Bd) was composed of microorganisms from genera *Defluviitoga* (19.4%), *Gracilibacter* (14.8%), *Cloacimonetes* (11.2%), *Marinospirillum* (9.3%), *Moorella* (9.1%), *Pseudomonas* (8.7%), *Proteiniphilum* (5.5%), and other genera (25.8%). As expected, microorganisms of these genera are known to be obligate anaerobes or facultative anaerobes, as there is no oxygen during the anaerobic digestion process. Jiang et al. [35] investigated the microbial diversity of 56 biogas plants, from feedstock (mix of manure, straw, vegetables, or sewage water [36]) to digestate. Among the top 50 genera found in digestate samples, authors reported microbial population from genera *Methanoculleus*, *Syntrophomonas*, *Desulfosporosinus*, *Sphaerochaeta*, and *Acholeplasma*. These anaerobic bacteria and archaea, also found in the digestate used in this work, had a higher relative abundance in digestate than in feedstock, demonstrating that they are active in the anaerobic digestion process. In addition, among genera found in our digestate, authors also reported populations from genera *Clostridium*, *Bacillus*, *Pseudomonas,* and *Acinetobacter*. Authors showed that types and microbiome of feedstock used in anaerobic digestion have a great influence on the digestate microbial diversity.

Tetraselmis genus was detected via chloroplastic DNA (25.6%) (Figure 3, Mb). Despite the high concentration of *N. oceanica* and *D. salina*, chloroplastic DNA was below 5% of the total number of reads (0.45% for *N. oceanica*, not detected for *D. salina*). As inoculum of microalgae were nonaxenic, bacteria were brought by the microalgal suspension. The microalgae cultures could have been carried out in the presence of antibiotics as in a previous work [20]. However, this does not reflect the conditions of a large-scale process. In addition, these bacteria bring an additional microbial diversity that potentially brings a huge diversity of degradation functions. These functions may be useful for wastewater bioremediation, to eliminate some compounds that microalgae cannot metabolize themselves. The populations of bacteria from microalgal suspensions were from the genera *Pseudomonas*

(19.3%), *Synechococcus* (18.4%), *Muricauda* (10.4%), *Planctomycetes* (6.3%), *Meridianimaribacter* (6.1%), *Aestuariivita* (5.1%), and other genera (8.9%). These microorganisms are known to be generally obligate aerobes or facultative anaerobes. It appears that one or several microalgae inoculum were contaminated with a *Synechococcus* cyanobacterium, as it was detected in high proportion (18.4%).

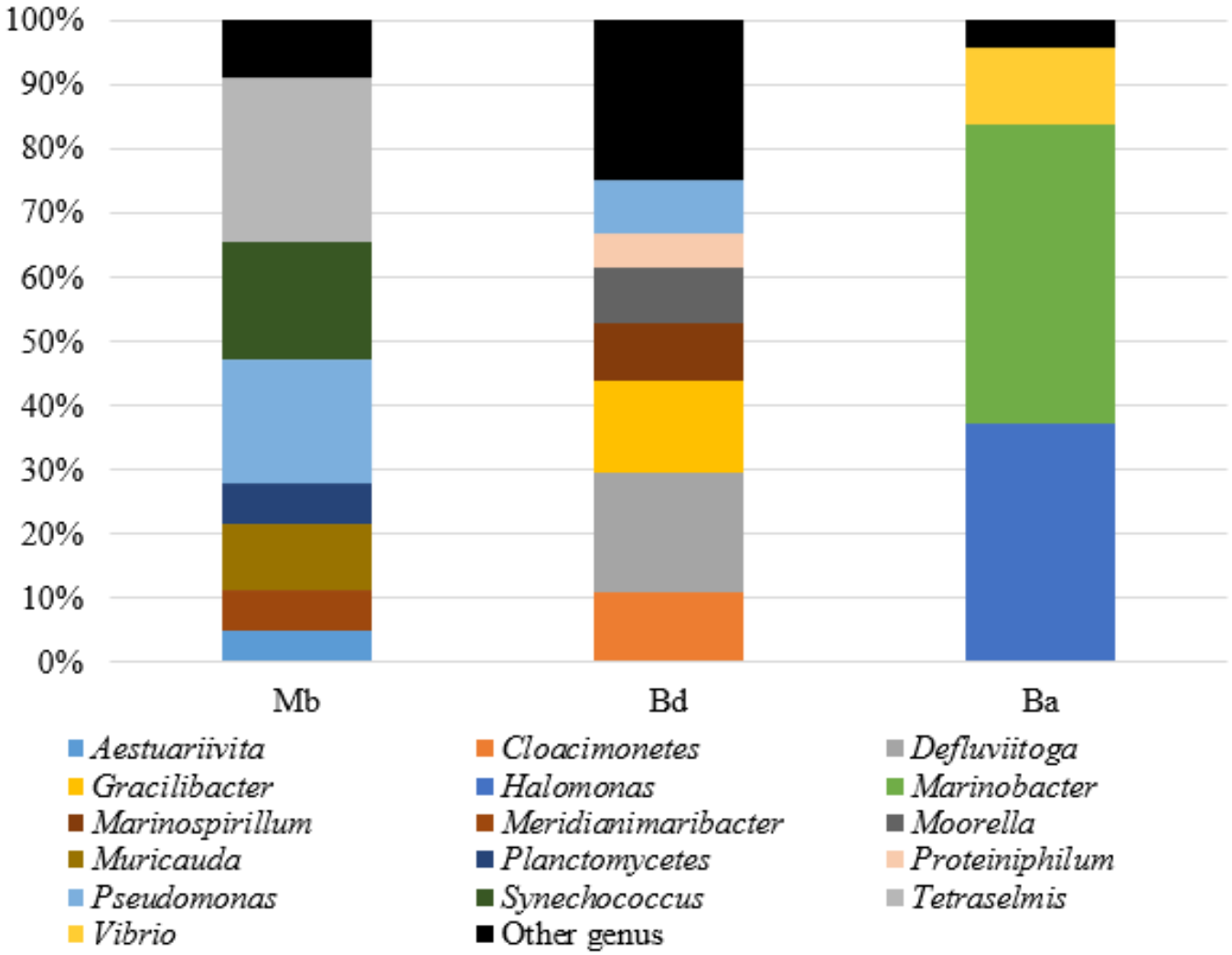

Figure 3. Major genera identified at day 0. Mb: microalgae and associated bacteria; Bd: bacteria from liquid digestate; Ba: aromatics-degrading bacteria.

Finally, *Marinobacter* (46.7%), *Halomonas* (37.1%), and *Vibrio* (12.0%) genera were well identified in the condition with the selected aromatic-degrading bacteria (Figure 3, Ba), with a small contamination from other genera (4.2%, mainly *Pseudomonas*), probably by cross-contamination from another condition or by free DNA from lysed cells which passed through the filter during medium preparation. For the other conditions, as expected, microbial diversities were mixes of the main genera found in the different populations (data not shown).

3.2.2. Active Populations

At day 2, 4, and 7, growth was maximal in all condition (Figure 2), and finished in conditions with only bacteria (Bd, Ba, Bd + Ba). RNA analyses were carried out to determine the active population in each condition. The most active genera detected (with more than 5% of the total number of read) are shown in Figures 4 and A1 of Appendix A.

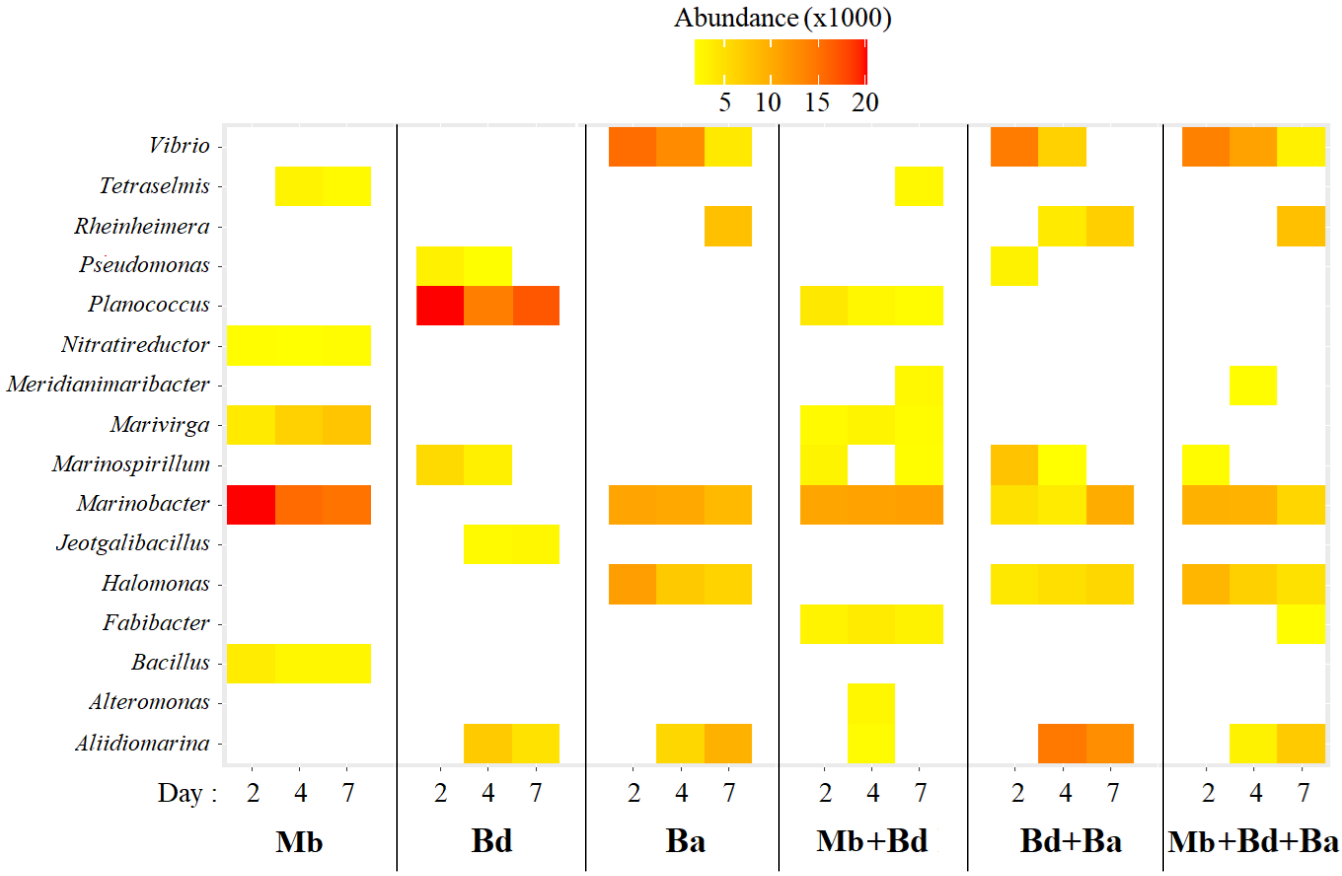

Figure 4. Heatmap of the major active genera identified in each condition at day 2, 4, and 7. Mb: microalgae and associated bacteria; Bd: bacteria from liquid digestate; Ba: aromatics-degrading bacteria; Mb + Bd: microalgae and associated bacteria + bacteria from liquid digestate; Bd + Ba: bacteria from liquid digestate + aromatics-degrading bacteria; Mb + Bd + Ba: microalgae and associated bacteria + bacteria from liquid digestate + aromatics-degrading bacteria.

Concerning bacteria from liquid digestate condition (Bd) at day 2 compared to day 0, active populations were different from microorganisms initially detected. Only populations from the genera *Pseudomonas* and *Marinospirillum* remained active in high proportion (>5%). Other genera, mainly obligate anaerobes (*Defluviitoga, Gracilibacter, Cloacimonetes, Proteiniphilum, Moorella),* were not detected or were in very low proportion. While representing less than 5% of the initial microbial diversity at day 0, bacteria from genus *Planococcus* became the major organisms with 61.4% of the population. Despite freezing and thawing the digestate prior to the experiments, bacteria grew in this condition. It can be hypothesized that the dissolved organic matter from liquid digestate acted as a cryoprotectant, protecting some bacteria. In the following days, *Planococcus* remained the dominant genus, and new genera were detected in high proportion, such as *Aliidomarina* (21.1%). *Aliidomarina* was not detected in high proportion at day 0 (<5%), but it became a major organism after one week of treatment, probably after salinity adaptation.

Concerning microalgae (Mb), all active population detected by RNA analyses at day 2 were not at day 0 (<5%) with the DNA analyses. *Marinobacter* became predominant in those 2 days (51.6%). It is interesting to note that the OTU representing the genus *Marinobacter* in this condition was not the same as in condition Bd, suggesting that they were two different *Marinobacter* populations, and they were not brought by cross-contamination. In addition, representatives of the genera *Bacillus*, *Marivirga,* and *Nitratireductor* could be detected in high proportion. In the following days, major microbial diversity remained globally the same, with the detection in high proportion of *Tetraselmis* (~7%). As *Tetraselmis* strain grew at higher concentration during the biological treatments with microalgae (Mb,

Mb + Bd, Mb + Bd + Ba), it was more detected than *Nannochlroropsis* (<5%) and *Dunaliella* (not detected) with the RNA analyses.

Concerning aromatic-degrading bacteria condition (Ba), the three selected genera (*Halomonas*, *Vibrio,* and *Marinobacter*) remained in high proportions, with the increase of Vibrio (from 12% at day 0 to 40% at day 2). After one week, when no more growth was detected (Figure 1), proportions of these three genera decreased due to the appearance in high proportion of *Aliidiomarina* and *Rheinheimera* genera. Bacterial populations of these genera took time to develop; they were detected in very low quantities at day 0. *Aliidiomarina* were probably brought by cross-contamination, as the same OUT was found in the condition Bd.

Concerning the condition with several communities (Mb + Bd, Bd + Ba, Mb + Bd + Ba), microbial diversity was greater (observed species between 70 and 130) but stayed in the same order of magnitude as the condition with bacteria from liquid digestate only (Bd), as the majority of the diversity came from liquid digestate (94 species observed). In the bioaugmented conditions (Bd + Ba, Mb + Bd + Ba), the same major active genera were identified as in the individual tests (e.g., *Halomonas*, *Aliidiomarina*, *Vibrio,* and *Marinobacter)*. No new genera were detected in addition. Nonetheless, proportions were generally lower as more different microorganisms were in the culture. Among the main genera, *Marinobacter* was found in similar proportion (13–29%) in all bioaugmented conditions. In condition Mb + Bd + Ba, the selected aromatic-degrading *Marinobacter* was predominant, as the *Marinobacter* from the microalgae suspension represented less than 1% of the total *Marinobacter* OTU. *Vibrio* was always highly active at day 2 before disappearing (from 36 to <10%), while *Halomonas* proportion remained constant (11–23%) during the first week of the biological treatment. In addition to these three genera, *Aliidiomarina* genus always became a major microorganism (>5%) after 4 days of culture in treatments with bacteria from liquid digestate or with aromatic-degrading bacteria, up to 19–33% after one week. Some populations from the genus *Aliidiomarina* are known to be able to develop in highly saline wastewater or soil [37], but this has not been reported as a hydrocarbons- or aromatic-degrading bacteria.

As PW used in this study was artificial, microbial populations naturally found in PWs were not investigated. Several studies reported the microbial diversity found in PWs. Among microorganisms identified by Grabowski et al. [38] in PWs, similar genera were found compared to this study (<5% for some of them), such as *Pseudomonas*. Lipus et al. [39] reported the presence of *Bacillus*, *Pseudomonas*, *Halomonas,* and *Psychrobacter* in PWs from Bakken shale (USA). While proportions were different, the presence of these same genera in the tests conducted in this study supports the bioremediation results observed during the biological treatments. Authors also reported the presence of obligate anaerobes that were present in digestate such as *Clostridium*, *Desulfosporosinus,* or *Methanoculleus*, showing that PWs could also bring a wide variety of population and functions. This also shows the importance of working on RNA in addition to DNA, to identify microorganisms that are active and not just present.

3.3. Bioremediation of Artificial-Produced Water Supplemented with Liquid Digestate

During the biological treatments, samples were analyzed weekly to determine NH_4^+, COD, and aromatic-compounds. These analyses were not carried out during the first week (in contrast to the RNA analyses) as the assimilation or elimination of these compounds can take more than a week during microalgae culture.

3.3.1. Ammonium Removal

To determine the nitrogen bioremediation capacity of microorganisms from microalgae suspension, liquid digestate, and aromatic-degrading bacteria suspension, soluble ammonium concentrations were followed during the cultures. Initial ammonium concentration was 129.4 $\pm$ 4.6 mgN.L^{-1} in all conditions. Removal percentages in each condition are presented in Figure 5.

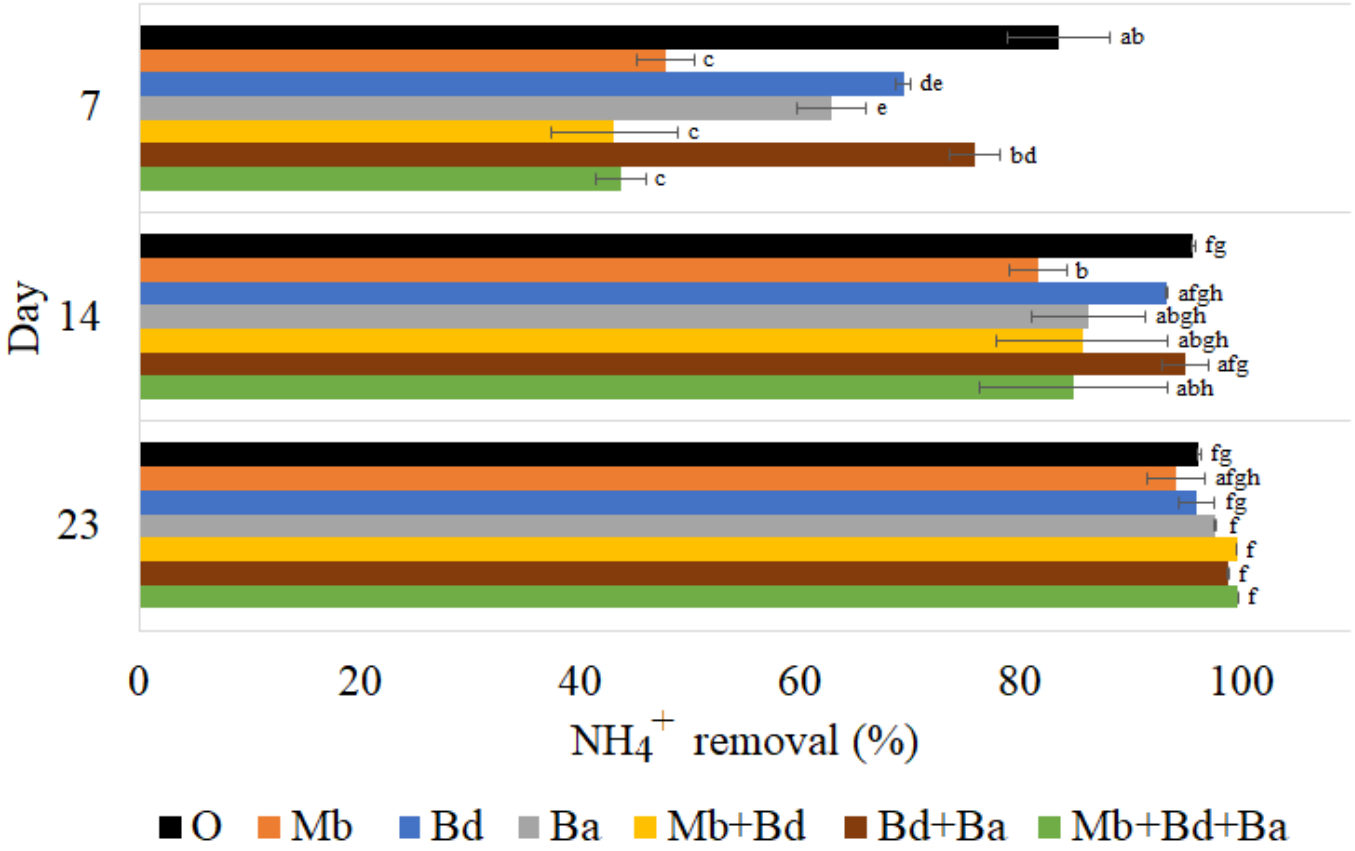

Figure 5. Soluble ammonium nitrogen removal at day 7, 14, and 23 for each condition. O: abiotic; Mb: microalgae and associated bacteria; Bd: bacteria from liquid digestate; Ba: aromatics-degrading bacteria; Mb + Bd: microalgae and associated bacteria + bacteria from liquid digestate; Bd + Ba: bacteria from liquid digestate + aromatics-degrading bacteria; Mb + Bd + Ba: microalgae and associated bacteria + bacteria from liquid digestate + aromatics-degrading bacteria. Values correspond to mean ± standard deviation, $n = 3$. The same letter next to bars indicates no significant difference between the tests (p-value > 0.05, all tests and all days considered).

Regarding abiotic condition (O), soluble nitrogen removal was high without microorganisms activity, with an elimination of up to 83% after 7 days, and 96% after 14 days. Considering only nitrogen removal at day 7, abiotic treatment appears to be even more effective than biological treatments. At pH 8.2, approximately 10% of the ammonia nitrogen is in the form of volatile ammonia. During the 23 days, ammonia was continuously volatilized as no biological activity modified the pH and no CO_2 was injected for this condition, leading to high nitrogen removal. For the other conditions, bacterial activity and/or CO_2 injections reduced the pH frequently around 7.5 and reduced ammonia volatilization. Another explanation could be that in the presence of micro-organisms, part of the ammonium (positively charged) is adsorbed on the surface of the cells (negatively charged), thus sequestering part of the ammonium ions and limiting their volatilization [40,41]. However, it has been shown that adsorption of ammonium decreased at high salinity, because sodium ions become too concentrated and compete for ammonium adsorption on cells [41].

Regarding other conditions at day 7, it appeared that microalgae reduced nitrogen removal, with 43–48% of removal in the presence of microalgae (Mb, Mb + Bd, Mb + Bd + Ba) against 63–76% for conditions with only bacteria (Bd, Ba, Bd + Ba). At day 14, nitrogen removal became more similar between the different conditions, with the lowest removal with microalgae alone (Mb, 82%), and the highest with bacterial treatment (up to 95%). At day 23, no significant difference could be observed between each biological treatment (94–100% ammonium removal). These results are consistent with nitrogen removal values

found in the literature that are commonly around 90–100% [13,14,16] with 10 to 30 days of microalgal treatments.

3.3.2. Chemical Oxygen Demand Removal

Similarly to ammonium removal, soluble COD concentrations were monitored to determine the bioremediation capacity of each population of microorganisms. Initial COD concentration was 540 ± 12 $mgO_2.L^{-1}$. Removal percentages in each condition are presented in Figure 6.

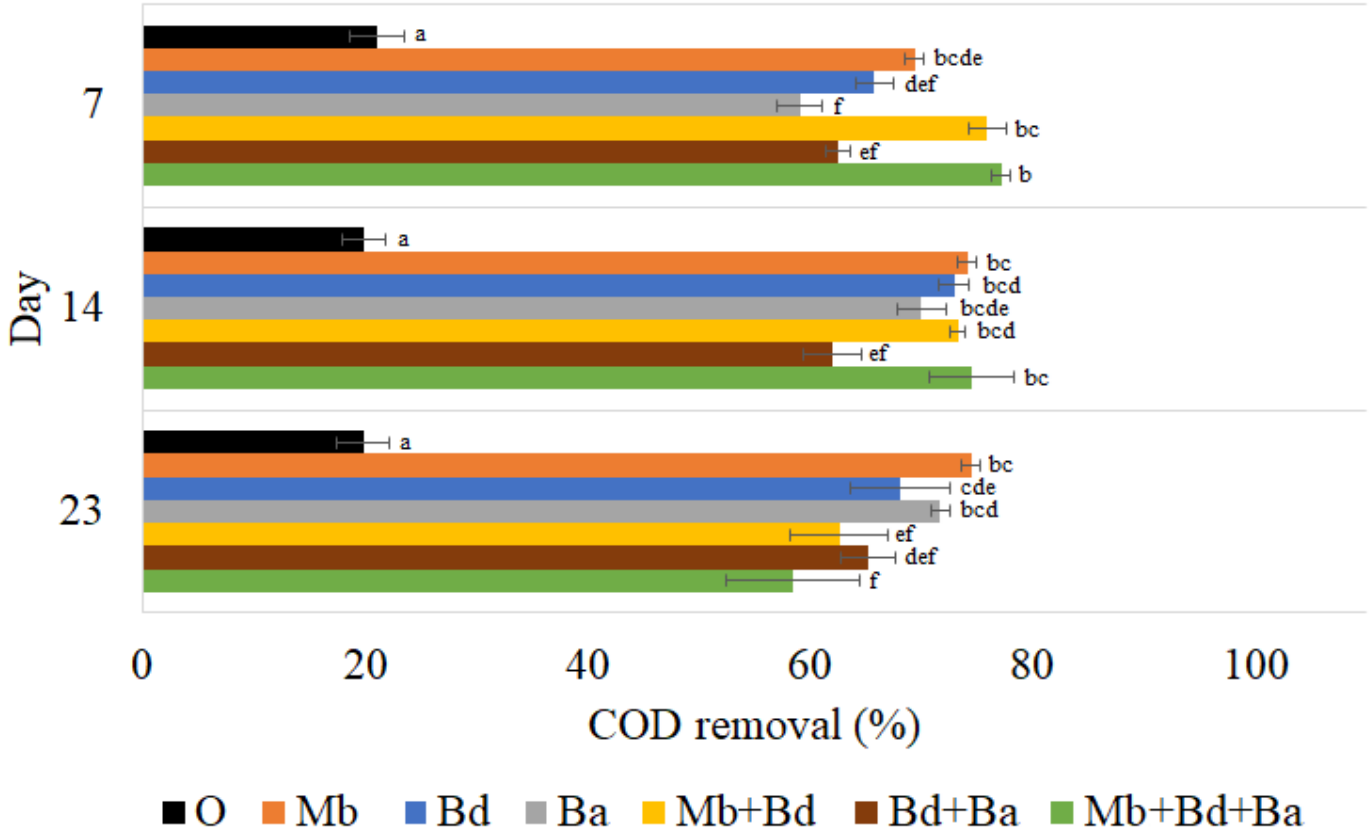

Figure 6. Soluble COD removal at day 7, 14, and 23 for each condition. O: abiotic; Mb: microalgae and associated bacteria; Bd: bacteria from liquid digestate; Ba: aromatics-degrading bacteria; Mb + Bd: microalgae and associated bacteria + bacteria from liquid digestate; Bd + Ba: bacteria from liquid digestate + aromatics-degrading bacteria; Mb + Bd + Ba: microalgae and associated bacteria + bacteria from liquid digestate + aromatics-degrading bacteria. Values correspond to mean ± standard deviation, $n = 3$. The same letter next to bars indicates no significant difference between the tests (p-value > 0.05, all tests and all days considered).

Regarding abiotic condition (O), it appears that 20% of COD from the medium can be volatilized with air bubbling. This percentage did not vary between day 7 and 23. Concerning biological treatment, 58 to 77% of COD were removed in 7 days, remaining the same after 14 and 23 days. While at 7 days the most effective treatments were with microalgae and microalgae with bacteria, after 14 days, the differences observed between each biological treatment were small or not significant. Finally, it appears that around 20% of the COD in this medium, composed of aPW, liquid digestate, and seawater, was recalcitrant COD and would need more advanced water treatments to eliminate it completely. Concerning microalgal treatments (Mb + Bd, Mb + Bd + Ba), COD removal decreased between days 14 and 23, suggesting that microorganisms produced more organic matter than they removed during the last week of culture, hypothetically in response to a lack of

an essential nutrient for growth. In the literature, COD removal ranging from 23 to 90% is reported for microalgal treatment, depending on the PW composition and the proportion used, with common values around 40–60% [11,13,15,16,42]. These values are consistent with bioremediation values found in this study.

3.3.3. Aromatic Compounds Removal

Aromatic compounds found in PWs such as BTEX, PAH, and phenolic compounds are dangerous/toxic and have to be treated, in addition to COD, to respect the environmental policy on the discharge of wastewater. In this work, the tested media contained 31% of the aPW, bringing approximately 16 mgC.L^{-1} of organic compounds in the form of aromatic compounds. Removal percentages in each condition are presented in Figure 7.

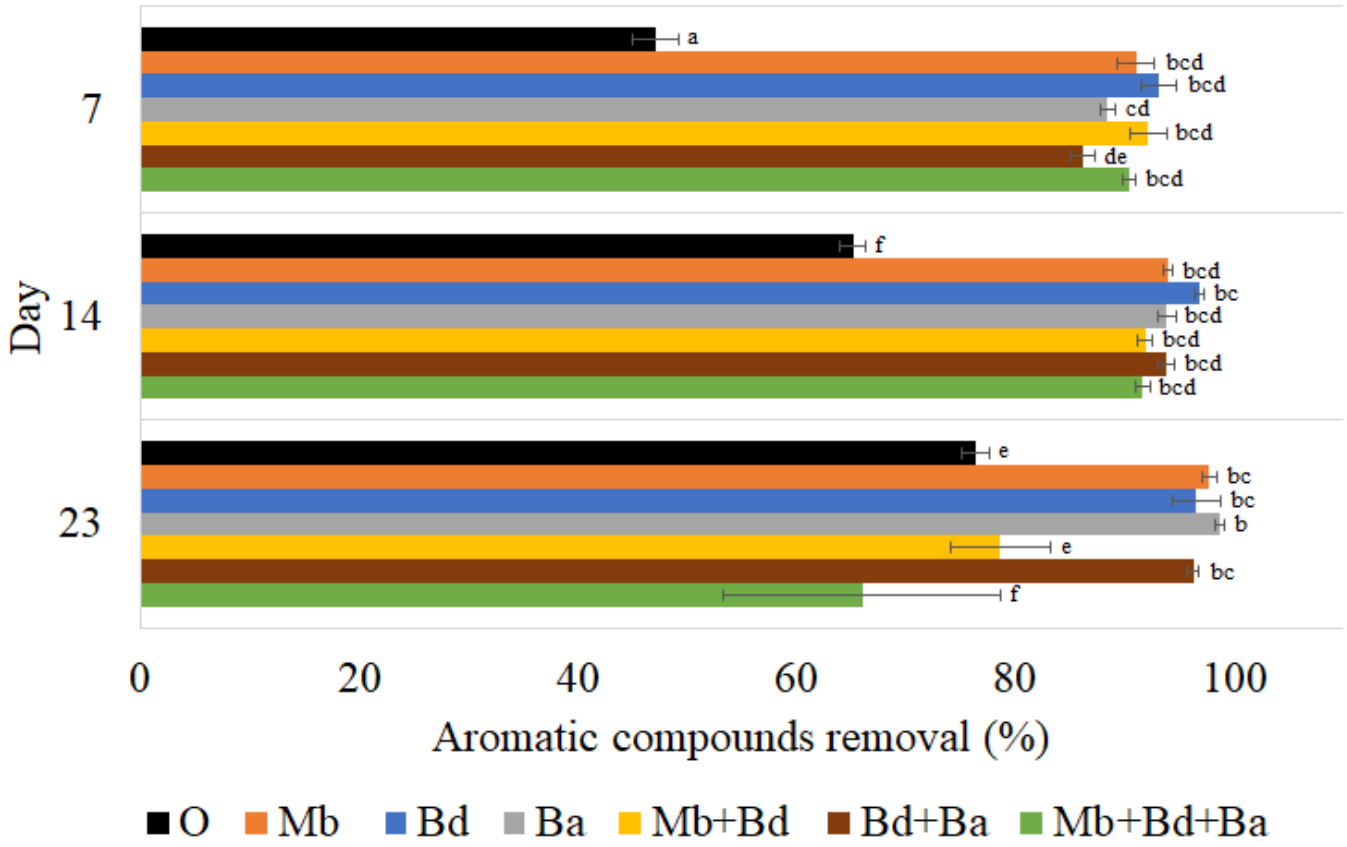

Figure 7. Aromatic compounds removal at day 7, 14 and 23 for each condition. O: abiotic; Mb: microalgae and associated bacteria; Bd: bacteria from liquid digestate; Ba: aromatics-degrading bacteria; Mb + Bd: microalgae and associated bacteria + bacteria from liquid digestate; Bd + Ba: bacteria from liquid digestate + aromatics-degrading bacteria; Mb + Bd + Ba: microalgae and associated bacteria + bacteria from liquid digestate + aromatics-degrading bacteria. Values correspond to mean ± standard deviation, $n = 3$. The same letter next to bars indicates no significant difference between the tests (p-value > 0.05, all tests and all days considered).

For the abiotic condition, aromatic compound concentrations decreased by 47, 65, and 75% at days 7, 14, and 23, respectively. It could be hypothesized that these aromatic compounds are volatilized, as aromatics such as BTEX, low-molecular-weight PAHs, and phenol are highly volatile (independently of the pH [43]). In addition, aromatic compounds could progressively be degraded, with products not detected by UV–Vis spectrophotometry, explaining that COD removal in the abiotic condition remained at 20%. For the biological treatment, no significant differences were observed among the various treatments, with 86–93% aromatic removal at 7 days and 92–99% at days 14 and 23. In treatments with

microalgae and bacteria from liquid digestate (Mb + Bd, Mb + Bd + Ba), decreases of aromatic removal were observed. It can be assumed that this is due to the production of some compounds that absorb slightly in the UV (240–300 nm) under these conditions. The removal of aromatic compounds in PW with microalgae alone is poorly documented in the literature. Previous work [20] investigated the toxicity of aromatic compounds on the same strain of microalgae. Results showed that in concentrations found in aPW, aromatic compounds such as benzene, naphthalene, and phenol were not toxic to *N. oceanica*, *D. salina*, and *T. suecica*. Marques et al. (2021) [17] determined a PAH elimination of 85% after 14 days' treatment using *N. oculata* microalgae, being similar to removal percentages found in this study. In a less complex medium, Takáčová et al. (2015) [44] studied the degradation of BTEX by microalgae *Parachlorella kessleri* in mineral medium spiked with 100 $\mu g.L^{-1}$ of each BTEX. Authors showed that *P. kessleri* was able to eliminate 40% of benzene, 40% of xylene, 30% of ethylbenzene, and 63% of toluene after 3 days' treatment, with a moderate impact on microalgae growth (13% inhibition), showing the possibility to apply a microalgae treatment to remove such compounds when using the right strains.

3.3.4. Bioremediation of Saline-Produced Water Supplemented with Anaerobic Digestate

Regarding the results about nitrogen, it appears that a biological treatment is not necessary to eliminate ammonium in water at a working pH around 8.3 (batch mode, seawater condition). Air bubbling was enough to remove up to 96% after 23 days. Concerning biological treatment, it appeared that bacteria were more accurate than microalgae to eliminate ammonium, as nitrogen elimination was faster in bacterial conditions (Bd, Ba, Bd + Ba). However, it cannot be determined if nitrogen fixation was quicker or if volatilization was higher, similarly to abiotic conditions. It is important to note that the microbial populations detected during the first week (thanks to RNA analyses) are not known to nitrify. Here, ammonia nitrogen is not oxidized to nitrite and nitrate but is incorporated into the biomass. Finally, bioaugmentation was not advantageous concerning nitrogen removal, as nitrogen removal was not significantly faster in conditions Bd + Ba and Mb + Bd + Ba. In all conditions, final ammonium concentrations were 3.2 ± 2.7 $mgN{\cdot}L^{-1}$.

Concerning COD, it appeared that the different microbial populations tested gave similar results, even when tested individually. A total of 58 to 77% of the COD was eliminated during the first week then remained stable, with final COD concentration of 179 ± 31 $mgO_2{\cdot}L^{-1}$. It is therefore not necessary to perform bioaugmentation, by mixing different type of microorganisms, to treat the dissolved COD in this type of medium.

Finally, regarding specifically aromatic compounds, as for COD, removal efficiencies were similar after a week and remained stable with at least 86% aromatic removal. Bioaugmentation is not needed to eliminate such quantities of aromatics in the tested conditions. Each condition had similar aromatic compound removal at day 7 and after. As shown in Figures 3 and 4, the absence of the three aromatic-degrading bacteria selected genera (*Marinobacter*, *Vibrio,* and *Halomonas*) in some conditions (Mb, Mb + Bd) showed that other microorganisms are able to degrade aromatic compounds. Among the genera presented in Figures 3 and 4, some bacterial populations from genera *Bacillus* and *Pseudomonas* have been reported as aromatic-degrading bacteria [45]. In addition, an OTU representing the genus *Marinobacter* was monitored in the microalgae condition Mb (a different OTU than the *Marinobacter hydrocarbanoclasticus* SP.17^T from condition Ba). Some *Marinobacter* strains can degrade aromatic compounds [45], and its presence in Mb conditions could potentially explain the decrease of aromatic compounds in conditions without selected bacteria in the group Ba.

Considering all these bioremediation results, it can be concluded that in the case of this artificial medium, bioaugmentation had no significant impact on the treatment of the medium, as removal efficiencies were not significantly better in the conditions with the aromatic-degrading bacteria. However, even if the bioremediation efficiencies were not better when performing bioaugmentation in this study, it is still interesting to have more genetic diversity in the microbial populations during a wastewater treatment, as a

diverse microbial population potentially provides more metabolic/physiological functions to eliminate atypical or uncommon compounds. In this way, liquid digestate is not only a nutrient source, but also a source of microbial diversity and, thus, degrading functions. The three aromatic-degrading bacteria selected for bioaugmentation did not provide any advantage in the case of the aPW with these concentrations of aromatic contaminants; however, they could be interesting in more contaminated PWs.

Concerning discharge regulation, Europe legislation [46] indicates that water after wastewater treatment must contain less than 125 $mgO_2 \cdot L^{-1}$, 10 $mgN \cdot L^{-1}$, and 1 $mgP \cdot L^{-1}$ to be released in natural environments. After a biological treatment of 23 days (batch mode), these objectives were achieved concerning nitrogen, but not for COD. An additional treatment would be necessary to decrease COD concentration below the legislation limit, for instance, with a treatment with aerobic activated sludges. As final COD concentration is not so far from the legislation limit, additional investigations should be carried out to obtain higher removal efficiencies and respect the legislation. In addition, as experimental results obtained at lab and batch scales are not always representative of those obtained at industrial scales, continuous or semicontinuous biological treatments with these microalgae/bacteria consortia in aPW, liquid digestate, and seawater should be performed in reactors commonly used for microalgal biomass production, to verify that similar bioremediation efficiencies would be obtained.

4. Conclusions

The objective of this study was to determine, at lab scale, the remediation efficiency of three origin of microorganisms: a consortium of microalgae and their associated bacteria, bacteria from liquid digestate, and aromatic-degrading bacteria selected to perform bioaugmentation. The medium was composed of artificial-produced water, real liquid digestate, and artificial seawater. Combinations of microorganisms from different origin with the selected aromatic-degrading bacteria were also realized to determine the effectiveness of bioaugmentation to treat this medium. Each population of microorganisms, and particularly in conditions Mb + Bd and Mb + Bd + Ba, achieved similar percentage of removal during the biological treatment, with 94–100%, 58–77%, and 86–93% of elimination for ammonium, COD, and aromatic compounds, respectively, after 23 days' treatment. Regarding in detail the populations present and active during the tests, the genus *Marinobacter* always appeared among the most active microorganisms. Results allowed us to conclude that in the case of this artificial medium, bioaugmentation had no significant impact on the treatment of the medium, as removal efficiencies were not significantly better in the conditions with the aromatic-degrading bacteria.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

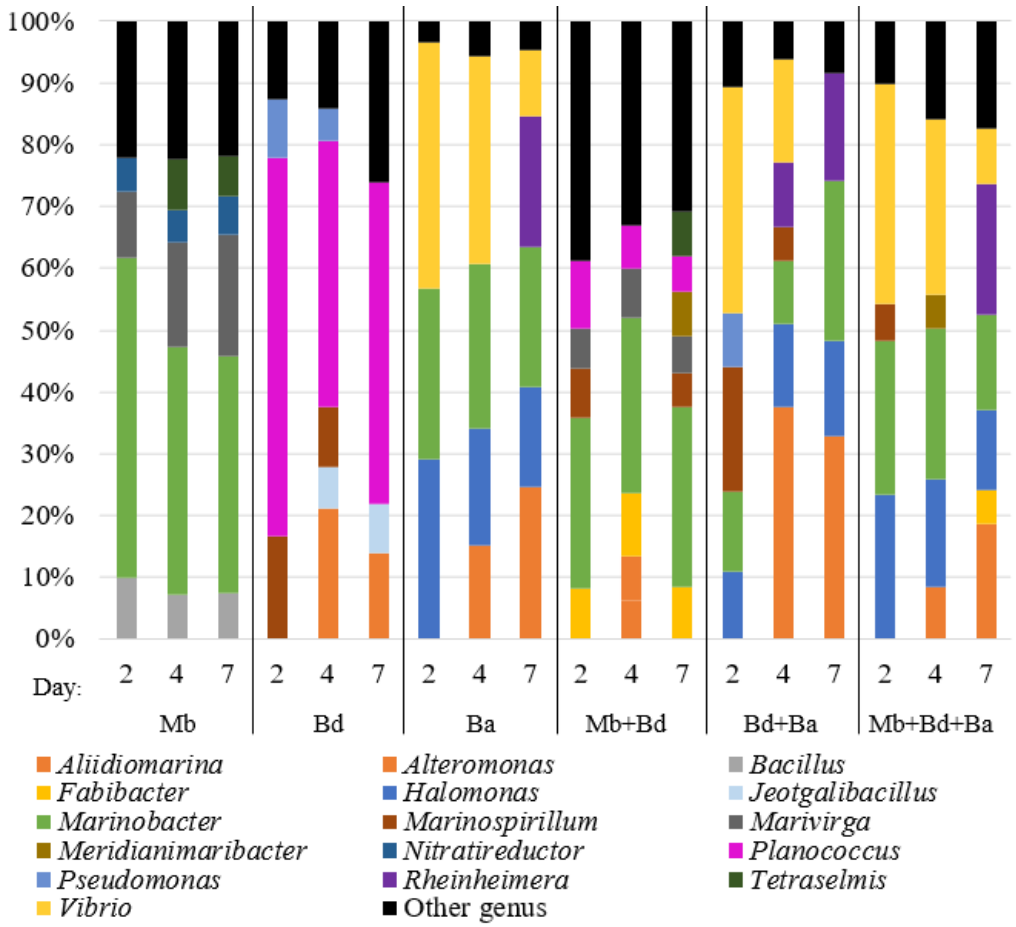

Figure A1. Bar plot of the major active genus identified in each condition at day 2, 4, and 7. Mb: microalgae and associated bacteria; Bd: bacteria from liquid digestate; Ba: aromatics-degrading bacteria; Mb + Bd: microalgae and associated bacteria + bacteria from liquid digestate; Bd+Ba: bacteria from liquid digestate + aromatics-degrading bacteria; Mb + Bd + Ba: microalgae and associated bacteria + bacteria from liquid digestate + aromatics-degrading bacteria.

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