






Bioaugmentation with *Advenella kashmirensis* for the treatment of a kraft pulp effluent by aerated lagoon

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ABSTRACT

Brazil is considered a world leader in paper production. It is estimated that the sector contributes 46.5% of the national gross domestic product, and its effluents have high concentrations of organic matter and recalcitrant compounds which can cause coloration and ecotoxicity, generating impact if not properly treated. A potential strategy for the treatment of this wastewater is bioaugmentation with native bacteria isolated from the industrial sludge and effluent, which, due to their potential metabolism adapted to adverse environmental conditions, may favor the removal of specific compounds. When associated with immobilization techniques, the inoculum can develop as a biofilm adhered to a support medium, which provides surfaces for adhesion, growth and survival of bacteria. We therefore investigated the treatment of Kraft pulp effluent by bioaugmentation with the autochthonous bacterium *Advenella kashmirensis* both immobilized in a spongy support medium - Aqua Porous Gel (APG) - and in free-swimming (planktonic) form, denominated Phase I and II, respectively. The investigation was performed in a bench-scale aerated lagoon system with untreated industrial effluent. The effect of the use of bacteria in bioaugmentation was assessed by the removal parameters of Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD₅), Color, Total Phenolic Compounds (TPC) and Lignin Compounds (LC). The results indicate that both Phases I and II were efficient for the treatment of COD, BOD₅ and color parameters. However, the biofilm form provided better stability to the biological system in the treatment of the Kraft pulp effluent.

Keywords: adhered, kraft effluent, SEM, suspended solids.

Bioaugmentação com *Advenella kashmirensis*, para tratamento de efluente de celulose kraft por lagoa aerada

RESUMO

O Brasil é considerado referência mundial na produção de papel, se estima que o setor contribui com 46,5% do produto interno bruto nacional, configurando uma manufatura extensiva cujos efluentes possuem concentrações elevadas de matéria orgânica e compostos



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recalcitrantes, que podem introduzir cor e ecotoxicidade, gerando impacto caso não se proceda o adequado tratamento do mesmo. Uma estratégia potencial para o tratamento desta água resíduária é a bioaumentação com bactérias nativas isoladas do lodo e efluente da indústria, estas devido ao potencial metabolismo adaptado às condições adversas do ambiente, podem favorecer a remoção de compostos específicos. Quando associada a técnicas de imobilização, o inóculo pode se desenvolver como biofilme aderido a um meio suporte, que proporciona superfícies para adesão, crescimento e sobrevivência das bactérias. Desta forma, foi avaliado o tratamento de efluente de celulose kraft por bioaumentação com bactéria autóctone *Advenella kashmirensis* imobilizada em meio suporte esponjoso do tipo aquaporousgel (APG), e sobre a forma de vida planctônica/livre, denominadas de fase I e II respectivamente, em sistema de lagoa aerada de escala de bancada com efluente industrial não tratado. O efeito do uso da bactéria na bioaumentação foi avaliado com efluente industrial não tratado. O efeito do uso da bactéria na bioaumentação foi avaliado pelos parâmetros de remoção de Demanda Química de Oxigênio (DQO), Demanda Bioquímica de Oxigênio (DBO₅), Cor, Compostos Fenólicos Totais (CFT) e Compostos Lignínicos (CL). Os resultados apontam que ambas as fases I e II, foram eficientes para tratamento dos parâmetros DQO, DBO₅ e cor. Entretanto, a forma de biofilmes conferiu melhor estabilidade ao sistema biológico no tratamento do efluente de celulose kraft.

Palavras-chave: efluente kraft, MEV, sólidos aderidos, suspensos.

1. INTRODUCTION

Kraft pulp accounts for 90% of global cellulose production (Del Rio *et al.*, 2022). Important for the world economy, the Kraft pulp industry generates effluents containing chemical oxygen demand (COD), biological oxygen demand (BOD₅), color and ecotoxicity (Ping *et al.*, 2019; Liang *et al.*, 2020), which are likely to impact the environment and human health without proper treatment (Lewis *et al.*, 2018; Ping *et al.*, 2019).

Alternatives for improving biological treatment systems for paper industry effluents have been investigated, such as bioaugmentation, which consists of selecting and adding microorganisms such as highly efficient bacteria to remove specific pollutants from the effluents (Lewis *et al.*, 2018; Liang *et al.*, 2020; Nunes, 2021; Verdel *et al.*, 2021).

Hypotheses suggest that bacteria that are isolated from polluted habitats, also known as autochthonous, have specific enzymatic systems that allow them to resist adverse environmental conditions (Kumar *et al.*, 2012). In addition, when adhered as biofilms to the surface of support media, such organisms can potentially withstand unfavorable conditions in the treatment system, such as temperature, pH, salinity and effluent load variations (Barwal and Chaudhary, 2014; Bayat *et al.*, 2015; Banerjee *et al.*, 2018).

The adhesion of the bacterial biofilm to the support medium depends on its characteristics of porosity, roughness and resistance to microbial degradation and abrasion; and such medium should preferably have a low cost (Bayat *et al.*, 2015; Peitz and Xavier, 2019). The AQUAPOROUSGEL® (APG) commercial sponge from Nisshinbo Chemical Inc is recommended for biofilm adhesion due to its high porosity, hydrophilic nature and stability (Peitz and Xavier, 2019; Subsanguan *et al.*, 2020).

In this context, since effluents from the pulp and paper industry correspond to extreme environments and are potentially capable of harboring microorganisms capable of purifying them (Lewis *et al.*, 2018), this work aimed to treat Kraft pulp effluent through bioaugmentation with autochthonous bacteria (*Advenella kashmirensis*) in the form of a biofilm adhered to APG compared to its non-immobilized application.

2. MATERIALS AND METHODS

The influent used for treatment in the aerated lagoon system inoculated with *A. kashmirensis* was kindly provided by an unbleached Kraft pulp mill in the metropolitan region of Curitiba, Brazil. We characterized and monitored three influent samples and the effluents from the aerated lagoon on laboratory scale by analyses of Chemical Oxygen Demand, Biological Oxygen Demand and Total and Volatile Suspended Solids according to APHA *et al.* (2017); Total and Volatile Adhered Solids adapted from APHA *et al.* (2017) and Melchioris (2019); Color (440nm) and Total Phenolic Compounds - TPC (215 nm) by Chamorro *et al.*, (2009) and Lignin Compounds - LC (UV280nm) according to the methodology by Çeçen (2003).

The study was carried out at the Wastewater Treatment Laboratory (LATAR - Laboratório de Tratamento de Águas Residuárias) of the Federal Technological University of Paraná (UTFPR) – Curitiba, Brazil, in two bench-scale Aerated Lagoons (AL) that had 1 L of useful volume each and operated in parallel (Figure 1). In the lagoon labeled Phase I, we added 90 units of APG support medium in a cubic format of 1cm³ with *A. kashmirensis* biofilm. In the AL labeled Phase II, we inoculated 10 ml of cell culture of *A. kashmirensis* that was isolated and identified by the Effluent Treatment Group (GTEF) from LATAR in the study by Silva *et al.* (2020). Both systems were subjected to continuous feeding with kraft samples. For the present study, the bacteria were cultured for reactivation and growth of isolated colonies according to the methodology described by Benson (2001).

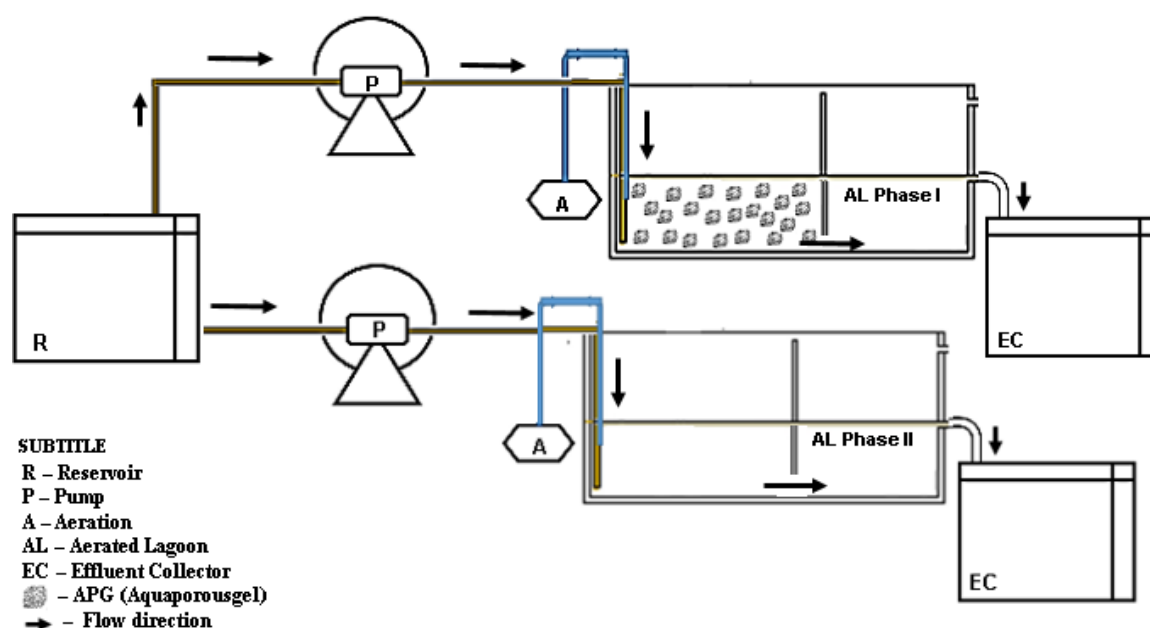


Figure 1. Aerated lagoon in lab scale.

The reactor was inoculated with cellulose industry sludge biomass at a concentration of 70 mgVSS.L⁻¹. An intermediate value among those used in biological systems (Von Sperling, 2014). Additionally, the kraft sample was supplemented with nutrients to achieve a COD:N:P ratio of 100:0.5:0.1, using 1.91 g L⁻¹ NH₄Cl and 0.56 g L⁻¹ K₂HPO₄.

To add the APG with adhered autochthonous bacteria in Phase I, it was necessary to previously perform a biofilm growth assay. This was structured in a 250 ml Erlenmeyer flask, consisting of 120 ml of sterile Nutrient Broth (NB) liquid medium, 10 ml of *A. kashmirensis* NB culture medium and 94 units of the APG support medium according to the methodology proposed by Gandhi *et al.* (2010). The bacterial culture medium was cultivated in a sterile penicillin-type flask containing 3 ml of NB medium and a scraping of a colony isolated in a

Petri dish with *A. kashmirensis* subculture, incubated for 24 hours in an oven at 28°C. The flask was covered with a foam top previously sanitized with ethanol instead of gauze, an adaptation promoted to maintain aeration in the flask since the chosen species is aerobic (Figure 2).

The biofilm growth test was operated for 21 days without agitation at room temperature (Figure 2). After the test period of the 94 APG units, 90 were inoculated in the Phase I Lagoon, 3 were used for analysis of adhered solids, and 1 unit was underwent a drying process and subsequently underwent gold metallization utilizing the Quanta Quorum Q150R ES equipment. These metalized samples were then subjected to Scanning Electron Microscopy (SEM) analysis at magnifications of 20x, 50x, 1000x, and 2000x, in the Multiuser Materials Characterization Center at UTFPR. APG samples were also collected from the lagoon at the end of the Phase I operating time.

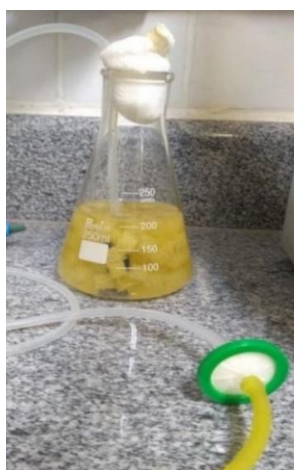


Figure 2. Experimental immobilization medium used in Phase I.

3. RESULTS AND DISCUSSIONS

3.1. Characterization of Kraft pulp industrial samples

Table 1 presents the characteristics of the industry samples which were used as influent of the AL treatment system.

Table 1. Characterization of the samples.

Parameter	Average value
BOD ₅ (mg.L ⁻¹)	148.27 ±18.08
COD (mg.L ⁻¹)	952.11 ±10.14
BOD ₅ /COD	0.16
TPC (UV 215nm)	306.56 ±9.38
Color (UV440 nm)	0.68 ±0.03
LC (UV 280 nm)	7.14 ±0.02

Nota: BOD₅ – Biochemical Oxygen Demand. COD – Chemical Oxygen Demand. TPC – Total Phenolic Compounds. LC: Lignin Compounds. Standard deviation values represent three industrial samples, each analyzed in triplicate.

It can be observed from Table 1 that the BOD₅/COD ratio calculated for the influent

samples was 0.16; therefore considered potentially recalcitrant in relation to Jordão and Pessoa (2017), who point out that indices of good biodegradability are related to values above 0.30 for this ratio.

When treating Kraft effluent from the same source as the sample in this study in an AL with Organic Loading Rate (OLR) of $0.2 \text{ kgCOD m}^{-3}\text{d}^{-1}$, and a BOD_5/COD ratio of 0.28, Nunes (2021) obtained global organic matter removal efficiencies reaching levels of 94% BOD_5 and 51% COD.

3.2. ALs operating parameters

Figure 3 shows the control parameters OLR, HRT (Hydraulic Retention Time), pH and temperature during the operating phases of the aerated lagoons. a) Phase I AL; b) Phase II AL.

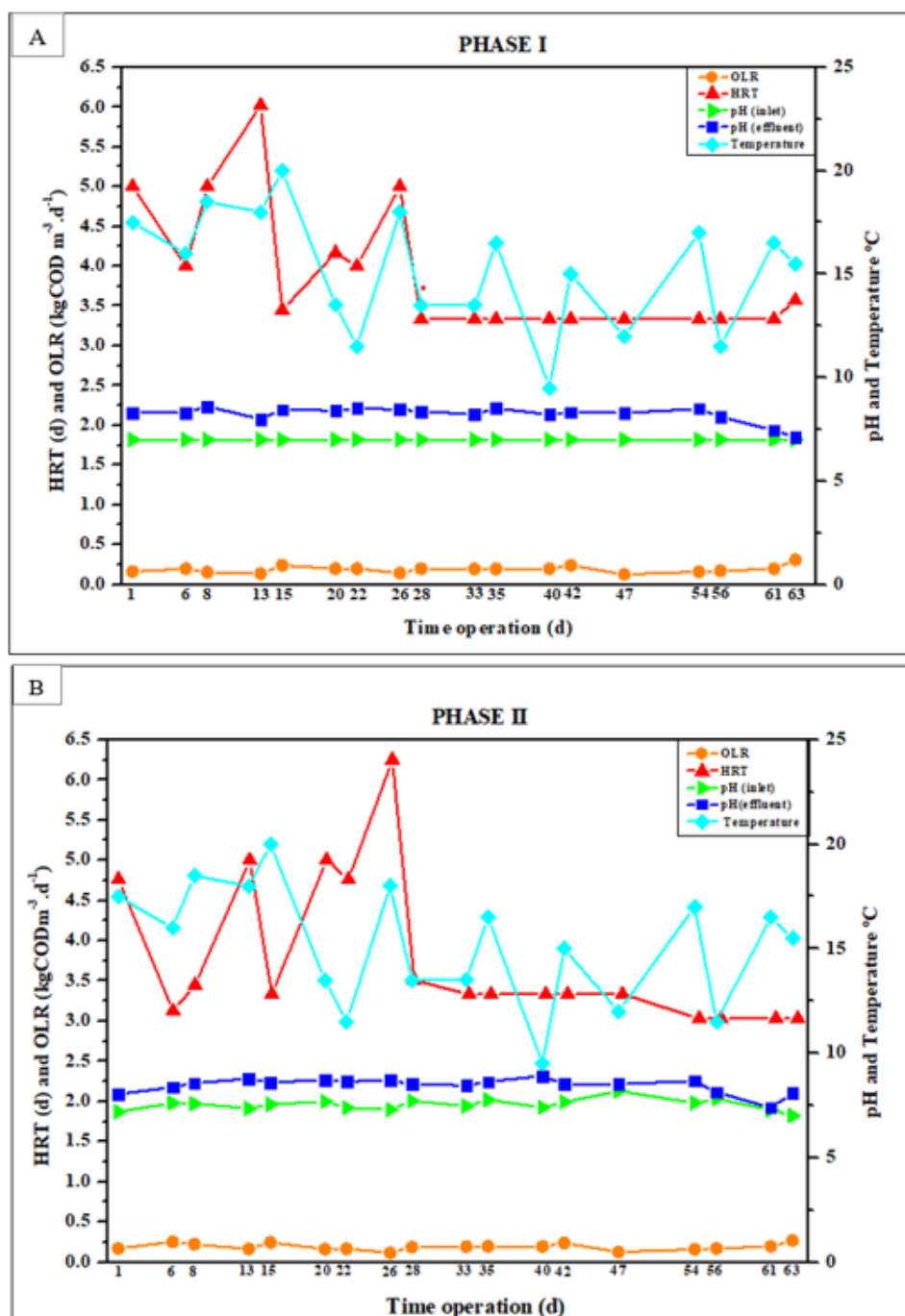


Figure 3. Treatment control parameters in ALs. a) Phase I AL; b) Phase II AL.

The average OLR applied in the experiments corresponded to $0.2 \text{ kgCOD m}^{-3}\text{d}^{-1}$, a value in line with the industry's reality, resulting in an average HRT of 3.78 d for Phases I and II.

The inlet pH was adjusted to 7.00 and during the operation, it tended to increase, with an average of 8.23 for Phase I and 8.46 for Phase II, consistent with what was observed by Pazda (2022) who obtained an average effluent pH of 8.1 ± 0.43 treating Kraft effluent in a Facultative Aerated Lagoon (FAL).

For Von Sperling (2014), the increase in pH observed in lagoon systems is obtained due to the formation of carbonate in anaerobic zones. Flemming *et al.*, (2016) describe the existence of functional gradients in the biofilm, where the population close to the surface in contact with the aqueous medium consumes O_2 at levels that hinder diffusion into the interior of the biofilm. In this way, the increase in pH may be related to the formation of anoxic zones in the internal environment of the support medium in Phase I.

3.3. Evaluation of treatment efficiency

Figure 4 presents the removal efficiencies calculated during the treatment of samples from the Kraft pulp industry in AL bioaugmented with *A. kashmirensis* in both phases. a) Phase I AL; b) Phase II AL.

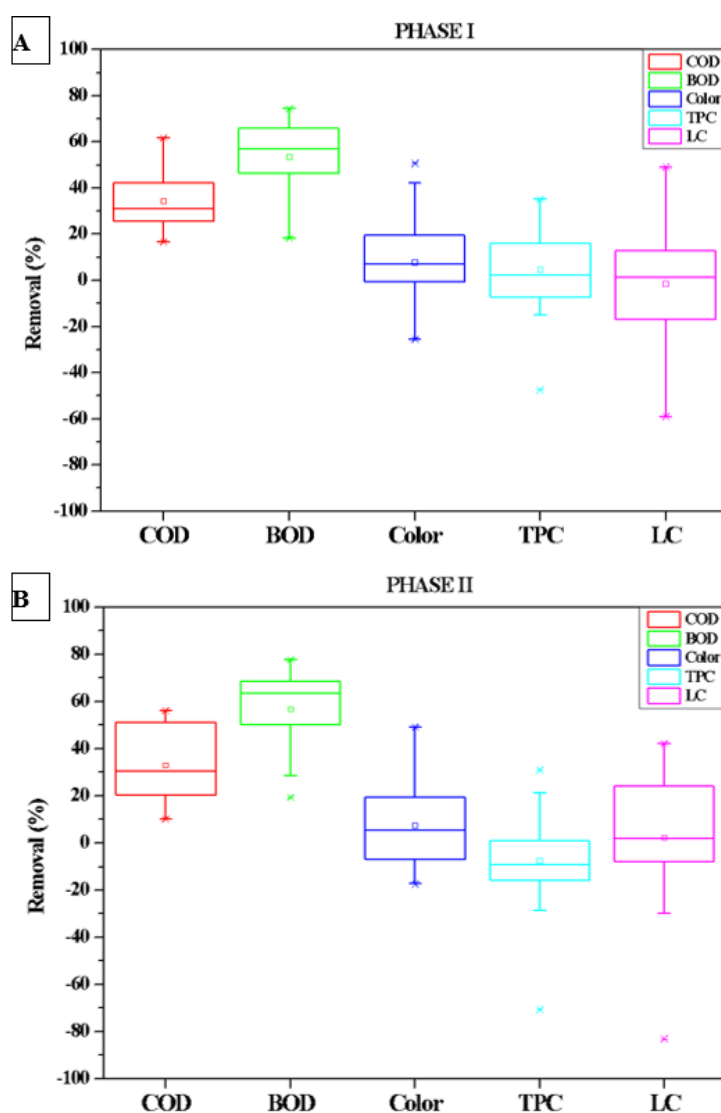


Figure 4. Removal efficiencies of physical-chemical parameters. a) Phase I AL; b) Phase II AL.

We obtained COD removal efficiency of 34% for Phase I and 33% for Phase II, values that did not differ statistically ($p > 0.05$). Vanzetto *et al.* (2014) obtained a COD removal efficiency of 48% treating Kraft effluent from the same source as the one in this research, using an MBBR reactor operated for 60 d with Kaldness K3 support medium.

The BOD₅ removal efficiency values found in Phase I corresponded to 53% and 57% for Phase II, values that did not differ statistically ($p > 0.05$) either. Melchioris (2019) obtained an average removal of this parameter of 51% after 60 d of operation when treating Kraft effluent in an MBBR reactor with APG support medium. After 120 d of operation, the average removal efficiency obtained corresponded to 82%. According to the author, this increase in removal efficiency is related to the adaptation and growth of the biomass in the support medium over time; therefore, the biofilm provides greater stability and improvement in biological treatments.

In addition to the system operating time, the HRT contributes to improving BOD₅ removal efficiency (RE). Gandhi *et al.* (2010) found a removal efficiency of 81% for this parameter with a HRT of 6 d treating cellulose effluent in a batch reactor filled with PUF support medium (polyurethane sponge) with *Bacillus subtilis* biofilm.

The color removal obtained in Phase I corresponded to 8%, while for Phase II the RE was 7%, which did not differ statistically from Phase I ($p > 0.05$). In the bioaugmentation assay using *Advenella kashmirensis* associated with biomass in an Erlenmeyer flask incubated in a shaker at 25°C, agitated at 100 rpm and with HRT of 11.6 hours, Silva *et al.* (2021) obtained a color removal potential of 60%. On the other hand, Peitz and Xavier (2019) observed a 38% increase in color in the study of treatment in a FAL with APG support medium associated with biomass.

The TPC removal found in Phase I was 5%, while for Phase II no removal efficiencies were obtained for this compound. Despite these differences, statistically, the results did not differ significantly at a 95% confidence level. Pazda (2022) obtained a removal rate of 8% for this compound when treating Kraft effluent from the same origin as the one in this research with the same C:N:P ratio in a FAL system.

As shown in Figure 4, lignin compounds were not removed in Phase I, whereas for Phase II the removal was only 2%, both being statistically similar at a confidence level of 95%. Peitz and Xavier (2019) obtained an increase of this compound, around 10%, treating Kraft effluent in AL with APG medium. Chamorro *et al.* (2009) also observed an increase in lignin compounds during treatment in an aerated lagoon.

Milestone *et al.* (2004) justify that when there are increments of lignin-derived compounds, they are related to the biological treatment carried out in FALs, in which molecules with high molecular weight are biotransformed and can repolymerize, forming new lignin-derived compounds.

The use of the *A. kashmirensis* species in effluent treatment processes is recent. The closest study in bioremediation using *A. kashmirensis* was conducted by Hao *et al.* (2013), focusing on the removal of petroleum. They employed the bacterium isolated from seawater, achieving a removal efficiency of 46%. For cellulose effluents, a good removal profile of specific compounds was observed, exceeding 60% color removal and 40% ligninic compound removal in the presence of biomass from the industrial plant, as presented by Silva *et al.* (2021).

Figure 5 presents the solids concentration profiles during the research phases a) Phase I AL; b) Phase II AL.

Regarding suspended solids (Figure 5a), for Phase I the concentration of VSS was 380 mg L⁻¹; and for Phase II, 415 mg L⁻¹. While the TSS content for Phase I was 1585 mg L⁻¹, for Phase II it was 1673 mg L⁻¹. With this result, we calculated the VSS/TSS ratio of the samples corresponding to 0.24 and 0.25 for Phases I and II, respectively. The results of Phase I were close to those observed by Melchioris (2019) in an experiment treating Kraft effluent in an MBBR reactor with APG support medium, in which the VSS concentration was 180 mg L⁻¹ after 60 days of system operation. Von Sperling (2014) attributes the VSS/TSS ratio to the

sludge stabilization tendency, indicating biomass associated with high sludge ages, which in the lagoon was greater than 50 d.

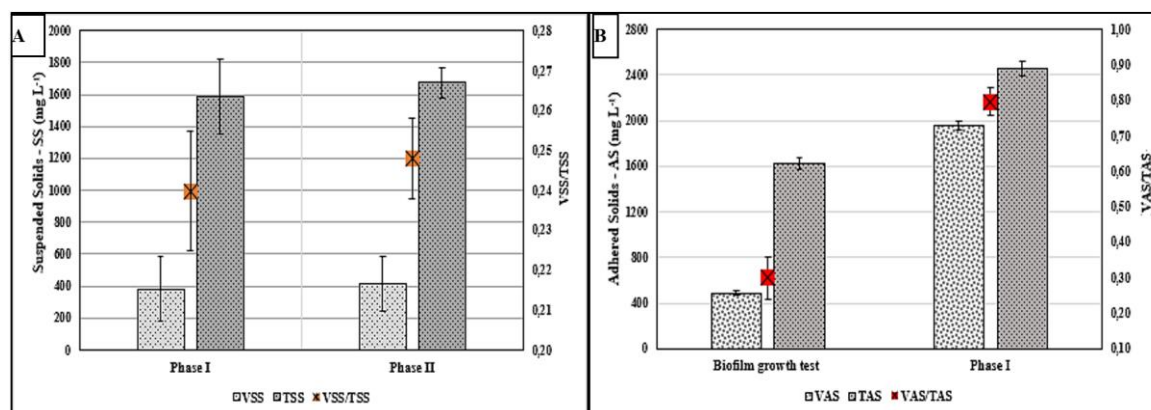


Figure 5. Suspended and Adhered Solid Content.

Figure 5b shows the concentration of solids retained in the APG support medium. The item “bacteria + APG” corresponds to the APG of the 21 day immobilization test for bacterial biofilm growth. In this test, we obtained a VAS of 486 mg L⁻¹; TAS of 1620 mg L⁻¹ and VAS/TAS of 0.30. For Phase I, where the APG units were previously immobilized with *A. kashmirensis* biofilm for 21 days, we obtained a total VAS of 1957 mg L⁻¹ and TAS of 2460 mg L⁻¹, which promoted a VAS/TAS of 0.80, a value that may indicate the tendency of the biomass to immobilization, where it is protected from oxidation levels inside the reactor.

According to Figure 5, one can infer the dynamics of the sludge in the lagoons, where the prevalence of biomass adhered to the APG occurs in relation to those suspended in the effluent. Such results may be related to the ability of the support medium to provide protection to unfavorable conditions, as pointed out by Barwal and Chaudhary (2014).

Melchioris (2019) investigated the solids adhered to the APG support medium used in an MBBR reactor, in which he obtained an average VAS of approximately 700 mg L⁻¹, with VAS/TAS ratio of 0.80.

3.4. SEM investigation of biofilm formation on the spongy support

Figure 6 shows the micrographs of the APG spongy support medium in order to verify its internal structures.

In Figure 6a, one can observe the APG sponge before its use in the aerated lagoon (50x magnification). In Figure 6b, it is possible to verify the internal three-dimensional structure of the APG, composed of several pores and internal walls that are necessary for adhesion and growth of microorganisms and biofilm (200x magnification). Subsanguan *et al.* (2020) point out that the surface area of the APG medium can reach 3000 m²m⁻³.

Figures 6c and 6d show micrographs of the APG in which the *A. kashmirensis* biofilm grew during 21 d. In Figure 6c, there are projections of the biofilm matrix at 200x magnification; the literature shows a beneficial relationship of less thick and more projected biofilm structures as they increase the liquid-biofilm interfaces, adsorption of molecules and diffusion of nutrients (Krsmanovic *et al.*, 2021). On the other hand, Image 6d (1000x magnification) shows a thicker conformation of the biofilm, possibly resulting from adhesion to the external part of the APG. As pointed out by Tsagkari and Sloan (2018), tension forces and turbulence in the aqueous medium can affect the physical structure of biofilms, with a tendency for their architecture to become more dense and cohesive to prevent their detachment.

Images 6e and 6f show the structure of the APG after the AL operation in Phase I, where we can observe great adhesion of biofilms to the spaces of the pores without, however, clogging

them. In Item 6e (50x magnification), we can verify different formats of adhered biofilms, but without evident stratification of a certain microbiological group in areas of the APG. In Image 5f (1000x magnification), it is possible to see a component that resembles the specialized reproductive structure of filamentous fungi, called conidia, which by adhesion to a substrate are germinated, originating hyphae (Aor *et al.*, 2018).

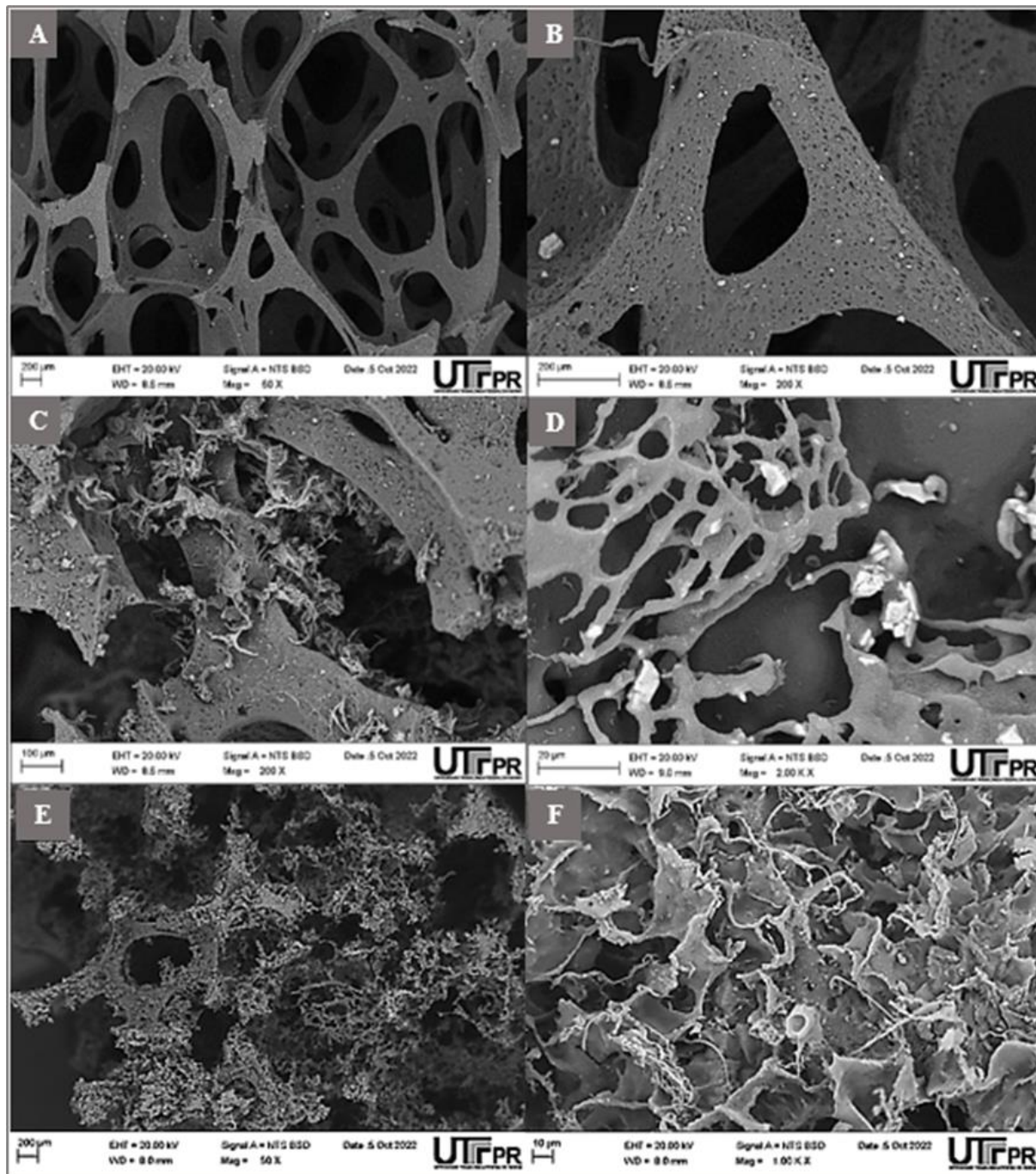


Figure 6. Scanning electron microscopy images of the APG support medium.

After 240 days operating the MBBR-APG reactor treating Kraft effluent, Melchioris (2019) also found the potential diversity of bacteria and fungi adhered to the support medium. According to the author, the pores of the APG support medium remained open, a characteristic that favored the performance of the biological treatment of the effluent, which corroborates the observations in the present investigation.

4. CONCLUSIONS

In this study, we evaluated the performance of the treatment of Kraft pulp effluent using an aerated lagoon system modified by the inoculum of the bacterial species *Advenella kashmirensis*.

In view of the global organic matter treatment, we obtained removal levels of 34% for COD and 53% BOD₅ in Phase I, and 33% COD and 57% of BOD₅ in Phase II; therefore, the conformation of the bacteria either as a biofilm or free-swimming did not lead to improvements in removal.

However, for specific compounds, the bioaugmentation of the bacteria adhered to the support medium in Phase I provided removal of color by 8% and 5% of TPC, results considered promising since they consist of removal rates of recalcitrant parameters, which may be a viable alternative that demands further investigation.

Through SEM analyses, it was possible to identify the formation of biofilm adhered to the *A. kashmirensis* APG as well as the biomass APG, without clogging of the pores of the support medium in up to 60d of system operation.

Considering the results, the bioaugmentation of the autochthonous species adhered as a biofilm constitutes a promising alternative to the biological treatment of effluent from the paper industry.

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