Microalgal-bacterial aggregates with flue gas supply as a platform for the treatment of anaerobic digestion centrate

Short title: Microalgal-bacterial aggregates for centrate treatment

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Abstract

BACKGROUND: Centrate treatment using microalgal-bacterial processes might be limited by the hydraulic retention time (HRT) required to achieve satisfactory COD and nutrients removal. Moreover, the poor settling of microalgal biomass still limits the technical and economic performance of microalgal-bacterial processes. In this work, the performance of microalgal-bacterial aggregates (MABAs) supplied with flue gas was investigated as an effective strategy to improve the treatment of centrate from anaerobic digestion of winery wastewater.

RESULTS: MABAs supplied with flue gas achieved maximum soluble COD, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$ and $N\text{-}NH_4^+$ removal efficiencies of 95%, 94%, 100%, and 100%, respectively, in 5-fold centrate dilution within 7 days of operation. Centrate turbidity or its components did not hinder the performance of the MABAs under the conditions tested and no aggregates were formed in controls without MABAs inoculation. The mean diameter of the MABAs after centrate treatment was the same or even larger than that of the aggregates of the inoculum. Scanning electron microscopy analyses showed that the liquid medium composition influenced the structure and the type of microalgal cells established in the MABAs.

CONCLUSION: MABAs-based centrate treatment supported by flue gas is a promising technology for improving COD and nutrients removal from centrate as well as further biomass harvesting.

Keywords: Centrate; Flue gas; Microalgal-bacterial aggregates; Nutrients removal; COD removal.

INTRODUCTION

Microalgal-bacterial processes have been reported as a suitable technology for the removal of organic matter and nutrients from centrates generated in sludge thickening or from anaerobic digestion processes.[1,2] Removal performances of total organic carbon, N, and P ranging from 75 to 100% have been consistently recorded in microalgal-bacterial systems treating centrate.[3-6] These processes are based on the aerobic oxidation of organic matter by heterotrophic bacteria, which produce a CO_2 that is taken up by microalgae. Thus, microalgal photosynthesis generates the O_2 required for COD removal.[7] Centrate treatment has been typically investigated in high rate algal ponds (HRAPs) devoted to biogas upgrading. However, the treatment of centrates and other high-strength wastewaters in HRAPs might be limited by the hydraulic retention time (HRT) required for achieving satisfactory COD and nutrients removal.[8] For instance, HRT of 10 – 73 days have been reported for achieving maximum COD and total nitrogen removal efficiencies of ~70% and ~85%, respectively, in HRAPs treating diluted centrate or diluted piggery wastewater.[3,9,10] Thus, strategies to improve the performance of microalgal-bacterial processes are still required when treating high strength wastewaters. Such strategies include the use of flue gas, which might boost the activity of both microalgae and aerobic bacteria due to the presence of CO_2 and O_2, respectively. Flue gas is a residual gas emitted from the combustion of fuels (including biogas) and is mainly composed of N_2 (68–79%), CO_2 (5–24%) and O_2 (7–17%). Flue gas is usually available in wastewater treatment facilities, and therefore, its use is a technically feasible option to improve COD and nutrients removal in microalgal-bacterial processes.[11,12] In fact, an enhancement in COD, total organic carbon and phosphorous removals by flue gas supply has been already reported in the treatment of low-strength wastewater in outdoors pilot HRAPs.[13] Nevertheless, as far as the authors know, the effect of flue gas supply on centrate

treatment has not been systematically studied to date. Moreover, the use of microalgae-bacteria aggregates (MABAs) has also been proposed as a strategy to improve the performance of microalgal-bacterial processes, since biomass harvesting can account for up to 40% of the total treatment costs.[2] The use of MABAs drastically improves the biomass settling velocity compared with dispersed microalgae cells,[14,15] thus enhancing biomass harvesting and the economic feasibility of the treatment process.[7,14,16]

In the present study, the use of MABAs supplied with flue gas was investigated as an effective strategy to treat high-strength centrate from the anaerobic digestion of winery effluents. Centrate from the anaerobic digestion of winery effluents was used as a model high-strength wastewater due to its industrial relevance and high organic matter content. Hence, the aim of this research was to assess the removal performance of soluble COD (sCOD), $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and PO_4^{3-} as well as to determine the impact of centrate dilutions and flue gas supply on the size and structure of MABAs.

MATERIALS AND METHODS

Centrate composition

The centrate was obtained from the digestate of an anaerobic reactor treating winery wastewater. The continuous stirred tank anaerobic reactor was operated with an organic loading rate of 10 g COD L^{-1} d^{-1} and an HRT of 8 days. The digestate was centrifuged at 3,500 rpm × 10 min. The resulting centrate (liquid fraction of the digestate) was characterized by sCOD, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ concentrations of: 27,250 ± 353 mg L^{-1}, 14.45 ± 0.05 mg L^{-1}, 30.5 ± 6.3 mg L^{-1} and 152.5 ± 10.6 mg L^{-1}, respectively. The sCOD in the centrate was mainly composed of residual ethanol (31.8%) and the following volatile fatty acids (VFAs): acetic acid (56.7%), butyric acid (6.4%) and propionic acid (5.1%). The digestate centrifugation protocol herein used did not remove completely the

solids from the centrate, which still contained total suspended solids (TSS) of 1,538 ± 43 mg L^{-1} and volatile suspended solids (VSS) of 1,162 ± 25 mg L^{-1}.

Inoculum

The inoculum consisted of MABAs obtained from an HRAP treating domestic wastewater and operated indoors under the following conditions: working volume of 50 L, average influent concentration of 500 ± 81.4 mg COD L^{-1}, HRT of 6 h, solids residence time of 12 h, light/dark periods of 12:12 h, and irradiance of 200 µmol m^{-2} s^{-1}. The average settling velocity of the MABAs was 3.7 ± 0.1 m h^{-1}. Microalgal-bacterial biomass used as inoculum in all the experiments was prepared as follows: 500 mL of culture broth were taken from the HRAP and biomass was allowed to settle for 30 min. Then, the supernatant was discarded to remove residual COD and nutrients. The original volume was replenished by adding a fresh BG-11 culture medium.

Chemicals and mineral salt medium

BG-11 mineral salt medium (UTEX Culture Collection of Algae) was used to dilute centrate and perform control tests. The medium had the following composition (mM): $NaNO_3$ (17.60), K_2HPO_4 (0.23), $MgSO_4$-$7H_2O$ (0.30), $CaCl_2$-$2H_2O$ (0.24), citric acid-$2H_2O$ (0.031), ferric ammonium citrate (0.021), Na_2EDTA-$2H_2O$ (0.0027) and Na_2CO_3 (0.19) and supplemented with 1 mL/L of the following trace metals solution (mM): H_3BO_3 (46), $MnCl_2$-$4H_2O$ (9), $ZnSO_4$-$7H_2O$ (0.77), Na_2MoO_4-$2H_2O$ (1.6), $CuSO_4$-$5H_2O$ (0.3), $Co(NO_3)_2$-$6H_2O$ (0.17). The chemicals used for the medium preparation were purchased from Sigma-Aldrich with a purity of at least 99%. CO_2 (Praxair, Querétaro, Mexico) with a purity of 99.9% was used to generate the synthetic flue gas used in the centrate treatment experiments.

Centrate treatment experiments

The experiments were performed in 500 mL glass bottles with a working volume of 300 mL. Centrate was diluted 5- and 10-fold with fresh BG-11 medium and inoculated with MABAs at initial VSS concentration of 50 mg L^{-1}. These experiments were conducted in triplicate. Control bottles (in duplicate) containing: (i) BG-11 medium and MABAs inoculum (50 mg VSS L^{-1}), and (ii) 10-fold diluted centrate without MABAs inoculum were also prepared. Control bottles with BG-11 medium were supplemented with acetate as sCOD source matching the same initial sCOD concentration of the control bottle with 10-fold diluted centrate. Due to the low nitrogen concentration in the centrate from the winery wastewater, $N\text{-}NO_3^-$ was supplied to bottles containing centrate matching the initial nitrogen concentration of the BG-11 medium. The initial pH in all bottles was adjusted to 7.5. The experiments were performed at 20 ± 2°C using light/dark periods of 12/12 h at 30 µmol m^{-2} s^{-1} of light intensity. Synthetic flue gas (78% N2, 19% O2 and 2% CO2) was continuously provided to all bottles using porous-stone diffusers at a gas flow rate of 90 mL min^{-1} (corresponding to 0.3 V_{gas} V_{liquid}^{-1} min^{-1}). In real-case scenarios, flue gas can be mixed with air to fulfill the O_2 and CO_2 supply required, thus achieving a composition similar to that studied in the present study (this is the case when the flue gas stream available is small compared with the volume of the photobioreactor). The experimental conditions set in test and control bottles are summarized in Table 1. Liquid samples were periodically taken for analysis of sCOD, nitrogen and phosphorus concentrations in each bottle.

Table 1. Experimental conditions set in centrate treatment experiments and control tests.

Experimental condition	**MABAs Inoculum**	**Initial COD (mg L^{-1})**	**Initial N-NH_4^+ (mg L^{-1})**	**Initial N-NO_3^- (mg L^{-1})**	**Initial P-PO_4^{3-} (mg L^{-1})**	**Initial ethanol (% COD)**
Centrate with 1/5 dilution (D 1/5 test)	Yes	5948 ± 111	2.7 ± 0.4	244.6 ± 41.1	24.7 ± 0.8	29
Centrate with 1/10 dilution (D 1/10 test)	Yes	3045 ± 233	1.2 ± 0.1	269.3 ± 17.6	14.9 ± 0.2	28
BG-11 control	Yes	2775 ± 198	0.5 ± 0.1	258.3 ± 61.9	19.0 ± 0.7	0
D 1/10 control	No	3342 ± 100	1.4 ± 0.1	268.8 ± 19.0	16.3 ± 0.7	26

MABAs size distribution

Images of the MABAs were obtained in a stereoscopic microscope (Stemi DV4, Carl Zeiss) equipped with an image acquisition system (LEICA ICC50 HD). Diameter distribution was determined by image analysis using the ImageJ software (version 1.52a). Gray balance was applied with a *Shangbang* threshold to identify aggregates by color saturation. The size of an aggregate was determined in terms of the Feret diameter (d_p), which is a parameter commonly used to characterize the size of heterogeneous particles, defined as the distance between two parallel tangents on opposite sides of the randomly oriented particle.[17] The Feret mean diameter (FMD) was determined as the average of all d_p values obtained for each experimental condition. Considering that the size of individual microalgae cells can range from 5 to 50 μm,[18] dispersed microalgal cells were discriminated in this analysis and only particles above 50 μm were considered as MABAs. At least 800 d_p values were considered to generate the FMD under each experimental condition.

Scanning electron microscopy (SEM)

The structure of MABAs was analyzed by scanning electron microscopy. Samples were fixed and dehydrated using the glutaraldehyde protocol.[19] Then, each sample was gold-covered using physical sputtering in a low vacuum coater. Images were obtained in a Zeiss EVO-50 microscope equipped with a Leica EM-ACE200 camera.

Analytical methods

Liquid samples were filtered (0.45 μm nylon membranes) before performing the analytical methods. Ethanol and initial VFA concentrations in the centrate were measured by GC-FID (7890 B, Agilent Technologies, Santa Clara, CA, USA) as described by

Carrillo-Reyes et al.[20] Soluble COD (sCOD) was measured using the colorimetric closed reflux method.[21] Soluble concentrations of $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ were measured by the salicylate method, the cadmium reduction method, and the molybdovanadate with acid persulfate digestion method, respectively.[21] TSS and VSS were determined according to standard methods.[21]

Statistical analyses

Differences among sCOD, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$ and $N\text{-}NH_4^+$ removal rates and Feret mean diameters under the different experimental conditions tested were determined by ANOVA tests followed by the post hoc Tukey's multiple comparisons test ($\alpha<0.05$) to identify specific means that are significantly different from each other. Statistical analyses were carried out in the GraphPad Prism software (version 7).

RESULTS AND DISCUSSION

Soluble COD removal

The removal of the complex sCOD present in the centrate, which included a mixture of VFAs and a relatively high content of ethanol that accounted for 28-29% of the initial sCOD supplied in test bottles, was assessed (Fig. 1). The D 1/5 tests contained a sCOD concentration of 5948 ± 111 mg L^{-1}, corresponding to an ethanol concentration of 1725 ± 32 mg L^{-1}. The microbial community was able to remove most of the sCOD within 7 days, supporting an average removal rate of 804.0 ± 16.9 mg L^{-1} d^{-1} and removal efficiency of 95% (Table 2). The D 1/10 tests contained a sCOD concentration of 3045 ± 233 mg L^{-1}, corresponding to an ethanol concentration of 852 ± 65 mg L^{-1}. Under these conditions, an average sCOD removal rate of 405.9 ± 33.4 mg L^{-1} d^{-1} was recorded, which is a half of the removal rate achieved in the D 1/5 tests. A high sCOD removal efficiency

of 93% was also observed in the D 1/10 tests. It must be noted that although D 1/5 tests were provided with twice sCOD concentration than the D 1/10 tests, the bottles with lower dilution contained a higher bacterial inoculum concentration coming from the centrate. Hence, besides the similar MABAs inoculum (50 mg SSV L^{-1}), D 1/5 tests contained an additional inoculum of 232 mg SSV L^{-1} from the centrate, while D 1/10 tests contained 116 mg SSV L^{-1}. Therefore, the higher sCOD removal rate recorded in the D 1/5 tests can be attributed to a higher sCOD concentration and a higher inoculum concentration coming from the centrate.

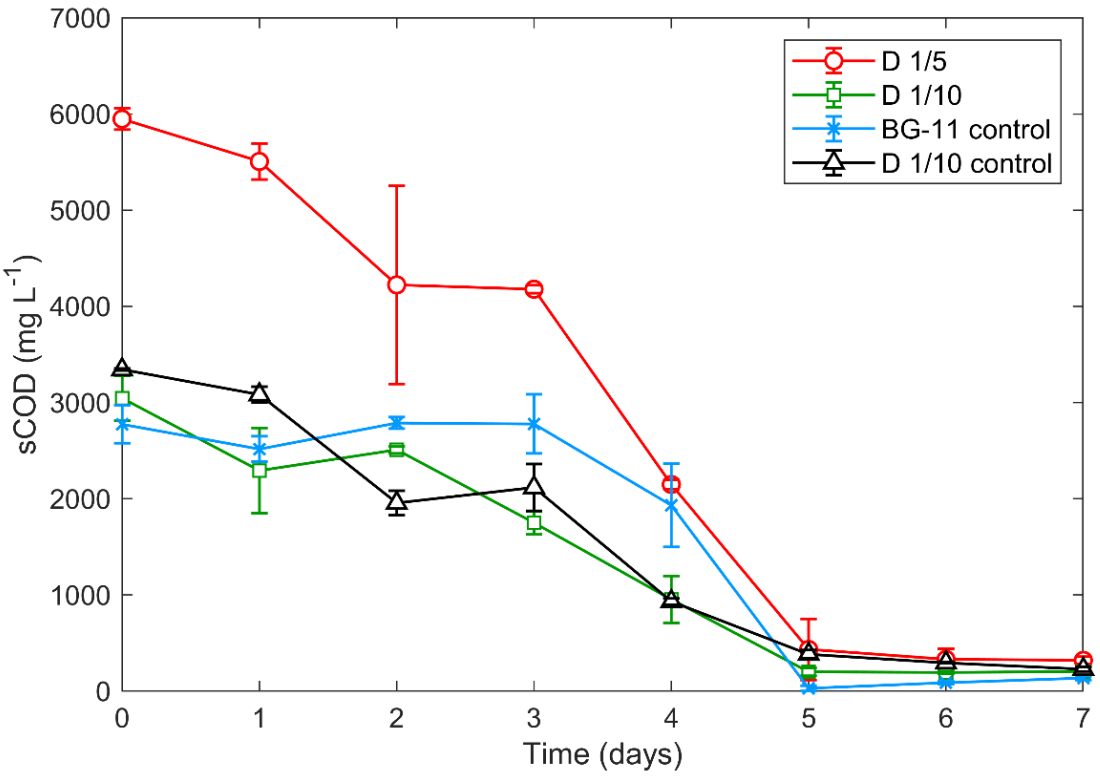

Fig. 1. sCOD concentration profiles of D 1/5 test (circles), D 1/10 test (squares), BG-11 control (asterisks) and D 1/10 control (triangles) during centrate treatment experiments.

Table 2. Removal performances of COD, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ determined in centrate dilutions and control tests.

Experimental condition	**COD**		**$N\text{-}NH_4^+$**		**$N\text{-}NO_3^-$**		**$P\text{-}PO_4^{3-}$**	
	Removal rate* (mg L^{-1} d^{-1})	Removal efficiency (%)	Removal rate* (mg L^{-1} d^{-1})	Removal efficiency (%)	Removal rate* (mg L^{-1} d^{-1})	Removal efficiency (%)	Removal rate* (mg L^{-1} d^{-1})	Removal efficiency (%)
D 1/5 test	804.0 ± 16.9	95	1.3 ± 0.2	100	33.0 ± 5.9	94	6.2 ± 0.2	100
D 1/10 test	405.9 ± 33.4	93	0.6 ± 0.1	100	32.7 ± 3.4	85	3.7 ± 0.1	100
BG-11 control	377.1 ± 28.4	94	0.5 ± 0.1	100	35.7 ± 8.8	96	4.8 ± 0.2	100
D 1/10 control	445.0 ± 3.7	93	0.7 ± 0.1	100	29.6 ± 1.9	76	4.1± 0.2	100

*Removal rates were calculated as $\frac{\Delta Concentration}{\Delta t}$, with Δt =7 days for COD and $N\text{-}NO_3^-$, Δt =2 days for $N\text{-}NH_4^+$ and Δt = 4 days for $P\text{-}PO_4^{3-}$.

Important insights on sCOD removal were also obtained from the control tests. BG-11 controls inoculated with MABAs and provided with acetate as sCOD source showed a similar removal performance than the D 1/10 tests, reaching a 94% removal efficiency. After statistical analysis (ANOVA + Tukey's test, both at α=0.05) no significant differences were found among the initial COD concentrations of the centrate 1/10 dilution, the BG-11 control, and the 1/10 dilution control. Besides MABAs, no additional inoculum was present in the BG-11 control. Thus, sCOD removal was carried out by the algal-bacterial community present only in the MABAs and it is worth noting that important sCOD removal in the BG-11 controls was observed only after 3 days of operation, while bottles containing centrate exhibited an important sCOD removal within the first 3 days. This suggested that the bacterial community in the centrate played a key role in sCOD removal. This was also supported by the fact that D 1/10 controls without MABAs inoculum exhibited a comparable sCOD removal to the D 1/10 tests. These results strongly suggest that heterotrophic bacteria present in the centrate played a key role in sCOD removal from centrate. Unlike biogas upgrading processes where the CO_2 absorbed in the liquid phase mainly boosts the growth of microalgae, when flue gas is supplied, the activity of both aerobic bacteria and microalgae is boosted since O_2 and CO_2 are provided, improving the removal of sCOD and nutrients, respectively.

Nutrients removal

The initial N-NH_4^+ concentration in the centrate represented only a small fraction of the nitrogen contained in all tests and bottles (Table 1). Thus, N-NH_4^+ was removed within the first two days of operation achieving a 100% removal efficiency regardless of the experimental conditions tested (Fig. 2A). Under the aerobic conditions used, N-NH_4^+ depletion was attributed to nitrification. On the other hand, D 1/5 tests supported an

1 average removal rate of 33.0 ± 5.9 mg $N\text{-}NO_3^-$ L^{-1} d^{-1} with a removal efficiency of 94%
2 (Table 2). Approximately 80% of the initial $N\text{-}NO_3^-$ supplied was removed within the
3 first 5 days of operation (Fig. 2B). D 1/10 tests showed a $N\text{-}NO_3^-$ removal rate comparable
4 with the D 1/5 tests, but a removal efficiency of 85%. The lower removal efficiency was
5 due to a slightly higher initial $N\text{-}NO_3^-$ concentration in the D 1/10 test bottles relative to
6 the D 1/5 tests. It is important to stress that microalgal growth, and therefore nutrient
7 removal, was not limited by sCOD removal since additional CO_2 was supplied through
8 flue gas.

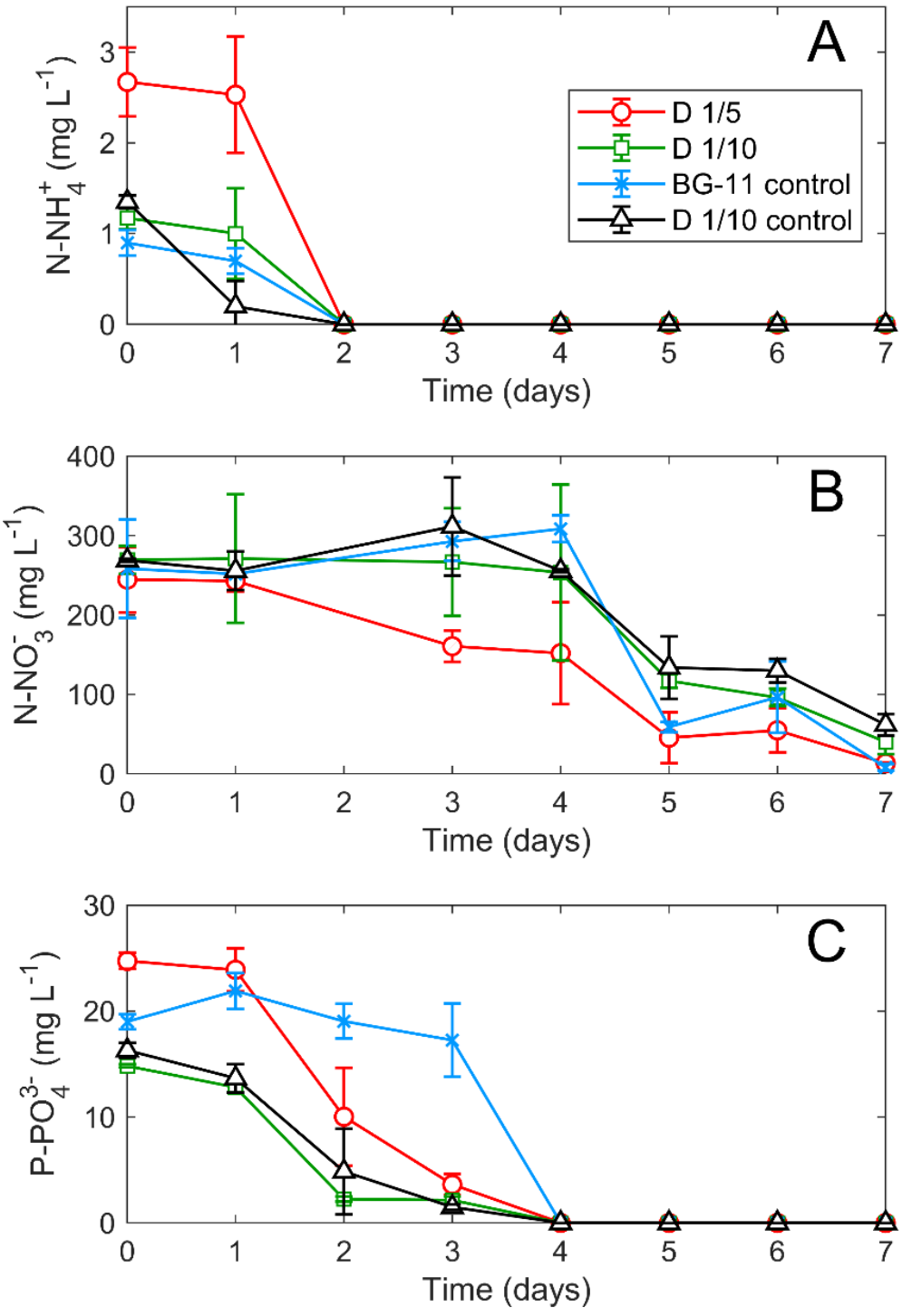

Fig. 2. Concentration profiles of (A) $N\text{-}NH_4^+$ (B), $N\text{-}NO_3^-$ and (C) $P\text{-}PO_4^{3-}$ during centrate treatment experiments in D 1/5 test (circles), D 1/10 test (squares), BG-11 control (asterisks) and D 1/10 control (triangles).

The BG-11 control supported a $N\text{-}NO_3^-$ removal rate of 35.7 ± 8.8 mg L^{-1} d^{-1}, comparable with that recorded in D 1/5 and D 1/10 tests (no significant differences among them). These results showed that when MABAs inoculum was provided the $N\text{-}NO_3^-$ removal rate was virtually the same, regardless of the culture medium used. Interestingly, the D 1/10 control tests without MABAs inoculum showed an average $N\text{-}NO_3^-$ removal only slightly lower than the bottles provided with MABAs inoculum, supporting a removal rate of 29.6 ± 1.9 mg $N\text{-}NO_3^-$ L^{-1} d^{-1}, which was not significantly different from the experiments provided with MABAs. Therefore, the addition of MABAs inoculum was not required for performing $N\text{-}NO_3^-$ removal from centrate. These results suggest that the centrate also contained a microalgal inoculum, which was enriched during the phototrophic conditions with the additional CO_2 supply from flue gas. Previous studies recently reported microalgal enrichment under phototrophic conditions using activated sludge from wastewater treatment as inoculum.[22, 23] Hence, it was not surprising to find out that the centrate herein used also contained microalgae.

Both centrate dilutions and the control tests achieved a 100% $P\text{-}PO_4^{3-}$ removal by day 4 (Fig. 2C). The $P\text{-}PO_4^{3-}$ removal rates ranged from 3.7 ± 0.1 to 6.2 ± 0.2 mg L^{-1} d^{-1}. The higher $P\text{-}PO_4^{3-}$ removal rate was achieved in D 1/5 tests since the initial concentration in these bottles was higher. The rapid decrease of $P\text{-}PO_4^{3-}$ could be attributed to surface cell adsorption rather than consumption as a result of the granular configuration of algal-bacterial biomass. It has been reported that $P\text{-}PO_4^{3-}$ uptake by microalgae is a two-stage kinetic process, surface cell adsorption being the first step of the $P\text{-}PO_4^{3-}$ uptake process.[24] This can also explain the further $N\text{-}NO_3^-$ consumption recorded after complete $P\text{-}PO_4^{3-}$ depletion.

Unlike sCOD removal where microalgae played a negligible role, nutrients were mostly removed by microalgal uptake.[25] In this context, the turbidity of the centrate might have

played a role in nutrients removal by affecting the light intensity available to the microalgal community.[26] In the present study, the $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ removal rates observed in the BG-11 control without turbidity were comparable with the removal rates recorded in the D 1/10 tests. Moreover, the superior $P\text{-}PO_4^{3-}$ removal rate recorded in the D 1/5 tests confirmed that no limitations due to light penetration occurred in none of the centrate dilutions tested. Thus, turbidity of the liquid medium was not a limiting factor for nutrients removal under the working conditions investigated since CO_2 was widely available for microalgal growth. Besides, the influence of the presence of ethanol in the cultivation medium on nutrients removal was also negligible under the concentrations tested (1,725 ± 32 mg L^{-1} and 852 ± 65 mg L^{-1} in D 1/5 and D 1/10 tests, respectively). A comprehensive review by Miazek et al.[27] showed that, in general terms, microalgal growth is not inhibited up to ethanol concentrations of 3,000 mg L^{-1}.

In this context, air supply has been proposed as a strategy to enhance nutrients removal from centrates or other nutrient-rich effluents in microalgal-bacterial photobioreactors. For instance, Morales-Amaral et al.[28] successfully operated aerated photobioreactors inoculated with *Muriellopsis* sp. and *Pseudokirchneriella subcapitata* under irradiance of 800 µmol m^{-2} s^{-1} (12 h a day) for centrate treatment (total N of 316 mg L^{-1} and total P of 35 mg L^{-1}). Under the best operational conditions tested at HRT of 3.3 days, nitrogen and phosphorus removal rates of 47.5 and 3.8 mg L^{-1} d^{-1} were reported, respectively. Ge and Champagne[4] treated centrate (total N of 230 – 480 mg L^{-1} and total P of 47 – 85 mg L^{-1}) in a microalgal-bacterial photobioreactor under irradiance of 60.5 µmol m^{-2} s^{-1} (24 h light cycles). The photobioreactor was aerated and operated at HRT of 12 days to achieve maximum nitrogen and phosphorous removal efficiencies of 90% and 98%, respectively. These nutrient removal performances are comparable with the results herein obtained. However, although the N removal rate of the present study was ~40% lower than that

reported by Morales-Amaral et al.[28], the irradiance here used was 27 times lower. Likewise, the HRT and irradiance set in the present study were 70% shorter and 50% lower, respectively, to achieve N and P removal efficiencies comparable with those reported by Ge and Champagne[4]. Therefore, the use of flue gas constitutes a promising strategy for the implementation of compact microalgal-bacterial processes for centrate treatment, which can be installed in locations with relatively low solar irradiance conditions.

MABAs size distribution

Biomass samples were collected at the end of the treatment tests and controls to evaluate the effect of the operating conditions on the size of the inoculated MABAs (Fig. 3). In the inoculum, 83% of MABAs were in the d_p range of 55 – 300 µm, while 17% were larger than 300 µm. This resulted in an FMD of 191.4 µm. In D 1/5 tests, 70% of MABAs were in the size range of 55 – 300 µm, while 30% were larger than 300 µm, yielding an FMD of 263.6 µm. The FMD recorded in D 1/5 tests was 38% higher compared with the FMD of the inoculum, this difference being statistically significant (Table 3). In D 1/10 tests, 88% MABAs were in the size range of 55 – 300 µm, while 12% were larger than 300 µm. This resulted in an FMD of 176.6 µm, which was not significantly different from the FMD recorded for the inoculum. In the BG-11 control lacking ethanol, 91% MABAs were in the size range of 55 – 300 µm, while only 9% of the MABAs were larger than 300 µm. This resulted in an FMD diameter of 155 µm, which was significantly lower than the FMD of the inoculum. Therefore, the conditions set in the BG-11 control decreased significantly the size of the MABAs (24% reduction relative to the inoculum). The D 1/10 controls without inoculum was unable to produce aggregates, the d_p values recorded were always $\leq$ 50 µm.

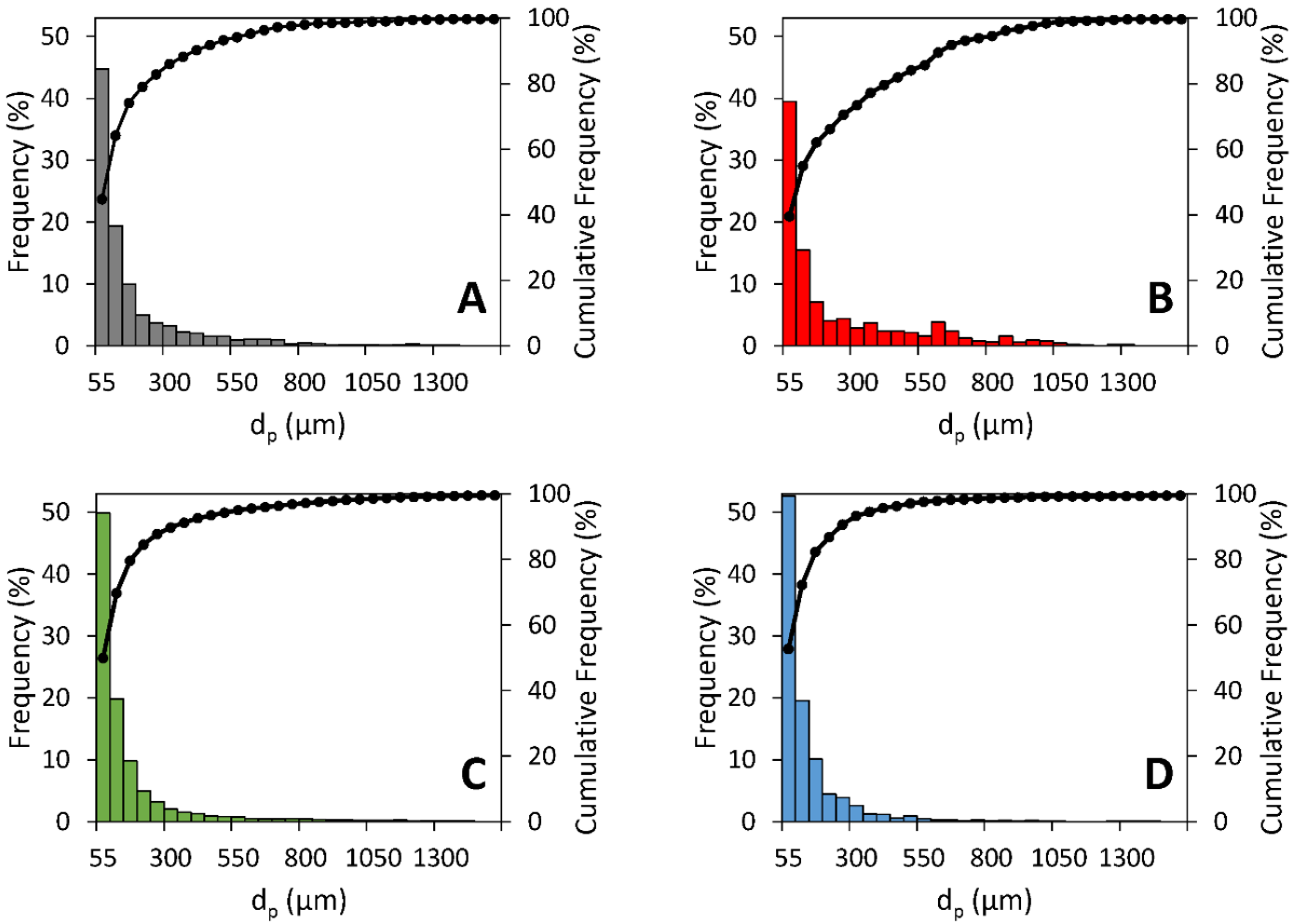

Fig. 3. MABAs diameters distribution in (A) inoculum and at the end of centrate treatment experiments in (B) D 1/5 test, (C) D 1/10 test and (C) BG-11 control.

Table 3. Tukey's multiple comparison test after ANOVA ($\alpha<0.05$) for the Feret mean diameters considering all d_p measured values in the inoculum, centrate dilutions, and control tests.

Condition 1 *vs* condition 2	**FMD (µm) Condition 1**	**FMD (µm) Condition 2**	***p*-value**	**Significant (α=0.05)**
Inoculum *vs* D 1/5 test	191.4	263.5	<0.0001	Yes
Inoculum *vs* D 1/10 test	191.4	176.6	0.1433	No
Inoculum *vs* BG-11 control	191.4	155.0	<0.0001	Yes
D 1/5 test *vs* D 1/10 test	263.5	176.6	<0.0001	Yes
D 1/5 test *vs* BG-11 control	263.5	155.0	<0.0001	Yes
D 1/10 test *vs* BG-11 control	176.6	155.0	0.0031	Yes

Biomass harvesting still constitutes one of the key challenges for the economic feasibility of large scale microalgal-bacterial processes.[29] Therefore, the presence of MABAs has a positive impact on the economic feasibility of the treatment process since enhanced biomass settling can be achieved without the addition of flocculants. In fact, the occurrence of MABAs can increase the biomass settling velocity by several orders of magnitude relative to dispersed microalgae cells.[7,14,16] Previous studies on wastewater treatment using microalgal systems reported MABAs diameters ranging from 100 to 5,000 µm,[7] while in the present study the diameters ranged from 55 to 1,300 µm. However, to the best of our knowledge, this is the first report on the successful MABAs-based centrate treatment supplied with flue gas.

The results obtained showed that even when the D 1/10 control was able to remove COD and nutrients at high efficiencies without MABAs inoculation, such conditions are not recommended for centrate treatment since aggregates were not formed. On the contrary,

when MABAs are inoculated, the FMD of the aggregates remained similar or even increased during centrate treatment. Interestingly, the FMD observed in the BG-11 control without centrate decreased relative to the FMD recorded in the MABAs inoculum. This strongly suggested that centrate components (i.e. ethanol) played a role in promoting microalgae-bacteria aggregation, which deserves further investigation. The dynamics of the FMD in long-term treatment experiments with MABAs also requires more research.

MABAs structure

SEM images were taken to assess qualitatively the effect of the different experimental conditions on MABAs structure (Fig. 4). Filamentous chlorophytes and diatoms were the main components of the MABAs present in the inoculum. Images at 1500× amplification showed the presence of *Anabaena*- and *Chlorella*-like microalgae in low abundances as well as bacterial growth on the surface of microalgal cells. The MABAs present in D 1/5 tests were characterized by a more compact structure with abundant filamentous chlorophytes. The 1500× amplification images showed that diatoms were very scarce, while *Chlorella*-like microalgae remained in the structure of the aggregates. The bacterial community in D 1/5 tests was more abundant than that observed in the inoculum. The filamentous chlorophytes in D 1/10 tests dominated the structure of the MABAs. In fact, the filaments were more elongated than those observed in D 1/5 tests. Images at 1500× amplification showed that filamentous chlorophytes were also more abundant inside the aggregates. Diatoms were scarce and *Chlorella*-like microalgae remained in a similar proportion than those observed in D 1/5 tests. Compared with centrate dilution tests, the MABAs in the BG-11 control were characterized by a lower abundance of filamentous chlorophytes and a higher abundance of *Chlorella*-like microalgae.

Interestingly, diatoms were more abundant in the BG-11 medium than in D 1/5 and D 1/10 tests, which suggested that ethanol in the centrate likely hindered the enrichment of diatoms. This is in agreement with Okumura et al.[30] who reported that diatoms are less tolerant of organic solvents such as ethanol than freshwater green algae and blue-green algae. Moreover, since the BG-11 controls were also supplied with flue gas, a negative impact of the CO_2 concentration on diatoms growth was ruled out.

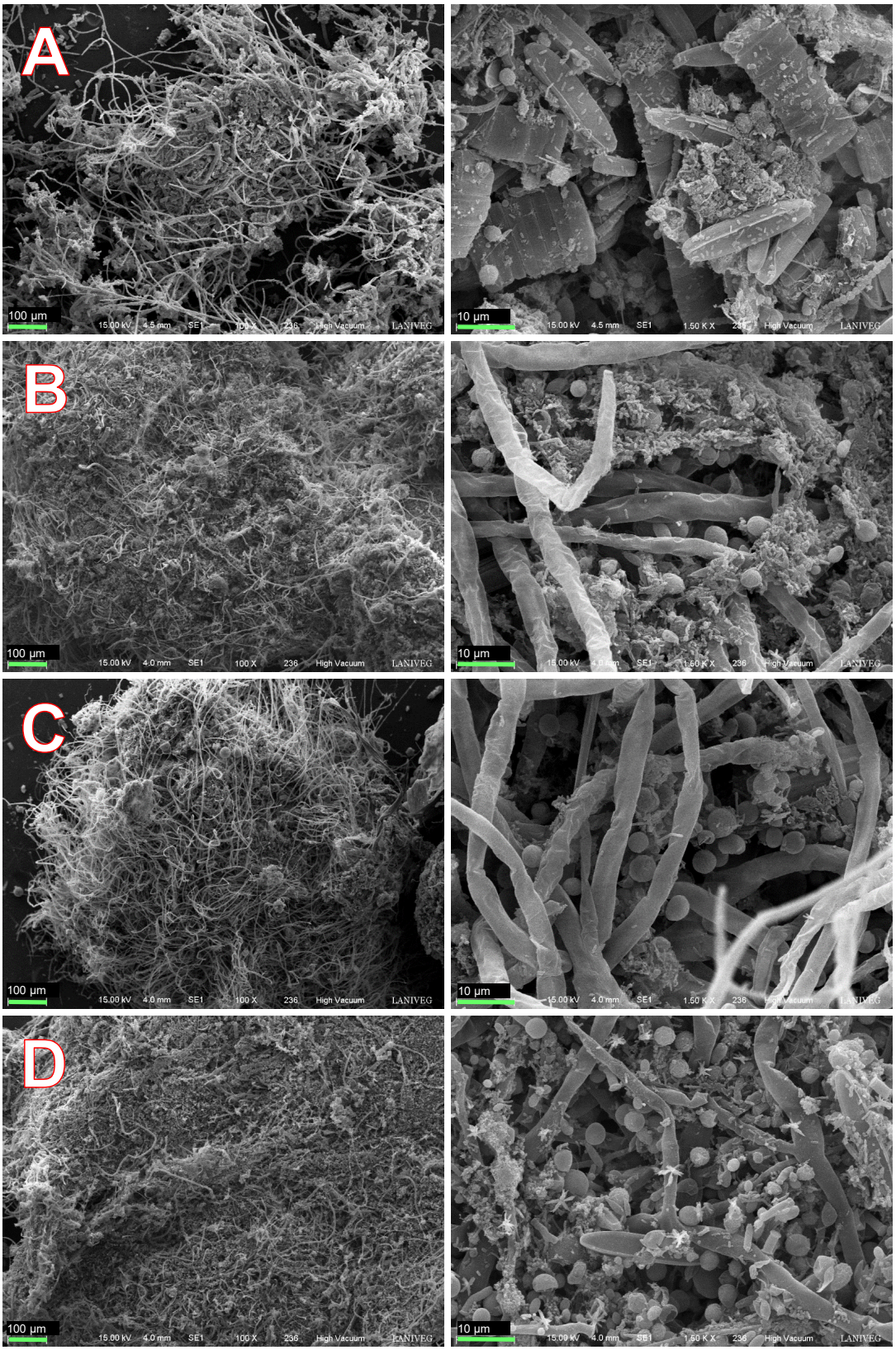

Fig. 4. Scanning electron microscopy images at 100× (left) and 1500× (right) magnifications of MABAs present in (A) inoculum, (B) D 1/5 test, (C) D 1/10 test, and (D) BG-11 control.

Microbial aggregation is a complex but common phenomenon in wastewater treatment processes. Several factors have been reported to influence microalgae-bacteria aggregation: bioreactor operation mode, the hydraulic retention time, light intensity, mixing conditions, presence of divalent cations (i.e. Ca^{2+} and Mg^{2+}) and inorganic/organic carbon concentration ratio.[7,14] It has been also reported that ethanol might trigger bacterial aggregation by stimulating the production of extracellular polymeric substances.[31] Thus, the ethanol present in the D 1/5 and D 1/10 tests likely played a role in maintaining MABAs of the same size or even larger than that observed in the inoculum. The fact that MABAs were not formed in the D 1/10 controls highlighted the relevance of inoculating the process with already formed aggregates since ethanol or other centrate components did not stimulate microalgae-bacteria aggregation in the short-term experiments herein performed.

CONCLUSIONS

Microalgae-bacteria aggregates supplied with flue gas were able to efficiently treat centrate from anaerobic digestion of winery wastewater. Maximum sCOD, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$ and $N\text{-}NH_4^+$ removal efficiencies of 95%, 94%, 100%, and 100%, respectively, were achieved within 7 days in 5-fold diluted centrate. Similar removal efficiencies were recorded in BG-11 controls, which confirmed that centrate turbidity or its components such as ethanol and VFAs did not hinder the performance of the MABAs under the conditions tested. D 1/10 controls showed that flue gas supply allowed efficient COD and nutrients removal even without MABAs inoculation. However, since no aggregates were formed in controls without MABAs inoculation such conditions are not recommended for centrate treatment due to the associated difficulties for biomass harvesting. The Feret mean diameter of the MABAs after centrate treatment was the same or even larger than

that of the aggregates present in the inoculum, which impacts positively on the economics of the treatment process. SEM analyses also showed that the liquid medium composition influenced the structure and the type of microalgal cells established in the MABAs. Although more research is needed in long-term experiments, the results herein obtained showed that MABA-based centrate treatment supported by flue gas could be implemented in locations with low solar radiation.

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Table 1. Experimental conditions set in centrate treatment experiments and control tests.

Experimental condition	**MABAs Inoculum**	**Initial COD (mg L^{-1})**	**Initial N-NH_4^+ (mg L^{-1})**	**Initial N-NO_3^- (mg L^{-1})**	**Initial P-PO_4^{3-} (mg L^{-1})**	**Initial ethanol (% COD)**
Centrate with 1/5 dilution (D 1/5 test)	Yes	5948 ± 111	2.7 ± 0.4	244.6 ± 41.1	24.7 ± 0.8	29
Centrate with 1/10 dilution (D 1/10 test)	Yes	3045 ± 233	1.2 ± 0.1	269.3 ± 17.6	14.9 ± 0.2	28
BG-11 control	Yes	2775 ± 198	0.5 ± 0.1	258.3 ± 61.9	19.0 ± 0.7	0
D 1/10 control	No	3342 ± 100	1.4 ± 0.1	268.8 ± 19.0	16.3 ± 0.7	26

Table 2. Removal performances of COD, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ determined in centrate dilutions and control tests.

Experimental condition	**COD**		**$N\text{-}NH_4^+$**		**$N\text{-}NO_3^-$**		**$P\text{-}PO_4^{3-}$**	
	Removal rate* (mg L^{-1} d^{-1})	Removal efficiency (%)	Removal rate* (mg L^{-1} d^{-1})	Removal efficiency (%)	Removal rate* (mg L^{-1} d^{-1})	Removal efficiency (%)	Removal rate* (mg L^{-1} d^{-1})	Removal efficiency (%)
D 1/5 test	804.0 ± 16.9	95	1.3 ± 0.2	100	33.0 ± 5.9	94	6.2 ± 0.2	100
D 1/10 test	405.9 ± 33.4	93	0.6 ± 0.1	100	32.7 ± 3.4	85	3.7 ± 0.1	100
BG-11 control	377.1 ± 28.4	94	0.5 ± 0.1	100	35.7 ± 8.8	96	4.8 ± 0.2	100
D 1/10 control	445.0 ± 3.7	93	0.7 ± 0.1	100	29.6 ± 1.9	76	4.1± 0.2	100

*Removal rates were calculated as $\frac{\Delta Concentration}{\Delta t}$, with Δt =7 days for COD and $N\text{-}NO_3^-$, Δt =2 days for $N\text{-}NH_4^+$ and Δt = 4 days for $P\text{-}PO_4^{3-}$.

Table 3. Tukey's multiple comparison test after ANOVA ($\alpha<0.05$) for the Feret mean diameters considering all d_p measured values in the inoculum, centrate dilutions and control tests.

Condition 1 *vs* condition 2	**FMD (µm)** **Condition 1**	**FMD (µm)** **Condition 2**	***p*-value**	**Significant** **(α=0.05)**
Inoculum *vs* D 1/5 test	191.4	263.5	<0.0001	Yes
Inoculum *vs* D 1/10 test	191.4	176.6	0.1433	No
Inoculum *vs* BG-11 control	191.4	155.0	<0.0001	Yes
D 1/5 test *vs* D 1/10 test	263.5	176.6	<0.0001	Yes
D 1/5 test *vs* BG-11 control	263.5	155.0	<0.0001	Yes
D 1/10 test *vs* BG-11 control	176.6	155.0	0.0031	Yes

Fig.1.

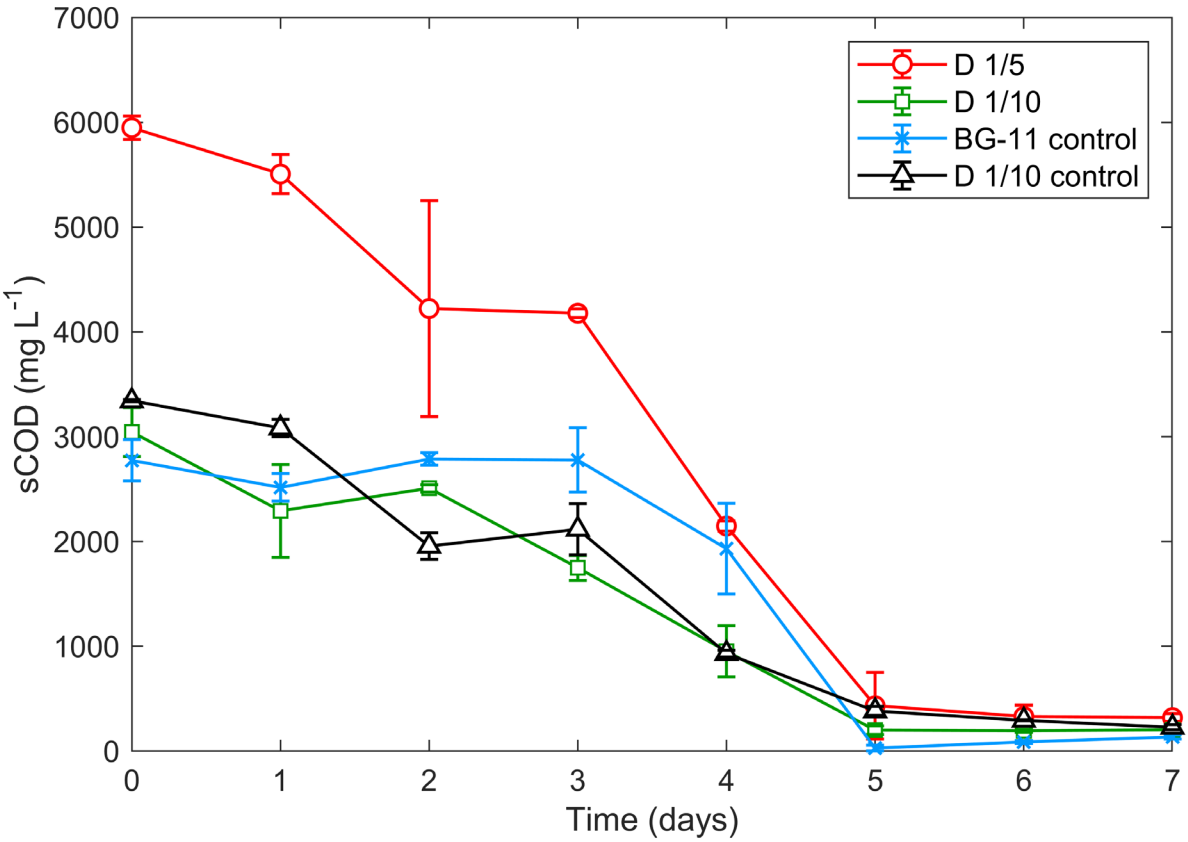

7000
6000
5000
4000
3000
2000
1000
0
sCOD (mg L-1)
0
1
2
3
4
5
6
7
Time (days)
D 1/5
D 1/10
BG-11 control
D 1/10 control

Fig. 2.

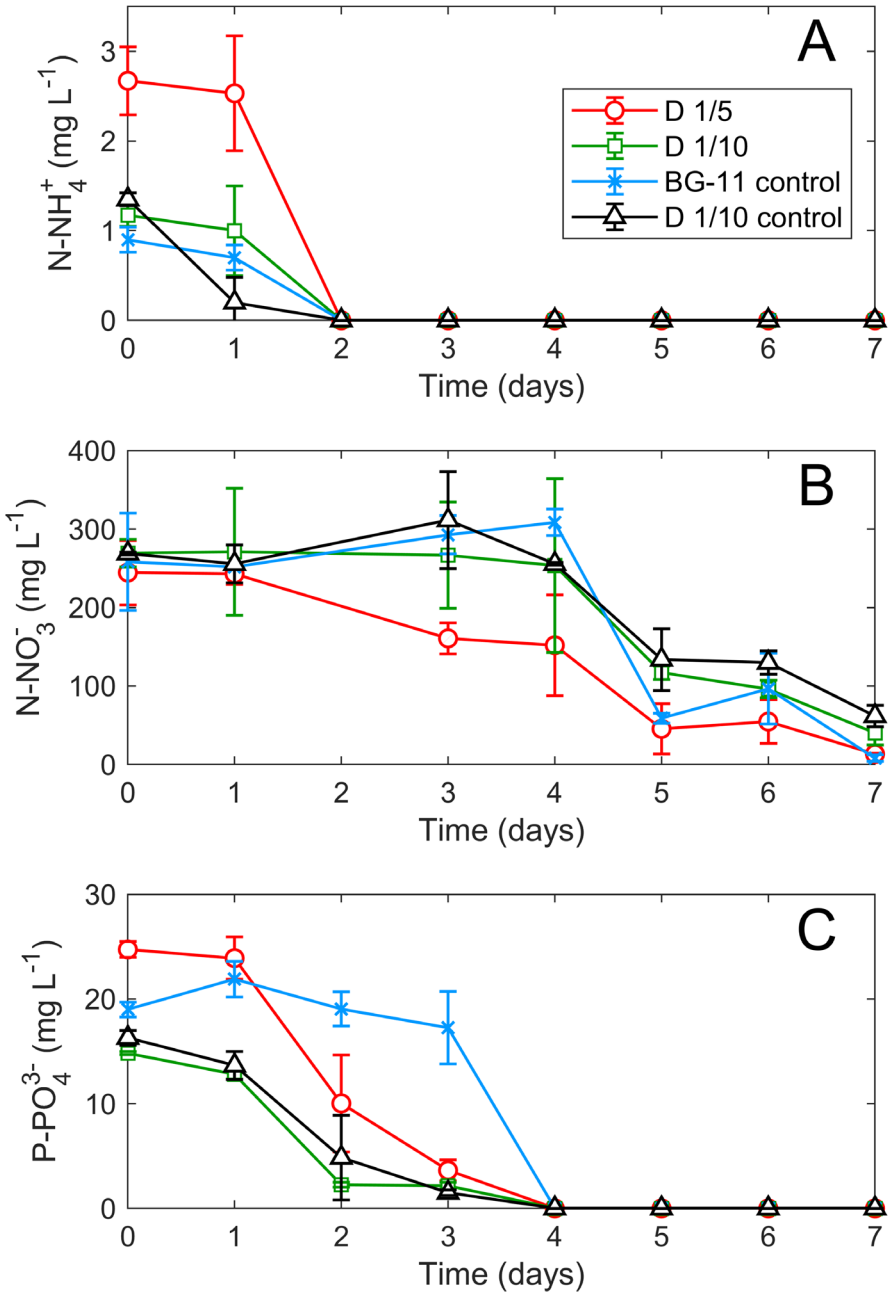

A
B
C
D 1/5
D 1/10
BG-11 control
D 1/10 control
N-NH4+ (mg L-1)
N-NO3- (mg L-1)
P-PO43- (mg L-1)
Time (days)
Time (days)
Time (days)

Fig. 3

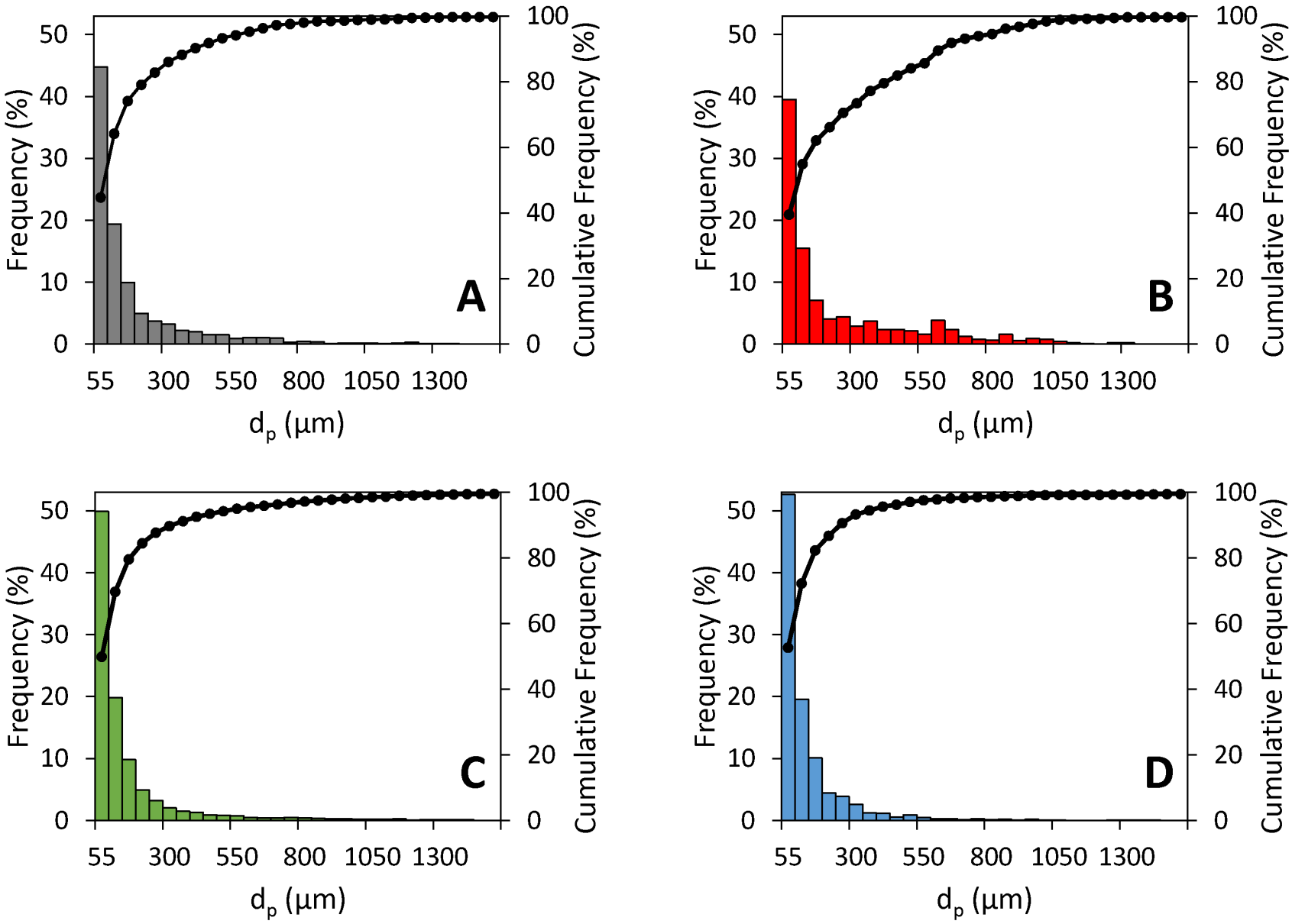
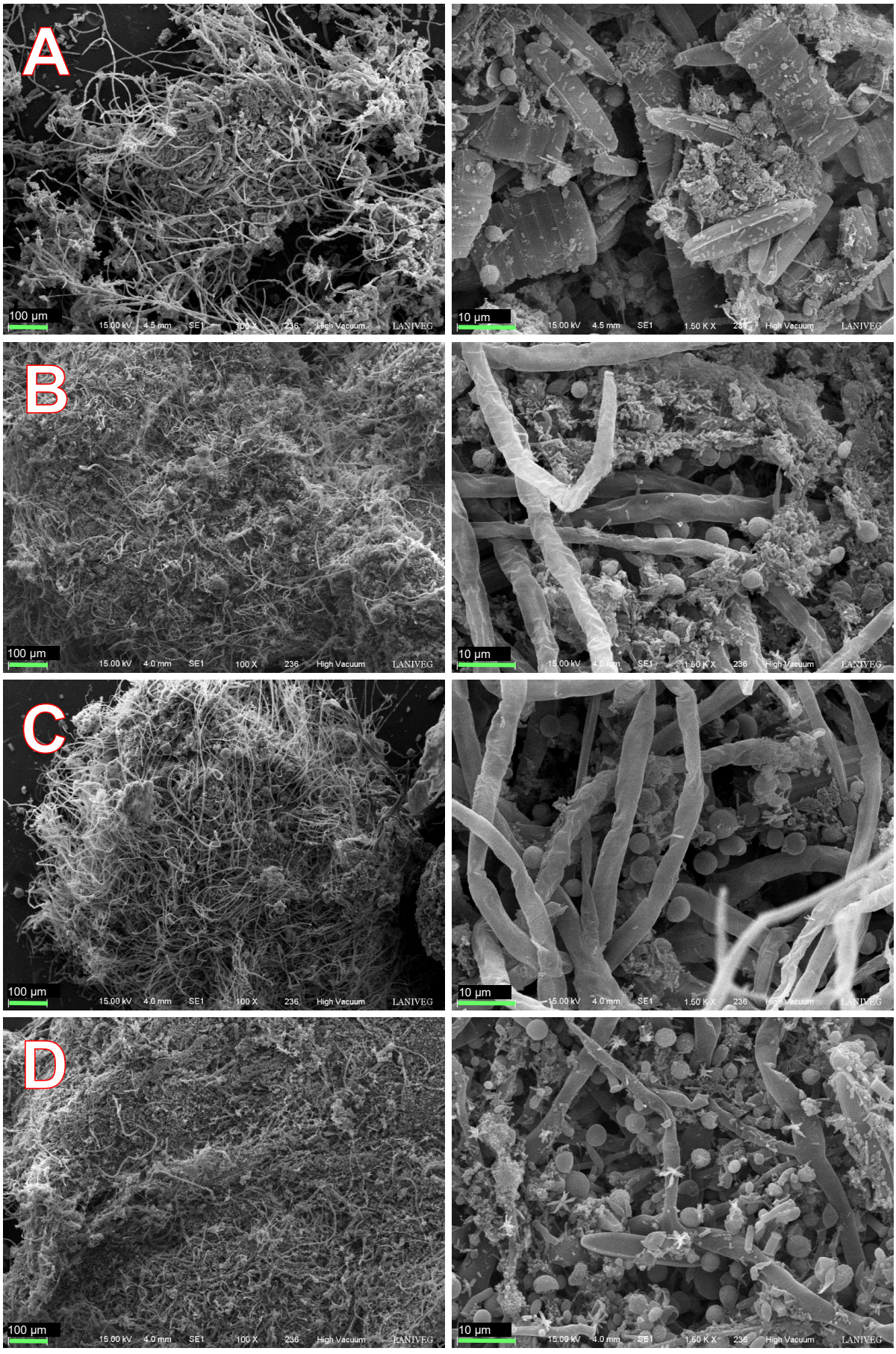

A
B
C
D
Frequency (%)
Cumulative Frequency (%)
d_p (µm)
0
10
20
30
40
50
0
20
40
60
80
100
55
300
550
800
1050
1300

Fig. 4


A
100 µm
15.00 kV 4.5 mm SE1 100 X 236 High Vacuum LANIVEG
10 µm
15.00 kV 4.5 mm SE1 1.50 K X 236 High Vacuum LANIVEG
B
100 µm
15.00 kV 4.0 mm SE1 100 X 236 High Vacuum LANIVEG
10 µm
15.00 kV 4.0 mm SE1 1.50 K X 236 High Vacuum LANIVEG
C
100 µm
15.00 kV 4.0 mm SE1 100 X 236 High Vacuum LANIVEG
10 µm
15.00 kV 4.0 mm SE1 1.50 K X 236 High Vacuum LANIVEG
D
100 µm
15.00 kV 4.0 mm SE1 100 X 236 High Vacuum LANIVEG
10 µm
15.00 kV 4.0 mm SE1 1.50 K X 236 High Vacuum LANIVEG