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Insights into bioflocculation of filamentous cyanobacteria, microalgae and their mixture for a low-cost biomass harvesting system

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ABSTRACT

Cyanobacteria and microalgae are considered as interesting feedstocks for either the production of high value bio-based compounds and biofuels or wastewater treatment. Nevertheless, the high costs of production, mainly due to the harvesting process, hamper a wide commercialization of industrial cyanobacteria and microalgae based products. Recent studies have found in autoflocculation and bioflocculation promising spontaneous processes for a low-cost and environmentally sustainable cyanobacteria and microalgae biomass harvesting process. In the present work, bioflocculation process has been studied for three different inocula: filamentous cyanobacteria, microalgae and their mixture. Their cultivation has been conducted in batch mode using two different cultivation media: synthetic aqueous solution and urban wastewater. The removal of nutrients and flocculation process performance were monitored during the entire cultivation time. Results have proved that bioflocculation and sedimentation processes occur efficiently for filamentous cyanobacteria cultivated in synthetic aqueous solution, whereas such processes are less efficient in urban wastewater due to the specific characteristics of this medium that prevent bioflocculation to occur. Besides different efficiencies associated to cultivation media, this work highlighted that bioflocculation of sole microalgae is not as effective as when they are cultivated together with filamentous cyanobacteria.

1. Introduction

Harvesting the microalgal biomass is challenging because of the significantly low biomass concentration in the culture medium (i.e. 0.5–5 g L^{-1}) as well as the small size of cells (i.e. 5-20 μm), which confer them poor settleability (Alam et al., 2016). Currently, no microalgae harvesting method can be considered economically feasible and, at the same time, efficient (Su et al., 2011; Ogbonna and Nwoba, 2021). In full scale systems, microalgae are commonly harvested using centrifugation but this method is particularly expensive because of the excessive capital costs and high energy demand, especially for low-value applications (Low-Décarie et al., 2011). In the framework of wastewater treatment, less expensive methods, such as chemically induced coagulation–-flocculation followed by solid/liquid gravity separation, can be convenient (del Rosario Rodero et al., 2020). Nevertheless, these processes could turn in an increase of sludge to be disposed and contamination of down-stream products, thus restricting the opportunities to valorise microalgae biomass (Liu and Vyverman, 2015). Actually, microalgae cultivation in wastewater is considered the sole economically feasible system to produce microalgae biomass to be converted into biofuels with minimum environmental impact (Nopens et al., 2001). Lowering harvesting costs is therefore considered a key factor for the development of sustainable full-scale production of microalgae biomass. Regarding this aspect, an attractive solution is represented by the spontaneous flocculation of the suspended biomass, through the occurrence of the autoflocculation and/or bioflocculation processes. Such spontaneous phenomena promote the gravity induced settling, thus resulting in a low cost as well as harmless to microorganisms biomass harvesting process. Moreover, as the use of any chemical based flocculants is not required, the spent cultivation medium can be eventually reused for several purposes or discharged into the environment with low or even no impact (Salim et al., 2011).

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Autoflocculation occurs when the suspended biomass flocculates around a nucleus composed of inorganic compounds, whereas bioflocculation refers to the spontaneous flocculation induced by organic secretions from cells (Ummalyma et al., 2017). Autoflocculation takes place at high pH values resulting from the consumption of dissolved carbon dioxide during photosynthesis: an increase of pH promotes the precipitation of calcium and phosphate based salts and microalgae cells act as solid supports and charge neutralizers for the precipitants. This specific feature makes the autoflocculation not feasible in all types of cultivation media; moreover, high pH values could undermine a further microalgae valorization (Christenson and Sims, 2011). Bioflocculation has been studied and observed for microalgae-bacteria, microalgae-fungi and microalgae-microalgae consortia (Alam et al., 2016), whereas it remains poorly investigated for cyanobacteria-microalgae consortium. However, it has been experimentally observed that in wastewater cultivation systems, microalgae show high settling efficiency when they form agglomerates with filamentous cyanobacteria (Su et al., 2011) and in our previous study, the filamentous cyanobacteria showed high bioflocculation performances when cultivated in synthetic medium (Iasimone et al., 2020). This finding suggests a possible bioflocculation process induced by synergetic interactions occurring between microalgae and cyanobacteria.

For this purpose, an experimental study was conducted on investigating the potential bioflocculation process occurring in consortia composed of sole filamentous cyanobacteria, sole microalgae and their mixture cultivated in two different media: a synthetic aqueous solution and urban wastewater. Batch cultivation tests were performed for 10 days and conducted by monitoring biomass productivity, nutrients removal, biomass flocculation efficiency and lipids accumulation in cells. Biomass flocculation was assessed by setting static hydraulic conditions. Furthermore, an innovative method based on image analysis was developed in order to monitor and control the flocculation performance. Finally, microscope screenings were carried out to study the interactions among cyanobacteria, microalgae and their mixture as well as their mutual dynamics.

2. Materials and methods

2.1. Microbial inocula

Two different microbial inocula were used for this study: filamentous cyanobacteria and microalgae.

The inoculum composed of filamentous cyanobacteria was selected from a native wastewater microbial polyculture, which was collected, as biofilm layer, from the effluent channel of the secondary clarifier of the urban wastewater treatment plant located in Isernia (Italy) and further cultivated in modified Bold Basal Medium (BBM) under controlled conditions. Microscope screenings showed that this inoculum was mainly composed of filamentous cyanobacteria, identified as *Pseudanabaena* sp. And *Leptolyngbya* sp. (Fig. 1) by their morphological features. The modified BBM was composed of the following elements: 250 mg L^{-1} $NaNO_3$, 25 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 75 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 75 mg L^{-1} K_2HPO_4, 175 mg L^{-1} KH_2PO_4, 25 mg.L^{-1} NaCl, 11.4 mg L^{-1} H_3BO_3, alkaline EDTA solution (50 mg L^{-1} EDTA, 31 mg L^{-1} KOH), acidified Iron solution (5 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 1 mg L^{-1} H_2SO_4), trace metals solution (8.8 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 1.4 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.7 mg L^{-1} MoO_3, 1.6 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.5 mg L^{-1} $Co(NO_3)_2 \cdot 6H_2O$, 8.4 mg L^{-1} $NaHCO_3$, 4.77 g.L^{-1} HEPES buffer (Low-Décarie et al., 2011). Inoculum cultivation was conducted in 500 mL glass flasks, filled with 200 mL of medium kept in agitation by an elliptic shaking table operating at 150 rpm. Cultivation was conducted at homogeneous and continuous light intensity of 100 ± 10 μmol m^{-2} s^{-1} (cool white fluorescent lamps) and constant temperature of 25 ± 1 °C. A standardized procedure (Liu and Vyverman, 2015) was followed to control the biomass productivity. Such procedure requires the re-inoculation of biomass every three days into a fresh cultivation medium.

Microalgae inoculum was collected from an outdoor microalgae raceway pond (22 m^3; 60 m^2) in operation at the "Laboratoire de Biotechnologie de l'Environnement" (LBE) located in Narbonne (France). Microscope screenings showed that this inoculum was mainly composed of *Chlorella* and *Scenedesmus* microalgae species (Fig. 1). The pond was continuously fed with a synthetic cultivation medium characterized by a chemical composition similar to the urban wastewater (Nopens et al., 2001). The inoculum was cultivated in modified BBM under the same operating conditions set for cyanobacteria inoculum.

2.2. Experimental setup

Cultivation tests were performed in 250 mL glass flasks with a working volume of 150 mL, covered with a cotton wool thick enough to avoid liquid loss and, at the same time, to enable gas exchange. The glass flasks were placed on an elliptic shaking table operating at 150 rpm. Tests were conducted in batch mode for 10 days, under the same operating conditions (i.e. light intensity and temperature) set for cultivating the two inocula. Two different cultivation media were tested: a synthetic aqueous solution (modified BBM) and a real urban wastewater. The synthetic aqueous solution was prepared as done to cultivate the inocula, whereas wastewater was collected at the entrance of the urban wastewater treatment plant of Narbonne (France) and, prior use, was firstly decanted and then filtered with 2.7 μm filter (Whatman glass-fiber filter, grade 50). After filtration, wastewater was enriched with 4.77 g L^{-1} of HEPES buffer in order to maintain a stable pH of 7.2 ± 0.3.

Both inocula were centrifuged for 20 min at 18500 rpm and the respective resulting pellets were re-suspended in 700 mL of the synthetic cultivation medium (modified BBM), thus obtaining two stock solutions used to inoculate tests labelled with C for cyanobacteria and M for microalgae respectively. Both solutions were characterized by an initial optical density (OD) of 0.05 abs, measured at 620 nm for cyanobacteria

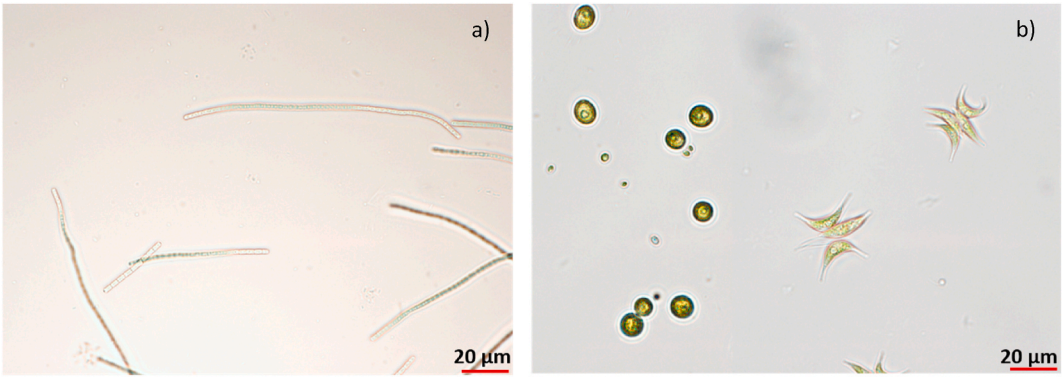

Fig. 1. a) Filamentous cyanobacteria (Pseudanabaena sp., Leptolyngbya sp.); b) Microalgae (Chlorella sp., Scenedesmus sp.).

and 660 nm for microalgae, respectively. From the two stock solutions, an equal volume was withdrawn and then homogeneously mixed up to obtain the third stock solution used to inoculate tests labelled with C&M for the mixed culture of cyanobacteria and microalgae. The same sequence of operations previously described for the synthetic cultivation medium was conducted using the filtered wastewater as cultivation medium, thus obtaining further three stock solutions used to inoculate tests with cyanobacteria (Cw), microalgae (Mw) and their mixture (C&Mw). The initial OD measured for cultivation tests fed with urban wastewater was equal to 0.1 abs at the specific wavelength for cyanobacteria and microalgae. The higher values of OD found for these tests were mainly caused by the higher initial turbidity in wastewater. During the experimental activity (at day 4), other two tests were carried out by mixing one of the three replicates of C with one of the three replicates of M in synthetic cultivation medium as well as in urban wastewater, thus generating other two specific tests named E and Ew, respectively. These latter tests were conducted in duplicate and they were used exclusively to assess the sedimentation process efficiency of the mixture formed by microalgae and cyanobacteria when these latter reached their exponential growth phase. A synthesis of the experimental design is reported in Table 1.

2.3. Analytical methods

Biomass productivity in tests C was monitored by OD at 620 nm as a proxy for phycocyanin content, which is proper of cyanobacteria biomass (Rippka et al., 1979). On the other hand, biomass productivity in tests M was monitored by OD at 660 nm as wavelength (Low-Décarie et al., 2011) and through measurements of total chlorophyll α (Chl α) (Ritchie R J, 2006). Biomass productivity in tests with mixed culture (i.e. C&M) was monitored considering the highest OD value between those measured at wavelengths of 620 nm and 660 nm. Such parameter (i.e. biomass productivity) was monitored every day by collecting samples of cultivation medium during the observation time. The total lipids content of each culture was monitored during the time as fraction (%) of lipid weight on dry cell weight, according to the sulpho-phospho-vanillin colorimetric method (Mishra et al., 2014). The dry cells weight (DCW) was determined using the method of suspended solid measurement (Xin et al., 2010). The biomass productivity (BP, mg $L^{-1}d^{-1}$) was calculated according to Equation (1):

$$BP = \frac{DCW_t - DCW_o}{t - t_0} \quad (1)$$

where DCW_0 (mgL^{-1}) is the biomass concentration at time t_0 (d) and DCW_t (mg L^{-1}) is the suspended solids concentration at any time t (d) > t_0 during the cultivation test.

The concentration of dissolved nutrients was measured using an ion chromatography system (ICS 3000 Dionex, USA): in particular, nitrogen (N) was monitored as nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+), whereas phosphorus (P) was monitored as phosphate (PO_4^{3-}). The nutrients removal rate R_i (mg $L^{-1}d^{-1}$) of the generic substrate i (i.e. $N–NH_4^+$, $N–NO_3^-$, $P-PO_4^{3-}$) dissolved in the cultivation medium was calculated according to Equation (2).

$$R_i = \frac{S_{0,i} - S_i}{t - t_0} \quad (2)$$

where $S_{0,i}$ (mgL^{-1}) is the initial concentration of substrate i at time t_0 (d), whereas S_i (mgL^{-1}) is the concentration of the same substrate i at any time t (d) > t_0 during the cultivation test.

Stoichiometry of nutrients consumption was evaluated according to the following Equation (3).

$$R\left(\frac{N}{P}\right) = \frac{N_{t0} - N_t}{P_{t0} - P_t} \quad (3)$$

where N_{t0} (mmolN L^{-1}) and P_{t0} (mmolP L^{-1}) are the total nitrogen (as $N–NH_4^+$ and $N–NO_3^-$) and thosphorus concentrations (as $P-PO_4^{3-}$) respectively, at initial time t_0 (d), while N_t (mmolN L^{-1}) and P_t (mmolP L^{-1}) are the nitrogen and phosphorus concentrations at any time t (d) > t_0 during the cultivation test.

All measurements were performed in triplicate for each test and for each observation day. From each group of three homogeneous measurements, the mean and the standard deviation were calculated.

2.4. Biomass flocculation assessment

Biomass flocculation efficiency was assessed through sedimentation tests. Such tests were performed in 100 mL glass tubes with a working volume of 50 mL filled with samples of cultivation medium collected from each batch test during the cultivation time. Tubes were placed vertically and kept stable in a box. Static and light-controlled conditions were maintained during the entire sedimentation test. Biomass flocculation was monitored by taking automatically pictures with a standard commercialized camera (Canon EOS 7D model, Japan) according to scheduled times. Pictures were then processed in order to evaluate the size of the “clarified area”, that is namely the zone of the cultivation medium where the concentration of biomass is lower than that estimated at time zero. The size of the “clarified area” was strictly related to the biomass flocculation efficiency and therefore, it can be reasonably considered as an indicator of the bioflocculation performance.

The pictures elaboration was conducted using Image J software (U. S. National Institutes of Health, Bethesda, Maryland, USA). Pictures were first homogenized in order to obtain the same light of background. After that, for each glass tube, a numerical matrix was generated in order to convert the picture of the cultivation medium into a sequence of numbers: a specific number was associated to each pixel depending on its colour (Supplementary material - section A). Not inoculated cultivation media at time t_0 were used to set the blank values and normalize the matrices. Finally, matrices were analysed in order to evaluate the size of the “clarified area” through the number of pixels whose values differed from the blank values by 60% at maximum. The threshold of 60% was chosen taking into account the variability (i.e. 0.60) of blank values at time t_0 for all the cultivation tests.

The microbial composition of the flocculated biomass was studied by analysing samples with an optical microscope (Olympus BX53F). Images

Table 1
–. Experimental plan.

Test	Cultivation medium	Inoculum	Initial OD	Light intensity	Temperature
			abs	(μmol m^{-2} s^{-1})	(°C)
C	Modified BBM	Cyanobacteria	0.05	100 ± 10	25 ± 1
M	Modified BBM	Microalgae	0.05	100 ± 10	25 ± 1
C&M	Modified BBM	Cyanobacteria mixed with microalgae	0.05	100 ± 10	25 ± 1
E	Modified BBM	Cyanobacteria mixed with microalgae	0.5	100 ± 10	25 ± 1
Cw	Urban wastewater	Cyanobacteria	0.1	100 ± 10	25 ± 1
Mw	Urban wastewater	Microalgae	0.1	100 ± 10	25 ± 1
C&Mw	Urban wastewater	Cyanobacteria mixed with microalgae	0.1	100 ± 10	25 ± 1
Ew	Urban wastewater	Cyanobacteria mixed with microalgae	0.5	100 ± 10	25 ± 1

were captured and stored using a camera (micro Olympus, DP 80). The morphological characterisation of the biomass was performed through comparisons between the pictures taken by the camera and those from databases of the international literature.

Finally, with the aim of enhancing the flocculation process, an amount of chitosan up to reach a concentration of 240 mg L^{-1} in the cultivation medium, was added to all tests. Chitosan is a natural, biodegradable, non-toxic, polycationic polymer, whose flocculating action has been studied for different microorganisms: in case of microalgae-bacteria consortium, the study performed by Gutiérrez et al. (2015), has proved that dosing chitosan at the concentration of 240 mg L^{-1}, almost 90% of biomass is recovered. The cationic polymer chitosan induces flocculation through a bridging mechanism (Lama et al., 2016).

3. Results and discussion

3.1. Biomass productivity and nutrients removal

Biomass productivity was evaluated in terms of OD and the relative trends for the different tests are plotted in Fig. 2 (similar trends were observed for Chl α). From this figure, it can be clearly noticed that: (i) biomass productivity was on average higher when urban wastewater was used as cultivation medium (Cw, Mw, C&Mw) rather than modified BBM (C, M, C&M); (ii) biomass productivity showed a similar trend until day 4 for both cultivation media, thus indicating that this initial growth phase was not dependent neither on microbial species nor on cultivation medium characteristics, but exclusively on the specific operating conditions such as light intensity (100 ± 10 μmol m^{-2} s^{-1}) and temperature (25 ± 1 °C); (iii) in modified BBM, the highest biomass productivity was achieved between day 3 and day 5; after this time range, the productivity showed a slight decrease up to turn into a negative value for test C; (iv) in urban wastewater biomass productivity showed similar trends for all the different cultures as proved by the OD values that increased progressively during the cultivation time, thus proving no relevant growth limitation.

Biomass productivity during cultivation time was calculated by equation (1), with DCW from time 0 to day 7 in tests with modified BBM and from time 0 to day 10 in tests with urban watewater (after day 7, tests C showed a decreasing trend). Results are summarized in Table 2 for each test. Tests conducted in urban wastewater (Cw, Mw, C&Mw) showed higher productivity than those conducted in modified BBM (C, M, C&M). Moreover, for both cultivation media, tests with the mixed culture (C&M, C&Mw) reached the highest productivity, accounting to 97.6 ± 4.9 mg L^{-1} d^{-1} and 129 ± 7 mg L^{-1} d^{-1} in modified BBM and in urban wastewater, respectively. Cyanobacteria and microalgae cultures showed similar productivity in modified BBM (66.6 ± 3.3 mg L^{-1} d^{-1} for C, 64.2 ± 3.2 mg L^{-1} d^{-1} for M) and in urban wastewater (119 ± 6 mg L^{-1} d^{-1} for C, 114 ± 6 mg L^{-1} d^{-1} for M).

Concentration of dissolved nutrients was monitored during the cultivation time. In this study, nutrients removal was mainly due to biotic absorption processes since the pH of both cultivation media was maintained at the value of 7.2 ± 0.3 by adding HEPES buffer, thus avoiding the abiotic removal processes through ammonia volatilization and phosphorus precipitation (Chiu et al., 2015). The reduced form of nitrogen (ammonium, NH_4^+) was mainly present in urban wastewater with the initial concentration of 56.2 ± 0.6 mg N–NH_4^+ L^{-1}, whereas the oxidised form (mostly nitrate, NO_3^-, as nitrite NO_2^- concentration was negligible during the whole cultivation time) was mainly present in the modified BBM with the initial concentration of 29.1 ± 0.2 mg N–NO_3^-. L^{-1} (Table 2). Trends of total Nitrogen (N_{tot}) removal are shown in Fig. 3a. Observing the curves, it can be noticed that the removal rate did not depend significantly on both inocula and cultivation media, as the total nitrogen removal rate was approximatively 5.5 ± 0.2 mg N $L^{-1}d^{-1}$ for all tests. Su et al. (2012) found a similar nitrogen removal rate for a mixed culture of microalgae cultivated in urban wastewater with an initial ammonia concentration of 48.9 ± 1 mg N L^{-1}. In this study, in tests fed with the modified BBM, nitrogen was depleted after 5 days of cultivation, whereas in tests fed with urban wastewater, it was completely absorbed at the end of the observation time (10 days). This aspect induces to understand the reason why the biomass productivity performed better in urban wastewater rather than in modified BBM (Fig. 2): in modified BBM, the nitrogen lack occurred already after day 5 and it was responsible for a progressive decrease of the productivity; in urban wastewater, nitrogen was available for a longer time, i.e. until day 10, thus promoting a significant rate of biomass productivity during the entire observation time. Anyway, in tests fed with modified BBM, the higher OD measured for microalgae compared to that for cyanobacteria (Fig. 2) after nitrogen depletion occurrence (i.e. day 5), denotes that microalgae own a better capability in storing nitrogen than cyanobacteria or, on the other hand, that a limited nitrogen availability affects the productivity for microalgae less than cyanobacteria. Moreover, the highest nitrogen availability in urban wastewater induced the highest biomass productivity (BP). These results are in agreement with those from the study conducted by Xin et al. (2010) on *Scenedesmus* sp.: authors observed a positive correlation between nitrogen availability and biomass growth and found the highest biomass production in tests

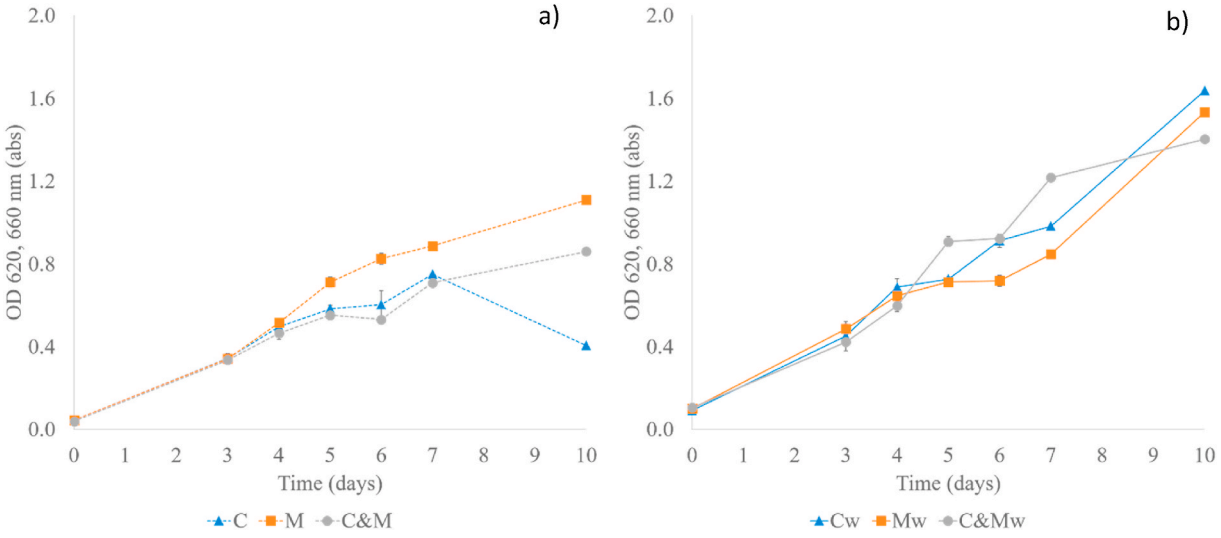

Fig. 2. Biomass productivity (with standard deviation) during the cultivation time for cultures: a) in modified BBM (cyanobacteria-C, microalgae M, mixed cyanobacteria and microalgae-C&M),b) in urban wastewater (cyanobacteria-Cw, Mw, C&Mw).

Table 2
Biomass productivity (BP), nutrients concentrations at time zero, nutrients removal rate and N/P ratio in modified BBM and in urban wastewater for different cultures.

	C	M	C&M	Cw	Mw	C&Mw
BP $_{overall}$ (mg L^{-1} d^{-1})	66.6 ± 3.3	64.2 ± 3.2	97.6 ± 4.9	119 ± 6	114 ± 6	129 ± 7
$N–NH_4^+$ t = 0 (mg L^{-1})	–	–	–	56.2 ± 0.6	56.2 ± 0.6	56.2 ± 0.6
R(N) (mg L^{-1} d^{-1})	–	–	–	5.6 ± 0.4	5.5 ± 0.7	5.5 ± 0.5
$N–NO_3^-$ t = 0 (mg L^{-1})	29.1 ± 0.2	29.1 ± 0.2	29.1 ± 0.2	–	–	–
$R(N^-)$ (mg L^{-1} d^{-1})	5.8 ± 0.2	5.5 ± 0.3	5.7 ± 0.2	–	–	–
$P\text{-}PO_4^{3-}$ t = 0 (mg L^{-1})	52.6 ± 0.4	52.6 ± 0.4	52.6 ± 0.4	5.9 ± 0.3	5.9 ± 0.3	5.9 ± 0.3
$R(P^-)$ (mg L^{-1} d^{-1})	4.43 ± 0.6	1.67 ± 0.3	1.96 ± 0.6	1.00 ± 0.1	1.45 ± 0.1	1.14 ± 0.2
$R(P\text{-}PO_4^{3-})$ (%)	25%	14%	14%	96%	100%	100%
N/P	1.2 ± 0.6	1.2 ± 0.6	1.2 ± 0.6	21.3 ± 0.9	21.3 ± 0.9	21.3 ± 0.9
R(N/P) (molN $molP^{-1}$)	3.53 ± 0.3	9.97 ± 0.5	12.3 ± 0.4	12.0 ± 0.6	10.1 ± 0.5	11.8 ± 0.7

where the initial nitrogen concentration was the highest.

As reported in the scientific literature, microalgae and cyanobacteria are capable to uptake nitrogen from different sources including ammonium, nitrate, nitrite and urea (Parmar et al., 2011; Eustance et al., 2013; Iasimone et al., 2017, 2018a). Nevertheless, ammonium is the preferred nitrogen source, since less energy is required for its uptake. Ruiz-Marin et al. (2010) reported that microalgae strains *C. vulgaris* and *S. obliquus* showed a stronger affinity for ammonium rather than any other form of nitrogen present in wastewater. From the experimental tests, it was therefore expected to find a higher total nitrogen removal rate in urban wastewater than in modified BBM. Such difference did not occur, thus it is reasonable to assert that in tests conducted in cultivation media containing a sole specific nitrogen form (i.e. nitrate in modified BBM and ammonium in urban wastewater), under specific light and temperature as well buffered pH conditions, the total nitrogen removal rate is not significantly depending on: (i) specific nitrogen form; (ii) type of inoculum; (iii) initial total nitrogen concentration. Moreover, the buffered pH condition prevented both cultivation medium acidification and alkalinisation, as result of biological nitrogen uptake from ammonium and nitrate, respectively (Eustance et al., 2013): this aspect contributed to keep stable the environmental conditions, thus avoiding stress in biomass metabolism.

Dissolved phosphorus was monitored as orthophosphate ion during the cultivation time for each test (Fig. 3b and c). In tests fed with the modified BBM (Fig. 3b), phosphorus removal occurred during the first 6 days, after that, a part of phosphorous was released. The highest phosphorus removal efficiency, accounting for 25% approximately (Table 2), was achieved in tests C. On the other hand, in tests fed with urban wastewater (Fig. 3c), phosphorus removal mainly occurred during the first 4 days and resulted completely absorbed into cells at the end of the cultivation time. Phosphorus removal rates are reported in Table 2 for each test: the highest values were achieved when modified BBM was used as cultivation medium and, more in detail, the highest removal rate of 4.43 ± 0.6 mg $P\text{-}PO_4^{3-}.L^{-1}$ d^{-1} was reached when cyanobacteria were used as inoculum (test C). Similar rates were obtained for both cultures separately cultivated in urban wastewater. Such different results are the direct consequence of the different biomass productivity observed in tests fed with the different cultivation media (i.e. modified BBM and urban wastewater, respectively). Indeed, in tests fed with the modified BBM, a decreasing biomass productivity is observed when the nitrogen was not available, thus stressful conditions for the biomass growth occurred and induced phosphorus release. On the contrary, in tests fed with urban wastewater, an increasing biomass productivity is noticeable during the entire observation time and this occurrence promoted the complete phosphorus uptake. As reported in the scientific literature, many microalgae strains can uptake phosphorus in amount much higher than required for their survival, thus storing the surplus amount into cells in form of polyphosphate (Poly-P) (Brown and Shilton, 2014). Finally, phosphorus uptake rate was higher when the initial phosphorus concentration was higher, contrary to what observed for initial total nitrogen concentration. Similar results were found by Zhu et al. (2015) that studied the phosphorus uptake in *Chlorella* sp. under nitrogen shortage conditions and for different initial phosphorus concentrations: such authors observed that the phosphorous uptake occurred mainly during the initial 2 or 3 days of cultivation in all tests; moreover, a total phosphorous removal was achieved in tests fed with the lowest initial phosphorus concentration (5.3 mgP L^{-1}), whereas phosphorus was not completely removed in tests fed with the highest initial phosphorus concentration (155.3 mgP L^{-1}). Analogous results for nitrogen (as nitrate) and phosphorus removal were obtained by Liu and Vyerman (2015) with *Pseudanabaena* sp. cultivated in synthetic cultivation medium where the N/P ratio was set equal to 1. Nevertheless, in their study, authors reported a lower biomass productivity (21.8 ± 0.7 mg L^{-1} d^{-1}) compared to this study, but it has to be considered that this value was referred to a photoperiod of only 12 h, while in this study, light was continuously supplied for 24 h per day. In similar conditions of light, Cai et al. (2013) studied the nitrogen and phosphorus removals conducted by various microalgae and cyanobacteria strains in the axenic batch

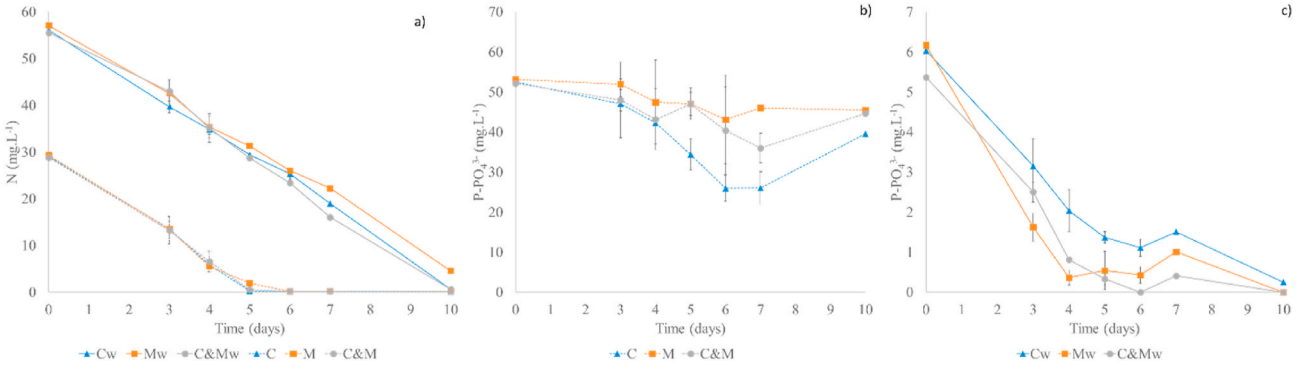

Fig. 3. a) Nitrogen concentration trend as nitrate ($N–NO_3^-$) and as ammonia ($N–NH_4^+$) during the cultivation time for cultures in modified BBM (C, M, C&M) and in urban wastewater (Cw, Mw, C&Mw). Phosphorus trend as phosphate ($P\text{-}PO_4^{3-}$) during the cultivation time for cultures: b) in modified BBM (C, M, C&M); c) in urban wastewater (Cw, Mw, C&Mw).

processes of different waste streams. Compared with results from the present work, authors found almost the same efficiency in removing total nitrogen, whereas less performance in removing phosphorus.

Finally, the stoichiometry of nutrients consumption, R(N/P), calculated according to equation (3) from day 0 to day 5 for each test, is reported in Table 2. Measurements were not extended longer than day 5 because the nitrogen was completely depleted by the 5th day of cultivation in tests fed with the modified BBM. When R is 1, nitrogen and phosphorus consumptions are equivalent; for values lower than 1, phosphorus removal is favoured, while for values higher than 1, nitrogen removal is higher than phosphorus. Results generally showed a higher nitrogen removal rate compared to phosphorus, according to the biomass stoichiometry of N:P = 16:1 (Geider and La Roche, 2002). However, the highest phosphorus uptake was achieved for cyanobacteria cultivated in modified BBM, thus indicating their higher demand of phosphorous compared to microalgae. Cyanobacteria growth was therefore favoured in modified BBM as this cultivation medium supplies a higher phosphorus amount compared to urban wastewater. This result is in agreement with the study conducted by Beuckels et al. (2015), which proved that the nitrogen amount in the biomass not only depends on the nitrogen supply, but also on the phosphorous supply in the cultivation medium. As consequence of this finding, models for microalgae nutrients uptake, based on a fixed Redfield stoichiometry, are not reliable for estimating the capacity of microalgae to remove nitrogen and phosphorous from wastewater (Parmar et al., 2011).

3.2. Lipid accumulation

High lipid content in microalgae strains is an essential prerequisite for their cultivation aimed at producing biofuels. A significant number of microalgae strains can store lipids up to 20–50% of their dry cell weight (DCW) (Iasimone et al., 2018b). In the present work, the highest lipid content of 22 ± 1.1% lipids/DCW was found in microalgae cultivated in urban wastewater (Fig. 4). Generally, microalgae can accumulate more lipids than cyanobacteria, that is the reason why the obtained results are in agreement of the expectations (i.e. the higher content of lipids for microalgae rather than cyanobacteria or their mixture).

Observing Fig. 4, it can be noticed that the highest accumulation of lipids is achieved when the condition of nitrogen shortage occurs. This aspect was already observed by Chiu et al. (2015), who found the highest lipid accumulation in microalgae cultivated in wastewater containing moderate concentrations of ammonium and phosphorus: small nitrogen availability (nitrogen shortage condition) contributes to lipids accumulation. In the present work, the lipids content obtained from microalgae strains was higher by 12% than values reported by Chinnasamy et al. (2010) from mixed cultures cultivated in untreated wastewater, whereas the lipids content was similar to the values reported by Kong et al. (2010) for *Chlamydomonas reinhardtii* cultivated in municipal wastewater (25.3% lipids/DCW). The interesting results obtained from this study when tests were conducted with microalgae in urban wastewater are confirmed by those reported in Chinnasamy et al. (2010) for mixed microalgae consortia: the authors found the highest lipids content in microalgae cultivated in wastewater rather than in BG11 synthetic medium. The higher content of lipids, obtained for microalgae cultivated in wastewater rather than in BBM could be explained by the particular growth condition offered by this medium in terms of nutrients content and for the presence of other microbial species that could have enhanced the lipid accumulation in the microalgal biomass.

3.3. Biomass flocculation

The occurrence of the spontaneous flocculation and its efficiency were investigated through pictures observation during sedimentation assays conducted for each cultivation test every day during the cultivation time. Images captured at time t = 0 and t = 50 min of the sedimentation assays are reported in the Supplementary material (sections B and C for cultivation tests conducted with modified BBM and urban wastewater, respectively).

Several studies have demonstrated that spontaneous flocculation of microalgae also can be induced by increasing the pH in the cultivation medium. This phenomenon is often known as autoflocculation. It has been proven that spontaneous flocculation at high pH is caused by chemical precipitation of calcium and/or magnesium salts or by precipitation of calcium phosphate (Semerjian and Ayoub, 2003; Vandamme et al., 2012). In this study, pH in both cultivation media was controlled by the HEPES buffer, which kept stable the pH at 7.2 ± 0.3. As consequence, the spontaneous flocculation phenomena observed in this study are occurrences of bioflocculation rather than autoflocculation.

Some species of microalgae own the natural skill to flocculate during growth in axenic culture medium. The genes responsible for self-flocculation in microalgae are still not completely known. Nevertheless, water-soluble extracts, polysaccharides and glycoproteins, simultaneously biosynthesized by microalgae, affect their self-flocculating properties since they are capable to form patches and/or bridges among cells by neutralizing negative charge, thus promoting self flocculation (Ogbonna and Nwoba, 2021). In this study, the self flocculation mechanism in the axenic culture of BBM was investigated for microalgae, for cyanobacteria and for their combination.

On the other hand, bacteria-microalgae symbiosis is a natural phenomenon that occurs in algal cultures and results in bioaggregates made

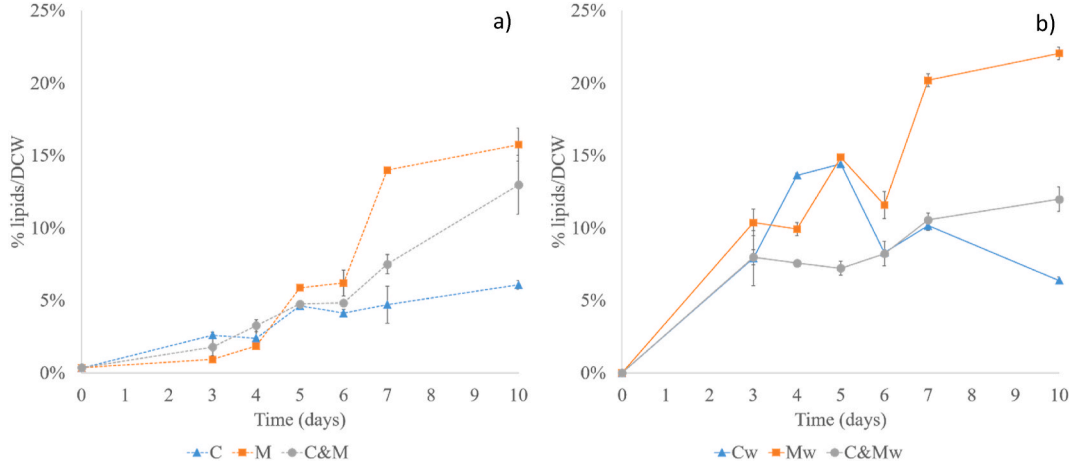

Fig. 4. –Lipids accumulation during the cultivation time for cultures: a) in modified BBM (C, M, C&M); b) in urban wastewater on the right (Cw, Mw, C&Mw).

of microalgal cells. Actually, bacteria can secrete biopolymers such as extracellular polysaccharides and gamma glutamate that own charged functional groups capable to neutralize the charge on the microalgal cells as well as form electrostatic patches and/or bridges (Mahata et al., 2021). In this study, the bacteria-based flocculants effect was investigated for cyanobacteria, microalgae and their mixture when cultivated in raw urban wastewater. Although cultivations of mixture made of bacteria with microalgae have been extensively studied in the literature, the role of cyanobacteria remains poorly investigated. In the current study, their role resulted crucial especially during the bridging and patching steps of flucculation since the long chains of filamentous cyanobacteria could have a key role in the formation of flocs.

3.3.1. Bioflocculation in modified BBM

In tests with modified BBM, bioflocculation was observed since day 4 for test C. The sedimentation process performance progressively improved from day 4 to day 7 as much as the size of cyanobacteria flocs increased during the same time lapse, as highlighted by images in Fig. 5. The sedimentation process efficiency resulted in being strongly correlated with floc size. Moreover, the increase of biomass concentration in the cultivation medium due to positive growth condition promoted the formation of significantly bigger sized flocs that settled with higher settling rate. Actually, a progressively wider zone of the "clarified area" was observed from day 4 to day 7 (Supplementary material – section B).

Tests M and C&M did not show any clear bioflocculation at day 4 (Supplementary material – section B) and, as a consequence, the third replicate of test C as well as M were mixed up in order to obtain test E in duplicate. The OD of the resulting mixture was 0.5 abs. These tests were aimed at assessing the occurrence of potential interactions between suspended microalgae and filamentous cyanobacteria in forming flocs when the microorganisms are in their exponential growth phase. Images captured by microscope for tests C&M and for tests E at day 5 (Fig. 6) clearly show a weak interaction between the two single cultures in tests C&M, whereas in tests E microalgae are homogeneously mixed with cyanobacteria, thus forming round shaped flocs. This aspect was responsible for improving the sedimentation performance in test E compared to test C&M (Supplementary material, section B, day 5). Finally, from day 6, in all cultivation tests the cultures reached the decline growth phase, as pointed out by the yellowish colour of the cultivation medium (Supplementary material, section B).

3.3.2. Bioflocculation in urban wastewater

In tests with urban wastewater, bioflocculation was observed since day 3 (Supplementary material, section C) for both tests Cw and C&Mw, whereas it was not evident for test Mw. The difference in results (i.e. an occurrence of bioflocculation in urban wastewater earlier than modified BBM and the occurrence of bioflocculation involving cyanobacteria in urban wastewater rather than in modified BBM) from tests in modified BBM and urban wastewater can be ascribed to the better biomass growth conditions in the latter cultivation medium that promoted a faster increase of biomass concentration and consequently induced the formation of big flocs for both tests (Cw and C&Mw). As done for tests conducted in modified BBM, at day 3 of cultivation time, one replicate of test Cw was mixed with one replicate of test Mw, thus obtaining test Ew that was conducted in duplicate. The initial OD of test Ew was 0.5 abs.

Cyanobacteria in test Cw did not show settling efficiency as high as in test C. This result is reasonably due to the specific configuration of aggregates that cyanobacteria formed in urban wastewater (Fig. 7a): in urban wastewater, the long filaments of cyanobacteria did not aggregate into flocs structures, as occurred in modified BBM, but formed clouded structures with low tendency to settle. This different configuration, observed by changing the cultivation medium, could be caused by colloidal solids, naturally present in urban wastewater, that negatively interacted with cyanobacteria, thus preventing the achievement of significant sedimentation efficiency. Indeed, as reported by Semerjian and Ayoub (2003), colloidal suspensions in urban wastewater consist of equally negatively charged solids that do not tend to aggregate because of repulsive forces. Such suspension is therefore stable and does not settle. In test Cw, the ionized functional groups composing the cell wall of cyanobacteria are responsible for charging negatively the same cyanobacteria and an electro-static repulsion force is therefore generated between cyanobacteria and colloids as well as between two or more cyanobacteria. Such condition prevents cyanobacteria to approach each other and aggregate spontaneously, according to van der Waals forces (Chatsungnoen and Chisti, 2016). To neutralize these negative charges, a cationic flocculant can be added to the suspension. Moreover, the effects of extracellular polymeric substances (EPS) is not negligible on bioflocculation performance since, in some specific conditions, they can negatively affect the process: an excessively relevant adsorption of the positively charged particles (i.e. EPS) on the negatively charged cells results in generating reversal repulsive forces as well (Taghavijeloudar et al., 2021).

Flocculants traditionally used in water and wastewater treatment are salts of either aluminium or iron, but these chemical compounds can have a detrimental effect on microalgae cultivation systems because they can contaminate the down-stream products, thus prejudicing the biomass valorization (Gutiérrez et al., 2015); moreover, flocculants increase the volumes of sludge produced (Riaño et al., 2012) and therefore the costs for its disposal. To overcome these disadvantages, chitosan biopolymer could be used for wastewater treatment. With the aim of improving the sedimentation process efficiency, an equal amount of chitosan (i.e. 240 mg L^{-1}) was added to all sedimentation assays at day 6. Indeed, in case of microalgae-bacteria consortium, the study performed by Gutiérrez et al. (2015) has proved that dosing 240 mg L^{-1} Chitosan, almost 90% of biomass is recovered; moreover, Vu et al. (2020) found an optimal dosage of Chitosan of 200 mg L^{-1} for *Chlorella* sp. The chitosan-induced flocculation resulted efficient for microalgae (80% of the water column was clarified) but had a detrimental effect on cyanobacteria and mixed cultures: the bioflocculation was even prevented (Supplementary material, section C). Moreover, Lama et al. (2016) studied the effect of chitosan on *Pseudanabaena* sp. flocculation and found interesting results when the authors added a smaller amount

Fig. 5. Microscope images of cyanobacteria in modified BBM at day 4, 6, 7 from left to right.

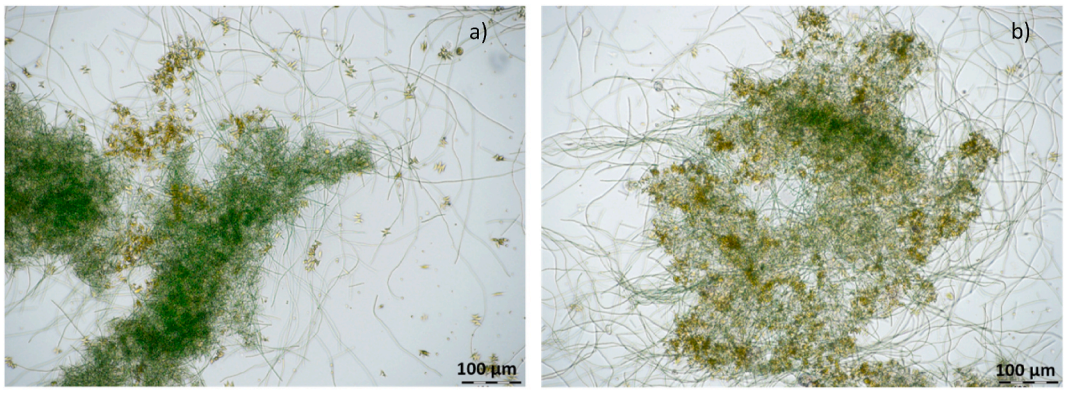

Fig. 6. Mixed cyanobacteria and microalgae cultures in modified BBM: a) C&M test; b) E test.

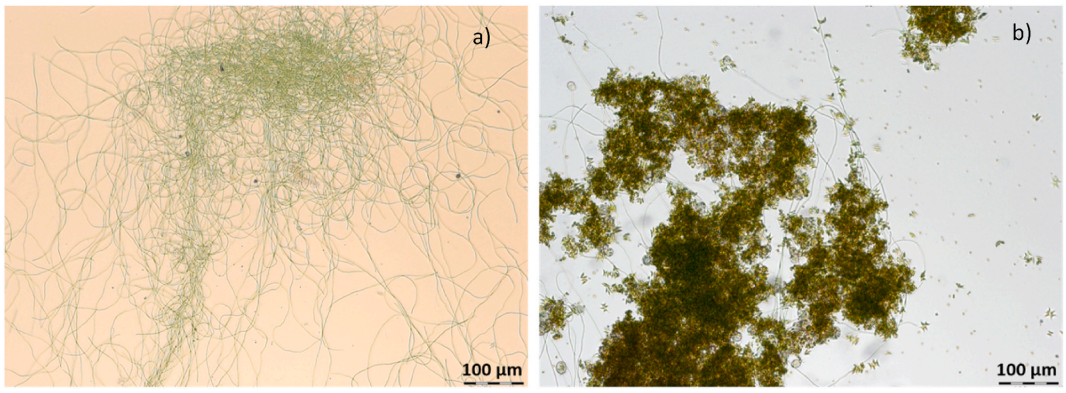

Fig. 7. Microscope images of: a)cyanobacteria aggregation in urban wastewater at day 3; b) microalgae aggregation in urban wastewater at day 7.

of chitosan, approximatively 80 mg L^{-1} in cultivation tests fed with a synthetic medium. Therefore, in the present work, the negative effect of chitosan on cyanobacteria flocculation could be reasonably due to the tested high dosage that increased the amount of total solids, thus reducing the efficiency of sedimentation process.

On the other hand, for microalgae cultivated in urban wastewater, bioflocculation took place at days 7 and 10, as highlighted by images captured with microscope (Fig. 7b). Bioflocculation can occur spontaneously in certain microalgae as consequence of an environmental stress (Ummalyma et al., 2017). Microalgae flocs settled in approximately 50 min, as showed by pictures reported in the Supplementary material, section C. Anyway, the supernatant was not clear as much as that observed for test Cw. Probably, the same stressful condition that induced the bioflocculation in microalgae caused, at the same time, the excretion of extracellular material that remained suspended in the cultivation medium, thus increasing its turbidity.

3.3.3. *Assessment of effect of bioflocculation on sedimentation process performance*

To assess the effect of bioflocculation on sedimentation process performance, a specific method was developed and described in the materials and methods section. The fraction of "clarified area" at the end of sedimentation tests (maximum time was set at 50 min) are reported in Fig. 8. Results from tests inoculated with sole microalgae are not

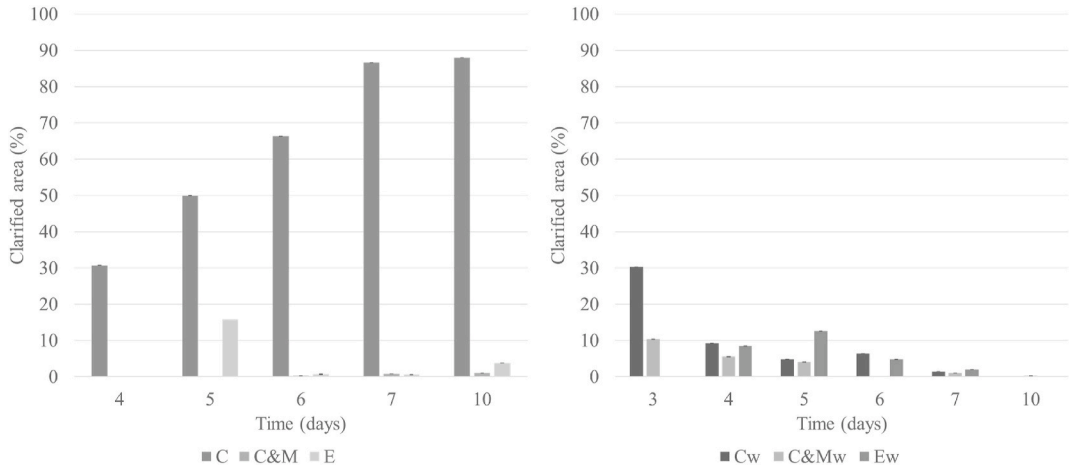

Fig. 8. Fraction (%) of "clarified area" at different days for tests in modified BBM (C, C&M, E) and in urban wastewater (Cw, C&Mw, Ew).

reported since the "clarified area" was not clearly appreciable. The widest "clarified area" was observed for test C: cyanobacteria disclosed better spontaneous flocculation and sedimentation performance during their exponential growth phase (days from 4 to 7), thus obtaining a size of the "clarified area" equal to 90% of the total sample volume. In tests fed with modified BBM, bioflocculation occurred also for mixed cultures in test E but only at day 5. In tests fed with urban wastewater, bioflocculation was observed for both cyanobacteria and mixed cultures inocula but resulted in being less effective, thus indicating that urban wastewater is not an appropriate medium to induce the bioflocculation. Moreover, the bioflocculation performance in urban wastewater generally decreased during the cultivation time for all tests, probably because of the increasing concentration of both colloids and extracellular material.

4. Conclusions

Biomass flocculation was studied in axenic culture medium (self flocculation phenomenon) and in raw urban wastewater (interaction with bacteria), resulting in the following main aspects:

- the occurrence of a bioflocculation phenomenon was clearly observed for cyanobacteria, whereas it was not evident for the microalgae species, *Chlorella and Scenedesmus* sp, cultivated in this study;
- in axenic culture medium, the effect of interactions between cyanobacteria and microalgae in forming flocs was more efficient when the different species were mixed at their exponential growth phase rather than when they were cultivated together from the beginning of the test. In the latter condition, cyanobacteria formed closed flocs structure without significantly incorporating microalgae. When bioflocculation occurs, the respective concentrations of microalgae and cyanobacteria present in the liquid phase will impact the concentrations obtained within the flocs;
- in raw urban wastewater, the flocculation phenomenon was different compared to axenic conditions: Cyanobacteria formed clouded structures, which remained suspended in the medium and were not capable to settle down. EPS and colloids were both responsible for this result since they promoted the bridging process but inhibited the patching process, thus preventing that cyanobacteria formed dense and settleable flocs as occurred in axenic culture medium. Moreover, although microalgae could be easily incorporated in the clouded flocs formed by cyanobacteria, the resulting aggregated structures are not capable to efficiently settle down, therefore the efficiency of the harvesting process results unsatisfactorily.

Author contribution

Floriana Iasimone: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. Jordan Seria: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. Antonio Panico: Methodology, Writing – original draft, Writing – review & editing.. Vincenzo De Felice, Supervision, Conceptualization, Project administrator. Francesco Pirozzi: Conceptualization, Supervision; Jean-Philippe Steyer: Supervision, Conceptualization, Methodology, Writing – original draft, Writing – review & editing,

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.111359.

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