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Surface physicochemical properties of selected single and mixed cultures of microalgae and cyanobacteria and their relationship with sedimentation kinetics

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Abstract

Background: Microalgae are photosynthetic microorganisms presenting a diversity of biotechnological applications. However, microalgal cultivation systems are not energetically and economically feasible. Possible strategies that can be applied to improve the feasibility of microalgal production include biofouling control in photobioreactors, the use of attached growth systems and bioflocculation. These processes are ruled by surface physicochemical properties. Accordingly, the surface physicochemical properties of *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, *Synechocystis salina* and *Microcystis aeruginosa* were determined through contact angle and zeta potential measurements. Additionally, mixed cultures of the selected microorganisms were performed. Sedimentation kinetics of the studied cultures was also evaluated to understand how surface physicochemical properties influence microalgal recovery.

Results: All studied microorganisms, except *S. salina*, presented a hydrophilic surface. The co-culture of *S. salina* with the other studied microorganisms resulted in a more hydrophobic algal suspension. Regarding zeta potential determinations, all studied suspensions presented a negatively charged surface (approximately -40.8 ± 4.4 mV). Sedimentation experiments have shown that all microalgal suspensions presented low microalgal recovery efficiencies. However, a negative linear relationship between microalgal removal percentage and free energy of hydrophobic interaction was obtained.

Conclusions: The evidence of a relationship between microalgal removal percentage and free energy of hydrophobic interaction demonstrates the importance of surface physicochemical properties on microalgal settling. However, the low recovery efficiencies achieved, as well as the high net zeta potential values determined, indicate that another factor to consider in microalgal settling is the ionic strength of the culture medium, which play an important role in suspensions' stability.

Keywords: Cyanobacteria; Microalgae; Mixed cultures; Sedimentation kinetics; Surface physicochemical properties

Background

Microalgal culturing has been the focus of several research studies worldwide, due to the wide variety of biotechnological applications described for these photosynthetic microorganisms [1,2]. When growing autotrophically, microalgae convert CO_2 (from atmosphere or flue gas emissions) into organic carbon compounds, thus reducing the CO_2 accumulation in the atmosphere [3-6]. Additionally, microalgae assimilate other compounds, such as nitrogen and phosphorus, frequently found in wastewaters, meaning that these microorganisms may play an important role in wastewater treatment processes [7-10]. Finally, microalgal biomass has several applications [1,11-13]: (i) human food and animal feed; (ii) production of cosmetics, drugs and functional food; and (iii) biofuels. However, microalgal cultivation still presents high process costs, which are mainly due to the low biomass productivities and the associated harvesting costs, accounting for 20% to 30% of biomass production costs [14]. Moreover, it requires large amounts of water and nutrients, which is the reason to be considered a process with high environmental impact [15].

To improve biomass productivities in microalgal photobioreactors (PBRs), new strategies should be adopted to

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avoid biofouling (the attachment of cells to the walls of PBRs). This phenomenon is responsible for lower biomass productivities, since the amount of light (the energetic source required for photosynthesis) that effectively penetrates into microalgal suspension is significantly reduced [16-18]. On the other hand, several authors have reported the growth of microalgae in biofilms. These immobilized growth systems facilitate further harvesting processes [7,16,19,20]. For both promotion and control of microalgal attachment, knowledge about surface physicochemical properties is essential [16,21,22]. Knowing the surface characteristics may also be very helpful in metabolite extraction. For example, the use of solvents with polarities similar to those of target metabolites may facilitate the contact between both, increasing extraction efficiency [23].

Regarding the harvesting process, currently applied methods include chemical coagulation/flocculation, gravity sedimentation, flotation, filtration, centrifugation and electrical-based processes [14,24,25]. Selection of an appropriate harvesting method depends on the end product, namely its value and properties [14]: it is important to consider the acceptable level of moisture, salt concentrations, cell damage and strain features, such as their density and size. Additionally, selection of an effective harvesting procedure must take into account that microalgal biomass must be further processed. Therefore, these procedures must not be toxic or contaminate microalgal biomass. It is also desirable that the selected harvesting method allows the recycling of the culture medium [25]. In this sense, bioflocculation appears as a viable alternative for the commonly used procedures. This method consists in the addition of other microorganisms or microbial metabolites to microalgal cultures, trying to stimulate microbial aggregation and flocs formation. The use of bioflocculation as harvesting method is more energetically efficient and reduces the impact of the addition of chemical flocculants to microalgal biomass, which tend to be an expensive and toxic alternative [26]. The ability of microorganisms to form flocs strongly depends on their surface physicochemical properties.

Information about the surface physicochemical properties of microalgae and about their interaction mechanisms may be very helpful in the development of energetically and economically feasible biomass production systems and harvesting procedures, as well as in the development of new strategies to prevent biofouling. This study provides information about the surface physicochemical properties of the microalgae *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* and the cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* determined experimentally, according to the method proposed by van Oss et al. [27-29]. Selection of these microorganisms was based on the following factors [30-33]: (i) these microalgae and cyanobacteria can be easily grown in laboratory cultures and (ii) several authors have reported the use of these microorganisms in a wide variety of biotechnological applications, such as CO_2 capture, wastewater treatment, biofuel production and synthesis of bioactive compounds. Additionally, algal suspensions resulting from the co-culture of different associations between the selected microorganisms were evaluated, to determine if these associations have influence on surface physicochemical properties and on their ability to form aggregates. Sedimentation kinetics of the studied cultures was also determined, to assess the influence of surface physicochemical properties on the recovery of microalgal biomass.

Methods

Microorganisms and culture medium

The microalgae *C. vulgaris* CCAP 211/11B and *P. subcapitata* CCAP 278/4 were obtained from the Culture Collection of Algae and Protozoa (UK), while the cyanobacteria *S. salina* LEGE 06079 and *M. aeruginosa* LEGE 91344 were obtained from the Laboratory of Ecotoxicology, Genomic and Evolution - CIIMAR (Centre of Marine and Environmental Research of the University of Porto, Portugal). Stock solutions of these microorganisms were prepared in OECD (Organisation for Economic Co-operation and Development) test medium [34], with the following composition (per litre): 250 mg $NaNO_3$, 12 mg $MgCl_2{\cdot}6H_2O$, 18 mg $CaCl_2{\cdot}2H_2O$, 15 mg $MgSO_4{\cdot}7H_2O$, 45 mg KH_2PO_4, 0.08 mg $FeCl_3{\cdot}6H_2O$, 0.1 mg $Na_2EDTA{\cdot}2H_2O$, 0.185 mg H_3BO_3, 0.415 mg $MnCl_2{\cdot}4H_2O$, 3 μg $ZnCl_2$, 1.5 μg $CoCl_2{\cdot}6H_2O$, 0.01 μg $CuCl_2{\cdot}2H_2O$, 7 μg $Na_2MoO_4{\cdot}2H_2O$ and 50 mg $NaHCO_3$. The culture medium was sterilized by autoclaving at 121°C for 15 min. The cells were incubated in 500-mL flasks at room temperature (24.0 ± 1.0°C), under continuous fluorescent light with an irradiance of 120 μE m^{-2} s^{-1} at the surface of the flasks. Agitation was obtained by bubbling atmospheric air (filtered through 0.22-μm cellulose acetate membranes, Orange Scientific, Belgium) in the bottom of the flasks.

Microalgal culturing in single and mixed cultures

Previously described stock solutions were used to prepare microalgal suspensions for further characterization of surface physicochemical properties and sedimentation kinetics determination. In these experiments, single cultures, as well as different associations between the selected microorganisms, were used. Batch experiments were performed in 500-mL flasks (VWR, Carnaxide, Portugal) with a working volume of 450 mL. Cells were cultivated for 7 days with an initial cell concentration of approximately 1.0×10^6 cells mL^{-1} (in mixed cultures, testing all possible associations of two microorganisms, the initial cell concentration was approximately 2.0×10^6 cells mL^{-1}). Temperature, light and agitation conditions were the same as for the stock solutions preparation. All the experiments were performed in duplicates under aseptic conditions.

Surface physicochemical properties

Zeta potential measurements

In the last day of each culture, microalgal suspensions were harvested, washed twice and resuspended in MilliQ water (Millipore, Billerica, MA, USA) to obtain a final concentration of about 5.0×10^6 cells mL^{-1}. Zeta potential was measured using a ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, UK). All the determinations were performed in a clear disposable zeta cell at approximately 25°C. Mean values of each studied suspension were obtained by at least triplicate measurements of two independent experiments.

Surface contact angle measurements

In the last day of each culture, microalgal suspensions were harvested, washed twice and resuspended in saline solution (0.85% *w*/*v* NaCl) to obtain a final concentration of about 5.0×10^6 cells mL^{-1}. Algal lawns were prepared by filtering the previously washed suspensions using 0.45-μm nitrocellulose membrane filters (Advantec MFS, Inc., Tokyo, Japan) until complete clogging of the membranes. Contact angle measurements were performed using the sessile drop method, as described by Busscher et al. [35]. The measurements were carried out at room temperature using water, formamide and α-bromonaphthalene (Sigma-Aldrich, Sintra, Portugal) as the reference liquids. Determination of contact angles was performed automatically using a model OCA 15 Plus (DataPhysics, Filderstadt, Germany) video-based optical contact angle measuring instrument, allowing image acquisition and data analysis. Contact angle measurements (at least 12 determinations for each liquid and for each algal suspension) were performed for two independent experiments.

Surface parameters and hydrophobicity determinations

After contact angle measurements, the values of surface hydrophobicity of the studied algal suspensions were determined using the approach of van Oss [36], which allows the assessment of the absolute degree of hydrophobicity of any surface in comparison with their interaction with water. In this approach, the degree of hydrophobicity of a given surface (*s*) is expressed as the free energy of hydrophobic interaction between two entities of that surface when immersed in water (*w*): ΔG_{sws}^{TOT}, in mJ m^{-2}. When $\Delta G_{sws}^{TOT} < 0$, the interaction between the two entities is stronger than the interaction of each entity with water and the material is considered hydrophobic. Alternatively, if $\Delta G_{sws}^{TOT} > 0$, the material is hydrophilic. ΔG_{sws}^{TOT} can be calculated through the surface tension components of the interacting entities, according to Equation 1 [27-29]:

$$\Delta G_{sws}^{TOT} = -2\left(\sqrt{\gamma_s^{LW}}-\sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+\gamma_w^-}+\sqrt{\gamma_s^-\gamma_w^+}-\sqrt{\gamma_s^+\gamma_s^-}-\sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz-van der Waals (LW) component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), being $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$. LW forces, usually attractive, result from instantaneous asymmetrical distribution of electrons in molecules (the higher the value of LW component, the more apolar is the surface and, therefore, the lower would be its affinity for polar liquids) [37]. Electron donor and acceptor parameters give information about the molecules present in the studied surface: higher γ^+ indicates the presence of positively charged molecules and higher γ^- indicates the presence of negatively charged molecules [36]. Acid-base (AB) forces result from electron transfer interactions between polar components of the involved surfaces. These interactions can be attractive (hydrophobic attraction) or repulsive (hydrophilic repulsion), depending on the free energy of hydrophobic interaction [37,38].

The surface tension components of a surface (*s*) were obtained by measuring the contact angles of three pure liquids (*l*), water and formamide (both polar) and α-bromonaphthalene (apolar), followed by the simultaneous resolution of three equations of the form Equation 2:

$$(1+\cos\theta)\gamma_l^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}}+\sqrt{\gamma_s^+\gamma_l^-}+\sqrt{\gamma_s^-\gamma_l^+}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$. The surface tension of liquid components was obtained from the literature [39]. The contact angle of the apolar liquid, α-bromonaphthalene, was used to quantify the apolar energy component γ_s^{LW}, since γ_l^- and γ_l^+ for this probe liquid are equal to zero. On the other hand, contact angles measured with the other probe liquids were used to determine the other surface parameters, γ_s^+ and γ_s^-.

Sedimentation kinetics

Microalgal recovery

In the last day of each culture, microalgal suspensions were also used to evaluate sedimentation kinetics. 1-mL samples were allowed to settle in polystyrene cuvettes at room temperature. Microalgal recovery within the settling period (8 h) was determined by measuring the turbidity (optical density (OD)) of the samples at 750 nm using a V-1200 spectrophotometer (VWR, Carnaxide, Portugal), according to the method proposed by Salim et al. [40]. Turbidity of the samples was measured at the same height in the cuvette. Microalgal removal percentage or microalgal recovery was calculated according to Equation 3 [40]:

$$\%R = \frac{OD_{750}(t_0)-OD_{750}(t)}{OD_{750}(t_0)} \cdot 100 \quad (3)$$

where $OD_{750}(t_0)$ is the turbidity of the sample determined in the beginning of microalgal settling and $OD_{750}(t)$ is the turbidity of the sample determined at time t.

Modelling microalgal sedimentation

Kinetic constants associated to the recovery of microalgal biomass were determined by fitting the experimental data, corresponding to the time-course evolution of microalgal removal percentages, to the Gompertz model, expressed by Equation 4 [41]:

$$y = a \cdot \exp(-\exp(b - ct)) \quad (4)$$

where y is the output value or microalgal removal percentage (%), a is the upper asymptote (%), b ($b > 0$) sets the displacement along the x-axis and corresponds to the lag time observed in the beginning of settling experiments (h) and c ($c > 0$) sets the tangent at the inflection point and corresponds to the sedimentation rate (h^{-1}). The kinetic parameters, a, b and c, were determined by minimizing the sum of squared residuals using the Solver supplement of Microsoft Excel 2013. The quality of the model fit was evaluated through analysis of the coefficient of determination (R^2).

Statistical analysis

Contact angles, surface tension parameters, zeta potential values and microalgal removal percentages were analysed using paired-samples t-test from the statistical software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Statistical tests were carried out at a significance level of 0.05.

Results and discussion

Surface physicochemical properties

The surface physicochemical properties determined using the approach of van Oss et al. [27-29] for the studied suspensions are shown in Table 1. Data is divided into single and mixed cultures, and the results are presented in the decreasing order of free energy of hydrophobic interaction (from the most hydrophilic to the most hydrophobic suspensions). Except for the microalga *C. vulgaris* and for the suspensions *P. subcapitata* + *M. aeruginosa* and *P. subcapitata* + *S. salina*, the contact angles measured with the polar probe liquids (water and formamide) were in agreement with the values of free energy of hydrophobic interaction, ΔG_{sws}^{TOT} . Higher contact angles were measured for microorganisms presenting more hydrophobic surfaces (lower ΔG_{sws}^{TOT} values). On the other hand, contact angles measured with the probe liquid α-bromonaphthalene for the different algal suspensions were not statistically different ($p > 0.05$).

Looking at electron donor and acceptor parameters, the values determined for the studied microorganisms ranged from 22.6 ± 3.2 to 57.4 ± 7.6 mJ m^{-2} and from 0.0 ± 0.0 to 0.5 ± 0.4 mJ m^{-2}, respectively. For the studied consortia, γ_s^- and γ_s^+ values ranged from 7.6 ± 2.2 to 53.0 ± 8.9 mJ m^{-2} and from 0.0 ± 0.0 to 0.9 ± 0.3 mJ m^{-2}, respectively. These components were not statistically different from those obtained for single cultures ($p = 0.61$ and $p = 0.99$, respectively). Higher values determined for γ_s^- indicate that the studied suspensions are electron donors [42] and may be attributed to the presence of excessive molecules of oxygen in the surface of the microorganisms and to the neutralization of γ_s^+ sites by the dominant γ_s^- sites through intermolecular and intramolecular interactions [36]. On the other hand, γ_s^+ values close to zero are in accordance with the van Oss et al. [27-29] model, which predicts almost the nonexistence of electron acceptor parameters [43]. According to Volpe and Siboni [43], the assumption of a monopolar surface is unrealistic. However, the authors concluded that the van Oss et al. [27-29] approach can be applied when the aim is to compare the ability of different microorganisms to interact with other microorganisms or surfaces. When the aim is to perform molecular interpretations, these parameters (determined through the van Oss et al. [27-29] method) should not be used. Additionally, plotting γ_s^- as a function of ΔG_{sws}^{TOT} resulted in a coefficient of determination (R^2) of 0.94, indicating that the van Oss et al. [27-29] approach can be correctly applied to determine the surface physicochemical properties of the selected algal suspensions. As for the contact angle measurements with the polar probe liquids, γ_s^- values correlated well with the free energy of hydrophobic interaction, since lower values of the electron donor parameter were observed for the microorganisms presenting lower ΔG_{sws}^{TOT}.

Regarding the Lifshitz-van der Waals component of the surface free energy, γ_s^{LW}, the values determined for the studied species were not statistically different ($p > 0.05$), ranging from 35.0 ± 1.5 to 35.9 ± 0.7 mJ m^{-2}. Similar results determined for this component indicate that cell wall composition of the studied microorganisms may not have significant differences. In the study performed by Ozkan and Berberoglu [44], differences in the LW component determined for green algae and diatoms were attributed to differences in the chemical composition of their cell walls. Several authors have reported that green algae, such as *C. vulgaris* and *P. subcapitata*, present cellulose-based cell walls, whereas diatoms present silica-based ones [45-48]. γ_s^{LW} values determined in this study for microalgae were very similar to those determined by Ozkan and Berberoglu [44] for green algae (ranging from 20.7 to 37.8 mJ m^{-2}). Additionally, γ_s^{LW} values determined for the studied cyanobacteria were also similar to those obtained for the cyanobacteria *Synechocystis* sp. and *Anabaena variabilis*

Table 1 Contact angles and surface physicochemical properties determined for each of the studied suspensions

Microalgal suspensions	Contact angles (°)			Surface tension parameters and free energy of hydrophobic interaction (mJ m^{-2})					Zeta potential (mV)
	θ_W	θ_B	θ_F	γ_s^{LW}	γ_s^{AB}	γ_s^-	γ_s^+	ΔG_{sws}^{TOT}	
Single cultures									
C. vulgaris	46.5 ± 2.8 a	37.1 ± 1.5 a	61.0 ± 0.3 a	35.0 ± 1.5 a	0.0 ± 0.0 a	48.7 ± 6.9 a	0.1 ± 0.1 a	52.5 ± 14.3 a	−35.4 ± 0.4 a
M. aeruginosa	44.8 ± 5.5 a	37.1 ± 1.6 a	36.4 ± 8.4 b	35.9 ± 0.7 a	10.7 ± 2.8 b	57.4 ± 7.6 a	0.5 ± 0.4 a	43.4 ± 15.5 a	−40.0 ± 2.4 a
P. subcapitata	48.7 ± 1.1 a	37.3 ± 2.2 a	45.8 ± 3.6 b	35.8 ± 1.0 a	5.4 ± 1.5 b	35.9 ± 1.7 b	0.2 ± 0.2 a	13.8 ± 3.8 b	−48.1 ± 0.9 b
S. salina	51.9 ± 8.0 a	37.3 ± 1.0 a	73.6 ± 1.1 a	35.8 ± 0.4 a	0.0 ± 0.0 a	22.6 ± 3.2 b	0.0 ± 0.0 a	−10.2 ± 6.6 b	−43.1 ± 2.4 b
Mixed cultures									
C. vulgaris + *M. aeruginosa*	41.9 ± 2.8 a	34.2 ± 4.4 a	51.6 ± 3.6 c	37.0 ± 1.8 a	0.5 ± 0.3 a	53.0 ± 8.9 a	0.0 ± 0.0 a	40.6 ± 11.8 a	−35.4 ± 1.0 a
C. vulgaris + *S. salina*	76.8 ± 4.2 b	35.3 ± 2.1 a	57.0 ± 3.1 c	36.6 ± 0.8 a	0.0 ± 0.0 a	52.2 ± 1.5 a	0.0 ± 0.0 a	40.1 ± 2.5 a	−45.1 ± 1.4 b
P. subcapitata + *M. aeruginosa*	41.9 ± 2.8 a	37.3 ± 0.9 a	35.5 ± 1.5 d	35.8 ± 0.4 a	11.7 ± 0.8 b	37.0 ± 4.8 b	0.9 ± 0.3 a	13.4 ± 7.2 b	−42.7 ± 1.4 b
C. vulgaris + *P. subcapitata*	57.0 ± 0.1 a	39.6 ± 0.1 a	56.0 ± 0.2 c	34.0 ± 0.1 a	0.0 ± 0.0 a	32.7 ± 0.3 b	0.0 ± 0.0 a	10.5 ± 0.5 b	−42.7 ± 1.5 b
P. subcapitata + *S. salina*	54.2 ± 1.9 a	37.6 ± 0.3 a	50.7 ± 1.1 c	35.6 ± 0.1 a	2.8 ± 0.5 c	32.0 ± 1.9 b	0.1 ± 0.0 a	8.3 ± 3.3 b	−40.5 ± 0.5 b
S. salina + *M. aeruginosa*	93.5 ± 3.3 c	35.0 ± 3.2 a	84.0 ± 1.3 a	36.7 ± 1.3 a	0.0 ± 0.0 a	7.6 ± 2.2 c	0.0 ± 0.0 a	−50.4 ± 8.6 c	−34.9 ± 1.0 a

Values are presented as the mean ± standard deviation of two independent experiments. Different letters within the same column represent statistically different values ($p < 0.05$). θ_W, contact angle with water; θ_B, contact angle with α-bromonaphthalene; θ_F, contact angle with formamide; γ_s^{LW}, Lifshitz-van der Waals component of the surface free energy; γ_s^{AB}, Lewis acid-base component of the surface free energy; γ_s^-, electron donor component; γ_s^+, electron acceptor component; ΔG_{sws}^{TOT}, free energy of hydrophobic interaction.

(28.3 and 37.0 mJ m^{-2}, respectively). γ_s^{LW} values determined for the different combinations of microalgae and cyanobacteria ranged from 34.0 ± 0.1 to 37.0 ± 1.8 mJ m^{-2}. These values were not statistically different ($p = 0.24$) from those determined for each individual strain, and in addition, no statistical differences ($p > 0.05$) were observed between the different studied consortia. As previously stated, similar results determined for this component indicate that cell wall composition of the studied microorganisms, even when grown in mixed cultures, may not have significant differences.

Values of γ_s^{AB} determined for the studied microalgae ranged from 0.0 ± 0.0 to 5.4 ± 1.5 mJ m^{-2}, whereas the same value determined for the cyanobacteria ranged from 0.0 ± 0.0 to 10.7 ± 2.8 mJ m^{-2}. The values obtained for microalgae were similar to those obtained by Ozkan and Berberoglu [44] for green algae (ranging from 0.0 to 5.1 mJ m^{-2}). Regarding the values obtained by the authors for cyanobacteria, γ_s^{AB} determined in this study for the cyanobacterium *S. salina* was equal to the one reported for *Synechocystis* sp. A γ_s^{AB} of 5.4 ± 1.5 mJ m^{-2} determined for the cyanobacterium *M. aeruginosa*, which is statistically higher than the one determined for *S. salina* ($p = 0.01$) may be related to the hydrophilic character of this cyanobacterium ($\Delta G_{sws}^{TOT} > 0$). The γ_s^{AB} values determined for the microalgae and for the cyanobacterium *M. aeruginosa* are a measure of the hydrophilic repulsion, since these microorganisms presented a hydrophilic surface. On the other hand, γ_s^{AB} determined for *S. salina* is a measure of hydrophobic attraction, due to the negative value of ΔG_{sws}^{TOT} determined for this cyanobacterium. The magnitude of AB interactions increases with increasing surface hydrophobicity (decreasing γ_s^+ and γ_s^-), and species having hydrophilic surface properties experience repulsive acid-base interactions [49]. γ_s^{AB} values determined for the studied consortia were not statistically different ($p = 0.64$) from those determined for microorganisms grown in single cultures, ranging from 0.0 ± 0.0 to 11.7 ± 0.8 mJ m^{-2}. Since positive ΔG_{sws}^{TOT} values were determined for all studied consortia, except for the one composed by both cyanobacteria, γ_s^{AB} values are a measure of hydrophilic repulsion. In the mixed culture of both cyanobacteria, γ_s^{AB} indicates the degree of hydrophobic attraction.

The values determined for the free energy of hydrophobic interaction have demonstrated that the microalgae and the cyanobacterium *M. aeruginosa* presented hydrophilic surfaces, while the surface of *S. salina* was considered hydrophobic. According to the determined values of ΔG_{sws}^{TOT}, the microorganisms can be listed by increased degree of hydrophobicity, as follows: *C. vulgaris*, *M. aeruginosa*, *P. subcapitata*, and *S. salina*. However, no statistical differences ($p = 0.60$) were observed in ΔG_{sws}^{TOT} values determined for *C. vulgaris* and *M. aeruginosa* (52.5 ± 14.3 and 43.4 ± 15.5 mJ m^{-2}, respectively). Negative values of ΔG_{sws}^{TOT} determined for *S. salina* suggest the potential of this microorganism to form flocs and, therefore, improve harvesting processes and also its ability to grow as sessile cells, which may have an application in biofilm formation for wastewater treatment and metabolite production. In mixed cultures, these values ranged from −50.4 ± 8.6 to

40.6 ± 11.8 mJ m^{-2}. Three distinct groups can be defined among the studied consortia: (i) the consortia composed of *C. vulgaris* + *M. aeruginosa* and of *C. vulgaris* + *S. salina* constitute the most hydrophilic group, with ΔG_{sws}^{TOT} values of about 40 mJ m^{-2}; (ii) the consortia of *P. subcapitata* + *M. aeruginosa*, *C. vulgaris* + *P. subcapitata* and *P. subcapitata* + *S. salina* constitute the group presenting a slightly hydrophilic surface (ΔG_{sws}^{TOT} values close to 10 mJ m^{-2}); and (iii) the more hydrophobic group, represented by the consortium of *S. salina* + *M. aeruginosa* (ΔG_{sws}^{TOT} of −50 mJ m^{-2}). These results have shown that the co-culture of a more hydrophobic microorganism with another one presenting a more hydrophilic surface results in a decrease in free energy of hydrophobic interaction values and, hence, in an increase in the degree of hydrophobicity. For example, the co-culture of the hydrophobic cyanobacterium *S. salina* with *C. vulgaris*, *M aeruginosa* and *P. subcapitata* resulted in a reduction of ΔG_{sws}^{TOT} values from 52.5 ± 14.3 to 40.1 ± 2.5, 43.4 ± 15.5 to -50.4 ± 8.6 and 13.8 ± 3.8 to 8.3 ± 3.3 mJ m^{-2}, respectively. The huge decrease of ΔG_{sws}^{TOT} observed for the co-culture of both cyanobacteria may be related to the high contact angles measured with polar probe liquids (θ_W and θ_F of $93.5° \pm 3.3°$ and $84.0° \pm 1.3°$, respectively), which are considered unusual [44]. The co-culture of the most hydrophilic microorganisms (*C. vulgaris* + *M. aeruginosa*) resulted in a hydrophilic surface, with ΔG_{sws}^{TOT} of 40.6 ± 11.8 mJ m^{-2}.

Regarding zeta potential values determined for the studied microorganisms, these values ranged from -35.4 ± 0.4 to -48.1 ± 0.9 mV. Zeta potential measurements give information about the charge of cell surfaces: negative or positive values of zeta potential depend on the functional groups present on cell surfaces and also on the pH of the culture medium [44]. Functional groups commonly found on cell surfaces include hydroxyl (-OH), carboxyl (-COOH) and amine ($-NH_2$) [44,50,51]. Since these groups are ionisable, when cells are exposed to low pH values, functional groups are protonated and net surface charge becomes positive, and on the other hand, when cells are exposed to high pH values, functional groups are deprotonated and the resulting net surface charge is negative. At the point of zero charge (PZC), corresponding to an intermediate pH, some groups are protonated while others are deprotonated and the surface charge is neutralized [52,53]. Negative values observed for the studied microalgae and cyanobacteria were expected, since the pH of the culture medium measured when the samples were collected was high (9.64 ± 0.65) and PZC reported for algae was approximately pH 3 [52], indicating that functional groups on the microorganisms' surface were deprotonated. Additionally, net zeta potential values give information about suspensions' stability. When absolute value of zeta potential is high, repulsive forces prevail over van der Waals forces, and hence, particles/cells are stable in the dispersed form. On the other hand, for low net zeta potential values, van der Waals forces (usually attractive) are higher than repulsive ones and the stability of the suspension is affected, resulting in the formation of aggregates and further settling [54,55]. Net zeta potential values determined for the studied microalgae and cyanobacteria are substantial, and therefore, it is expected that the microorganisms keep stable in suspension. These results are in accordance with the non-evidence of flocs formation in the studied cultures, especially in that of the hydrophobic cyanobacterium *S. salina*. Therefore, zeta potential measurements are very important for a better understanding of the interactions among microalgae and cyanobacteria. Although microorganisms may present hydrophobic surfaces, their ability to form aggregates or to attach to surfaces may be strongly affected by the ionic strength of the culture medium. As for single cultures, zeta potential values determined for the studied consortia were negative, indicating an overall surface charge negative. In fact, the pH of the culture medium in these cultures was about 9.50 ± 0.33, which is higher than the reported PZC for microalgae (pH 3). Therefore, functional groups on microorganisms' surface may be deprotonated, conferring a negative surface charge. Zeta potential values determined ranged from -34.9 ± 1.0 to -45.1 ± 1.4 mV. These values were not statically different ($p = 0.66$) from those determined for single cultures, which may be related to similar ionic strengths of the culture medium, since both single and mixed cultures were cultured in OECD test medium [34] with the same composition. Additionally, no statistical difference ($p > 0.05$) was observed in zeta potential values for the studied microalgal associations. Net zeta potential values determined were also high, determining the stability of the suspensions and, therefore, the non-evidence of aggregates formation. This information may be very useful in the design of new strategies to control biofilm formation and development and to improve microalgal harvesting.

Determining the surface physicochemical properties of different consortia has shown that the co-culture of microorganisms with different degrees of hydrophobicity or hydrophilicity can alter the physicochemical properties of the single-cultured microorganisms. These findings can be very important in several applications, such as promoting aggregates formation and, therefore, bioflocculation and biofilm formation and, on the other hand, prevent and control biofouling. However, it is very important to take into account the ionic strength of the culture medium, since this parameter influences surface charge and, hence, the higher or lesser extent of electrostatic repulsions.

Sedimentation kinetics

Microalgal removal percentages determined for single and mixed cultures within the 8-h settling period are

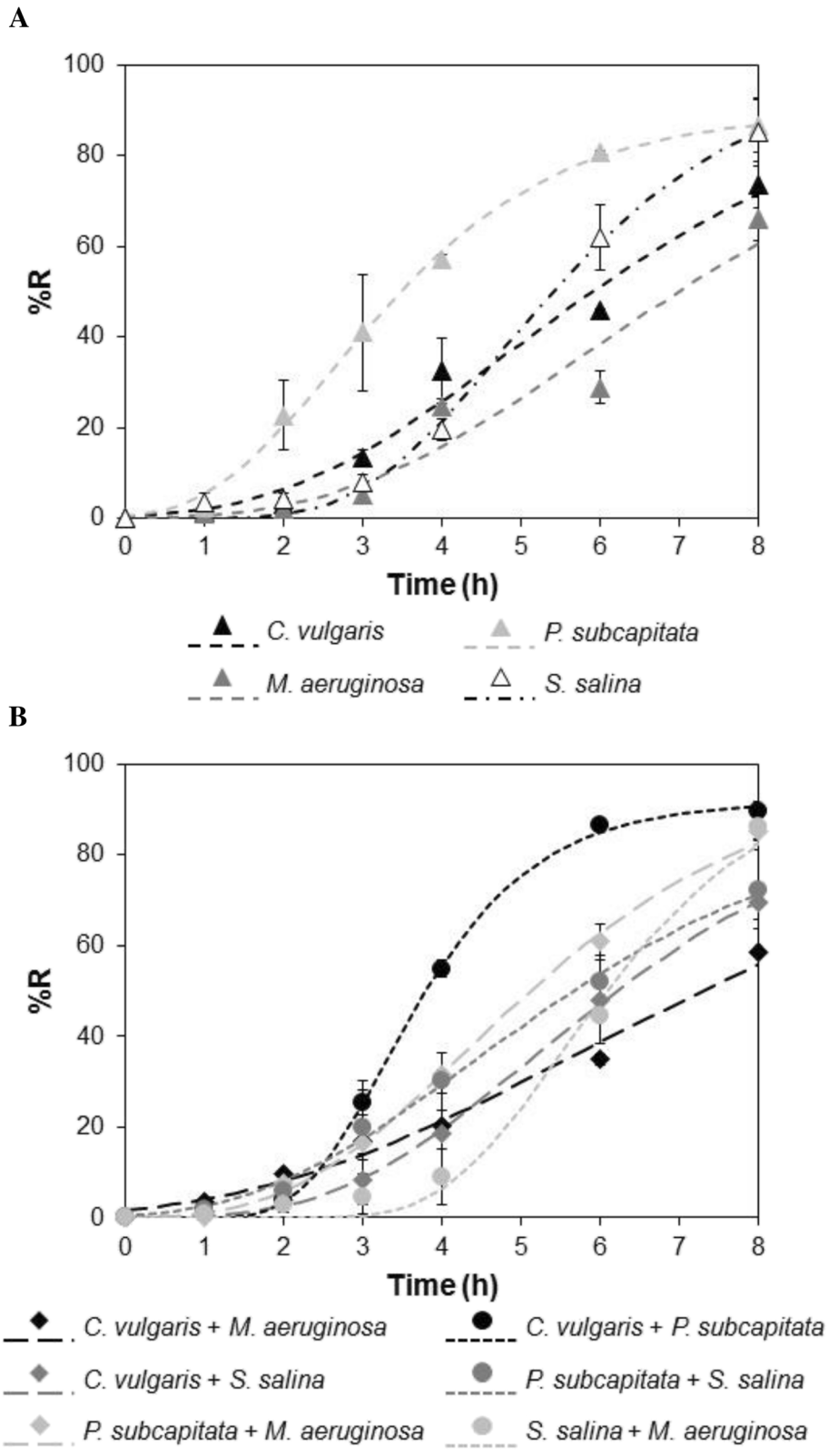

Figure 1 Microalgal removal percentages determined within the settling period for each of the studied suspensions. (**A**) Single cultures and (**B**) Mixed cultures. Error bars correspond to the standard deviation of two independent experiments. Dashed lines represent the model fit of the Gompertz model to the experimental data.

presented in Figure 1A and B, respectively. Analysis of these data indicates that sedimentation rates of the studied algal suspensions are very low. After 8 h of settling, maximum removal percentages achieved in single and mixed cultures were 86.6% ± 6.0% and 89.6% ± 2.2%, respectively. Additionally, as it is possible to see from Figure 1A and B, all microalgal suspensions presented a lag time, starting to sediment after, at least, 1 h of the beginning of the experiment. These results are in accordance with the zeta potential values determined for these suspensions. As it was referred in the 'Surface physicochemical properties' section, when absolute value of zeta potential is high, repulsive forces prevail over van der Waals forces, and hence, particles/cells are stable in the dispersed form. Similar removal percentages have been reported by Salim et al. [40] and Manheim and Nelson [56] when harvesting microalgae through bioflocculation. Higher removal percentages can be achieved using chemical coagulants/flocculants. In the study performed by Papazi et al. [57], chemical coagulants, such as aluminium and ferric sulphate, were used to harvest the microalga *Chlorella minutissima*, resulting in a removal percentage of 80% after 2 to 4 h of settling. In the study performed by Garzon-Sanabria et al. [58], after 1 h of settling, approximately 90% of the microalga *Nannochloris oculata* was recovered from the culture medium. These authors used aluminium chloride as coagulant.

Relationship between surface physicochemical properties and microalgal settling

Table 2 presents microalgal removal percentages determined after 8 h of settling and the kinetic parameters obtained through the model fit of the Gompertz model to the experimental data. Looking at R^2 values determined through the model fit (values close to 1) and to the model curves superimposed to the experimental data (in Figure 1A and B), it is possible to conclude that the Gompertz model can be correctly applied to describe microalgal recovery in the studied conditions. The sedimentation kinetic parameters determined through this model confirm the low settleability of the studied microalgal suspensions, which may be related to the high net values determined for zeta potential. According to these data, lag times, *b*, observed in the beginning of the sedimentation process were high, ranging from 1.0 to 3.0 h for single cultures and from 1.7 to 4.0 h for mixed cultures. Additionally, low sedimentation rates, *c*, were determined for both single and mixed cultures: 0.33 to 0.63 h^{-1} and 0.24 to 0.94 h^{-1}, respectively. Although microalgal recovery using these suspensions was not effective, the values determined through contact angle measurements can be correlated with sedimentation results. From Tables 1 and 2, it is possible to observe that, in general, an increase in the degree of hydrophobicity (lower ΔG_{sws}^{TOT} values) corresponds to an increase in microalgal removal percentages, % *R*, and in sedimentation rates, *c*. Higher removal percentages for the most hydrophobic surfaces were expected since, as it was stated in the 'Surface parameters and hydrophobicity determinations' section, when ΔG_{sws}^{TOT} decreases, the interaction between the two entities is stronger than the interaction of each entity with water. Therefore, particles in suspension tend to form aggregates, improving settling rates. In fact, plotting removal percentages determined

Table 2 Sedimentation kinetics determined for each of the studied suspensions

Microalgal suspensions	% R^a	Gompertz model parameters			
		a (%)	*b* (h)	*c* (h^{-1})	R^2
Single cultures					
C. vulgaris	73.6 ± 5.2 a	100.0	2.0	0.35	0.990
M. aeruginosa	66.1 ± 4.8 a	100.0	2.8	0.33	0.965
P. subcapitata	86.6 ± 6.0 b	89.7	1.0	0.63	0.999
S. salina	85.0 ± 7.5 b	100.0	3.0	0.56	0.997
Mixed cultures					
C. vulgaris + *M. aeruginosa*	58.6 ± 0.8 c	100.0	1.7	0.24	0.993
C. vulgaris + *S. salina*	69.6 ± 13.7 a	95.3	2.7	0.41	0.999
P. subcapitata + *M. aeruginosa*	85.2 ± 2.2 b	100.0	2.1	0.45	0.999
C. vulgaris + *P. subcapitata*	89.6 ± 2.2 b	91.8	2.2	0.94	1.000
P. subcapitata + *S. salina*	72.4 ± 8.7 a	82.1	1.7	0.38	0.998
S. salina + *M. aeruginosa*	86.1 ± 2.7 b	100.0	4.0	0.66	0.994

Microalgal removal percentage was determined after 8 h of settling, and recovery kinetic parameters were determined through the Gompertz model. [a]Values are presented as the mean ± standard deviation of two independent experiments. Different letters within the same column represent statistically different values ($p < 0.05$). % *R*, microalgal removal percentage determined after 8 h of settling; *a*, upper asymptote; *b*, displacement along the *x*-axis or lag time ($b > 0$); *c*, tangent at the inflection point or sedimentation rate ($c > 0$); R^2, coefficient of determination obtained through the model fit of Gompertz model to the experimental data.

after 8 h of settling as a function of the free energy of hydrophobic interaction suggests a negative relationship between these parameters. By excluding the values corresponding to the three most hydrophobic surfaces and to the most hydrophilic one, a linear relationship between microalgal removal percentage and free energy of hydrophobic interaction was obtained (R^2 = 0.91). These results show the importance of studying surface physicochemical properties to understand the flocculation process and improve harvesting procedures.

Conclusions

This study presented a broad characterization of surface physicochemical properties of microalgae and cyanobacteria, as well as how these properties vary when these microorganisms are co-cultured. Regarding the surface properties of single cultures, *S. salina* presented a hydrophobic surface, suggesting the ability of this microorganism to form aggregates. However, macroscopic observations have shown no evidence of aggregates formation, which was confirmed through the study of sedimentation kinetics. This might be due to the high net zeta potential values determined, which are responsible for the stability of the suspensions in their dispersed form. The co-culture of different microalgae and cyanobacteria has shown that when a more hydrophobic microorganism is co-cultured with another one presenting a more hydrophilic surface, free energy of hydrophobic interaction tends to decrease, resulting in a more hydrophobic surface. This study has also shown a negative linear correlation between microalgal removal percentages and free energy of hydrophobic interaction, reinforcing the importance of surface physicochemical properties on the sedimentation process. Due to the low recovery efficiencies achieved, another factor to consider in the prevention or promotion of cell attachment is the ionic strength of the culture medium, since this parameter strongly influences surface charge.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ALG carried out the experimental assays and statistical analysis and drafted the document. CF helped in the experiments regarding contact angle measurements. JAL was involved in zeta potential determinations and respective analysis. JCMP participated in the design of the study and coordination. MS conceived the study and participated in its coordination. All authors read and approved the final manuscript.

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