



# Estimating fractal dimension of microalgal flocs through confocal laser scanning microscopy and computer modelling



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## A B S T R A C T

Flocculation followed by settling is gaining momentum as a means to concentrate microalgal biomass due to the low investment and operation costs of the process. Microalgal flocculation can be further optimized by knowing the relationship between the hydrodynamic conditions applied in the process and the geometric properties of the flocs, namely characteristic size and fractal dimension,  $D_f$ , given that settling rate is highly dependent on these two parameters. Current methods to characterize the geometry of flocs rely on estimating the 2D fractal dimension from microscopic images, which may result in inaccuracies caused by the overlapping or superimposition of aggregate structures prompted when the image of a 3D object is projected on the plane, and due to the fact that the estimation performed is dependent on the orientation of the particle during image acquisition. The present paper describes a new procedure to estimate  $D_f$  of *Chlorella sorokiniana* aggregates by correlating the 2D fractal dimension of the real aggregates microscopic images with the 2D fractal dimensions of computer generated flocs of prescribed 3D geometry. This procedure avoids the inaccuracies entailed with floc imaging and those due to the random orientation of the floc during image acquisition.

## 1. Introduction

The cost associated to harvesting remains one of the critical factors that hinder the mass production of microalgae as industrial feedstock. Flocculation followed by settling is gaining momentum as a means to concentrate microalgal biomass due to its low investment and operation costs [1]. In order to increase the profit margins expected from microalgal biomass production at industrial level, it is desirable to optimize the harvesting process to the greatest possible extent. Such optimization can be achieved by selecting a flocculant that offers a good compromise between effectiveness and price, and once the flocculant is selected, by optimizing the flocculation and settling processes themselves. While the literature offers numerous references describing the flocculation of various microalgal strains employing flocculation agents of different chemical nature [2], the optimization of flocculation coupled with settling has not enjoyed the same degree of attention. Among the different aspects likely of being optimized, of especial relevance are mixing and settling velocity [3]. Settling velocity is dependent on particle size and morphology, the latter through the fractal dimension ( $D_f$ ). For a fixed settler and operating conditions, settling velocity will be higher at larger floc size. For a given floc size, settling velocity

generally decreases with increasing  $D_f$  values. The relation between these two parameters is however complex, for the aggregate translational hydrodynamic radius is a function of its geometry [4]. Understanding the relationship between mixing conditions and floc geometry may help to find an optimum compromise between stirring requirements and settling velocity in flocculation coupled with settling processes. The present work contributes to the above idea by proposing a method to estimate the 3D geometry of microalgal aggregates produced through flocculation.

The geometric characterization of particle aggregates is generally carried out through the estimation of the 2D fractal dimension ( $D_2$ ) of their microscopic images, either taken with confocal microscopy or with online microscopy [5,6]. Although providing some information on the shape of the aggregates, both approaches present drawbacks that limit their use as predictor of settling velocity. First, there is no general correspondence between  $D_2$  and  $D_f$  [7]. On the other hand, both confocal microscopy and online microscopy yield a 2D projection of a 3D object, which implies a loss of information of the object's geometry. Therefore, in the image, some 3D structures of the aggregate will be overlapped, i.e. not visible, while others will appear superimposed on the projection plane, creating thus a distorted profile of the object.

Abbreviations: AS, aluminium sulphate; CCA, cluster-cluster aggregation; CLSM, Confocal laser scanning microscopy;  $D_2$ , 2D fractal dimension;  $D_f$ , fractal dimension; FD, fractal dimension; G, velocity gradient; MFD, maximum Feret diameter; MFDD, maximum Feret diameter distribution; PFD, projected fractal dimension; PAC, polyaluminium chloride; Re, Reynolds number

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Given that  $D_2$  is obtained from the relation of scale and perimeter of the floc projected, its estimation based on the floc profile will lead to inaccurate results. Finally, the  $D_2$  of flocs is dependent on the orientation of the particle at the moment of taking its image or projection [8], thus the fractal dimension based on processing online microscopy images of the aggregates suspension may yield inaccurate results. Confocal laser scanning microscopy (CLSM) avoids the randomness associated to floc orientation to a great extent given that the flocs tend to settle on the orientation offering the maximal area to the ground of the cuvette or glass where they are deposited. Nevertheless, when flocs analysed are large, confocal laser fails to penetrate all planes in the sample and is therefore unable to resolve the complete 3D geometry of the aggregate. The present work aims to overcome the limitations cited above by devising a new method to estimate the 3D fractal dimension of microalgal flocs that combines confocal microscopy images of real flocs and computer generated models of cell aggregates. Our process involves measuring the maximum Feret diameter (MFD) and  $D_2$  fractal dimension of microalgal aggregates on their CLSM images and generating several populations of virtual flocs with different input  $D_f$  having their sizes coerced to the MFDs measured on the real flocs. The average  $D_f$  of the real flocs is then estimated to be equal to the input  $D_f$  of the virtual population of aggregates having the average  $D_2$  closest to that measured on CLSM images. A comparison of the methods referred above and the one developed in the investigation described in this paper is given in Table 1.

## 2. Materials and methods

### 2.1. Microalgal cultures

Microalgal culture samples were taken from a 5.5 L photobioreactor (PBR) operated with a strain of *Chlorella sorokiniana* (CCAP No. 211/8 K) grown in tris-acetate-phosphate medium (TAP medium) [9]. The PBR was maintained at a temperature between 23 and 25 °C and was aerated with 0.2 µm filtered air at 2 L min<sup>-1</sup>. pH was maintained at 7.5 through an on-demand automatic CO<sub>2</sub> supply (0.2 L min<sup>-1</sup>). Light was provided by means of four fluorescent cool white light bulbs in a 12 h cycle. The culture was maintained at 0.2 g L<sup>-1</sup> dry biomass concentration.

### 2.2. Flocculation

Medium molecular weight chitosan (Sigma-Aldrich 448877, CAS Number 9012-76-4) was employed as flocculation agent. The flocculant solution was prepared by dissolving the chitosan in a solution of glacial acetic acid 1% vol. The solution was mechanically stirred at 400 rpm for 1 h and left to settle for 24 h.

Flocculation essays were carried out in 600 mL beakers with 200 mL of microalgal culture. Stirring was achieved by means of a four-blade 45 pitch impeller of diameter 4.95 cm ( $N_p = 1.27$ ). Three different stirring speeds were considered, namely 200, 350 and 500 rpm, which

**Table 1**

Comparison of the different optical methods to estimate the fractal dimension of microalgal flocs.

Fractal dimension estimation method	Composite profile error	Orientation error	Direct estimation of $D_f$	Image acquisition time
Online microscopy	YES	YES	NO	FAST
Confocal microscopy	YES	NO	NO	SLOW
Confocal microscopy and computer modelling	NO	NO	YES	SLOW

corresponded to Reynolds numbers  $8.2 \times 10^3$ ,  $1.4 \times 10^4$  and  $2.0 \times 10^4$  and velocity gradients (G) 263, 611 and 1043 s<sup>-1</sup> respectively. In each case a dose of 5 ppm of chitosan solution was added to the stirred microalgal sample. After 2 min of stirring, 0.5 mL of a commercial dispersant (Nopco ESA 120) was added to the flocculating system to prevent aggregation after sampling.

### 2.3. Confocal laser scanning microscopy image acquisition

Aggregate microscopic images were taken with a confocal laser scanning microscope Olympus FV1200, using a laser excitation of 405 nm and receiving the emission from 594 nm. No especial sample preparation was used for image acquisition: a drop of the microalgal sample in consideration was placed on a slide by means of a broad-tipped Pasteur pipet to avoid damaging the aggregates. The flocs were left to settle at the bottom of the slide before image acquisition so that they most likely present the maximal surface area projected on the horizontal plane.

### 2.4. Fractal analysis of the microscopy images

Image processing was carried out with the Fiji distribution of ImgeJ 1.151 h. Each image stack acquired was projected on the Z plane and the resulting image was made binary for further 2D analysis. The flocs of each image were automatically selected and stored in single files by means of a script. The MFD of each floc was automatically measured. The estimation of  $D_2$  was done through the BoneJ plugin Fractal Dimension tool applying 18 different box sizes, from 200 to 6, with a reduction rate of 1.2.

### 2.5. Generation of fractal-like virtual flocs

A cluster-cluster aggregation model was employed to simulate the fractal growth of microalgal flocs. The fractal-like nature of particle aggregates can be described by the following scaling power-law:

$$N = k_f \left( \frac{R_g}{a} \right)^{D_f}$$

where  $N$  is the number of particles forming the aggregate,  $k_f$  is the fractal pre-factor,  $R_g$  is the gyration radius of the floc,  $a$  is the mean diameter of the primary particle, and  $D_f$  is the fractal dimension. In order to ensure constant geometric properties, the above scaling Law must be fulfilled all along the process of generation of virtual aggregates. Filippov et al. [10] proposed an expression to guaranty the fulfilment of the scaling Law at all steps in the process of generating an aggregate from the combination of two smaller clusters (CCA aggregation model).

$$\Gamma^2 = \frac{a^2(N_1 + N_2)}{(N_1 N_2)} \left( \frac{N_1 + N_2}{k_f} \right)^{\frac{2}{D_f}} - \frac{N_1 + N_2}{N_2} R_{g1} - \frac{N_1 + N_2}{N_1} R_{g2}$$

In the above expression,  $\Gamma$  is the distance between the centres of masses of the combining clusters,  $N_1$  and  $N_2$  are the number of particles in each of the small clusters,  $k_f$  is the fractal pre-factor,  $R_{g1}$  and  $R_{g2}$  are the radii of gyration of each cluster. Skorupski et al. [11] devised an algorithm to merge virtual clusters based on eq. 2. The key steps in the algorithm is to randomly select two particles among the most external cells in the combining clusters and perform the necessary translations and rotations to achieve the merging through the selected cells without any particle being overlapped. In the present work, we applied the mentioned algorithm with small modifications, namely in the generation of the random 3D direction on which the clusters are combined, and in the fact that we recalculated the masses centre of the aggregate being generated at each growth step. The modified merging method was incorporated into an algorithm to grow a cluster up to a prescribed characteristic size with a given fractal dimension. Sintering was not

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