COMPOSITION AND RHEOLOGICAL PROPERTIES OF MICROALGAE SUSPENSIONS: IMPACT OF ULTRASOUND PROCESSING

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Abstract

In this study the rheological properties of aqueous suspensions of three microalgae species, *Nannochloropsis gaditana*, *Scenedesmus almeriensis* and *Spirulina platensis*, were investigated as a function of solids content, and related to their composition and microstructure. In addition, the impact of ultrasound processing on their structuring ability was also studied. The less rigid character of the *Spirulina platensis* cell walls (with very low carbohydrate contents) and the presence of extracellular components promoted cell-cell interactions, yielding suspensions which showed a shear thinning behaviour at lower concentrations than *Nannochloropsis gaditana* and *Scenedesmus almeriensis*. It is noteworthy that the three species showed different viscoelastic properties at 25 wt. % total solids. *Spirulina platensis* suspensions showed a more elastic behaviour and lower frequency dependence, characteristic of weak gels, whilst *Nannochloropsis gaditana* and *Scenedesmus almeriensis* behaved more like viscous liquids. The ultrasound treatment did not affect the cell wall integrity, but it promoted the release of intracellular components (some of which could have been partially degraded) and disrupted physical interparticle interactions in *Nannochloropsis gaditana* and *Scenedesmus almeriensis*. This has an impact on the rheological properties, increasing the viscosity of *Nannochloropsis gaditana* suspensions, while the viscosity of *Scenedesmus almeriensis* suspensions was reduced. The outcomes of this work give insights into the exploitation of these microalgae species in soft materials for food, pharma and other technological applications.

Keywords: microalgae; suspensions; rheology; cell wall polysaccharides; ultrasounds

1. Introduction

Microalgae are a renewable biomass source which in the last decades have found applications within very diverse sectors such as the production of biofuels, the food industry, pharma and cosmetics [1, 2]. In particular, there is an increased interest in the use of microalgae in food products due to their high nutritional value, which is based on their high protein content, their interesting lipid profile and the presence of bioactive compounds such as carotenoids, phycobilins, polysaccharides, vitamins, and sterols [2, 3]. Although microalgae are mainly commercialised as dietary supplements, more and more food products containing microalgae, such as pasta, snack foods, candy bars or gums, and beverages, have been recently launched into the market [4-7]. To make better use of the nutritional benefits of microalgae, more research is needed to understand their impact in food structure and processability. For instance, it has been recently demonstrated that *Spirulina platensis* microalgae interfere with the gelatinization process of starch, having an impact on the properties of extruded starch foams [8, 9]. Rheological properties of microalgae suspensions are relevant for food processability and for the textural properties of food systems. The existence of microalgae species with distinct cell wall composition and microstructure brings the possibility of producing microalgae-based foods with a broad range of rheological characteristics.

The rheological properties of suspensions depend on several factors, among them the solid particle concentration described by the particle volume fraction [10]. The maximum particle volume fraction, which is the number of particles that can be packed in a certain volume, depends on the particle properties such as the size, morphology and hardness. Other factors influencing the rheology of suspensions are the particle size distribution, interparticle forces, and the viscosity of the continuous liquid phase. Whilst the mechanics of dilute and

semi diluted suspensions are well understood, there are still many uncertainties for concentrated suspensions [11]. Experimental and theoretical studies on systems ranging from monodisperse spherical particles at high concentrations to polydisperse suspensions with different morphologies have been carried out for more than fifty years [12-14]. However, the application of such models to microalgae suspensions is challenging due their high polydispersity, particle softness, the presence of crystalline and amorphous states, and the often highly non-spherical shape of the particles. Nevertheless, there are a number of studies regarding rheological properties of microalgae suspensions in the literature showing that below a critical concentration, most microalgae suspensions display Newtonian behaviour and above a certain concentration, some microalgae suspensions exhibit non-Newtonian fluid behaviour [15-19]. Whilst the cell wall composition of many land plants is well established, the cell wall composition and structure of the hundreds of thousands of microalgae species remains widely unexplored. Microalgae species which have been investigated present distinct molecular components, intra- and intermolecular linkages, and cell wall microstructure. For instance, microalgae belonging to the *Nannochloropsis gaditana* species have been reported to present a bilayered cell wall composed of a cellulosic inner wall protected by an outer hydrophobic algaenan layer [20]. In contrast, the cell wall from *Spirulina platensis* has been seen to present a structure of four layers, mainly composed of proteinaceous material [21]. The different composition and structure of microalgae cell walls have a strong impact on their integrity and susceptibility of intracellular components to be released during processing. As a result, an incipient amount of work is being developed to explore efficient methods for the disruption of microalgae cell walls and subsequent extraction of intracellular bioactive compounds [22-25]. Amongst the different disruption methods, ultrasound treatments have attracted a great deal of interest since they are able to disrupt cells with less energy loss compared with high-shear force

methods [26]. The ability of ultrasound treatments to disrupt cell walls from various microalgae species has been shown to be useful to increase the extraction yield of different compounds such as lipids and proteins [22, 27-29]. On the other hand, the structural changes and the release of intracellular components induced by cell wall disruption treatments are also expected to affect the rheology of treated microalgae suspensions.

In this study the rheological properties of aqueous suspensions of three microalgae species, *Nannochloropsis gaditana*, *Scenedesmus almeriensis* and *Spirulina platensis*, were investigated and related to their composition and microstructure. Furthermore, the impact of ultrasound processing on their structuring ability was also studied. To do this, shear viscosity and viscoelasticity measurements, as well as compositional and microstructural analyses, were carried out before and after ultrasound processing. The outcomes of this work give insights into the exploitation of these microalgae species for food, pharma and other technological applications.

2. Materials and methods

2.1 Materials

Three different microalgae species, i.e. *Nannochloropsis gaditana*, *Spirulina platensis* and *Scenedesmus almeriensis*, in the form of dry powders, were kindly donated by Dr. Ación from the University of Almeria (Spain). The *Spirulina platensis* biomass was produced using a raceway open photobioreactor, while *Nannochloropsis gaditana* and *Scenedesmus almeriensis* were produced using closed photobioreactors. The obtained biomass was collected by centrifugation and subjected to freeze-drying. The freeze-dried samples, coded as *Nannochloropsis gaditana*, *Spirulina platensis* and *Scenedesmus almeriensis* were stored in the fridge until further use.

Sulphuric acid (99.2-101%), methanol, sodium carbonate, gallic acid (97.5-102.5%), potassium persulphate ($\geq 99\%$), phosphate buffered saline tablets, acetyl chloride, ABTS ((2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))) ($\geq 98\%$) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (97%) were obtained from Sigma-Aldrich (Spain). The Folin-Ciocalteu reagent, modified Lowry reagent and bovine serum albumin were obtained from the “modified Lowry protein assay kit” purchased from Thermo Fisher scientific (Spain).

2.2 Ultrasound treatment

The three microalgae species were subjected to an ultrasound treatment, following the protocol previously described in [30]. Briefly, the microalgae powders were dispersed in distilled water at a concentration of 0.4g/L. The suspensions were then treated with a UP-400S ultrasound equipment (Hielcher GmbH, Germany), applying a power of 400W and a frequency of 24 kHz for 5 min. After that, the suspensions were freeze-dried and the resulting powders were stored in the fridge until further use. These samples were coded as *Nannochloropsis gaditana*-US, *Spirulina platensis* -US and *Scenedesmus almeriensis*-US.

2.3 Preparation of microalgae suspensions

Microalgae suspensions at different concentrations (1, 5, 10, 15, 20 and 25 % wt.) were prepared by adding the required amount of microalgae powder to milli Q water and gently mixing with a magnetic stirrer, leaving the samples to fully hydrate overnight. After that the rheological, microscopical and particle size experiments were performed.

2.4 Chemical composition of microalgae before and after ultrasound treatment

2.4.1 Carbohydrate content

The carbohydrate composition in the samples was determined after acid methanolysis of the dry microalgae powders, following the method described in [31]. This technique is not able to cleave crystalline polysaccharides. As cellulose is present in some of the tested microalgae, a two-step sulphuric acid hydrolysis was also performed [32] as to determine the total glucose, and cellulose was calculated as the difference [33]. The samples were then analysed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with a 940 IC system (Metrohm) equipped with a Matrosep Carb 2 column (4×250 mm, Metrohm). Control samples of known concentrations of mixtures of glucose, fucose, galactose, arabinose, xylose, mannose or glucuronic were used for calibration (Sigma). All experiments were carried out in triplicate.

2.4.2 Protein content

Samples were analysed for total nitrogen content using an Elemental Analyser Rapid N Exceed (Paralab S.L., Spain). About 100 mg of each of the powdered samples were pressed to form a pellet which was then analysed using the Dumas method, which is based on the combustion of the sample and subsequent detection of the released N_2 [34]. The total protein content was calculated from the nitrogen content multiplied by a factor of 6.27 in the case of *Spirulina platensis* and 5.95 for *Nannochloropsis gaditana* and *Scenedesmus almeriensis* [35, 36].

2.4.3 Lipid content

The lipid content was estimated gravimetrically after performing the Soxhlet extraction of ca. 3 g of dry microalgal material with 800 ml of toluene/ethanol 2:1 (v/v) during 24 h.

2.4.4 Ash content

The ash content was determined by dry biomass calcination of ca. 2 g of dry microalgae powder in muffle furnace, according to the standard TAPPI T211 om-07 method.

2.4.5 Phenolic content

Total phenolic content was estimated by the Folin-Ciocalteu colorimetric assay [37]. Briefly, Folin-Ciocalteu reagent was diluted 1:10 with distilled water and 1 mL of the final dilution was mixed with 0.2 mL of the extract sample (microalgae suspensions in distilled water at a concentration of 5 mg/mL) at room temperature. Finally, 0.8 mL of sodium carbonate (75 mg/mL) were added and the samples were heated up to 50°C during 30 minutes. Absorbance values were read at 750 nm. A calibration curve was built using gallic acid as the standard, and the total phenolic content was expressed as mg of gallic acid (GA)/g extract. All determinations were carried out in triplicate.

2.5 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) radical cation scavenging activity

The ABTS^{•+} radical cation scavenging activity of the microalgae before and after ultrasound processing was determined according to [37]. Briefly, 0.192 g of ABTS were dissolved in 50 mL of PBS at pH 7.4 and mixed with 0.033 g of potassium persulfate overnight in the dark to yield the ABTS^{•+} radical cation. Prior to use in the assay, the ABTS^{•+} was diluted with PBS for an initial absorbance of $\sim 0.700 \pm 0.02$ (1:50 ratio) at 734 nm, at room temperature. Free radical scavenging activity was assessed by mixing 1.0 mL diluted ABTS^{•+} with 10 μ L of microalgae suspensions (5 mg/mL in water) and monitoring the change in absorbance at 6 minutes. A calibration curve was developed by using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox). The antioxidant capacity was

expressed as mg Trolox equivalents (TE)/g extract. All determinations were carried out in triplicate.

2.6 Fourier transform infrared spectroscopy (FT-IR)

Freeze-dried microalgae powders were analyzed by FT-IR in attenuated total reflectance (ATR) mode using a Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were recorded at 4 cm⁻¹ resolution in a wavelength range between 400-4000 cm⁻¹ and averaging a minimum of 32 scans.

2.7 Particle size analysis

The particle size distribution of microalgae suspensions (5% in water) before and after ultrasound treatment was determined by laser diffraction using a Malvern Mastersizer 3000 apparatus equipped with a Hydro MV accessory for liquid samples (Malvern Instruments, UK). A refractive index of 1.4683 and an absorption index of 0.01 were used.

2.8 Optical microscopy

Aqueous microalgae suspensions at a concentration of 5% were analysed by optical microscopy. Digital images were taken using an Eclipse 90i Nikon microscope (Nikon corporation, Japan) equipped with 5-megapixels cooled digital colour microphotography camera Nikon Digital Sight DS-5Mc. Acquired images were analysed and processed by using Nis-Elements Br 3.2 Software (Nikon corporation, Japan).

2.9 Confocal laser scanning microscopy (CLSM)

Microalgae powders, before and after ultrasound treatment, were dispersed in water to produce 5% suspensions. Hydrophobic components, such as lipids, were stained with Nile

Red before being imaged with a CLSM (Leica TCS SP5, Heidelberg, Germany). The objective used was a HCX PL APO CS 63.0x1.20 WATER UV with zoom 4. The 488 nm line from an argon laser was used for excitation and two channels of were used for emission, 580–606 nm for lipids/hydrophobic components (green) and 670–695 nm for chlorophyll autofluorescence (red). The image resolution was 1024×1024 pixels.

2.10 Rheological characterization

The rheological properties of the microalgae aqueous suspensions were measured at 25°C in a strain-controlled ARES G2 rheometer (TA Instruments, New Castle, Delaware, USA) equipped with parallel plates (40 mm in diameter) and a solvent trap to prevent evaporation. The gap was set to 0.5 mm. Shear viscosity measurements were performed from 0.1 to 100 s⁻¹. Small amplitude oscillatory shear experiments were carried out from 0.1 to 10 rad·s⁻¹ at 0.5 % strain. The strain was selected from the lineal viscoelastic region (LVR) obtained from strain sweeps from 0.01 to 1000% at 10 rad·s⁻¹. Duplicates were measured for each concentration.

2.11 Statistical analysis

Data analysis was carried out using Statgraphics Stratus by Statgraphics Technologies, Inc. One-way analysis of variance (ANOVA) was done to determine the significant differences between sample means, at a significance level of $P < 0.05$. Mean comparisons were performed by the Tukey Test.

3. Results and Discussion

3.1 Chemical composition of microalgae before and after ultrasound treatment

The composition of the three different microalgae species was characterised before and after being subjected to the sonication treatment and the results are summarised in Table 1. As observed, the three different microalgae species presented very distinct composition, with ashes (~36%) and proteins (~27%) being the main components of *Spirulina platensis*, while *Nannochloropsis gaditana* was mainly composed of lipids (~41%) and carbohydrates (~33%), and *Scenedesmus almeriensis* contained a high amount of carbohydrates (~44%) and proteins (~30%). Although similar trends in terms of major components have been reported in the literature for these species, their specific amounts are highly variable depending on the cultivating and harvesting conditions. For instance, *Spirulina platensis* microalgae have been described to be mainly composed of proteins, which typically range from 40% to 70% [38, 39], while carbohydrates (8-16%) and lipids (4-11%) are minor constituents [38]. However, some studies stated that *Spirulina platensis* cells cultivated in nitrogen-deficient media [40, 41] and genetically-modified species with higher growth rates [42] possess lower protein content than the wild type microalgae. *Scenedesmus almeriensis* has been reported to be composed mainly of proteins and carbohydrates [43], while similar protein, lipid and ash contents to the ones provided in Table 1 have been previously reported for *Nannochloropsis gaditana* [44]. It should be noted that very low (or even nil) polyphenol contents were determined for the three microalgae species. This does not necessarily mean that the microalgae did not contain phenolic compounds, as bioactive compounds are mainly located intracellularly and thus, cannot be released towards the liquid medium when the cell walls are intact, as previously pointed out [45].

As shown in Table 1, the application of the ultrasound treatment resulted in a relative increase in the amount of quantified polyphenols. The amount of lipids, on the other hand, was substantially decreased in *Nannochloropsis gaditana*, while it was not significantly

affected in *Spirulina platensis* and *Scenedesmus almeriensis*. The amount of carbohydrates, proteins and ashes were not determined after the ultrasound treatment since these were based on destructive methods and therefore, the estimated values should be the same irrespective of the sample treatment. Although it should be taken into account that the contents provided in Table 1 are relative amounts (and, therefore, it is not possible to discuss the results in terms of absolute values), the results suggest that the cell wall structure was damaged to some extent by the treatment, enabling the release of a certain amount of intracellular components; however, it seems that the treatment did not produce a complete rupture of the cell walls since only minor compositional changes took place. This is in agreement with a previous study which reported that even though sonication could increase the antioxidant capacity of several microalgae species, other methods such as ultraturrax homogenization were more effective for cell wall disruption [45]. It should also be noted that substances such as pigments and fatty acids may have been degraded upon sonication. In fact, several studies have reported that the pigments present in microalgae are easily degraded due to temperature, light or other microorganisms, while polyunsaturated fatty acids may undergo radical-induced degradation upon sonication [46, 47]. This might explain the decrease in the amount of ashes and lipids observed for some of the microalgae species. In particular, the lipid decrease in *Nannochloropsis gaditana* may be attributed to the higher content in polyunsaturated fatty acids (which are less stable) of this species [48].

The antioxidant activity of the microalgae suspensions was in the range of 20-56 $\mu\text{mol TE/g}$ sample. Greater antioxidant capacities than the ones estimated in this work have been described for extracts rich in bioactive components. For instance, antioxidant activities of 100-600 $\mu\text{mol TE/g}$ sample have been described for *Scenedesmus obliquus* extracts rich in carotenoids [49] and *Nannochloropsis gaditana* extracts rich in carotenoids, chlorophylls

and polar lipids have been reported to possess antioxidant capacities of 300-800 $\mu\text{mol TE/g}$ sample [50]. This indicates that most of the compounds responsible for the high antioxidant potential of microalgae are intracellular components. As deduced from Table 1, the antioxidant capacity after the sonication treatment, increased in the case of *Spirulina platensis* while it decreased for *Nannochloropsis gaditana* and *Scenedesmus almeriensis*. The explanation for these results is quite complex, since the antioxidant potential may be linked to the presence of polyphenols or other bioactive components, such as sulphated polysaccharides, peptides and aminoacids [51-53]. Thus, it is not possible to unequivocally link the effect in the antioxidant capacity of the samples to the release or degradation of specific components.

Table 1. Composition and antioxidant capacity of the microalgae before and after ultrasound treatment . Data shown as mean $\pm$ SD, n=3.

	Carbohydrates (%)	Proteins (%)	Lipids (%)	Ashes (%)	Polyphenols (mg GA/g sample)	Antioxidant Capacity ABTS ($\mu\text{mol TE/g}$ sample)
<i>Spirulina platensis</i>	8.0 ± 1.2^a	26.8 ± 0.1^{bc}	18.8 ± 5.9^a	36.0 ± 0.2^d	0.9 ± 0.4^a	56.6 ± 0.5^d
<i>Nannochloropsis gaditana</i>	32.5 ± 2.9^b	22.8 ± 0.2^a	40.9 ± 1.3^b	15.6 ± 0.1^{ab}	n.d.	31.6 ± 2.0^c
<i>Scenedesmus almeriensis</i>	44.1 ± 5.5^c	30.2 ± 0.3^d	19.7 ± 3.8^a	19.4 ± 0.6^{bc}	n.d.	20.0 ± 1.3^b
<i>Spirulina platensis</i> -US	---	---	13.7 ± 4.1^a	---	8.0 ± 0.9^b	77.9 ± 1.3^e
<i>Nannochloropsis gaditana</i> -US	---	---	32.0 ± 9.6^a	---	1.6 ± 0.3^a	23.1 ± 1.3^b
<i>Scenedesmus almeriensis</i> -US	---	---	25.8 ± 7.7^a	---	1.6 ± 0.5^a	10.2 ± 0.9^a

Values with different letters are significantly different ($p \leq 0.05$).

The FT-IR spectra from the microalgae before and after ultrasound treatment are shown in Figure 1. As observed, the spectra from *Scenedesmus almeriensis* and *Nannochloropsis gaditana* were very similar, showing some minor differences in the relative intensity of the peaks appearing within the region 830-1475 cm^{-1} . In particular, differences could be noted within the range of 1300-1100 cm^{-1} , corresponding to C-O-C stretching vibrations of polysaccharides, which could be ascribed to the distinct carbohydrate composition of *Scenedesmus almeriensis* and *Nannochloropsis gaditana*. It should be noted that the band located at 1260 cm^{-1} , characteristic of sulphate ester groups [54], was only visible in *Nannochloropsis gaditana*, indicating the presence of sulphated polysaccharides, as previously described [15]. The three microalgae species showed the two bands characteristic from the presence of proteins, located at 1640 cm^{-1} (amide I) and 1540 cm^{-1} (amide II). The broad band in the range of 3500-3000 cm^{-1} , corresponding to the hydroxyl and N-H stretching of carbohydrates and proteins, was also detected in all the samples. The greater lipid and carbohydrate content in *Scenedesmus almeriensis* and *Nannochloropsis gaditana* was evidenced by the appearance of the C=O stretching band at 1740 cm^{-1} (not detected in *Spirulina platensis*) and by the greater intensity of the sharp bands at 2920 cm^{-1} (associated to C-H symmetric stretch) and 2852 cm^{-1} (associated to C-H asymmetric stretch). The latter two bands have been related to the presence of long aliphatic chains of algaenan, i.e. straight-chain highly saturated aliphatic compounds, in *Nannochloropsis gaditana* [55]. Some changes in the FT-IR spectra of the microalgae were seen after the sonication treatment. In the case of *Spirulina platensis*, the position and relative intensity of some peaks within the region of 800-1200 cm^{-1} were slightly modified in the ultrasound-treated sample. In particular, the band located at 1420 cm^{-1} , assigned to the C-H bending from alkanes [56], became broader after sonication, which may indicate structural changes in the apolar

fraction. Furthermore, a broad band at 1090-1030 cm^{-1} , attributed to C–O–P stretching [57], became more intense in the treated sample, which could be related to the release of small peptides. Moreover, a clear change in the O-H and N-H stretching band (at $\sim 3000 \text{ cm}^{-1}$) was seen upon the ultrasound treatment. Specifically, this band appeared sharper, which is typical of protein-rich spectra, probably indicating ultrasound-induced protein extraction, as previously described in different aquatic biomass materials [58, 59].

Figure 1. ATR-FTIR spectra from microalgae before and after ultrasound treatment.

3.2 Carbohydrate composition

The cell walls from most microalgae species are known to be mainly composed of polysaccharides. The specific carbohydrate composition is crucial to determine the integrity of cell walls and their susceptibility to disruption upon different treatments [24, 25, 60]. Thus, the carbohydrate composition of the three microalgae used in this work was studied

and the results are compiled in Table 2. The most abundant carbohydrates in most microalgae species are mainly glucose (21%–87%), galactose (1%–20%), and mannose (2%–46%) and varying amounts (0%–17%) of arabinose, fucose, rhamnose, ribose, and xylose [35]. The very low carbohydrate content in *Spirulina platensis* (about 8% of the total mass) was represented by glucose and minor amounts of galactose and rhamnose, in agreement with previous literature [61, 62]. The glucose content is most probably derived from an energy reserve, glycogen-type glucan [63, 64], while galactose and rhamnose derive from exopolysaccharides [65, 66]. The rhamnose-containing exopolysaccharide, often described as spirulan, has been attributed a relatively high molecular weight, a pseudoplastic, polyelectrolytic nature, besides several health-promoting properties [67, 68]. The minor presence of this component might nevertheless contribute to the rheological properties of the suspensions. The low carbohydrate content in *Spirulina platensis* is not surprising, since these microalgae are known to present thin and fragile peptidoglycan cell walls [69]. In the case of *Nannochloropsis gaditana*, a relatively high amount of cellulose was present, as opposed to the other two microalgae. *Nannochloropsis* spp. microalgae have bilayered cell wall structure consisting of a cellulosic inner wall which is protected by an outer hydrophobic algaenan layer [55]. Algaenan consists of aliphatic, partially esterified carbohydrates of different length, which are not accounted for in the current analysis [70]. The non-cellulosic glucose units may arise from the energy reserve glucan chrysolaminarin, while minor contents of galactose, manose and rhamnose in similar ratios have also been reported to be present as part of the cell wall [71-73]. In contrast, *Scenedesmus almeriensis* was mainly composed of glucose, mannose and galactose. Several *Scenedesmus* species have been shown to possess a rigid wall constituted of glucose, mannose and galactose [74], while low molecular weight exopolysaccharides have been reported to contain fucose, rhamnose and glucosamine sugar units [75] and promote flocculation, foaming and adhesion [75, 76].

Glucose units can also arise from a starch-like reserve polysaccharide [73]. The higher carbohydrate content in *Nannochloropsis gaditana* and *Scenedesmus almeriensis* can be correlated to their more rigid cell wall structures, less susceptible to damage by sonication than those found in *Spirulina platensis*. This would explain the greater release of polyphenols in the latter after the ultrasound treatment.

Table 2. Carbohydrate composition of the microalgae. Data shown as mean $\pm$ SD, n=3.

	Carbohydrate concentration (mg/g)		
	<i>Spirulina platensis</i>	<i>Nannochloropsis gaditana</i>	<i>Scenedesmus almeriensis</i>
Fucose	5 $\pm$ 1	7 $\pm$ 1	12 $\pm$ 2
Rhamnose	11 $\pm$ 1	10 $\pm$ 1	6 $\pm$ 1
Arabinose	<1	<1	<1
Xylose	5 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 1
Galactose	10 $\pm$ 1	31 $\pm$ 1	19 $\pm$ 3
NCG (*)	30 $\pm$ 2	147 $\pm$ 5	303 $\pm$ 37
Cellulose	3 $\pm$ 1	91 $\pm$ 5	11 $\pm$ 2
Mannose	9 $\pm$ 3	25 $\pm$ 2	77 $\pm$ 7
Uronic acids	6 $\pm$ 2	9 $\pm$ 1	9 $\pm$ 2

(*) NCG: Non-cellulosic glucose

3.3 Particle morphology and size distribution of aqueous suspensions

Light and confocal laser scanning microscopy analyses were carried out to characterise the morphology of microalgae cells in aqueous suspensions. Note that in the micrographs from confocal laser scanning microscopy auto-fluorescence from chloroplasts is shown in red and

hydrophobic components which stained for Nile Red, such as lipids, are shown in green. In the case of *Spirulina platensis* (Figures 2A and 2G), different types of particles could be visualised: oval-shaped cells (average cell size of 4.6 μm), tubular particles (pointed out with arrow in Figure 2A) and a background of amorphous material. A tubular morphology has been consistently reported in the literature for different *Spirulina* species [77-79], and has been ascribed to the association of cells attached by their walls to form colonies. The fact that unicellular fragments were mainly observed could be due to disassociation of individual cells as a result of the stirring process applied during sample preparation. *Nannochloropsis gaditana* cells presented a globular shape (cf. Figures 2B and 2H) similar to that previously reported for other *Nannochloropsis* species [80, 81], with an average cell diameter of 1.9 μm (cf. Table 3). In contrast, *Scenedesmus almeriensis* cells presented a more oval-like morphology with a larger average cell size of 4.8 μm (cf. Figures 2C and 2I), in agreement with previous studies [82, 83]. As deduced from the images corresponding to the ultrasound-treated microalgae (Figures 2D-F and 2 J-L), cell disruption did not appear to take place, as most cells seemed to remain intact and the average cell size was not significantly affected (cf. Table 3). However, in the case of *Nannochloropsis gaditana* and *Scenedesmus almeriensis*, individual cells appeared to be more homogeneously dispersed in the ultrasound-treated material, as opposed to the untreated microalgae, where the cells tended to be associated in small clusters. The *Spirulina platensis* cells seemed to be surrounded by extracellular material, likely containing lipids, represented in green in the confocal images (Figure 2G). Unstained samples of *Spirulina platensis* did not show any fluorescence in the amorphous material, hence excluding the possibility of the green colour coming from auto-fluorescence. This background network was disrupted by the ultrasound treatment (Figure 2D).

Figure 2. Light microscopy (A-F) and confocal scanning laser microscopy (G-L) images of 5% microalgae suspensions in water. CSLM shows autofluorescence from chlorophyll (red) and lipids/hydrophobic components (green).

The particle size distribution and the average particle size and specific surface area were determined by laser diffraction (cf. Figure 3 and Table 3). The estimated values for the surface weighted mean particle size ($D[3,2]$) were within the same range as the sizes estimated from the light microscopy images. It should be noted that the $D[3,2]$ values were slightly larger likely due to the presence of extracellular material attached to the cell surface, which is not accounted for when estimating particle size from light microscopy. The smaller average particle size corresponded to the *Nannochloropsis gaditana* microalgae, which also presented a larger specific surface area. On the other hand, the sonication treatment did not have a significant impact on the average particle size of *Spirulina platensis* and *Nannochloropsis gaditana* cells, in agreement with the microscopy results, while a minor effect was observed for *Scenedesmus almeriensis* cells, with a reduction in their surface weighted mean size and increasing their specific surface area. The obtained particle size distributions (cf. Figure 3) indicated the coalescence of microalgae cells into clusters, fact that has been previously reported in the literature for several microalgae species [84-86]. While the *Spirulina platensis* and *Scenedesmus almeriensis* cells seemed to flocculate forming a major population of clusters with sizes of ca. 33-44 μm and 14-18 μm , respectively, a more heterogeneous size distribution was observed in the case of *Nannochloropsis gaditana*. In that case, three different populations with size ranges of ca. 1-2 μm (single cells), 65-88 μm and 462-628 μm were detected in the untreated microalgae. After subjecting the *Nannochloropsis gaditana* cells to the ultrasound treatment, the distribution of the two larger-sized populations was significantly modified, with most of the

clusters being within the range of 65-88 μm . Although less evident, the size distribution in *Scenedesmus almeriensis* was also modified after the sonication treatment, suggesting a fewer amount of larger sized clusters. The greater extent of cluster disaggregation in *Nannochloropsis gaditana* and *Scenedesmus almeriensis*, as compared with *Spirulina platensis* cells, may be related to the presence of extracellular material, acting as a glue, in the latter.

Figure 3. Particle size distribution of untreated and ultrasound-treated microalgae. (A) *Spirulina platensis*, (B) *Nannochloropsis gaditana* and (C) *Scenedesmus almeriensis*.

Table 3. Specific surface area and particle size determined from the DLS measurements (D [3,2]: surface weighted mean; D [4,3]: volume weighted mean) and from the optical microscope images (D). Data shown as mean $\pm$ SD, n=3.

	Specific surface area (m ² /g)	D [3,2] (μm)	D [4,3] (μm)	D (μm)
<i>Spirulina platensis</i>	0.52 $\pm$ 0.01 ^a	11.5 $\pm$ 0.3 ^c	32 $\pm$ 2 ^{ab}	4.6 $\pm$ 2.0 ^a
<i>Nannochloropsis gaditana</i>	0.84 $\pm$ 0.02 ^{cd}	7.2 $\pm$ 0.2 ^a	83 $\pm$ 9 ^c	1.9 $\pm$ 0.6 ^a
<i>Scenedesmus almeriensis</i>	0.74 $\pm$ 0.02 ^b	8.1 $\pm$ 0.2 ^b	22 $\pm$ 1 ^{ab}	4.8 $\pm$ 1.2 ^a
<i>Spirulina platensis</i> -US	0.55 $\pm$ 0.03 ^a	10.8 $\pm$ 0.6 ^c	31 $\pm$ 3 ^b	3.4 $\pm$ 1.9 ^a
<i>Nannochloropsis gaditana</i> -US	0.80 $\pm$ 0.02 ^c	7.5 $\pm$ 0.2 ^{ab}	80 $\pm$ 4 ^c	1.5 $\pm$ 0.5 ^a
<i>Scenedesmus almeriensis</i> -US	0.89 $\pm$ 0.01 ^d	6.8 $\pm$ 0.1 ^a	17 $\pm$ 1 ^a	5.1 $\pm$ 1.7 ^a

Values with different letters are significantly different ($p \leq 0.05$).

3.4 Rheological properties of aqueous suspensions

3.4.1 Flow behaviour of microalgae suspensions

The flow behaviour of microalgae suspensions at different concentrations is shown in Figure 4, represented as log-log plots of shear viscosity vs. shear rate. As it can be observed, at 1 wt.% concentration, the suspensions of the three species showed a Newtonian behaviour, with viscosity values close to that of water (1 mPa·s). As the concentration increased, the behaviour shifted from Newtonian to shear thinning (i.e. decreased viscosity with increased shear). This was already apparent at 5 wt.% for *Spirulina platensis* (Figure 4A), whilst for *Nannochloropsis gaditana* (Figure 4B) and *Scenedesmus almeriensis* (Figure 4C) shear thinning behaviour was observed at higher concentrations of 15 wt.% total solids. Previous studies have shown that *Nannochloropsis spp.* suspensions behave as shear thinning fluids

at 25 wt.% [18, 87] whilst at 8 wt.% they have Newtonian behaviour [15], indicating that the critical concentration lays somewhere between 8 wt.% and 25 wt.%, in agreement with our results. Shear thinning behaviour could be due to interactions between microalgae cells (intact or broken) and soluble polymers released from the microalgae into the liquid phase. The presence of this shear thinning behaviour at lower concentrations for *Spirulina platensis* could be due to the extracellular polysaccharides (such as spirulan) and lipids observed by CSLM (Figure 2G).

Table 4 shows the fitting parameters of the shear stress vs. shear rate curves for 25 wt.% total solids using a power-law model. All the suspensions showed $n < 1$, with values between 0.27 and 0.59. The lowest values corresponded to the *Spirulina platensis* suspension, with $n = 0.27 \pm 0.01$. *Spirulina platensis* and *Scenedesmus almeriensis* showed a higher consistency compared to *Nannochloropsis gaditana* suspensions, suggesting that the contribution of hydrodynamic forces was higher in the former. In Figure 4D it can be observed that the viscosity vs. concentration behaviour of the three microalgae species falls under a master curve up to 20 wt.%, whilst at 25 wt.% the curves start to deviate from each other, with *Nannochloropsis gaditana* having the lowest viscosity and *Scenedesmus almeriensis* the highest viscosity. The increase of viscosity with concentration follows an exponential growth with an exponent of $n = 0.28 \pm 0.01$ ($R^2 = 0.98 \pm 0.01$). Broader particle size distributions typically lead to lower viscosities, since small particles can fit in the voids created by larger particles. Furthermore, they will act as a lubricant; when flow starts, the large particles will flow over the small particles decreasing the viscosity of the system. In the case of microalgae, the shape, density, surface roughness and deformability of the cells would influence the interaction between particles and, thus, the maximum volume occupied by the particles. On the one hand, *Nannochloropsis gaditana* presented the more rigid cell

walls, based on the measured cellulose content (Table 2) and in agreement with previous studies [20]; therefore, *Nannochloropsis gaditana* cells would lead to high particle phase volumes since the particles could not deform to accommodate for other particles. On the other hand, *Nannochloropsis gaditana* cells had the smallest average particle size and the broadest particle size distribution (cf. Table 3 and Figure 3) and this could lead to a lower particle phase volume and, thus, to lower viscosity. *Scenedesmus almeriensis* had similar average particle size and particle size distribution than *Spirulina platensis*; however, the higher viscosity of the former indicates that the particle volume fraction occupied by *Scenedesmus almeriensis* cells is larger than that of *Spirulina platensis* cells. This could be due to the more rigid cell walls in *Scenedesmus almeriensis* (cf. Table 2). To sum up, and similarly to what has been shown for suspensions of other plant materials such as fruits and vegetables [88-90], at low concentrations, the rheology of microalgae suspensions is mainly controlled by the rheology of the liquid phase and the particle volume fraction [91]; whilst at high concentrations the viscosity depends on the deformability of closely-packed microalgae cells, which in turn is a function of cell wall composition and structure.

Figure 4. Shear viscosity at different concentrations for A) *Spirulina platensis*, B) *Nannochloropsis gaditana* and C) *Scenedesmus almeriensis*. The change from Newtonian to shear thinning behaviour is clearly seen at different concentrations for each species. D) Shear viscosity (selected at 40 s⁻¹) as a function of microalgae concentration (% wt.) and master curve ($n = 0.28 \pm 0.01$, $R^2 = 0.98 \pm 0.01$) showing a general behaviour for these three microalgae species with deviation at higher concentrations. Data shown as mean $\pm$ SD at each shear rate, $n=3$.

Table 4. Parameters obtained from fitting the log-log shear stress vs shear rate plots to a Power Law ($\sigma = K\dot{\gamma}^n$) model. σ represents the shear stress, K the consistency coefficient, $\dot{\gamma}$ the shear rate and n the flow index. Parameters obtained from fitting the elastic modulus

G' vs angular frequency ω to power law model ($G' = a\omega^b$). The quality of the fitting is represented by R^2 . nd=not detected. Tan(delta) values of the 25 wt. % suspensions.

	$\sigma = K\dot{\gamma}^n$,			$G' = a\omega^b$			
	Consistency coefficient (Pa s ⁻ⁿ)	Flow behaviour (-)	R^2	a	b	R^2	Tan (delta) at 0.5 rad s ⁻¹ G''/G'
<i>Spirulina platensis</i>	17.40 ± 1.32	0.27 ± 0.01	0.98	170 ± 12.5	0.16 ± 0.02	0.97	0.25 ± 0.08
<i>Nannochloropsis gaditana</i>	3.62 ± 0.88	0.59 ± 0.01	0.99	5.70 ± 0.90	0.42 ± 0.05	0.98	1.02 ± 0.00
<i>Scenedesmus almeriensis</i>	21.40 ± 0.50	0.41 ± 0.01	0.99	55.6 ± 7.01	0.40 ± 0.11	0.97	0.72 ± 0.13

3.4.2 Viscoelastic properties of microalgae suspensions

To better understand the differences in rheological properties at high concentrations, the viscoelastic properties of the suspensions at 25 wt.% were investigated. Figure 5 shows the viscoelastic moduli (G' and G'') vs. frequency. At this concentration, the *Spirulina platensis* suspensions showed an elastic behaviour ($G' > G''$) over the whole range of frequencies. *Scenedesmus almeriensis* suspensions also showed a predominant elastic behaviour, especially at higher frequencies, whilst *Nannochloropsis gaditana* showed a slightly predominant viscous behaviour ($G' < G''$) at high frequencies. The frequency dependency of G' was fit to a power law model ($G' = a\omega^b$) and values of fitting parameters are shown in Table 4. Suspensions of *Nannochloropsis gaditana* and *Scenedesmus almeriensis* showed similar frequency dependence with b values of ca. 0.40, whilst *Spirulina platensis* suspensions showed a lower frequency dependence with an exponent of 0.16. The exponent value lower than 2 as well as the $G' > G''$ and $\tan(\delta) < 1$ indicate that the *Spirulina platensis* suspension behaved as a weak gel, while the *Nannochloropsis gaditana* suspension showed a predominant viscous behaviour ($\tan(\delta) > 1$) [92]. The values of the fitting

parameter a were in the order *Spirulina platensis*>*Scenedesmus almeriensis*>*Nannochloropsis gaditana*, suggesting that the strongest network was present in the *Spirulina platensis* suspensions. On the contrary, the weakest network was present in the *Nannochloropsis gaditana* suspensions, in agreement with their most viscous nature.

Figure 5. Frequency sweeps of 25% wt. suspensions of *Spirulina platensis*, *Nannochloropsis gaditana* and *Scenedesmus almeriensis*. Data shown as mean $\pm$ SD at each angular frequency, $n=3$.

The complexity of these materials makes it difficult to underpin the main reason for having networks of different strength, since covalent, non-covalent, as well as electrostatic interactions, might be occurring between the microalgae cells and/or with the extracellular components in the liquid phase. Based on the composition, the presence of exopolysaccharides (either loosely attached to the cell wall or released to the liquid phase), which contained rhamnose and galactose in *Spirulina platensis* and rhamnose and fucose in

Scenedesmus almeriensis, could explain the stronger nature of the networks in these suspensions, whilst the rigidity of the *Nannochloropsis gaditana* cells, mainly containing cellulose, would lead to weaker networks.

3.4.3 Impact of ultrasound processing on rheological properties

Compositional and microscopy analyses suggested that ultrasound processing even though not disintegrating the cell walls, changed the microalgae suspensions to some extent. In *Nannochloropsis gaditana* and *Scenedesmus almeriensis*, cell clusters were separated into single cells (cf. Figure 2) and intracellular components (like proteins or peptides) were released towards the liquid medium. The effect of this on the rheological properties of aqueous suspensions was also investigated, and the results for 25 wt.% suspensions of ultrasound-treated microalgae are presented in Figure 6. The ultrasound treatment did not have a significant impact on the viscosity of *Spirulina platensis* suspensions, whilst it increased the viscosity of *Nannochloropsis gaditana* suspensions and decreased the viscosity of *Scenedesmus almeriensis* suspensions. The increase in viscosity could be related to a higher viscosity of the liquid phase due to the release of cell components in the case of *Nannochloropsis gaditana*, whilst in *Scenedesmus almeriensis* a narrower particle size distribution as result of ultrasound processing (cf. Figure 3), could explain the lowest viscosity of these suspensions. In terms of viscoelastic properties, ultrasounds increased the G' (elastic modulus) and G'' (viscous modulus) of *Spirulina platensis* and *Nannochloropsis gaditana*, whilst a trend towards a decrease in the viscoelastic moduli was observed for the *Scenedesmus almeriensis* suspensions. An increase in the interactions between cells and the cell components released to the liquid phase could contribute to a higher viscoelasticity in the *Nannochloropsis gaditana* and *Spirulina platensis* suspensions. On the other hand, the disaggregation of cell clusters could explain the weakening effect in the *Scenedesmus*

almeriensis suspensions, due to the reduction of cell-cell interactions, which contribute to the elasticity of the system.

Figure 6. A) Viscosity of 25% wt. microalgae suspensions (at 10 s⁻¹) before and after ultrasound treatment. B) Viscoelastic moduli of 25% wt. microalgae suspensions, G' (elastic

modulus) and G'' (viscous modulus) selected at 10 rad s^{-1} , as result of ultrasound treatment. Data shown as mean $\pm$ SD, $n=3$.

4. Conclusions

The rheological properties of aqueous suspensions from three microalgae species, before and after being subjected to an ultrasound treatment, were investigated and related to their composition and physico-chemical properties. The composition of the three microalgae was substantially different: *Spirulina platensis* was mainly composed of ashes ($\sim 36\%$) and proteins ($\sim 27\%$), while *Scenedesmus almeriensis* ($\sim 44\%$ carbohydrates and $\sim 32\%$ proteins) and *Nannochloropsis gaditana* ($\sim 41\%$ lipids and $\sim 33\%$ carbohydrates) were richer in carbohydrates. In particular, *Nannochloropsis gaditana* contained a relatively high amount of cellulose, providing greater rigidity to the cell walls. On the other hand, exopolysaccharides were mainly composing the carbohydrate fraction in *Spirulina platensis* and *Scenedesmus almeriensis*. The presence of extracellular material was especially evident in the *Spirulina platensis* microalgae; this explained the fact that *Spirulina platensis* suspensions presented shear thinning behaviour at lower concentrations than *Scenedesmus almeriensis* and *Nannochloropsis gaditana*, as well as the weak gel behaviour of the 25 wt. % *Spirulina platensis* suspensions. The viscosity of the three microalgae species followed the same master curve up to 20 wt. % total solids; however, at 25 wt. % the curves started to deviate from each other, with *Nannochloropsis gaditana* having the lowest viscosity and *Scenedesmus almeriensis* the highest viscosity. These results suggest that at low concentrations the rheology is mainly controlled by the liquid phase and the particle volume fraction; whilst at high concentrations the viscosity of the microalgae suspensions depends on the deformability of closely-packed microalgae cells, determined by their cell wall composition and structure. Remarkably, the different behaviour of 25 wt. % *Spirulina*

platensis (weak gel behaviour) and *Nannochloropsis gaditana* (viscous liquid behaviour) suspensions, suggests potential different applications for these two species.

The application of the ultrasound treatment did not produce cell wall disruption, but it seemed to promote the release of a certain amount of intracellular components; however, some of these components, such as the more unstable polyunsaturated fatty acids, may have been degraded upon the treatment and, thus, only a minor effect in the antioxidant capacity of the microalgae was observed. The treatment did not significantly impact the particle size of the microalgae, producing only a minor decrease in the cell size of *Scenedesmus almeriensis* cells. On the other hand, ultrasounds were able to disrupt the cells' clusters in *Scenedesmus almeriensis* and *Nannochloropsis gaditana*. All these factors affected the rheology of the ultrasound-treated suspensions, increasing the viscosity of *Nannochloropsis gaditana* suspensions, while the viscosity of *Scenedesmus almeriensis* suspensions was reduced. Furthermore, the viscoelastic moduli of *Spirulina platensis* and *Nannochloropsis gaditana* suspensions were increased, which could be attributed to greater interactions between the cells and the extracellular material; however, the heterogeneity and complexity of these systems makes it difficult to correlate the rheological changes to one single factor.

These results demonstrate that it is possible to produce microalgae suspensions with very different rheological properties, for specific applications, by selecting the appropriate species. Moreover, the application of mild ultrasound treatments may also be an efficient approach to modify the rheological properties of microalgae suspensions.

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Conflict of interest

The authors declare that they have no conflict of interest.

Informed consent, human or animal rights

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Declaration of authors agreement

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