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# **Effect of ultrasonication, high pressure homogenization and their combination on efficiency of extraction of bio- molecules from microalgae *Parachlorella kessleri***

Rui Zhang<sup>1\*</sup>, Nabil Grimi<sup>1</sup>, Luc Marchal<sup>2</sup>, Nikolai Lebovka<sup>1,3</sup>, Eugène Vorobiev<sup>1</sup>

<sup>1</sup>Sorbonne University, Université de Technologie de Compiègne, ESCOM, EA 4297 TIMR, Centre de  
recherche Royallieu - CS 60319 - 60203 Compiègne cedex, France

<sup>2</sup>LUNAM Université, CNRS, GEPEA, Université de Nantes, UMR6144, CRTT, Boulevard de  
l'Université, BP 406, 44602 Saint-Nazaire Cedex, France ;

<sup>3</sup>Institute of Biocolloidal Chemistry named after F. D. Ovcharenko, NAS of Ukraine, 42, blvr.  
Vernadskogo, Kyiv 03142, Ukraine

\*Corresponding Author Address:

Mrs. Rui Zhang

Sorbonne University, Université de Technologie de Compiègne, ESCOM, EA 4297 TIMR, Centre de  
recherche Royallieu - CS 60319 - 60203 Compiègne cedex, France

E-mail address: [rui.zhang@utc.fr](mailto:rui.zhang@utc.fr)

## Abstract

The disintegration efficiencies of microalgal *Parachlorella kessleri* cells by ultrasonication (US) and high pressure homogenization (HPH) treatments were investigated. The applied procedures included individual US, individual HPH, or combined US followed by HPH treatments. The test concentrations of cell suspensions were 1% and 10% dry matter. The microstructures of cell suspensions were analyzed using scanning electron microscopy, light microscopy and light scattering techniques. Extraction was characterized by the ionic,  $Z_i$ , carbohydrate,  $Z_c$ , protein,  $Z_p$ , and pigment (dyes),  $Z_d$ , extraction indexes. Application of US treatment (1% dry matter, 400 W, 30 min) gave  $Z_i \approx 0.10$ ,  $Z_c \approx 0.45$ ,  $Z_p \approx 0.16$ , and application of HPH treatment (1% dry matter, 400 bar, 4 passes) gave  $Z_i \approx 0.10$ ,  $Z_c \approx 0.20$ , and  $Z_p \approx 0.11$ . In both cases, the efficiency of extraction can be arranged as follows:  $Z_i < Z_p < Z_c$ . Application of a preliminary US treatment with 10% dry matter and a final HPH treatment with 1% dry matter allows increasing the extraction efficiency and decreasing the energy consumptions. For example, US (1% dry matter, 400 W, 30 min) + HPH (1% dry matter, 1200 bar, 4 passes) treatment ( $\approx 105.6$  kJ/g dry matter) gave  $Z_i \approx 0.49$ ,  $Z_c \approx 0.69$ , and  $Z_p \approx 0.32$ , whereas US (10% dry matter, 400 W, 30 min) + HPH (1% dry matter, 1200 bar, 4 passes) treatment ( $\approx 53.8$  kJ/g dry matter) gave  $Z_i \approx 0.76$ ,  $Z_c \approx 0.83$ ,  $Z_p \approx 0.74$ .

**Keywords:** Microalgae; *Parachlorella kessleri*; Ultrasonication; High pressure homogenization; Selective extraction; Bio-molecules

## Nomenclature

$A$	absorbance of pigment peaks
$c$	specific heat capacity of suspension, J/g °C
$C$	concentration of carbohydrate, mg/g dry matter
$C_m$	concentration of suspension, % dry matter
$C_p$	concentration of protein, mg/g dry matter
$d$	diameter of particles, $\mu\text{m}$
$E$	specific energy consumption for HPH and US, kJ/g dry matter
$m$	mass of suspension, g
$N$	number of HPH passes
$p$	homogenizing pressure, bar
$P_a$	actual power of US, W
$P_g$	generator power of US, W
$t$	time of US, min
$\Delta T$	temperature elevation, °C
$V$	relative volume, %
$Z_c$	carbohydrate extraction index
$Z_d$	pigment (dye) extraction index
$Z_i$	ionic extraction index
$Z_p$	protein extraction index

### Abbreviations

<b>HPH</b>	high pressure homogenization
<b>PSD</b>	particle size distribution
<b>SEM</b>	scanning electron microscopy
<b>U</b>	untreated
<b>US</b>	ultrasonication

### Greek symbols

$\rho$	density of suspension, kg/m <sup>3</sup>
$\sigma$	electrical conductivity (mS/cm)
$\lambda$	wavelength of absorbance

### Subscripts or superscripts

$i$	initial
$f$	final
$v$	violet absorbance
$r$	red absorbance

# 1. Introduction

Nowadays, increased interest has been focused on the development of emerging technologies for the total recovery of bio-molecules from marine substrates like microalgae. Microalgae have high growth rates and photosynthetic efficiencies. They are also rich in valuable bioactive components, such as proteins, lipids, polyunsaturated fatty acids, carbohydrates, pigments and polyphenols [1–3], that can be used in food, feed, cosmetics, pharmaceutical and biofuel industries [4–6].

The microalga *Parachlorella kessleri* (*P. kessleri*) is a unicellular freshwater organism (Chlorophyta). It is a near spherical cell with a diameter of 2.5-10  $\mu\text{m}$  and has thick rigid cell walls (60-80-nm) [7,8]. *P. kessleri* can rapidly accumulate biomass, starch, proteins and lipids [9–11]. It was demonstrated that under unfavourable growth conditions (lack of light, nutrient stress, nitrogen starvation), the culture can accumulate large amounts of energy-rich compounds, such as triglycerides (TAG) and starch [12]. However, these valuable compounds are enclosed in intracellular vacuoles and chloroplast, protected by the rigid cell walls and membranes [13], thus greatly limiting their recovery during the process of extraction. From the oldest techniques (e.g. decoction and maceration) to conventional extraction techniques (e.g. Soxhlet), large volumes of aqueous or organic solvents are used, depending on the polarity of the target compounds [14]. In addition, these methods suffer from some disadvantages, such as their small scale, long extraction time and low process efficiencies, the co-extraction of undesirable components, and they require more complex downstream separation steps [15].

Effective recovery methods of intracellular contents from microalgae have been reported. These methods can be chemical, enzymatic, or different physical treatments, such as ultrasonication (US), microwaves, pulsed electric fields, and mechanical stresses (high pressure homogenization (HPH),

bead milling (BM) etc...) [13,16–19]. The efficacy of *P. kessleri* cell disintegration by US treatment was evaluated by laser light scattering methods [20]. In the US treatment, the cell walls can be damaged by the bursting of cavitation bubbles outside the cells and the development of extremely high pressures. The effective time of ultrasonication was dependent on the growth phase of *P. kessleri* cells. In the stationary-phase, the cell walls were more resistant to the US treatment and the disruption effect was decreased with an increase in the cell concentration. For the nitrogen-starved *P. kessleri* cultures, effects of disruption by HPH and BM on the profile of fatty acids and lipids composition have been investigated [21,22]. The efficiency of HPH and BM can be explained by high-pressure shears, elongations, turbulences, and cavitations. The proportions of amphiphilic free fatty acids and lysophosphatidylcholine were higher in HPH than in BM. HPH disruption techniques applied at a pressure of 1750 bar (4 cycles) to the strain *P. kessleri* UTEX2229 allowed the extraction of 65% of the total lipids and 46% of the TAG [22]. However, the mechanical techniques are highly energy consuming and require a specific energy consumption of at least 33 kJ/g dry matter [23]. In addition, a growing number of studies have been recently conducted on combined methods to achieve synergy, thereby increasing the yield of extraction. For example, the use of US in combination with microwave irradiation enhanced oil production from *Chlorella vulgaris* was studied [24]. According to Tavanandi et al [25], a combined US + freezing and thawing method obtained the highest yield (91.62%) of C-Phycocyanin from *Arthrospira platensis*, while US (43.05%) or freezing and thawing alone (62.56%). Cho et al. [26] used a combined conventional Floch method with HPH treatment (1200 psi, 35 °C), which can easily destruct the rigid cell walls of microalgae and release the intact lipids with minimized extraction time and temperature.

The efficiency of extraction of bio-molecules from *P. kessleri* cultures can be improved by using

advanced protocols based on US, HPH treatments or their combinations. However, to the best of our knowledge, such studies on *P. kessleri* cultures are scarce at the present. The aim of this work was to study the impact of different individual and combined US and HPH protocols on extraction efficiencies of ionic components, proteins, carbohydrates, and pigments from microalga *P. kessleri*. This study investigated the dependence of recovery behaviors of ionics, carbohydrates, proteins, and pigments on the extraction protocols that were used. The microstructure of cell suspensions was observed. The distribution functions of cells were determined. Finally, the extraction efficiency in dependence of specific energy consumption and concentration of suspension were discussed.

## 2. Materials and methods

### 2.1 Chemicals

Sulfuric acid, D-glucose, phenol and bovine serum albumin (BSA) standard were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The other chemicals that were used were analytical grade.

### 2.2 Microalgae

Microalgae *P. kessleri* used in this study were provided by AlgoSolis (Saint-Nazaire, France). They were produced in one step and in batch mode in a flat panel airlift photobioreactor (PBR) (6 L) at  $25 \pm 0.5$  °C. Culture homogenization was achieved by sterile air injection at the bottom of the PBR. The pH and temperature were measured using Mettler probe (Mettler Toledo SG 3253 sensor). The value of pH 7 was adjusted with CO<sub>2</sub> bubbling, and constant light was provided by an LED array panel. The harvested culture was centrifuged and stored at -20 °C until use.

The moisture content of *P. kessleri* was  $\approx 87\%$ . The biomass pastes were first thawed at ambient

temperature and then diluted with deionized water in order to prepare different suspensions with a final biomass concentration,  $C_m$ , of 1% and 10% dry matter (hereinafter %), respectively. All extractions were performed using 500 g of the suspensions.

### 2.3 Extraction procedures

**Fig. 1** presents the schematic of applied extraction techniques for the recovery of bio-molecules from *P. kessleri* biomass. The applied procedures include US treatment (S procedure), HPH treatment (P procedure) and US treatment followed by HPH treatment (combined S + P procedure). In control experiments, untreated (U procedure) suspensions were also analyzed.

For the S procedure, 1% or 10% suspensions were used. For the P procedure, the instrument's operating conditions restrict the maximum concentration and 1% suspensions were always used. For the combined S (10%) + P (1%) procedure, a dilution step was carried out after the S procedure.

For the S procedure, the UP-400S ultrasound processor (Hielecher GmbH, Germany) was used. It was operated at a constant frequency of 24 kHz. The ultrasound probe with a diameter of 14 mm and a length of 100 mm was plunged into a beaker, containing 500 g of suspensions. The time of US,  $t$ , and generator power (declared),  $P_g$ , were varied within the ranges 0-30 min (0, 5, 10, 20, 25 and 30 min), and 0-400 W (0, 100, 200 and 400 W) respectively. The actual ultrasonic power,  $P_a$ , was estimated from the temperature elevation,  $\Delta T$ , in sample using the following equation:

$$P_a = mc\Delta T/t \quad (1)$$

where  $c$  ( $\approx 4.18 \text{ J/g}^\circ\text{C}$ ) is the specific heat capacity of the suspensions. For the applied procedures,  $P_a \approx 28 \pm 1 \text{ W}$  ( $P_g = 100 \text{ W}$ ),  $P_a \approx 77 \pm 2 \text{ W}$  ( $P_g = 200 \text{ W}$ ), and  $P_a \approx 156 \pm 4 \text{ W}$  ( $P_g = 400 \text{ W}$ ).

Specific energy consumption,  $E$  (kJ/g dry matter), was calculated for S procedure as follows:

$$E = P_a t / (C_m m). \quad (2)$$

For example, for  $C_m = 1\%$  suspension and  $t = 30$  min, the values of  $E \approx 10$  kJ/g dry matter ( $P_g = 100$  W),  $E \approx 27.6$  kJ/g dry matter ( $P_g = 200$  W), and  $E \approx 56$  kJ/g dry matter ( $P_g = 400$  W). For  $C_m = 10\%$ , and  $t = 30$  min,  $E \approx 5.6$  kJ/g dry matter ( $P_g = 400$  W). Note that heating of the same suspension from 20 to 100 °C requires  $\approx 33.4$  kJ/g dry matter. Therefore, the suspensions were immersed in a cooling bath to avoid overheating. The maximum temperature increase during the US at 400 W for 30 min does not exceed 45 °C. Moderate temperatures were used to avoid thermal degradation of organic compounds as well as provide an efficient application of US [27,28]. In principle, the prolonged US treatment can cause degradation of targeted compounds. Note that no specific reaction products after sonication (5 to 55 min) applied to the isolated phenolic compounds of apple pomace were previously observed [27].

For the P procedure, 500 g of suspensions were homogenized in a NS 100L-PANDA 2K two-stage high pressure homogenizer (Niro Soavi S.p.A., Parma, Italy). The average throughput of the equipment was 10 L/h. The homogenizing pressure,  $p$ , was fixed in the range of 400 to 1200 bar (1 bar =  $10^5$  Pa). The number of passes ( $N$ ) was varied from 1 to 10. The initial temperature of suspensions before P procedure was 22 °C and the temperature elevation after HPH treatment never exceeded 30 °C. Before each pass through the homogenizer, the suspension was cooled to 22 °C.

Specific energy consumption,  $E$  (kJ/g dry matter), for P procedure was estimated as follows [29]:

$$E = pN/C_m\rho \quad (3)$$

where  $p$  is the pressure of treatment (Pa),  $N$  is the number of passes, and  $\rho$  ( $\approx 10^6$  g/m<sup>3</sup>) is the density of the suspensions. For example, at  $C_m = 1\%$  suspension and  $N = 4$ , we have  $E \approx 16$  kJ/g dry matter (400 bar),  $E \approx 32$  kJ/g dry matter (800 bar), and  $E \approx 48$  kJ/g dry matter (1200 bar).

## 2.4 Characterization

The characterization measurements were done at the same temperature (22 °C). In this work, to maximize the disintegration of the suspension, the P procedure with  $p = 1500$  bar,  $N = 10$ , and  $E \approx 150$  kJ/g dry matter was always applied.

### 2.4.1 Ionic components

The degree of extraction of ionic components was characterized using electrical conductivity disintegration index  $Z_i$  (ionic extraction index) [30]:

$$Z_i = (\sigma - \sigma_i) / (\sigma_f - \sigma_i), \quad (4a)$$

where  $\sigma$  is the electrical conductivity of suspensions and the subscripts  $i$  and  $f$  refer to the initial and final (maximum) values, respectively. In experiments with maximally disintegrated 1% suspension, we have obtained the value of  $\sigma_f$  equal to  $0.91 \pm 0.01$  mS/cm.

The above equation gives  $Z_i = 0$  for the untreated and  $Z_i = 1$  for the maximally disintegrated suspensions.

The electrical conductivity was measured using a conductivity meter InoLab pH/cond Level 1 (WTW, Weilheim, Germany).

### 2.4.2 Microstructure of cell suspensions

Scanning electron microscopy (SEM) images of cell suspension were obtained at 2500 folds magnification using a QUANTA 250 FEG equipment (FEI Company, France) at 20 kV accelerating voltage. For SEM investigations, the microalgal cells were fixed with buffered aldehyde and in osmium tetroxide, then dehydrated in ethanol, dried with an air dryer, mounted on a specimen stub, and coated with carbon [31]. Optical microscopy images of suspensions were obtained at 40 folds magnification using a VisiScope light microscope (VWR, Italy). In each experiment, 18 images from three different

samples were analyzed.

### 2.4.3 Particle size disruption

The particle size distribution (PSD) was measured in the diapason 0.01-3000  $\mu\text{m}$  using laser diffraction Malvern Mastersizer 2000 instrument (Malvern, Orsay, France). Before injection into Malvern cells, the suspensions were carefully stirred. The PSD was calculated using the original Malvern software.

### 2.4.4 Analyses of supernatant

The suspensions were centrifuged using a mySPIN6 Mini Centrifuge (Thermo Fisher Scientific, China) at 6000 rpm (2000 g) for 10 min. The supernatants were used for further chemical analysis. All chemical analyses were based on color reactions with reagents and absorption spectra that were measured using a UV/VIS spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA).

The content of water-soluble carbohydrates was determined using a phenol-sulfuric acid method [34]. D-glucose was used as a standard. The color reaction was initiated by mixing 1 mL of supernatants (diluted if required) with 0.1 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid (Sigma-Aldrich, France). The reaction mixture was kept at 20 °C for 20 min. Absorbance was measured at 490 nm and the concentration of carbohydrates,  $C$ , was evaluated.

The concentration of proteins,  $C_p$ , was determined by means of Bradford method [35]. The diluted supernatant (0.1 mL) was mixed with 1 mL of Bradford Dye Reagent (Thermo Fisher, Kandel, Germany) using the vortex mixer VX-200 (Labnet International, France) and kept at 22 °C for 5 min. The absorbance was measured at 595 nm. BSA was used for the calibration of the instrument.

Absorption spectra were measured in the wavelength range of 350-900 nm against the blank (with

the precision of  $\pm 1$  nm). The content of pigments was estimated spectrophotometrically by analysis of the absorbance of peaks,  $A$ , at the wavelengths of  $\lambda_v \approx 400$  nm (violet) and  $\lambda_r \approx 680$  nm (red). These peaks can be attributed to the absorbance of carotene and chlorophylls dyes [16] (For more details see Fig. S1 in the Supplementary materials).

Based on the measured values of  $C$ ,  $C_p$  and  $A$ , the following carbohydrate, protein and pigment (dye) extraction indexes were defined:

$$Z_c = (C - C_i)/(C_f - C_i), \quad (4b)$$

$$Z_p = (C_p - C_p^i)/(C_p^f - C_p^i), \quad (4c)$$

$$Z_d = (A - A_i)/(A_f - A_i), \quad (4d)$$

where the  $i$  and  $f$  refer to the initial and final (maximum) values, respectively.

In experiments with maximally disintegrated 1% suspension (for the P procedure with  $p = 1500$  bar and  $N = 10$ ), we obtained the following maximum values:  $C_f = 1335.5 \pm 10.6$  mg/g,  $C_p^f = 834.1 \pm 8.2$  mg/g,  $A_f = 0.777 \pm 0.01$  ( $\lambda_v = 400$  nm), and  $A_r = 0.356 \pm 0.01$  ( $\lambda_r = 680$  nm).

## 2.5 Statistical analysis

Each experiment was repeated at least three times. Data are expressed as mean  $\pm$  standard deviation. The error bars, presented on the figures, correspond to the standard deviations. One-way analysis of variance was used for statistical analysis of the data with the help of OriginPro 8.0. A probability value (p value) of less than 0.05 was considered statistically significant.

## 3. Results and discussion

### 3.1 Cell structure and distribution of particle sizes in suspensions

Fig. 2 presents SEM images of untreated (U) microalgae cells (a), and cells obtained after treatment of suspensions ( $C_m = 1\%$ ) using S (400 W, 30 min) (b), and S + P (1200 bar,  $N = 4$ ) (c)

procedures. The SEM images for U and S samples were rather similar. Cells have near spherical shape and their mean size  $\approx 4 \mu\text{m}$ . Small interspaces and holes were observed and some cells were damaged. The U sample images reflect the effects of harvesting (Fig. 2a). For the S sample, the shear stress that is externally released by cavitation during US treatment can introduce interspaces, holes and microfractures in cells and produce shrinkage (Fig. 2b). However, these effects were not clearly visible in SEM images. For the S + P sample, most cells were damaged and the cell walls' integrity was almost completely lost (Fig. 2c).

The effects of U, S and S + P procedures on the microstructure of cell suspensions were elucidated using the data on the PSD (Fig. 3). The PSD revealed the presence of a bimodal distribution of untreated (U) cells with the small and large peaks located at  $\approx 4 \mu\text{m}$  and  $\approx 45 \mu\text{m}$ , respectively (Fig. 3a). We assume that the smaller peak corresponds to the individual cells of *P. kessleri*, whereas the larger peak corresponds to the agglomerated cells that formed clusters. The bimodal distributions that reflect agglomeration were also observed for microalgae *Nannochloropsis oculata* [32] and yeast cells [33]. As previously conjectured, the aggregates can represent microalgae flocks formed by the harvesting mechanism used (flocculation) [32].

Fig. 3b presents data on the PSD for the 1% suspensions treated using S (400 W, 30 min) + P (400-1200 bar,  $N=4$ ) procedures. For the S + P (400 bar) sample, a single peak with median diameter located at  $\approx 3.4 \pm 0.1 \mu\text{m}$  was observed. Therefore, it can be concluded that this treatment procedure allows complete disaggregation of the agglomerated cells in untreated suspension. For the S + P (800 bar) sample, a single peak with median diameter located at  $\approx 2.7 \pm 0.1 \mu\text{m}$  was observed and an increase of  $p$  (S + P (1200 bar) sample) resulted in a noticeable increase of the content of cell debris with a size  $\leq 1 \mu\text{m}$ .

**Fig. 3c** compares data on the PSD for 1% and 10% suspensions treated using the S (400 W, 30 min) and S (400 W, 30 min) + P (1200 bar,  $N=4$ ) procedures. Note that for individual US (S samples), for both 1% and 10%, single peaks with median diameters located at  $\approx 3.8 \pm 0.1 \mu\text{m}$  were observed. These values are smaller than  $4.0 \pm 0.1 \mu\text{m}$  for intact cell, this can reflect the effects of US on the structure of cell walls. The increase of the suspension concentration resulted in a decrease of peak height and the minor broadening of the peak.

For the combined procedure (S + P samples), the PSD revealed the presence of the multimodal distributions with peaks located at  $\approx 0.5, 2.7$  and  $17.4 \mu\text{m}$  which correspond to the formation of cell debris, damaged cells, and conglomerates of cell, respectively. The observed re-aggregation of cells can be a result of cell-cell walls adhesion at high pressures during the HPH treatment. Note that for the S and S + P samples, the observed effects were more prominent for more concentrated suspensions (**Fig. 3c**).

**Fig. 4** compares optical microscopy images of untreated (U) suspensions (a), and suspensions after treatment using S (400 W, 30 min) (b), and S + P (1200 bar,  $N=4$ ) (c) procedures. The concentration of suspensions was 1%. The obtained images supported the PSD data related to the presence of agglomerated cells in the untreated suspension (**Fig. 4a**) and their complete disaggregation after the application of S procedure (**Fig. 4b**). The application of the combined S + P procedure resulted in the appearance of cell debris, small aggregates, and some resistant cells remained undamaged (**Fig. 4c**).

### 3.2 Extraction of bio-molecules

**Fig. 5** shows changes of ionic,  $Z_i$ , carbohydrate,  $Z_c$ , protein,  $Z_p$ , (a), and pigment,  $Z_d$ , (b) extraction indexes in the course of the US treatment (S procedure) at different US powers (0-400 W). The values

of all extraction indexes increased with the increase of US power and extraction time,  $t$ , and the maximum degrees of extraction were obtained by using the highest power and the longest applied extraction time,  $t = 30$  min.

The obtained data evidenced that even at  $t = 30$  min all the measured parameters were still far from the saturated values. The time changes of  $Z_d$  at wavelength of  $\lambda_v = 400$  nm and  $\lambda_r = 680$  nm were rather similar, but they were more pronounced at  $\lambda_v = 400$  nm (Fig. 5b). Note that extracted dyes (carotene and chlorophylls) are practically insoluble in water. These changes can reflect an increase in the concentration of dye binding molecules that are soluble in water, which supports the presence of dyes in water. The stabilization of dyes in water can be supported by the formation of dye-macromolecular water-soluble complexes [36].

Extraction for 30 min at 400 W resulted in  $Z_i \approx 0.10$ ,  $Z_c \approx 0.45$ ,  $Z_p \approx 0.16$  (Fig. 5a). The obtained values can be arranged in the following order:

$$Z_i < Z_p < Z_c, \quad (5)$$

The data evidenced that the highest extraction efficiency was obtained for carbohydrates and the lowest was obtained for ionic components. This can be explained by the release of a certain amount of extracellular polysaccharides present in the cell walls of microalgae [37,38].

The estimated indexes for pigments are  $Z_d \approx 0.174$  ( $\lambda_v$ ), and  $Z_d \approx 0.077$  ( $\lambda_r$ ) and their ratio  $r = Z_d(\lambda_v)/Z_d(\lambda_r)$  was  $\approx 2.2$ . Note that the value of  $Z_d(\lambda_v)$  was comparable with the value of  $Z_p$ . Therefore, we can speculate that stabilization of dyes in water reflects a release of water-soluble proteins.

Fig. 6 shows changes of ionic,  $Z_i$ , carbohydrate,  $Z_c$ , protein,  $Z_p$ , (a), and pigment,  $Z_d$ , (b) extraction indexes for P procedure at different applied pressures (400-1200 bar). The maximum degrees of extraction were obtained by using the highest values of  $p$  and  $N$ . Extraction for  $N = 10$  at  $p = 1200$  bar

resulted in  $Z_i \approx 0.62$ ,  $Z_c \approx 0.87$ ,  $Z_p \approx 0.71$  (Fig. 6a). Note that for the P procedure, the obtained values of the extraction index can be arranged in the same order as the S procedure (Eq. 5). The estimated indexes for pigments are  $Z_d \approx 0.99$  ( $\lambda_v$ ), and  $Z_d \approx 0.45$  ( $\lambda_r$ ) and their ratio  $r = Z_d(\lambda_v)/Z_d(\lambda_r)$  was also  $\approx 2.2$ , the same was observed in the S procedure. Note that the application of the first four passes ( $N \leq 4$ ) allowed obtaining a noticeable degree of extraction and further passes resulted in insignificant supplementary effects at high specific energy consumption. Therefore, in further experiments the protocols with  $N = 4$  were applied.

The obtained data shows that both individual S and P procedures allow an increase of extraction of bio-molecules. The order of these extraction efficiencies are represented in Eq. (5). However, on one hand, the individual S procedure is not very efficient for damaging cells and it results in moderate extraction yields. On the other hand, the individual P procedure can lead to the intensive generation of cell debris and degradation of bio-molecules with increased energy consumption and elevation of temperature [37]. Application of procedures that combine the important qualities of US and HPH treatments can be attractive in terms of extraction yield, selectivity and energy consumption.

Fig. 7 compares the ionic,  $Z_i$ , carbohydrate,  $Z_c$ , and protein,  $Z_p$ , extraction indexes versus the specific energy consumption,  $E$ , using S, P and S + P procedures for diluted suspensions with  $C_m = 1\%$ . The time of US was  $t = 30$  min, and number of passes of HPH was  $N = 4$ . The extraction efficiency of ionics and proteins is higher for HPH (P procedure) than for US (S procedure) treatment at the same energy consumption. However, for carbohydrates, the effect depends upon energy. For example, for the same energy consumption of  $E = 15$  kJ/g dry matter, the carbohydrates extraction with US was higher ( $Z_c \approx 0.3$ ) than with HPH ( $Z_c \approx 0.2$ ) (Fig. 7). The extraction of bio-molecules for combined S + P procedure can display synergetic behaviour. For example, individual S (400 W) and P (400 bar)

procedures were ineffective for the extraction of ionic components, and gave  $Z_i \leq 0.10$ . However, combined S (400 W) + P (400 bar) procedure gave  $Z_i \approx 0.37$  (Fig. 7). Similar behaviour was also observed for combined S (400 W) + P (800, 1200 bar) procedures.

For the extraction of carbohydrates, the combined S + P procedure was less effective. For example, the individual S (400 W,  $\approx 56$  kJ/g dry matter) and P (400 bar,  $\approx 16$  kJ/g dry matter) procedures for extraction of carbohydrates gave  $Z_c \approx 0.45$  and  $Z_c \approx 0.20$ , respectively, whereas the combined S + P procedure (400 W, 400 bar,  $\approx 73.5$  kJ/g dry matter) gave  $Z_c \approx 0.49$ . However, the combined S + P procedure at higher pressure (400 W, 1200 bar,  $\approx 105.6$  kJ/g dry matter) gave  $Z_c \approx 0.69$ .

Moreover, the extraction of proteins for combined S + P procedure was ineffective when compared with individual P (800, 1200 bar,  $N=4$ ) procedure. For example, extraction of proteins using P (1200 bar,  $N=4$ ) procedure gave  $Z_p \approx 0.57$  at  $E \approx 48$  kJ/g dry matter, and extraction using S (400 W) + P (1200 bar) gave  $Z_p \approx 0.32$  at  $E \approx 105.6$  kJ/g dry matter (Fig. 7). This possibly reflects a degradation of proteins or their irreversible binding to the cell wall provoked by US. Therefore, the application of individual S, P, or combined S + P procedures should be done based on the most suitable extraction selectivity, purity of extract and energy consumptions.

Note, that considerable specific energy consumptions were obtained for diluted suspensions ( $C_m = 1\%$ ). For concentrated suspensions, the extraction can be more effective in terms of energy consumption per g dry matter. Fig. 8 compares extraction behaviour with application of combined S + P procedure. In these experiments, the concentration was  $C_m = 10\%$  in the preliminary S (400 W, 30 min,  $\approx 5.6$  kJ/g dry matter) procedure and it was  $C_m = 1\%$  in the final P (400-1200 bar,  $N=4$ ) procedure. After the S procedure, the extraction indexes were  $Z_i \approx 0.18$ ,  $Z_c \approx 0.44$ ,  $Z_p \approx 0.09$ . Note that for concentration of  $C_m = 1\%$ , the similar preliminary S (400 W, 30 min,  $\approx 56$  kJ/g dry matter)

procedure gave  $Z_i \approx 0.10$ ,  $Z_c \approx 0.45$ ,  $Z_p \approx 0.16$ . Application of the final P procedure at  $p = 400$  bar ( $E \approx 21.8$  kJ/g dry matter) increased the level of  $Z_i$  up to  $\approx 0.76$  ( $p < 0.05$ ) and no further increase in  $Z_i$  was observed with an increase of  $p$  or  $E$  (Fig. 8). However, for carbohydrates and proteins, the extraction indexes  $Z_c$  and  $Z_p$  continuously increased with the increase of  $p$  or  $E$ . Finally at the pressure of 1200 bar ( $E \approx 53.8$  kJ/g dry matter), they reached values of  $Z_c \approx 0.83$  and  $Z_p \approx 0.74$ . These values can be compared with the maximum values of extraction indexes  $Z_i \approx 0.49$ ,  $Z_c \approx 0.69$ , and  $Z_p \approx 0.32$ , obtained using S (1%, 400 W, 30 min) + P (1%, 1200 bar,  $N = 4$ ) procedure ( $E \approx 105.6$  kJ/g dry matter). Therefore, the preliminary ultrasonication of more concentrated suspensions allowed increasing the extraction efficiency and decreasing the energy consumptions.

## 4. Conclusion

The application of individual S, P, or combined S + P procedures for ionic, carbohydrates, proteins and pigments recovery requires taking into account the selectivity of extraction, purity of extracts and energy consumptions. The S procedure allowed the disaggregation of cells and it can affect the structure of the cell walls. The P procedure was always applied to the 1% suspension and it also has supplementary effects on the damage of cells. This procedure can produce impurities, cell debris and provoke re-aggregation of cells. The concentration of the treated suspensions is also important. For diluted suspension ( $C_m = 1\%$ ), application of individual S or P procedure allowed extraction of components that can be arranged in the following order:  $Z_i < Z_p < Z_c$ . For combined S + P procedure, synergetic behaviour was observed during the extraction of ionic components, and it was absent for the extraction of carbohydrates. However, it was negative for the extraction of proteins. This reflects the formation of cell wall protein complexes induced by the disruption of cell walls during preliminary ultrasonication. Obtained data also allowed speculation that stabilization of dyes in water can reflect

the release of water-soluble proteins. The preliminary ultrasonication of more concentrated suspensions ( $C_m = 10\%$ ) followed by HPH of 1% suspensions allowed increasing the extraction efficiency and decreasing the energy consumptions.

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## **Declaration of contributions**

All authors have worked in the conception and design of the study. RZ performed experiments, preliminary analyzes of the results. NG designed the protocol and supervised the work. LM has provided the studied materials and discussed the results. RZ, NG, NL, EV have realized the interpretation of data, drafted and the revised the manuscript.

## **Conflict of interest statement**

We declare that this manuscript has not any potential financial or other interests that could be perceived to influence the outcomes of the research.

## **Statement of informed consent, human/animal rights**

No conflicts, informed consent, human or animal rights applicable

## **Declaration of authors**

All authors have approved the manuscript and agree with peer review process and its submission to Algal Research

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## Figure captions

**Fig. 1.** Schematic of applied extraction procedures for bio-molecules recovery from *P. kessleri* biomass.

**Fig. 2.** Scanning electron microscopy (SEM) images of untreated cells (U) (a), and the cells obtained after treatment using US treatment (S procedure) (400 W, 30 min) (b), and S + HPH treatment (P procedure) (1200 bar, 4 passes) (c) procedures. The suspension's concentration was 1% dry matter.

**Fig. 3.** Particle size distributions (PSD) of untreated suspensions (U) (a), and the suspensions obtained after treatment using US treatment (S procedure) (1% dry matter, 400 W, 30 min) + HPH treatment (P procedure) (1% dry matter, 400-1200 bar, 4 passes) (b), and S (1% or 10% dry matter, 400 W, 30 min) + P (1% dry matter, 1200 bar, 4 passes) procedures (c).

**Fig. 4.** Optical microscopy images of untreated suspensions (U) (a), and the suspensions obtained after treatment using US treatment (S procedure) (400 W, 30 min) (b), and S + HPH treatment (P procedure) (1200 bar, 4 passes) (c) procedures. The suspension's concentration was 1% dry matter.

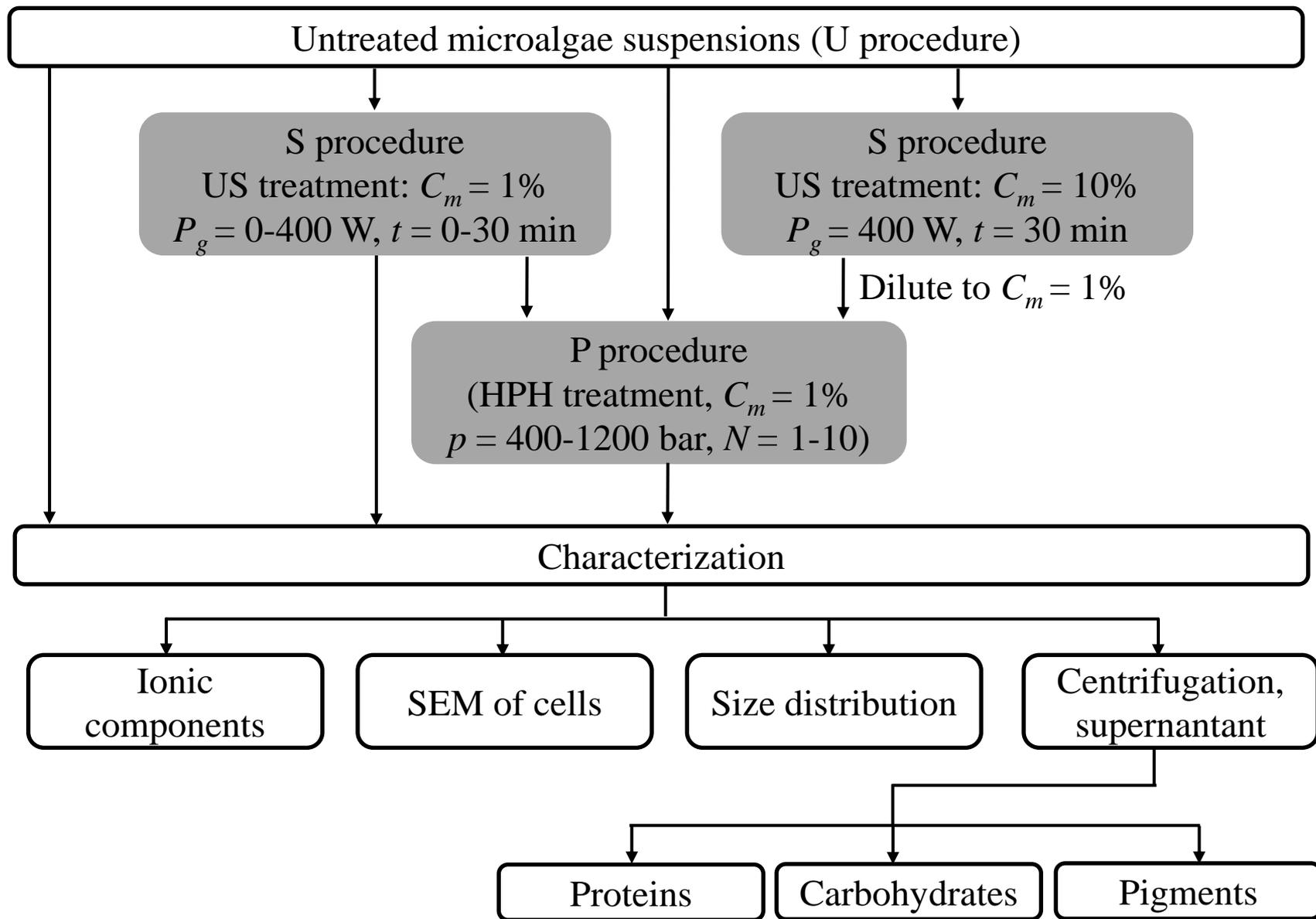
**Fig. 5.** Ionic ( $Z_i$ ), carbohydrate ( $Z_c$ ), protein ( $Z_p$ ) (a), and pigment ( $Z_d$ ) (b) extraction indexes versus the time ( $t$ ), for US treatment at different powers (0-400 W). The suspension's concentration was 1% dry matter, and the value of  $Z_d$  was measured at two different wavelengths  $\lambda_v = 400$  nm (violet) and  $\lambda_r = 680$  nm (red).

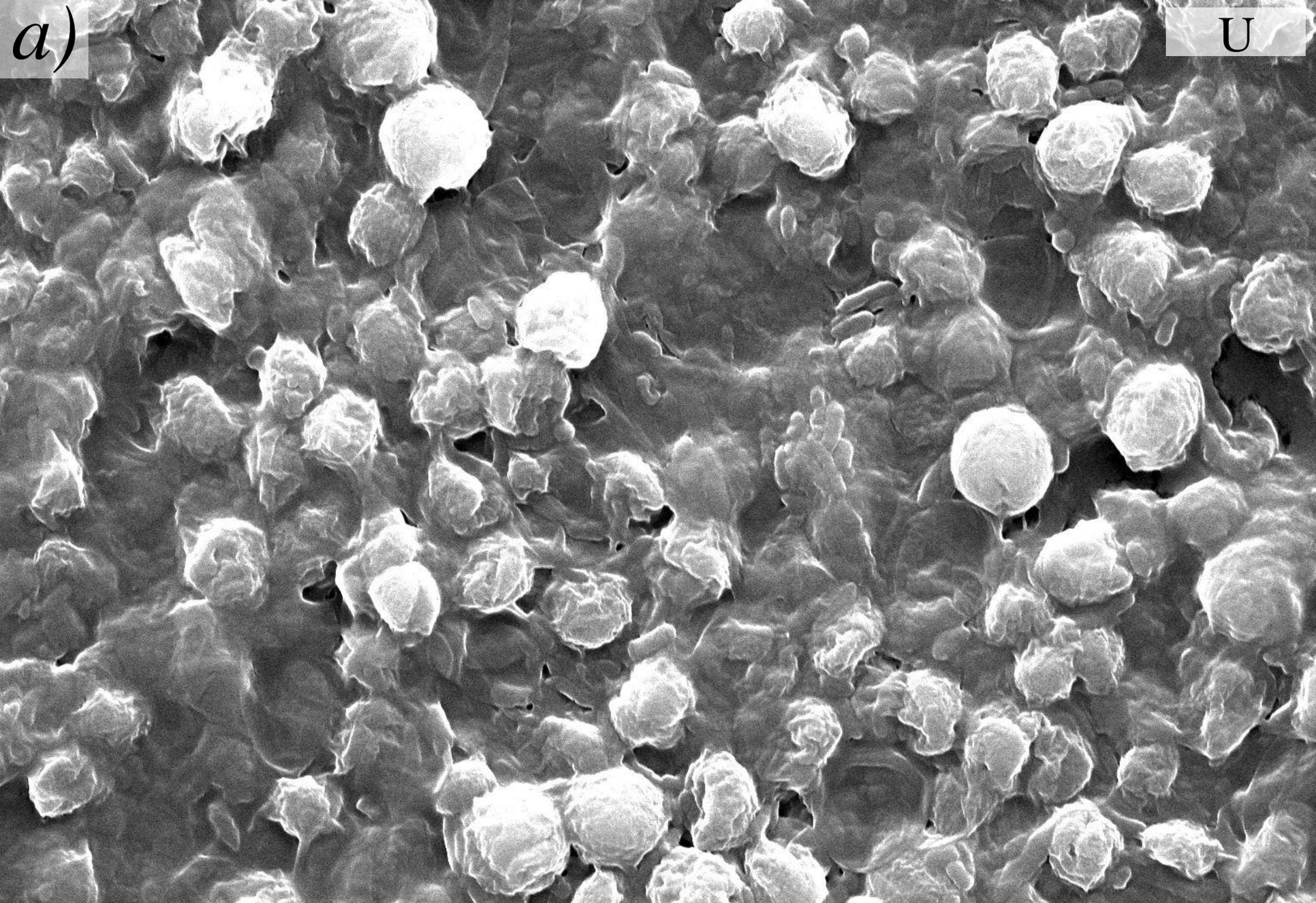
**Fig. 6.** Ionic ( $Z_i$ ), carbohydrate ( $Z_c$ ), protein ( $Z_p$ ) (a), and pigment ( $Z_d$ ) (b) extraction indexes versus the number of HPH passes ( $N$ ) at different pressures (400-1200 bar). The suspension's concentration was 1% dry matter, and the value of  $Z_d$  was measured at two different wavelengths  $\lambda_v = 400$  nm (violet) and  $\lambda_r = 680$  nm (red).

**Fig. 7.** Ionic ( $Z_i$ ), carbohydrate ( $Z_c$ ) and protein ( $Z_p$ ) extraction indexes versus the specific energy consumption ( $E$ ), for treatment using individual US treatment (S procedure) (100-400 W, 30 min),

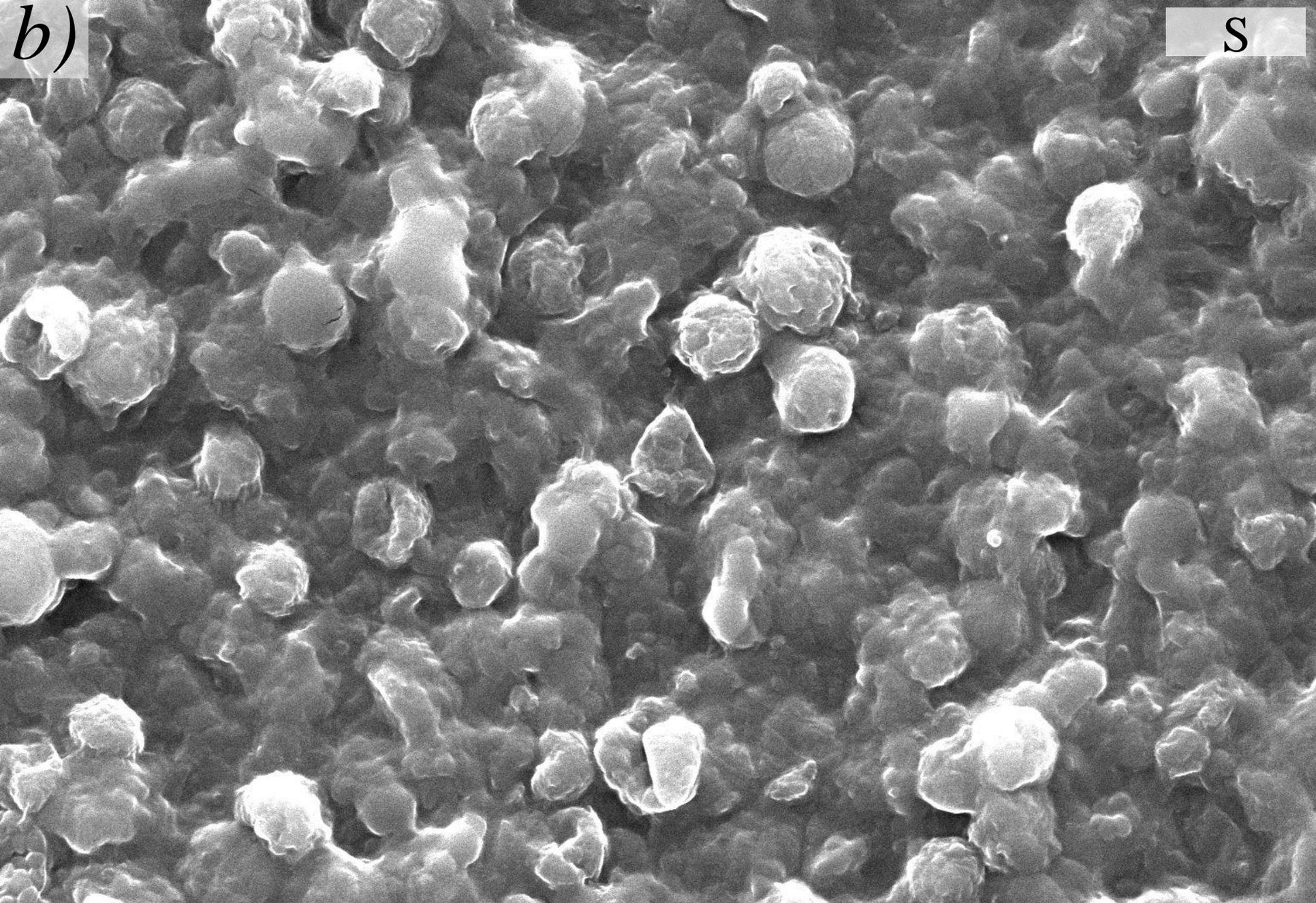
HPH treatment (P procedure) (400-1200 bar, 4 passes) and combined S (400 W, 30 min) + P (400-1200 bar, 4 passes) procedures. The suspension's concentration was 1% dry matter.

**Fig. 8.** Ionic ( $Z_i$ ), carbohydrate ( $Z_c$ ) and protein ( $Z_p$ ) extraction indexes versus the specific energy consumption ( $E$ ), using individual US treatment (S procedure) (10% dry matter, 400 W, 30 min), and combined US treatment (S procedure) (10% dry matter, 400 W, 30 min) + HPH treatment (P procedure) (1% dry matter, 400-1200 bar, 4 passes). Significant differences ( $p < 0.05$ ) between different treatments are represented by different letters.

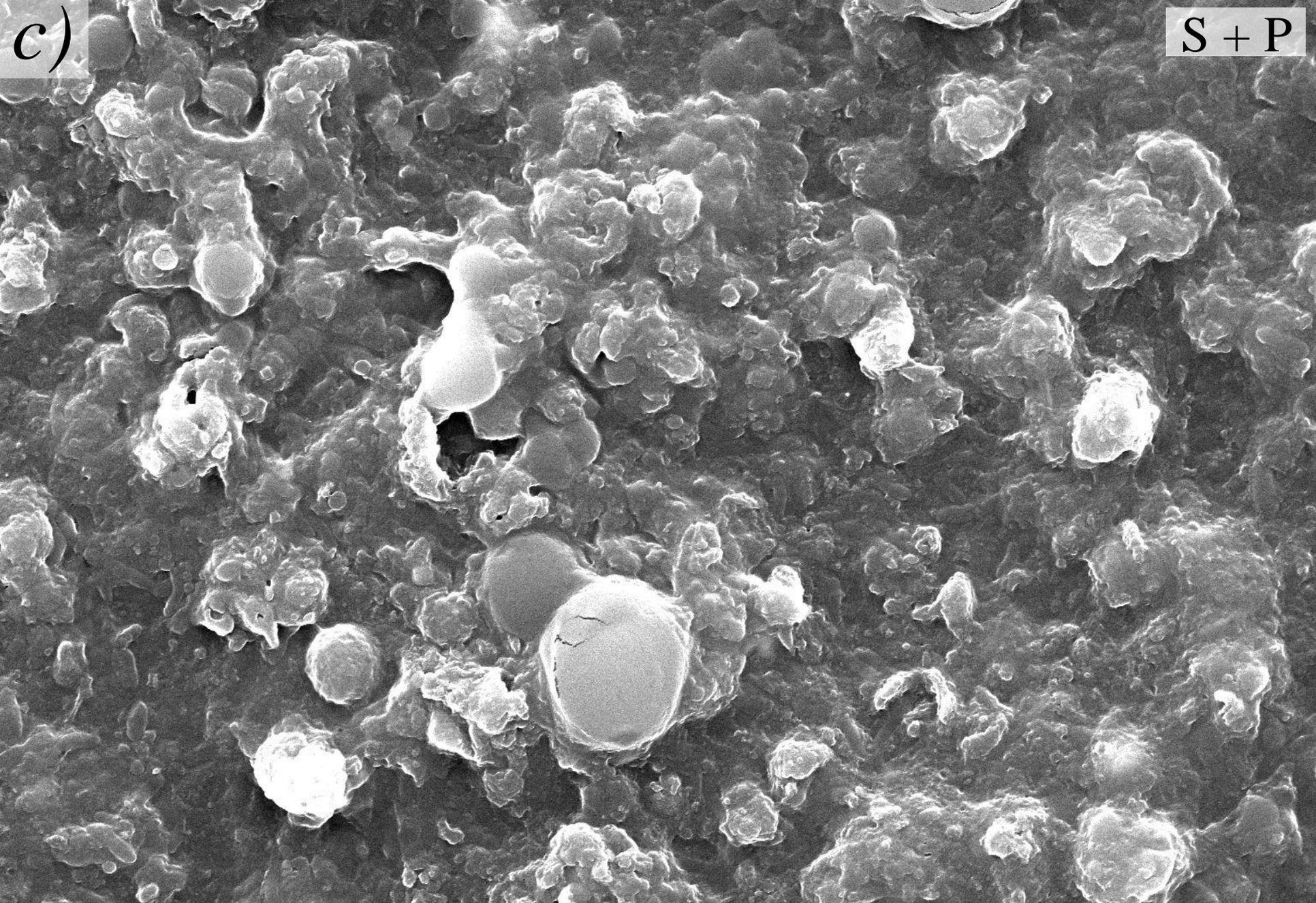




	HV	mag 只	det	mode	WD	HFV	pressure	10 μm	
	20.00 kV	2 500 x	ETD	SE	9.7 mm	50.8 μm	3.19e-5 Torr	Quanta FEG 250	

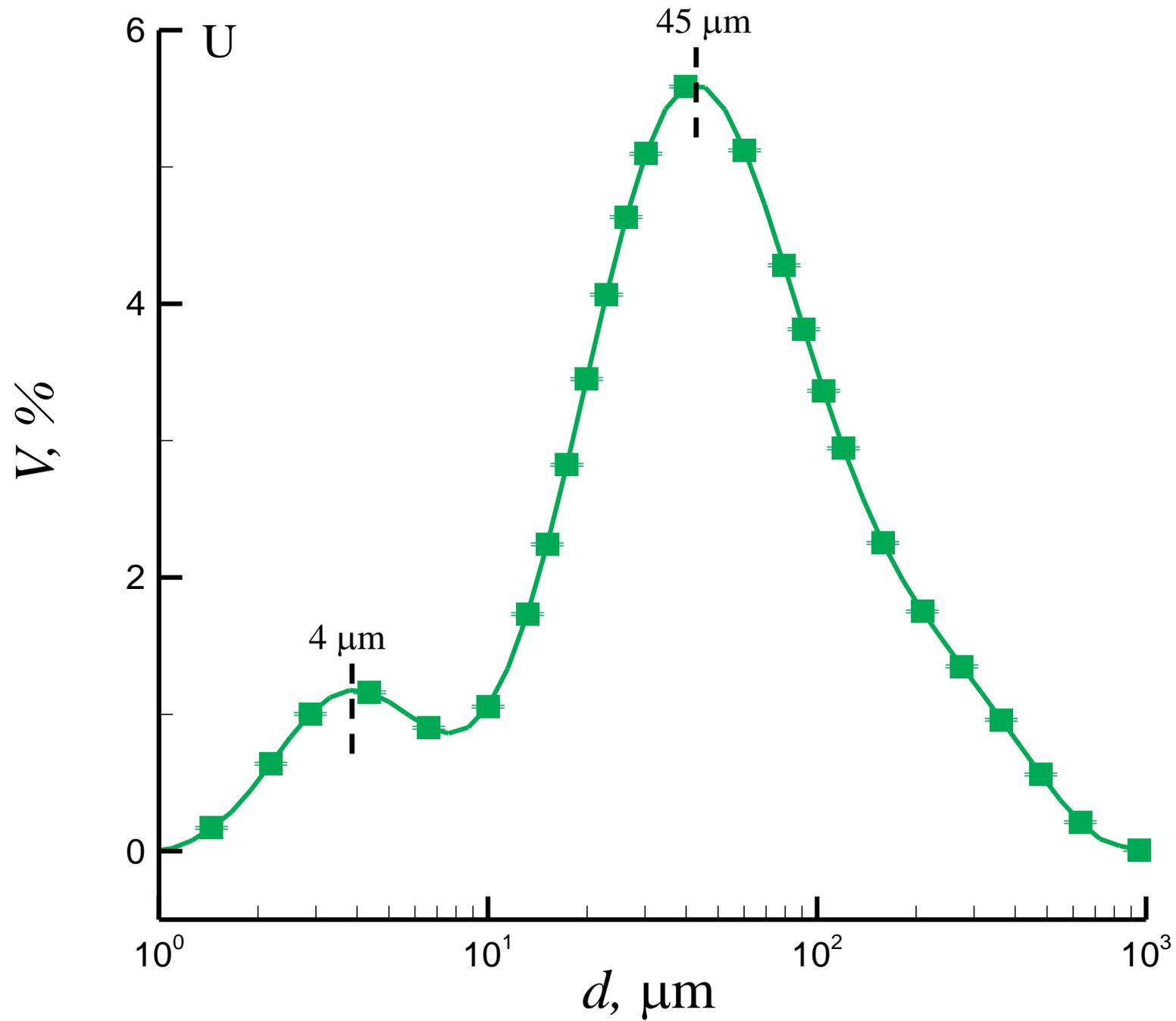


	HV	mag	det	mode	WD	HFV	pressure	10 $\mu$ m
	20.00 kV	2 500 x	ETD	SE	9.7 mm	50.8 $\mu$ m	1.74e-5 Torr	

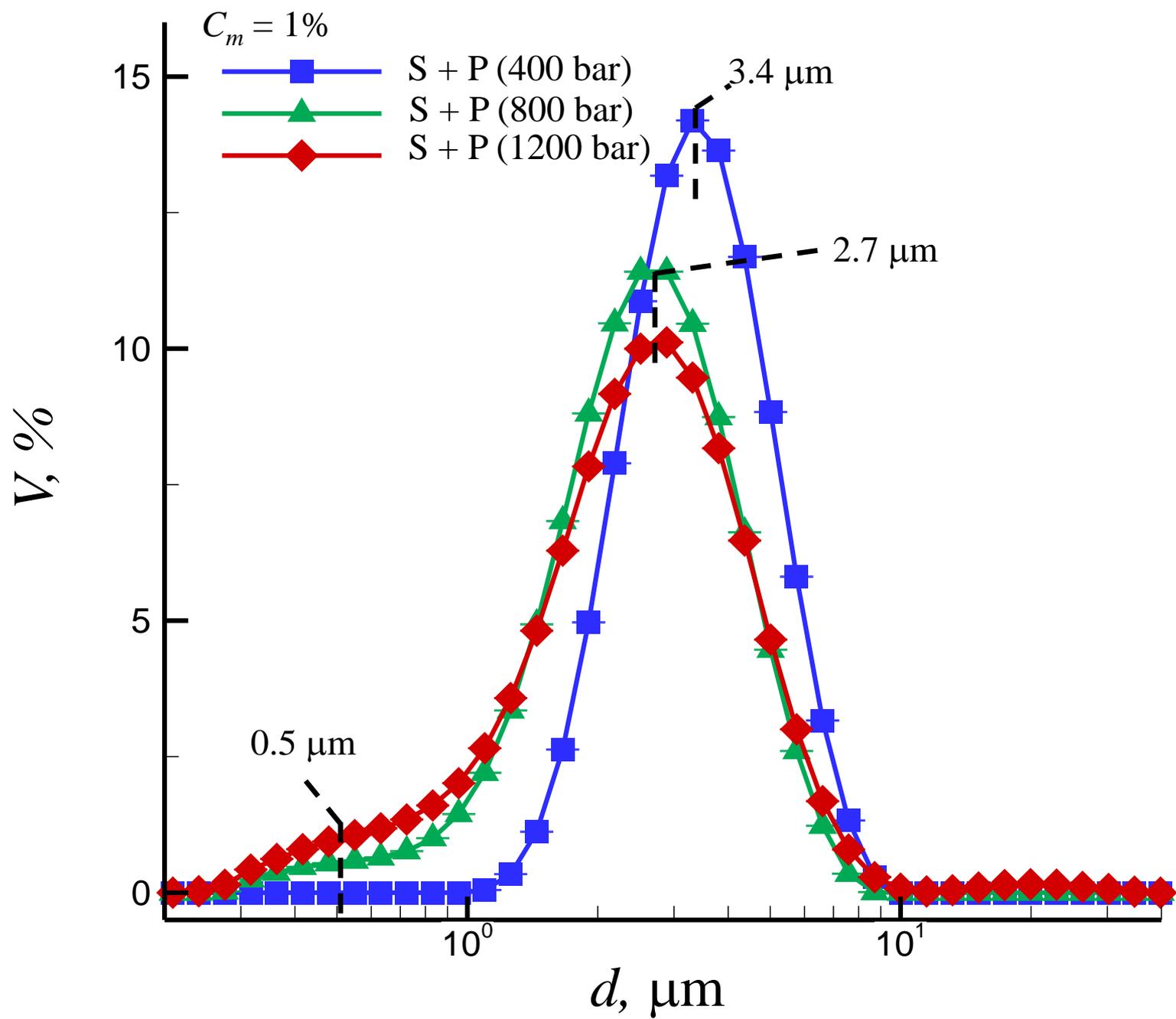


	HV	mag	det	mode	WD	HFV	pressure	10 $\mu$ m
	20.00 kV	2 500 x	ETD	SE	9.5 mm	50.8 $\mu$ m	7.73e-6 Torr	

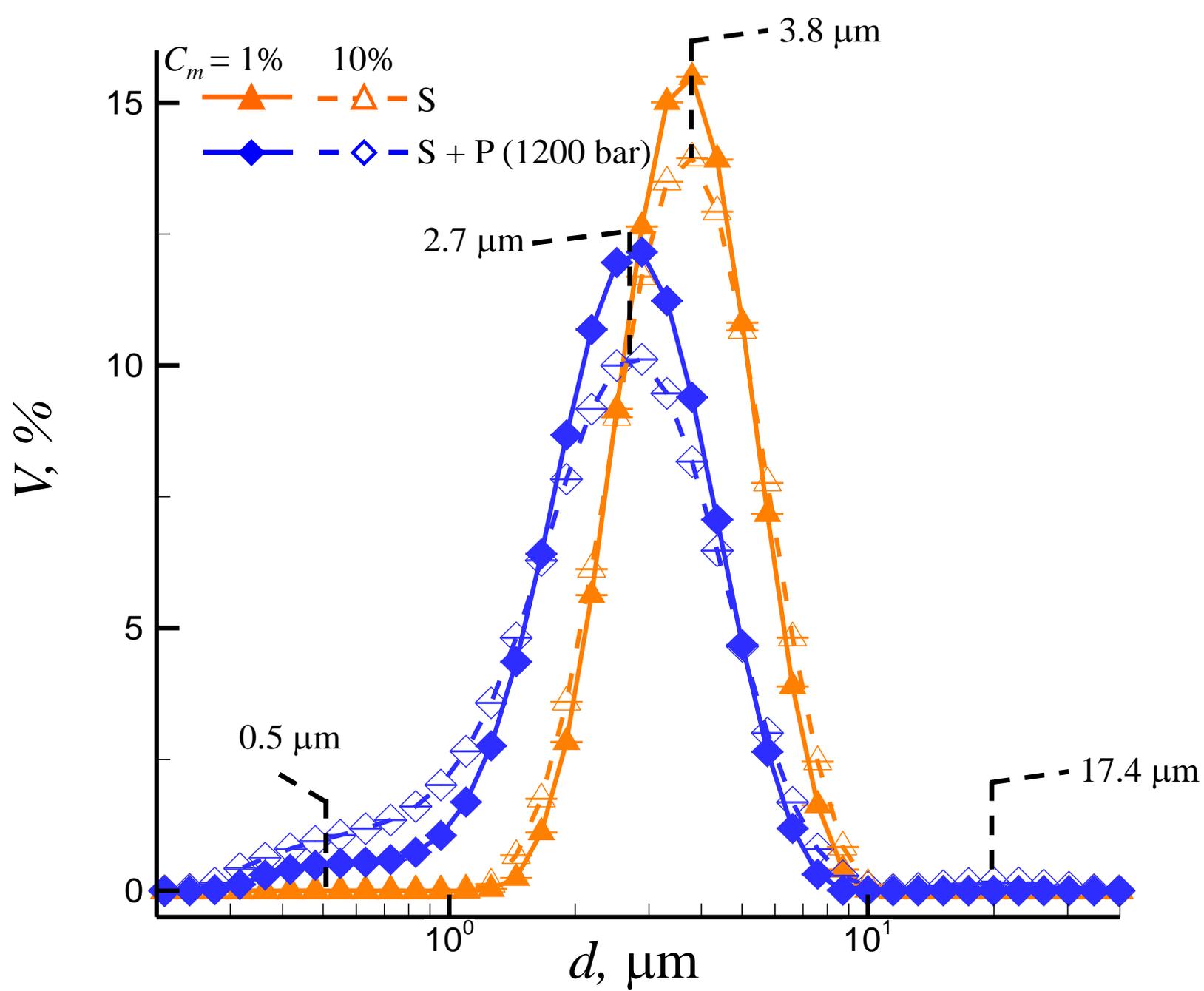
a)



b)

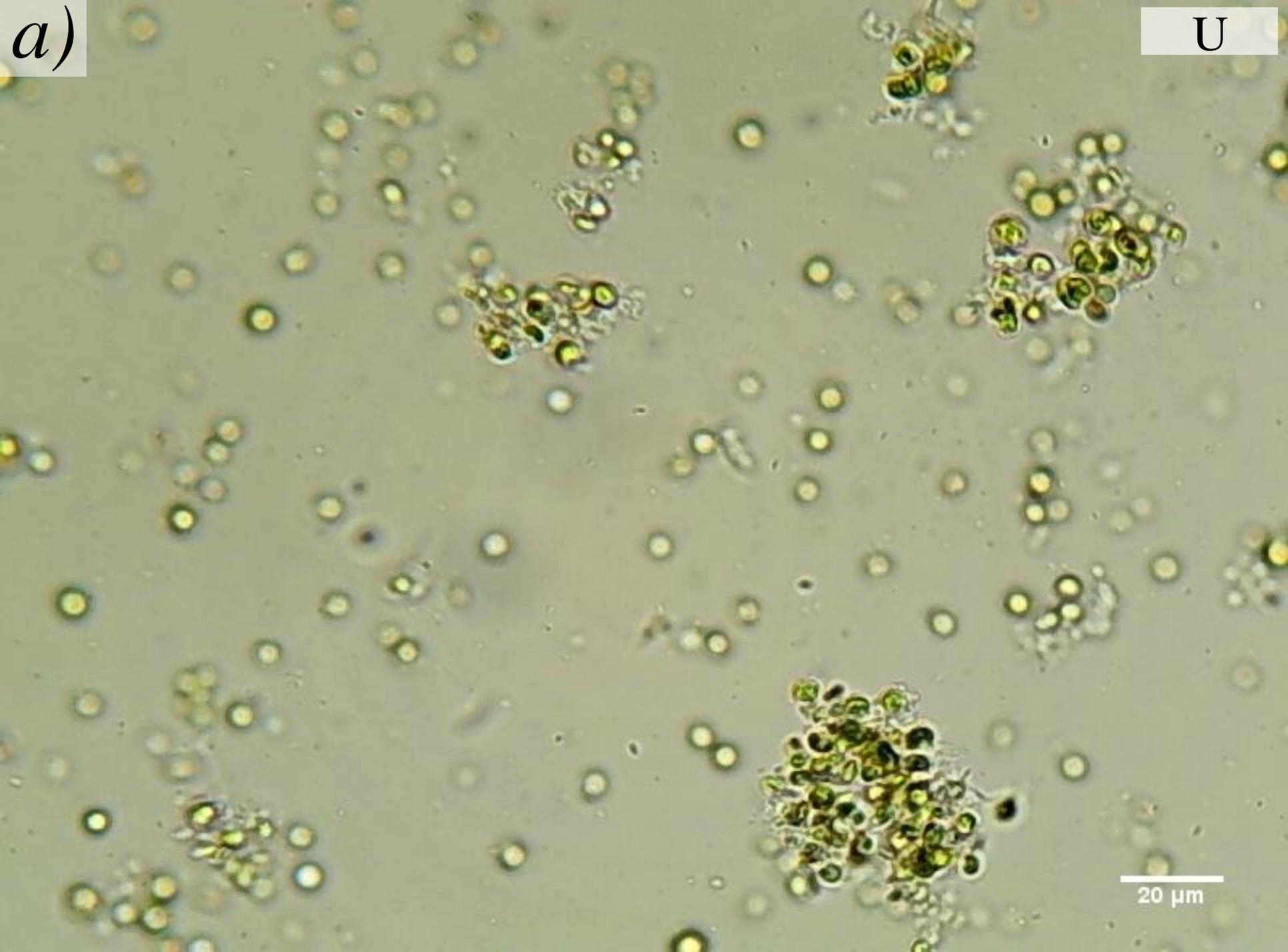


c)



*a)*

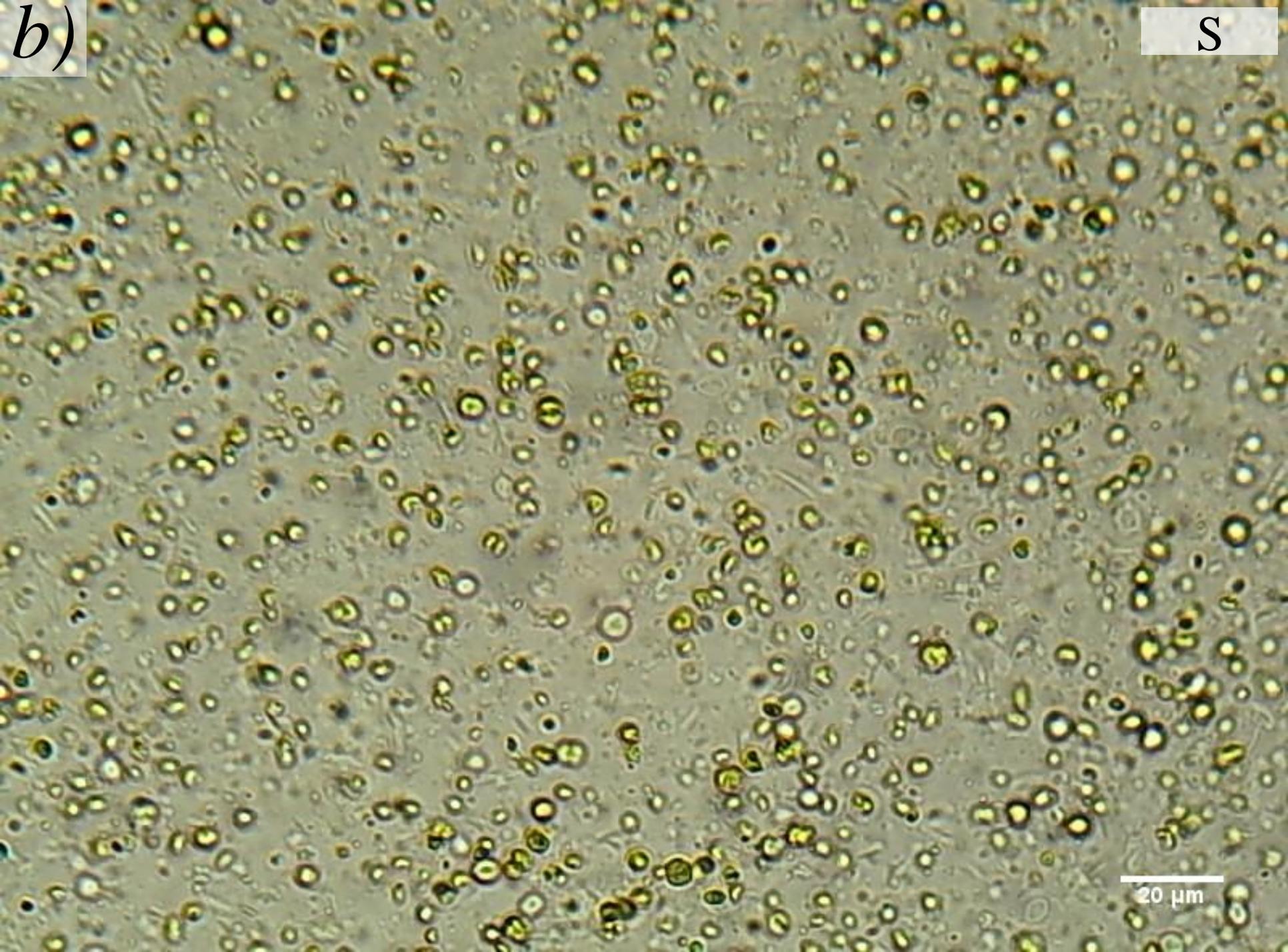
U



20 μm

*b)*

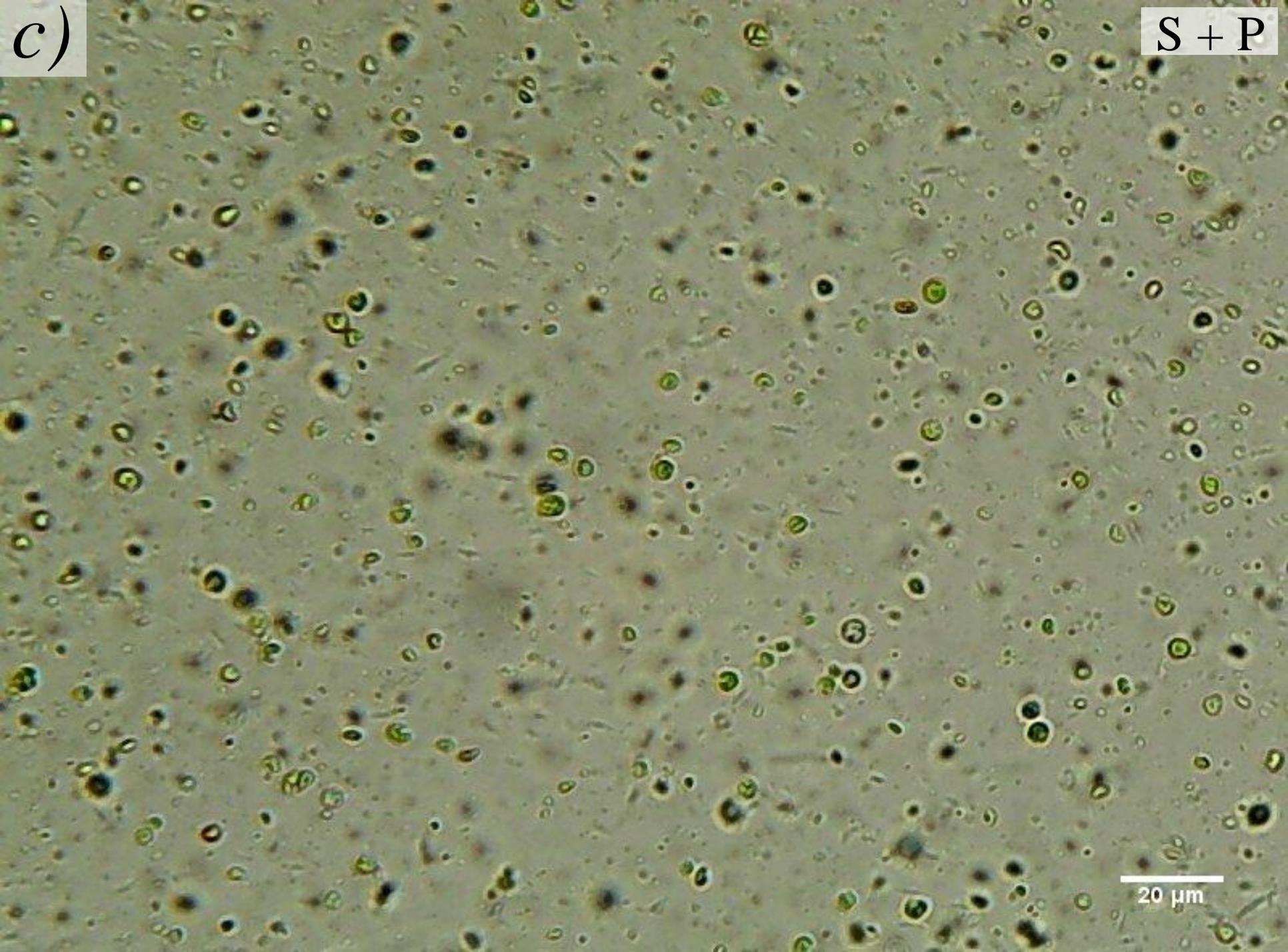
S



20  $\mu\text{m}$

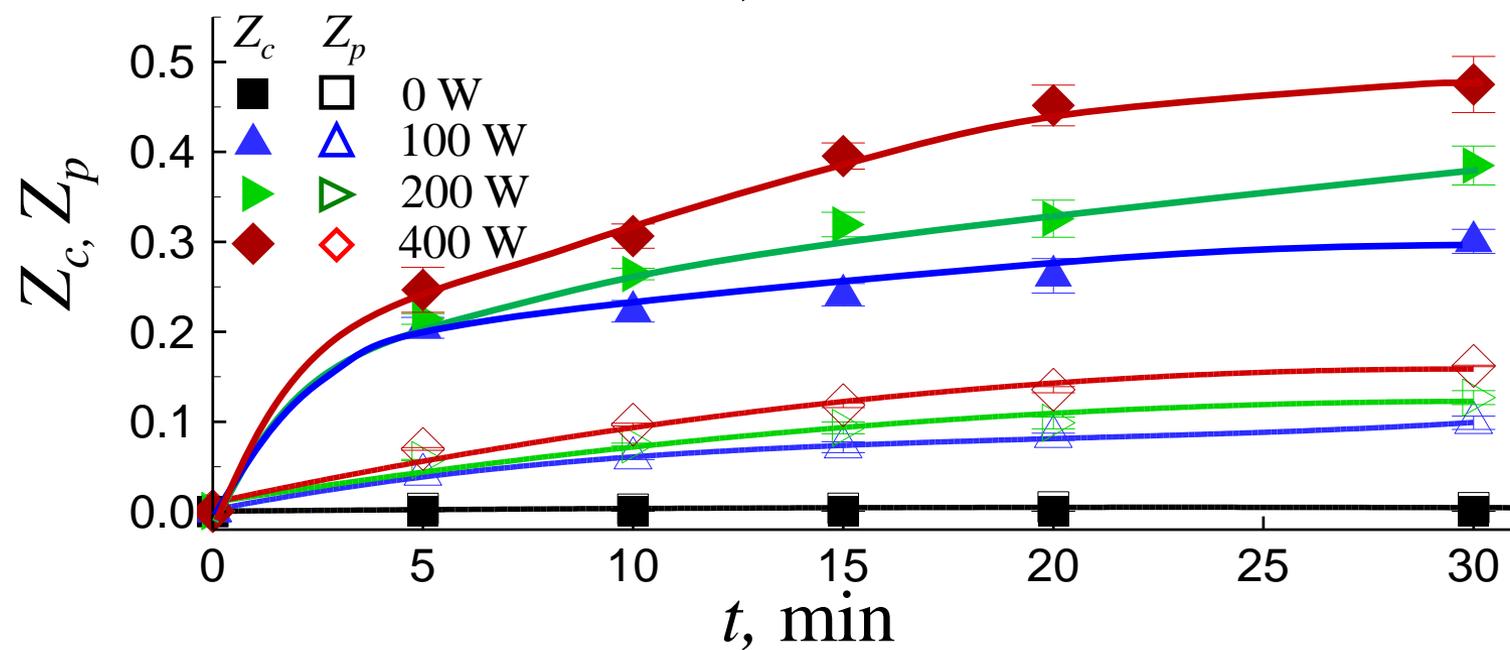
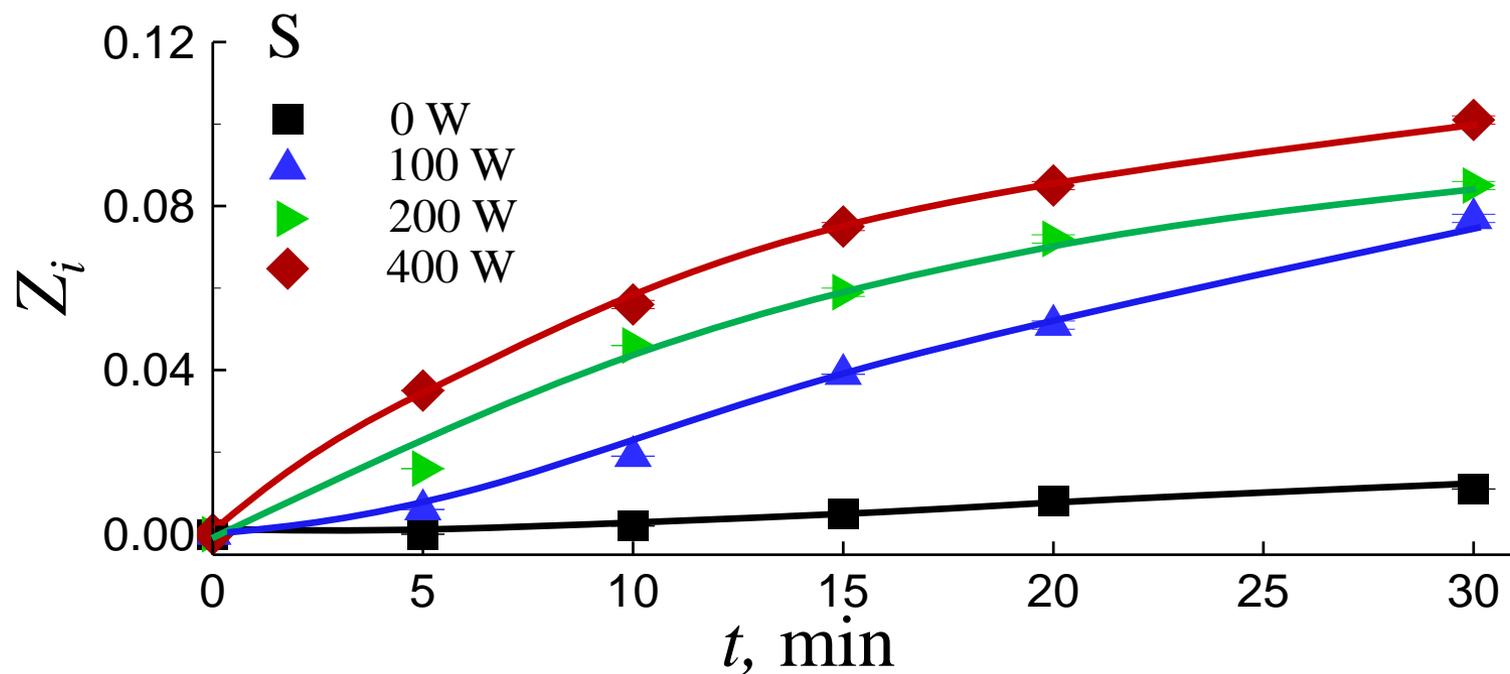
c)

S + P

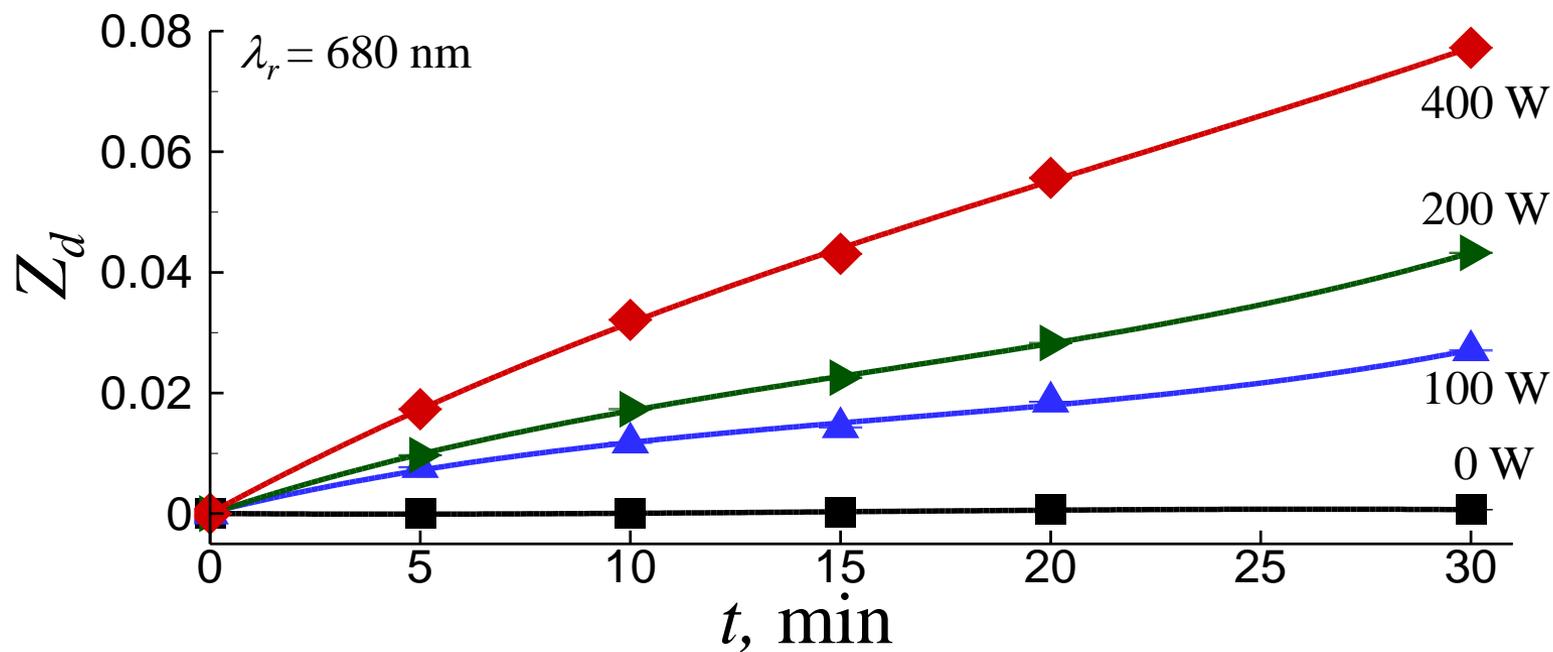
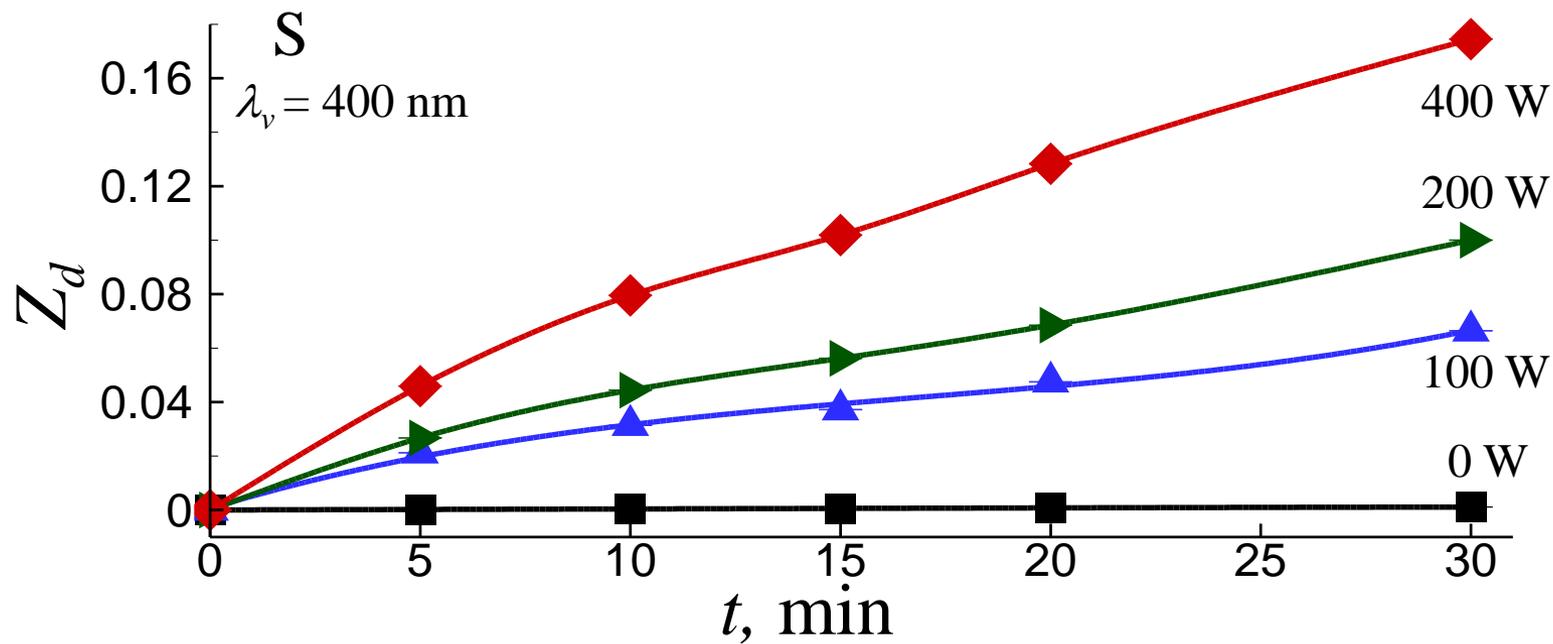


20 μm

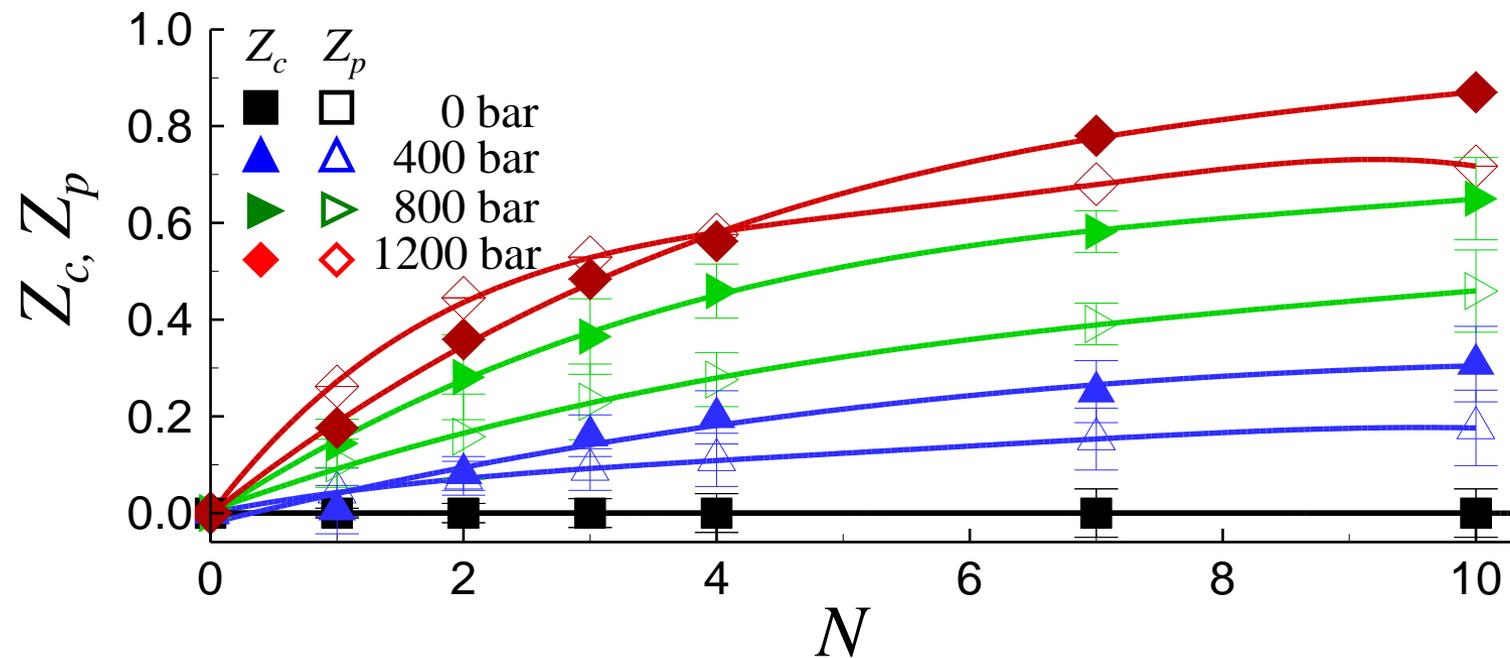
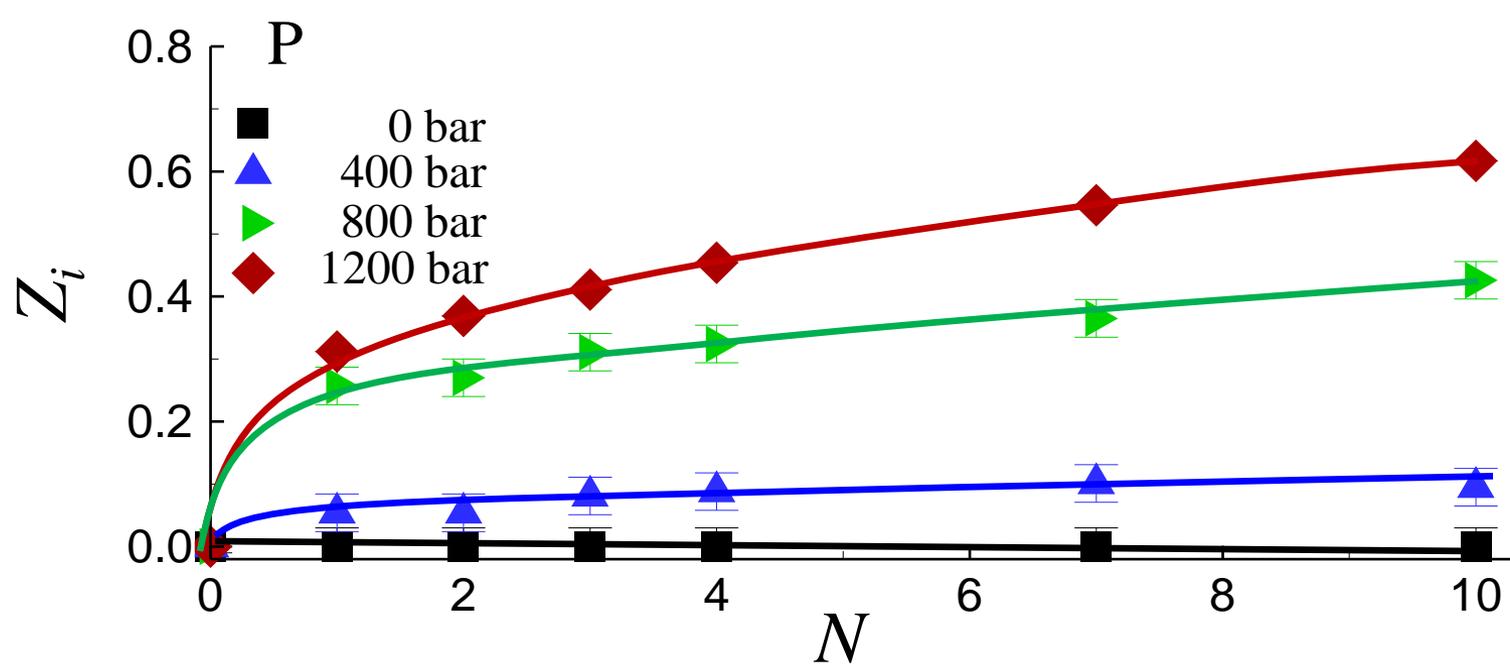
a)



b)



a)



b)

