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Morphological and cellular organization of green microalgae to cope with cold stress in subarctic environment

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ABSTRACT

Microalgae are one of the most widely dispersed living organisms on Earth and can be found even in extreme environments. Especially in such habitats, the microalgal cell wall plays an essential role as it is the first barrier in continuous contact with the surrounding and changing environment. In cold conditions microalgae can show changes in their morphology, also known as phenotypic plasticity which is the ability of an organism to show different phenotypes when exposed to different environmental conditions. In addition, presence or absence of algaenan is thought to be responsible for increase/decrease permeability and stiffness of the cell wall. The aim of this work was to evaluate and compare how microalgae cells can modify their cell wall components and cellular morphology under low-temperature conditions (5 °C) and differ during the exponential and stationary phases. Four microalgae species were studied: *Coelastrella* sp. 3–4, *Chlorella vulgaris* sp. 13–1, *Haematococcus pluvialis*, and *Scenedesmus* sp. B2–2, which were isolated from subarctic locations.

Using a histochemical approach in conjunction with light microscopy, cell features such as size, organization and cell wall ornamentation were evaluated. Staining procedures showed changes in biochemical components such as pectins and presence or absence of exopolysaccharides and lipids. Results showed that *Coelastrella* cultures did not grow under low-temperature conditions. However, *Chlorella vulgaris, Haematococcus pluvialis* and *Scenedesmus* species demonstrated a slower growth rate, bigger and rounded cell-shape during cold condition. Furthermore, the latter microalgal strain also showed modification in algaenan presence as one of the main components in cell wall architecture, which can be related to the permeability of cell wall. Changes in other features such as cell organization and cell wall ornamentation were investigated.

1. Introduction

Phenotypic plasticity is referred to the ability of a single genotype to show two or more phenotypes induced by environmental factors [1]. This biological phenomenon can be found in a variety of organisms, from plants to vertebrate animals [2–4] providing them an adaptive advantage to increase their chances of evolutionary success in the environment. The adaptation translates into morphological, biochemical and physiological changes resulting in a different appearance in a specific habitat.

Microalgae are phototrophic organisms and one of the most widely distributed groups of organisms on Earth, capable of living in environments with extreme growth conditions such as high salinity [5], high CO_2 levels [6], pH [7], temperature and light [8].The latter two are closely related to arctic and subarctic zones, where there is a large fluctuation of temperature and light intensity during the seasons of the year.

Cold environments are described as those in which temperature decreases and remains around 0 °C for long periods during the year. Such environments exist in a large part of the Earth's surface and therefore surviving in such environments is a challenge for lifeforms. Psychrotolerant organisms are those that can live in habitats with temperature fluctuations occurring between 0 and 30 °C. Moreover, due to climate change, events with extreme temperatures are more common every year. Different adaptative mechanisms have been described in microalgae that help them survive in these conditions. *Chlamydomonas nivalis* increases different compounds such as extracellular polysaccharides and carotenoids to facilitate the adaptation [9]. *Coccomyxa subellipsoidea C-169* produces anti-freeze proteins to resist low temperatures (cold tolerant) [10]. Under cold conditions, the photosynthesis in vascular

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plants decreases and the growth rate also slows down [11–13].

In contrast to vascular plants, microalgae mostly present a single lifestyle. Therefore, there is a need for them to have a resistance that should act as a physical barrier to protect their cells against external and changing environments. Depending on the species, cell walls are mostly composed of complex polysaccharides and glycoproteins. Regarding organization, cell walls are organized in different layers, which can change during the cell cycle. Accordingly, cell wall resistance to extreme environmental conditions is crucial for the survival of these photosynthetic organisms. It has been documented that microorganisms possess adaptive strategies to maintain the fluidity of membranes at low temperatures such as changes in fatty acids profile [14–16]. *Klebsormidium* cell walls are coated with extra-layers as a reinforcement under freezing conditions [17]. Cell wall resistance can be classified into two types according to their chemical composition [18,19]. Algaenan is a key compound that can make the difference in cell wall strength and permeability as shown in recent studies [20–22]. Algaenan, also known as a sporopollenin, is a biopolymer with a complex composition that stands out as a highly acid-base resistant material. In most of the cell walls of green coccoid microalgae, the algaenan is part of the trilaminar structure-type (TLS) cell wall, which is made up of sandwich-like layers that are 10–20 nm thick. The outer layers have a high electron density while the inner layer has a low electron density that provides a protective layer against external biotic or physicochemical factors [23]. The knowledge of the presence, function and composition of the algaenan is essential to identify an efficient method for cell wall disruption as part of pre-treatment processes in bio-refineries. For example, microalgae are known to store high-value and interesting compounds for the biotechnology industry, such as pigments, lipids, and proteins, among others [23,24]. The presence/absence of algaenan can vary greatly within the same microalgal genus [25], as in the case of the genera *Chlorella* and *Scenedesmus* [22,26,27] as well as in *Haematococcus* [28–30]. However, there is a lack of knowledge about the changes in algaenan in algal cell walls because of cold stress.

Umeå is in the subarctic zone in Northern Sweden (63° 49′42 N, 20°15′34 E), where the average temperature during the winter can reach below −10.7 °C [31]. Some microalgal species isolated in the Umeå region belong to the *Scenedesmus* and *Chlorella* genera, and *Coelastrella* was for the first time identified in North Sweden by Ferro et al. [32].

The clade Chlorophyta encompasses most of the green algae and the majority are unicellular organisms. Due to their rapid growth, high production of biomass and valuable compounds there has been an increased biotechnological interest for these organisms in recent decades. Within the clade, the genera *Chlorella, Scenedesmus, Haematococcus, Botryococcus* and *Chlamydomonas* have received a particular interest for bio-refineries and biotechnological companies.

Chlorella is one of the most difficult genera to classify due to great variety of shapes, organization, and number of pyrenoids. Previous studies have reported an average size of 3.5-5 μm [33]. However, the structure and composition of the cell wall is challenging to characterize, and it is a subject of study for many research groups and industry. The species of the genus *Coelastrella* are unicellular coccoid or elliptical shaped cells. The uniqueness of this genus is the presence of ornamentation on their cell wall with numerous meridional ribs [34,35]. On the other hand, *Scenedesmus* is mostly represented by coenobium of 2 or 8 ellipsoid cells, enveloped by a TLS-type cell wall [36]. *Haematococcus pluvialis* is one of the strains with the greatest biotechnological interest because of its production and storage of astaxanthin in the cytoplasm, a natural and high-value carotenoid. Its cell wall is characterized by high resistance and low permeability, thus increasing the costs of cell disruption in extraction processes. *Haematococcus pluvialis* present round and unicells and they can show two types of morphologies, flagellate and aplanospore along their cell cycle. Regarding cell wall architecture, *H. pluvialis* also present TLS type cell wall, which includes up to 5 stages in its cell wall layer distribution throughout its life cycle. [29].

Currently, a limited number of studies have focused on understanding the adaptation of microalgal cell walls to cold environments. Furthermore, there is a scarcity of knowledge regarding the specific impact of these adaptations on the presence or absence of compounds such as polysaccharides, pectins, and lipids, as well as their micro-distribution within the cell. In this work we have therefore employed a microscopy approach in combination with different histochemical techniques to study microalgae *Coelastrella* sp. 3–4, *Scenedesmus* sp. B2–2, *Chlorella vulgaris* sp. 13–1 and *Haematococcus pluvialis*. The stains selected for this study work were Ruthenium Red, Toluidine Blue O (TbO), Crystal Violet and Sudan Black B (SBB) because of their minimal requirement of biological material, ease of use, speed of application, and the ability to provide direct results in less than 1 h for Ruthenium Red, TbO, and SBB, and within 24 h for Crystal violet. As a result, it becomes possible to rapidly visualize the key compounds above-mentioned and their micro-distribution at the cellular level.

A comparative analysis was conducted for investigating different morphological and biochemical strategies adopted by these microalgae to cope with the cold environment including changes occurring during exponential and stationary phases.

2. Material and methods

2.1. Microalgae cultivation

The microalgal strains studied were *Coelastrella* sp. (3–4), *Scenedesmus* sp. (B2–2), *Chlorella vulgaris* (13−1) that were isolated and genetically characterized by Ferro et al. [32], and *H. pluvialis* strain isolated at Swedish University of Agricultural Sciences in Umeå (F. Gentili pers. comm.) and genetically characterized by Martínez et al. [37]. Microalgae species were cultivated in transparent glass bottles containing 500 mL Bold's basal medium (BBM) and placed in a plant growth cabinet (Conviron A1000, Conviron, Winnipeg, Canada). Growth parameters include a control condition (25 °C, 18:6 [L: D] at 130 μmol m^{-2} s^{-1}) and cold conditions (5 °C, 18:6 [L: D] at 130 μmol m^{-2} s^{-1}). The culture bottles were shaken at 120 rpm with a bubbling rate of 0.1–0.2 L air/min. All experiments were performed in three biological replicates.

2.2. Sample collection

Optical density (OD) of the cultures was measured using an UV–Vis spectrophotometer (Biotek Epoch 2 Microplate Reader, USA) at wavelength of 750 nm. The microalgae in control condition were harvested during their exponential growth phase (12 days) and at stationary phase (21 days). However, cultures grown under cold temperature were harvested at exponential and stationary phase after 40 and 55 days, respectively. The specific growth rates for each microalgal specie were calculated using the formula $\mu = \ln(N_2/N_1)/ (t_2 - t_1)$, where μ is the specific growth rate, and N_1 and N_2 are the biomass at time 1 (t_1) and time 2 (t_2), respectively.

The cultures were collected by centrifugation at 4000 *g* for 5 min. After harvesting, microalgae cells were washed three times with MiliQ water flash-frozen with liquid nitrogen and stored at −80 °C until microscopy studies.

2.3. Light microscopy

Histochemical microscopy techniques were carried out to investigate presence and micro-distribution of cell wall compounds and evaluate their resistance during control and cold conditions. For all the staining techniques, 10 μg ultra-concentrate sample was used for each microalgal strain. The presence/absence of algaenan was performed using Crystal violet staining according to Zych et al. [26] with a 0.2 % (*w/v*) aqueous final concentration and 24 h incubation under continuous agitation. 0.02 % (w/v) aqueous Ruthenium red and metachromatic dye of 1 % Toluidine blue O (TbO) in 0.1 % Borax solution (w/v) were used to

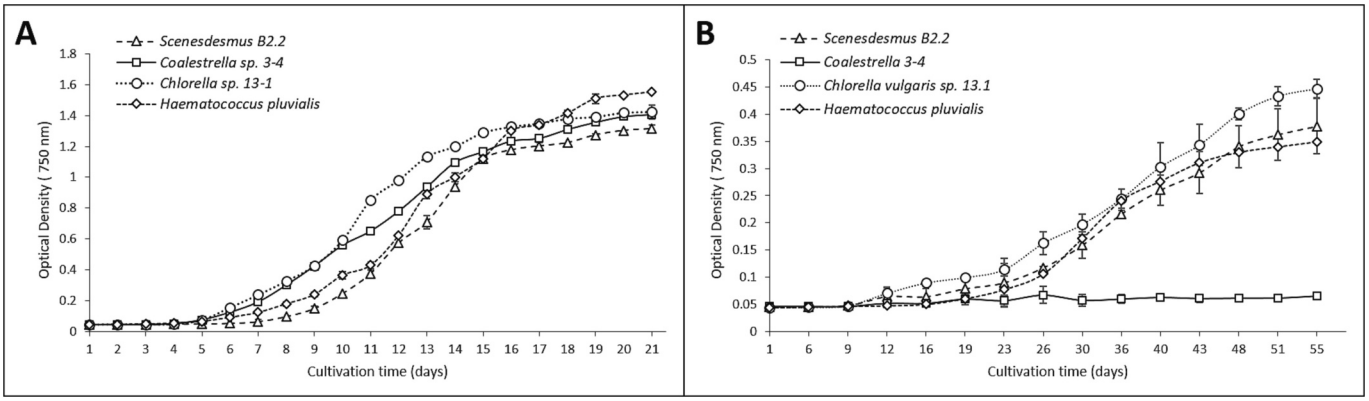

Fig. 1. Comparison of growth curves for selected sub-arctic microalgae under different temperatures of 25 °C (A) and 5 °C (B).

investigate pectin-rich material and anionic polysaccharides respectively [38]. The samples were stained, using Ruthenium red or TbO for 15 min under gentle agitation. Three washes with distilled water and centrifugation (4000 *g*, 5 min) were performed after each staining with Crystal violet, Ruthenium red and TbO.

For localization of lipids, 0.5 % Sudan black B (SBB) in 70 % ethanol solution (*w/v*) was utilized. Samples were pre-fixed with 70 % ethanol for 1 min before staining using Sudan black B. After centrifugation (4000 *g*, 5 min), the pellet was incubated with SBB for 30 min with continuous gentle agitation, washed 3 times with 70 % ethanol followed by a final wash with MiliQ water.

All stained samples were placed onto glass slides, mounted in glycerol (50 % *v/v*) and examined using a Leica DMLB light microscope (LM) with digital images recorded using an Infinity X-32 camera (DeltaPix, Denmark). All micrographs were analyzed (e.g., counting stained cells, size etc.) using Image J as image analysis software [39].

3. Results and discussion

3.1. Effects of cold condition on growth and cellular organization of microalgal strains

The growth of the selected species was evaluated at two temperatures conditions (25 and 5 °C) to investigate their physiological responses of temperature tolerance. In this study, all microalgal strains had a normal growth at 25 °C as expected showing typical s-shaped growth curves (sigmoidal curves) with a similar growth rate (25 °C) (Fig. 1A), giving daily growth rate (μ) values of 0.365 for *C. vulgaris* and 0.371 for *H. pluvialis*. *Scenedesmus* sp. exhibited the highest growth rate with a value of 0.432 while *Coelastrella* sp. had the lowest growth rate of 0.182. However, although *C. vulgaris*, *Scenedesmus* sp. and *H. pluvialis* grew at low temperature conditions, no growth for *Coelastrella* sp. 3–4 cultures were observed during the cultivation at 5 °C showing an approximately constant daily growth rate between 0.000 and 0.009. The growth rates of the other three microalgae during the low temperature were observed to be 0.053 for *C. vulgaris*, 0.035 for *H. pluvialis* and 0.046 for *Scenedesmus* sp. B2–2. The values showed that their growth rates were reduced by ca 10 times under low temperature condition when compared to that of the control condition (Fig. 1A vs 1B). For example, the midpoint of the exponential phase under control condition was reached at ca 12 days. However, the same growth rate did not reach until 36 days after inoculation at 5 °C condition. A similar result was observed for the stationary phase with 16 and 51 days under control- and cold conditions respectively (Fig. 1A vs 1B). Moreover, cold condition also had effects on biomass production which was reflected in OD values (i.e., y-axis of Fig. 1A, B), measured at 750 nm. The growth curves showed that exponential and stationary phases reached 0.8–1 and 1.2–1.5 OD respectively under optimal conditions (Fig. 1A). However, the cultures grown under cold condition showed much low values of ca 0.2 and 0.35–0.45 OD for exponential- and stationary phases respectively indicating negative effect of cold stress on the biomass production (Fig. 1 B).

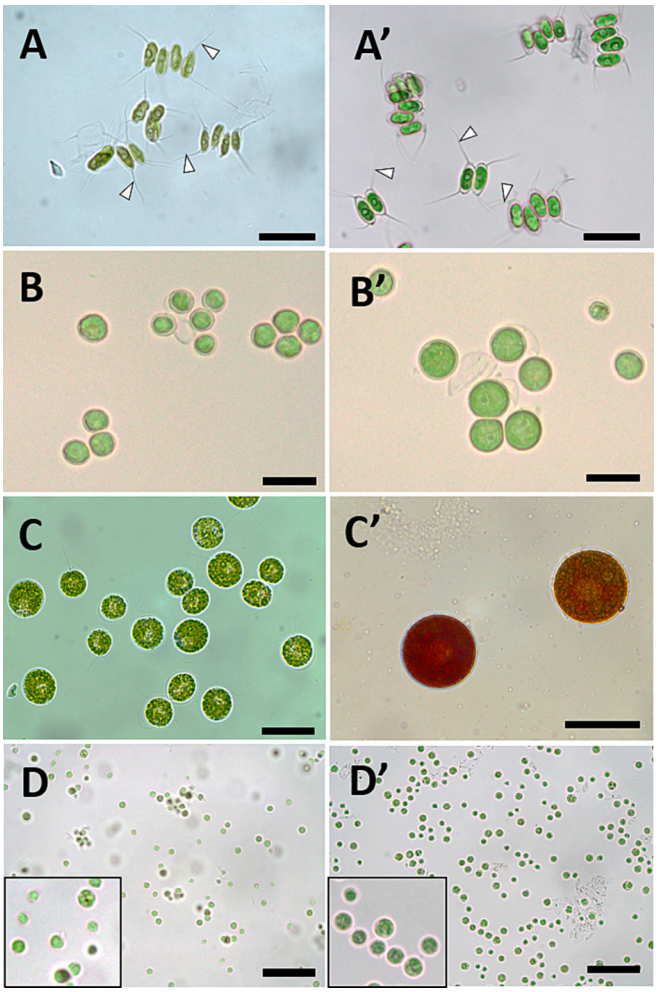

Fig. 2. Light micrographs of sub-arctic microalgae isolated under control conditions (25 °C) at exponential (A, B, C and D) and stationary stages (A', B′, C′ and D′): *Scenedesmus* sp. B2–2 (A, A') in which white arrowheads indicate typical terminal spines in the coenobium. *Coelastrella* sp. 3–4 (B, B′), *Haematococcus pluvialis* (C, C′) and *Chlorella vulgaris* sp. 13–1 (D, D′) Insets in the Figs. D and D′ show high magnification images of the same relevant micrograph providing a better perspective of the cell shape. Bars: A-B, A'-B′, C and D-D′ 20 μm; C′, 15 μm.

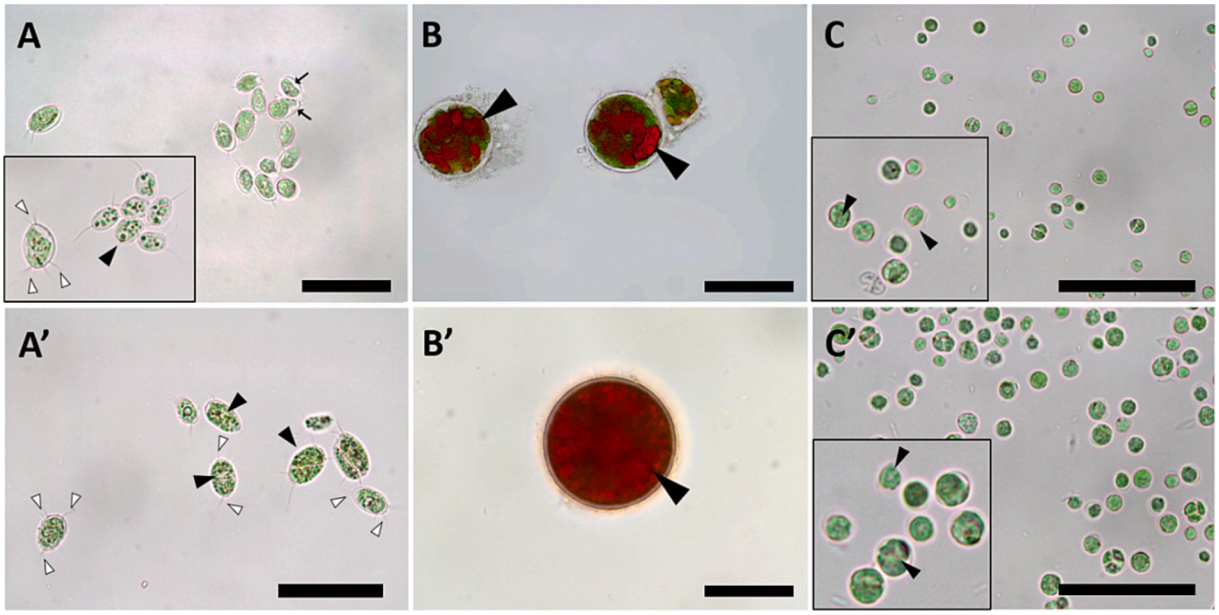

Fig. 3. Light micrographs showing microalgae cultivated at cold conditions (5 °C) at exponential (A, B and C) and stationary stages (A', B′ and C′): *Scenedesmus* sp. B2–2 (A, A'), *Haematococcus pluvialis* (B, B′) and *Chlorella vulgaris* sp. 13–1 (C, C′) Insets in the Figs. A, C and C′ show high magnification images of the same relevant micrograph providing a better perspective of the cell shape and details of the intracellular compartment. Bars: A-C, A'-C′20 μm. black arrowheads indicate intracellular disorganization, white arrowheads indicate spines, black arrows indicate separation between protoplast and cell wall.

Fig. 2 shows phenotypes of the selected microalgae and their cellular organization under optimal condition at 25 °C. However, under low temperature condition we observed an intracellular disorganization in *Scenedesmus*, *H. pluvialis and C. vulgaris* (arrowheads in Fig. 3), shapeless chloroplasts and an increase of granules/vesicles in their cytoplasm (black arrowheads in Fig. 3 A-A'), which could be a consequence of the accumulation of energy storage components such as lipids and starch granules under stress conditions. It is reported that cold exposure in *Chlamydomonas reinhardtii* causes to increase number of starch granules in the cytoplasm and a disorganized chloroplast after 120 h exposure to low temperature [15]. Further, *Scenedesmus* sp. B2–2 showed a separation of protoplast and cell wall (arrows in Fig. 3A).

Besides intracellular disorganization, *H. pluvialis* also showed a visible increase of astaxanthin accumulation in the intracellular space (arrowheads in Fig. 3B-B'). This phenomenon is associated with a reduction in chlorophyll levels caused by low temperature, likely due to the suppression of chlorophyll biosynthesis and related enzymes under cold stress [40]. Fang et al. [41] suggested that degradation products of chlorophyll could potentially be utilized for astaxanthin biosynthesis. Moreover, this morphology is normally found in mature cells in the stationary phase under control condition when the nutrient content starts to become limited. The effect has previously been reported in *H. pluvialis* isolated from Svalbard where more than 80 % of cells were red colored at 4–6 °C [42].

3.2. Cell size and micro-structural changes

Results of the LM provided information on cellular shapes, cell organization, size and stress-responses including the effects of cold condition on the cellular morphology of the four microalgae strains. *C. vulgaris* sp. 13–1, *Coelastrella* sp. 3–4 and *H. pluvialis* showed a spherical cell-shape while *Scenedesmus* sp. B2–2 was the only strain that mostly presented as 4–cells coenobium organization having four long spines at the lateral cells in the coenobium (arrowheads in Fig. 2).

All strains from the two growth stages (exponential and stationary), under control- and cold conditions were examined for their cell size variation. As expected, all microalgal strains increased their cell size (diameter) between exponential and stationary phase as part of their life cycle and cell- maturation (Fig. 2). For example, *H. pluvialis* and *Coelastrella* increased their cell size by 54 and 43 % respectively compared with *C. vulgaris* and *Scenedesmus* that increased relatively at 9 and 12 % respectively (Table 1).

Table 1
Cell size (diameters) of four microalgae in two different stages (exponential and stationary) and at different growth temperatures (25 °C as a control and 5 °C as cold conditions). (*) Measures were performed in length side. "-" indicates no grown cultures. N (sample size) =30 cells per each microalgal strain, condition and growth phase. Measurements expressed in mean ± deviation standard.

Microalgae strain	Condition	Exponential phase	Stationary phase
Coelastrella sp. 3–4	Control	4.39 ± 1.11	8.41 ± 1.75
	Cold	–	–
Haematococcus pluvialis	Control	11.74 ± 2.57	15.11 ± 2.32
	Cold	20.26 ± 2.44	33.46 ± 2.14
Chlorella vulgaris sp. 13–1	Control	2.52 ± 0.40	3.60 ± 0.44
	Cold	3.12 ± 0.23	5.41 ± 0.65
Scenedesmus sp. B2–2	Control	6.96 ± 0.79 (*)	7.82 ± 0.62 (*)
	Cold	8.52 ± 0.62 (*)	8.21 ± 1.12 (*)

It should be highlighted that no growth was observed with *Coelastrella* under cold condition in contrast with the other three strains of *Scenedesmus*, *H. pluvialis* and *C. vulgaris*, which were growing in cold condition showing an increase in cell size in both growth stages (Fig. 3). The effect is previously explained by Atkinson [43] based on the temperature-size rule, which explains how most of the organisms show larger body/cell size to minimize their surface area to volume ratio, an example of phenotypically plastic response. Table 1 shows that the cell diameter of *C. vulgaris* in a stationary phase increased from ~3.5 μm at 25 °C to ~5.5 μm during 5 °C growth condition. *Scenedesmus* sp. varied its size at exponential phase from ~7 μm under control condition to ~8.5 μm at low temperature. Similar results are also observed by Lindberg et al., [44] where they compare *C. vulgaris* 13–1 and *Scenedesmus* sp. B2–2 grown at 22 and 5 °C. *H. pluvialis* had the most significant size differences between exponential- and stationary stages showing ~11 μm and ~ 15 μm respectively while the cultures grown at 5 °C had a mean cell size of 20 μm and 35 μm at exponential- and stationary phase respectively. In some cases, it reached around 55 μm cell

size indicating that it tripled its cell size compared to control condition (Table 1).

Another important finding of the present work is the change in cell organization between 25 and 5 °C. As previously mentioned, *C. vulgaris* sp. and *H. pluvialis* conserved their single-cell arrangement at low temperature (Fig. 3). *Scenedesmus* sp. was organized as a coenobium with four ellipsoidal cells, which is their typical morphology under optimal conditions. Nevertheless, it showed changes in cell organization, i.e., from 4-cell coenobium organization to mostly unicellular cells under cold condition following Atkinson's theory [41]. These morphological modifications in the two environmental conditions can clearly be visualized in Fig. 2A-A' vs Fig. 3A-A'. In line with our results, a reduction in coenobium number is also reported with the same *Scenedesmus* specie by Lindberg et al., [44] and Ferro et al., [45], who reported the presence of all possible combinations of 1, 2, 3 and even 4 cells.

Another example of phenotypic plasticity observed in this study was the modification of cell wall ornamentation, i.e., spine number and their arrangements within the organism (white arrowheads in Fig. 2A-A' vs Fig. 3A-A'). Under control condition *Scenedesmus* is characterized as a coenobium with 4 spines, one at each of the four corners. Previous studies show that *Scenedesmus* modifies its morphology in response to a wide variety of external factors [46–48]. Regarding the spine's length, present results indicated that *Scenedesmus* sp. has spines with an average length of 9 μm under control condition, but there was a shortening to an average of 3 μm under 5 °C growth condition. Lindberg et al., [42] show that most of *Scenedesmus* coenobium/cells were spineless under similar low temperature condition. Further, some studies also report microalgae cells could produce shorter bristles or even spineless cells when the temperature changes considerably [49]. Some species of the genera *Scenedesmus* are organized in coenobium, but those coenobia can turn mostly into unicell organization type under stress conditions. Egan and Taylor [50] categorize coenobium up to four unicell types within the genera *Scenedesmus* sp. We found that *Scenedesmus* sp. B2–2 mostly presented as 4-cell coenobium at 25 °C but changed into unicells at 5 °C condition as categorized by Egan and Taylor [50]. The unicellular morphology is characterized by an ovoid shape, with one or more spines at each pole. Similar phenotype can also be observed with most of the *Scenedesmus* cells grown under low temperature condition in this work. Similar features are also observed in *Scenedesmus communis* and *S. armatus* [50], where a multi-spined unicell formation was observed at low temperatures. This morphology could presumably be a result of the fact that the extracellular layer, which holds the coenobium together in optimal condition, was likely not produced at low temperatures and thus do not deactivate those spiny spots. Eventually, all cells showed spines instead of just the two typical terminal cells in the coenobium. These modifications in morphological features may explain why they grow more slowly in comparison with other strains of microalgae, which are generally round and with no detailed cell wall ornamentation.

Accordingly, *Scenedesmus* sp. B2–2 is one of the four selected microalgal strains that showed a greater degree of phenotypic plasticity at low temperature observed during the present study; an adaptation that characterizes the *Scenedesmus* genus.

3.3. Cold triggers micro-morphological/cell wall histochemical changes in subarctic microalgae

Cell wall resistance was investigated in the four microalgal species under cold condition. Algal cell wall is the first barrier in contact with the surrounding environment and thus cell wall should have features that protect the organisms against biotic and abiotic stress factors. Dunker and Wilhelm [22] demonstrate that Crystal violet, Ruthenium red and TbO dyes can be used to classify cell wall resistance as high, medium, and low, respectively based on the degree of permeability to these dyes.

Similar histochemical microscopy techniques employed using Crystal violet, Ruthenium red, TbO and SBB stains for improve our understanding on how cell wall biopolymers/biochemical components vary among the four microalgae provided interesting results. Although crystal violet stain is mostly used to categorize Gram bacteria, there has been some studies where Crystal violet is used to evaluate algaenan content and its role in the permeability of the cell walls of microalgal species [26]. Since the staining technique is subject to cell wall disturbance and may lead to false positives, only if 100 % of the cells are stained is considered absence of algaenan as a cell wall component. Further, Ruthenium red is a standard dye used in plant tissues for staining pectic substances red/pink and has previously been used to stain pectins of red algae [51]. However, the reactivity of ruthenium red, and thus the staining intensity, is thought to depend on the methyl-esterification of the pectin compound with more intense staining for low methyl-esterified pectins and less intense the high methyl-esterified pectins [52].

The TbO is a metachromatic dye that can react with different chemical components of cells differently giving different colors depending on the component of the sample to which it is bound. For example, it can stain acidic polysaccharides generating a light to bright pinkish-purple stain while nucleic acids in greenish blue [53–55]. However, TbO can show no change of color or orthochromasy during staining that could be due to low concentration of the target compound [55]. On the other hand, SBB is used to stain lipids and perhaps the most popular of all Sudan dyes as a simple fat stain. However, there remains an unsatisfactory compromise with the stain concerning maximum staining intensity and minimum lipid loss; some phospholipids, free cholesterols and free fatty acids tend to dissolve in the dye solution (e.g., ethanol used in the dye) [54,56].

The presence/absence of algaenan in cell walls was evaluated by Crystal Violet staining. Results showed that *Coelastrella*, *Scenedesmus* and *H. pluvialis* were generally not stained with crystal violet (Figs. S1B—B′, S2B—B′, S3B—B′ respectively), indicating they contained algaenan in their cell walls confirming previous finding [29,57–59]. However, during the current experiments, it was always observed a small percentage of stained cells in these microalgae strains. This may be due to some damage in the cell wall, collapsed cells [26] or cells with under-developed cell wall architecture affecting cell wall permeability. Nevertheless, we obtained a negative result regarding algaenan presence in *C. vulgaris* cell wall where 100 % of cells were completely stained (Fig. S4B—B′). This is because the dye can penetrate cells that do not contain algaenan in their cell walls and completely stain the cells [26]. Moreover, crystal violet staining was also performed with growth cultures under low temperature condition to visualized changes in the cell wall permeability and *Scenedesmus* was the only investigated microalgae that showed changes in the staining pattern; a complete staining of all cells under low temperature (Fig. S5B—B′). This suggested that the presence of algaenan as a part of their cell wall composition was affected by the low temperature condition which can be related with the observed change in the cell wall permeability leading to the stained cells.

Presence of pectic substances and their micro-distribution in algal cell walls were investigated using Ruthenium red staining. Results clearly showed the presence of pectins in *Coelastrella*, as a part of the mother cell wall enveloping the daughter cells in autospores (arrowheads in Figs. S1 C-C′) especially in exponential growth stage under control condition. *Coelastrella* showed positive staining of both the colonies' wall and cell walls of daughter cells with higher staining intensity at the area joining two daughter cells together (white arrowheads in Fig. S1C). This can be explained as pectins are involved in cell wall expansion [59] and responsible for holding cells together, especially daughter cells [60]. Presence of pectins in *Scenedesmus* was observed between coenobial cells, with strong staining reaction at the regions joining cells together within a four-celled coenobium (Fig. S2 C-C′). Similar results have been found previously in other species of *Chlorella* [61–63] and *Scenedesmus* [64–66]. However, the presence of pectic substances was not detected in *H. pluvialis* cells under control conditions

Table 2
Overview of investigated microalgal strains with respect to factors related to cell wall resistance. The factors were evaluated by histochemical microscopy techniques. Cell wall resistance of the four microalgae was evaluated at two growth stages (exponential- and stationary data separated by "/", respectively) and different growth temperatures (25 °C as a control and 5 °C as cold conditions). Cell wall resistance was classified based on [22] indications. "(+)" more than 75 % cells are stained "(−)" less than 25 % cells are stained, "(±)" approx. 50 % cells are stained. (*) staining but no specific compounds. "Nd" no data shown. LR = low resistance, MR = medium resistance and HR = high resistance.

Microalgae strains	Algaenan presence		Crystal Violet		Toluidine Blue O		Ruthenium Red		Sudan Black		Cell wall definition	
	Control	Cold	Control	Cold	Control	Cold	Control	Cold	Control	Cold	Control	Cold
Coelastrella sp. 3–4	+	nd	± / ±	nd	±*/ ±*	nd	+ / ±	nd	± / -	nd	MR	nd
Scenedesmus sp. B2–2	+	−	± / ±	+ / +	±*/ ±*	+*/+*	+ / +	- / -	- / -	± / ±	HR	MR
Chlorella vulgaris sp. 13–1	−	−	+ / +	+ / +	+ / +	+ / +	- / +	- / +	- / -	± / ±	LR	LR
Haematococcus pluvialis	+	+	- / -	- / -	- / +	- / -	- / -	- / -	± / +	- / -	HR	HR

(Fig. S3 C-C′). The pattern of pectin presence and their micro-distribution changed when growing under low temperature condition in *Scenedesmus*, *H. pluvialis and C. vulgaris* (arrowheads in Fig. S5 C-C′, S6 C-C′ and S7 C-C′, respectively). Here, the concentration of pectic substances as part of the cell walls seems to decrease as reflected in their staining intensity (e.g., Figs. S2 C-C′ vs S5 C-C′). *Scenedesmus* was the most representative example, as it changed from a coenobial to a unicellular lifestyle, where the pectic layer that enveloped the coenobium disappeared under these stress conditions (Fig. S5 C-C′).

Anionic polysaccharides in the microalgal cell walls can be studied using TbO which is a polychromatic dye having a high affinity for anionic/acidic groups (i.e., carboxylate, phosphate or/and sulfate) of polysaccharides. The stain has a metachromatic behavior where different colors or shades are generated when the dye binds with different chemical components of cells [67]. In the current work, we examined the localization of anionic polysaccharides in the four microalgae cell walls using the dye and a positive staining result was observed indicating the presence of extracellular polysaccharides in *H. pluvialis* and *C. vulgaris* under control condition (Fig, S3D—D′ and S4D—D′, respectively). Surrounding cell walls of the two green microalgae were stained pinkish purple/purple possibly suggesting the presence of anionic polysaccharides in the extracellular matrix. Although no presence of anionic polysaccharides was detected in *Scenedesmus* and *H. pluvialis* under cold conditions (Fig.S5D-D′ and Fig. S6D—D′, respectively), *Chlorella*, under the same conditions, showed not only anionic polysaccharides presence in their cell walls but also increase in amounts as reflected in intense staining reaction of the cell walls (Figs. S4D-D vs S7D—D′).

Nevertheless, completely stained cytoplasms (orthochromasia staining) were observed with TbO staining of *Coelastrella* under 25 °C (Fig. S1D—D′) and a very weak staining can be seen inside the cells mostly as small spots in *Scenedesmus* sp. B2–2 in both temperatures (25 and 5 °C) (Fig. S2D—D′ and S5D—D′, respectively). Cell walls of these two strains did not stain with TbO but intracellular organelles or cytoplasms were stained blue with non-specific affinity. Barcyte et al., [68] reported similar results in *Cylindrocystis* (Streptophyta) in which green algae cells are stained with TbO but only small structures like polyphosphate grains are stained with intense blue. As discussed, TbO staining produces a basic reaction, which can stain several types of compounds and intracellular components such as mitochondria, nucleus, lysosomes resulting an unspecific staining.

Further, fats can be localized using Sudan black B (SBB) which is a general stain for localizing all lipids especially neutral lipids forming black/black-blue complexes. However, it has not been widely used as a general histochemical analytical method for microalgae [69,70]. After staining with SBB, presence of lipid and/or their micro-distribution were determined in *Coelastrella* autospores grown in exponential growth phase under the control condition (25 °C) (white arrows in Fig. S1E), but similar detection was not found in the stationary stage (Fig. S1E'). Despite the same temperature condition, no staining reaction was observed with *Scenedesmus* and *C. vulgaris* cells (Fig. S2E-E' and S4E-E', respectively). Nevertheless, some black complexes were observed in the intracellular space in both *Scenedesmus* and *C. vulgaris* cells growing under cold condition at both growth stages (arrowheads in Fig. S5E-E' and S7E-E', respectively). In contrast, although small accumulations of lipids were detected in *H. pluvialis* cells in control condition in both exponential and stationary phases (arrowheads in Fig. S3E-E') opposite results were found in cultures grown under cold condition; no positive staining observed possibly indicating less/no lipid accumulation in their cells (Fig. S6E-E'). Furthermore, no substantial differences were observed between the two growth stages, exponential and stationary, in any of the microalgae strains used. The observed accumulation of lipids in cells could be related with the phenomena previously mentioned regarding energy storage components during stress conditions. Altogether, our results revealed differences in the presence/absence of bioactive compounds and their micro-distribution depending on the microalgal strain, they were harvested in the growth stage under. Data from all the histochemical microscopy techniques were summarized in Table 2.

4. Conclusion

In summary, we exploited different histochemical staining techniques with light microscopy to gain better understanding of cell wall main components including other biochemical compounds, such as algaenan, pectins, anionic polysaccharides and lipids, with respect to their presence/absence and extra- or intracellular micro-distribution in four microalgal species isolated in sub-arctic locations: *Chlorella vulgaris* sp. 13–1, *Coelastrella* sp. 3–4, *Scenedesmus* sp. B2–2 and *Haematococcus pluvialis*. The study elucidated (a) how biochemical characteristics of unicellular microalgae cell walls can be changed under different growth stages and at different temperatures; (b) how different layers of the cell wall may be involved in cell organization at different stages of the life cycle; and (c) how micro-structural and biochemical characteristics are affected under abiotic stress conditions such as exposure to cold temperatures (5 °C). Results showed cellular location and the behavior of those biochemical compounds of the four microalgae at different growth conditions while no growth development was detected in *Coelastrella* under cold conditions. The four microalgae showed micro-morphological changes such as cell size and shape, cell organization, cell wall ornamentation with *Scenedesmus* sp. B2–2, which turned coenobium to unicellular organization and multi-spined because of low temperature stress. Each microalga strain demonstrated different pathways to tolerate stress condition. Our results may provide important clues for meeting current challenges on cell disruption during biotechnology and bio-refineries, for example, cold exposure can change cell wall composition and micro-structure making them more accessible to upstream and downstream processes.

CRediT authorship contribution statement

María González-Hourcade: Conceptualization, Methodology, Investigation, Writing- original draft & editing. **Dinesh Fernando**: Conceptualization, Formal analysis, Methodology, Resources, Review &

editing. Funding acquisition. **Francesco Gentili**: Conceptualization, Formal analysis, Methodology, Resources, Review & editing. Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2023.103254.

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