University of South Bohemia in České Budějovice

Faculty of Science

The fitness of three strains of the alga *Chromera velia* - Salinity and pH

Bachelor Thesis

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Annotation

This bachelor thesis demonstrates the ecological fitness of the alga *C. velia* and its three strains in different pH and salinity. Lipidomic analysis together with statistical analyses revealed significant differences among tested *C. velia* strains. Lipidic profile of all three strains was affected rather with salinity than pH level.

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Kateřina Kabeláčová

Abstract

This bachelor thesis is dealing with the research of the alga *Chromera velia*. This alga is the closest known relative photosynthetic organism to obligate parasites from the group apicomplexa. Parasites during their life cycle have to survive in different environments inside hosts. They go through changes in temperature, salinity and pH level. Since the *C. velia* is relative to these parasites they should have in common the resilience. Thus, the growing ability of three strains of the *Chromera velia* was tested through cultivation in environments with a wide range of salinity and varying pH levels. The growing curves were obtained by spectrophotometer TECAN. Results show the ability of *C. velia* to sustain growth almost in all environments. In addition, to assess the influence of tested physical factors to the primary metabolism of the organism the lipidomic analysis was performed by HPLC ESI MS method. The ability to store high amounts of triacylglycerols makes *C. velia* a potential candidate for biotechnological applications.

Keywords

Chromera velia, autotrophy, heterotrophy, parasitism, fitness, cultivation, fatty acids, biotechnology

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CONTENT

1 INTRODUCTION

This bachelor thesis was developed in the Laboratory of Evolutionary Protistology, which belongs under the Institute of Parasitology in České Budějovice as a part of the Biological centre of Czech Academy of Science. There were compared algae isolated from the original culture of *C. velia* collected from Sydney Harbour (CCMP2878), derived culture from the *Vitrella brassicaformis* culture (CCMP3155) in the Laboratory of Evolutionary Protistology with working name "*Chromera tropica"* and the alga isolated from a culture sample taken from One Tree Island location close to the south-western coast of Australia.

C. velia is a free-living green-brown alga, mostly associated with stony corals. After the alga discovery and description by Moore et al,. (2008), they assumed that *C. velia* is the missing link in the phylogenetic tree of the superphylum Alveolata and is the closest photosynthetic ancestor to obligate intercellular parasites of the apicomplexan group (Moore at al., 2008). Further exploration of morphological and phylogenetical characteristics confirmed the hypothesis that apicomplexan parasites have evolved from symbiotic algae. When the specialised organelle – apicoplast in apicomplexan parasites was described, scientists proposed that the ancestor of the parasites was photosynthetic and lived as a mutualistic symbiont within invertebrate animals, like the way zooxanthellae dinoflagellates are symbionts in corals and other organisms (McFadden and Walker, 1997). Since is the *C. velia* closes photosynthetic relative to Apicomplexa its genetic equipment should provide a broad spectrum of the mechanism how to cope with different physical conditions as salinity or pH level.

The main factors influencing marine algae growth and distribution are light, temperature, nutrients, and salinity. Salinity is a local parameter with high variability, especially in coastal regions (Kirst, 1989). Osmoregulatory systems are essential for algae dealing with the water stress e.g. salinity fluctuation, desiccation or freezing (Yancey et al., 1982; Yancey 2005). Osmoregulation is achieved by the accumulation of inorganic ions as K^+, Na^+, Cl^- and NO_3^- (Davison, 1985), but the concentration of other ions especially Mg^{2+} and Ca^{2+} doesn't cause any effect on the osmoregulation process (Kirst, 1989). However, high accumulation of intracellular salts due to the osmotic stress seriously affects metabolic function and maintenance of proper transmembrane potentials

(Yancey, 1982). Another mechanism to achieve the osmoregulation is via osmotic organic solutes (osmolyte) systems, also called as “compatible-soluble” (Borowitzka, 1974) and do not interfere with macromolecules and transmembrane potentials even in high concentration. The major osmotic compounds in eukaryotes are restricted to few groups of organic small molecules: polyols (polyhydric alcohols), free amino acids, urea, and methylamines. One example of a polyol as an osmolyte is glycerol occurring in the phylogenetic distant organism (Chlorophytes, insects, crustaceans, and vertebrates) which points to a high degree of convergent evolution of the “compatible solubles” (Yancey et al., 1982; 2005). However, the most abundant polyol in nature is considered mannitol. Mannitol is a polyol related to the aldohexose mannose which is synthesized by bacteria, fungi, Apicomlexa (Michalski et al., 1992; Rohloff et al. 2004), Haptophytes (Obata, 2013), green algae (Wegmann, 1986), red algae (Eggert et al., 2006), brown algae (Reed et al., 1980), lichens, and higher plants (Perri et al., 2018).

The pH value has a significant effect on the physiology of microorganisms, especially metabolism. Protonation and deprotonation affects protein conformation and thus activity of enzymes. In addition to metabolic pathways, it also influences the throughput of the substance over membranes. If the molecule loses the charge, it can better pass through the membrane, on the contrary, if it gets it, it loses the ability of spontaneous diffusion [1]. Several studies have focused mostly on acid tolerant and acidophilic algae species. They can maintain a constant, neutral, cytosolic pH over a wide range of external pH values (Lane and Burris, 1981; Gimmler and Weis, 1999). The algae have developed several strategies, such as a maintaining positive membrane potential and a positive charge outside the cytosolic membrane (Remis et al., 1994), a decreased permeability of the cytosolic membrane for protons or an active proton pumping activity (Gross, 2000; Gimmler, 2001).

Studies focusing on cultivation and *C. velia* analysis discovered its ability to store high amounts of fatty acids in form of storage lipids triacylglycerols (TGs) (Woo et al., 2015). Furthermore, comparison of fatty acids amounts in *C. velia* with another well-known oleaginous alga *Nannochloropsis oculata* shows the higher content of TGs in *C. velia* (Schneedorferová, 2014). *C. velia* seems to be a candidate for biotechnological research, therefore, could be compared with other microalgae which are already used in cosmetics, pharmaceutics and nutrition (Bellou et al., 2014). In past few decades,

scientists have been also focusing on the use of microalgae as a new source of alternative energy to replace fossil fuels (Milano at al., 2016).

Recently the study dealing with the *C. velia* fatty acid content and growth rate during different salinities, light quality and intensity, was published (Lukeš et al., 2017). However, Lukeš et al. (2016) study covers only original *C. velia* strain (CCMP2878). This project is focused on the comparison of three *C. velia* strains in different salinities and novel pH experiment.

2 CHROMERA VELIA

Chromera velia (Fig. 1) is a free-living spherical green-brown alga which was first found in 2001 associated with the stony corals *Plesiastrea versipora* from Sydney Harbor, New South Wales, Australia, and *Leptastrea purpurea* from One Tree Island, Great Barrier Reef, Queensland, Australia (Moore, 2006; Moore et al., 2008).

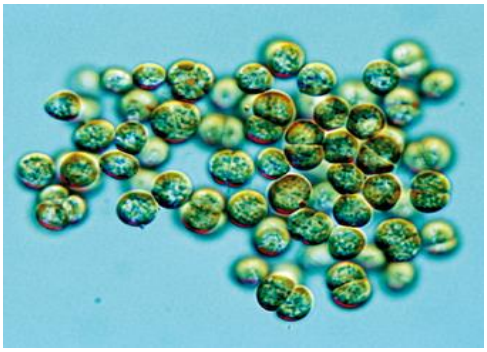

Figure 1 *Chromera velia*. [2]

C. velia was isolated by Moore, (2006) during attempts to culture *Symbiodinium* which belongs to the major group of endosymbiotic dinoflagellates. Phylogenetic analysis of this green-brown alga found that it holds a special relationship in the phylogenetical tree. It was found that *C. velia* is related to dinoflagellates (Janouškovec et al., 2010) which form a large and diverse group of algae that live in freshwater and marine environments. About half of the dinoflagellates are photosynthetic, while the other half is entirely heterotrophic or even parasitic (Gornik et al., 2015).

Unexpectedly, the other closest known relative to *C. velia* is a large and diverse group of obligate intracellular parasites which belongs to the phylum Apicomplexa. Many parasites of this group cause serious diseases. They are infecting a diverse range of hosts from marine invertebrates, amphibians, reptiles, birds to mammals including humans. The well-known disease malaria is caused by *Plasmodium spp.*, toxoplasmosis is caused by *Toxoplasma gondii*, and coccidiosis in poultry is caused by *Eimeria* (Oborník et al., 2009). Furthermore, Woo et al. (2015) revealed evolutionary path from *C. velia* to apicomplexans via genomic research.

With this special evolutionary position between dinoflagellates and apicomplexan parasites (Fig. 2), it leads to the thought that *C. velia* would have biochemical processes similar to both groups. As the apicoplast is important for the life of apicomplexan parasites, it has serious potential in medical science to be a drug-target. *C. velia* provides the perfect model organism to gain more knowledge about the apicoplast and biochemical pathways in apicomplexans in the hope to find better therapeutic methods (Weatherby and Carter, 2013).

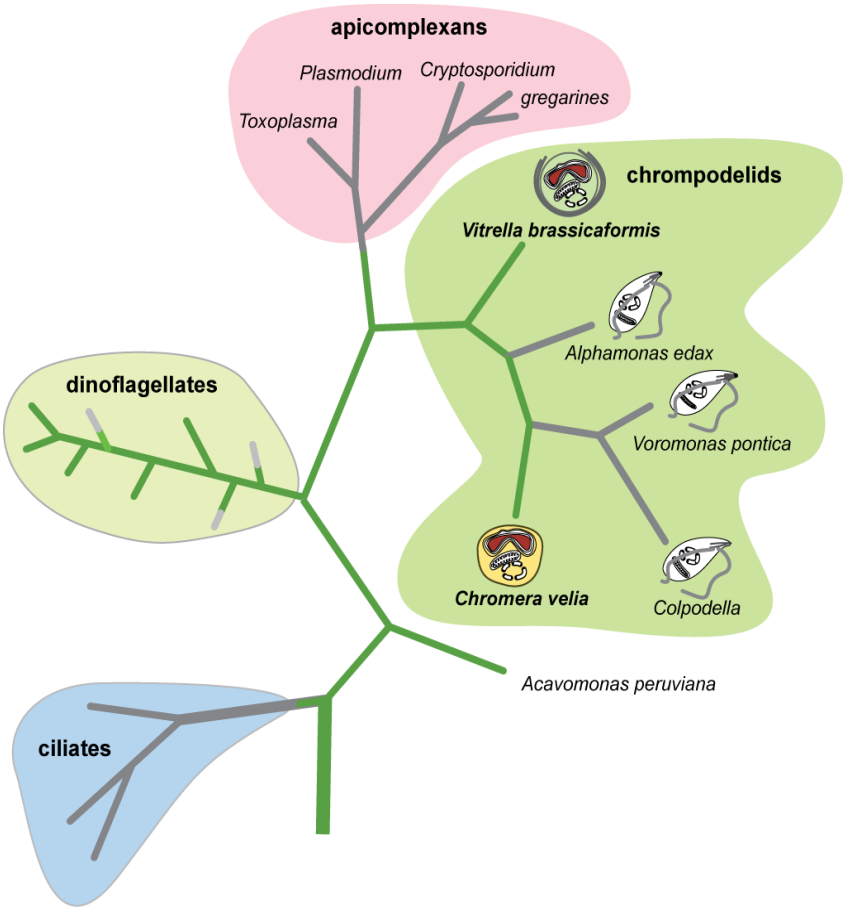

Figure 2 Phylogeny of chrompodelids and their relatives (Oborník and Lukeš, 2015).

2.1 Biological characteristics

There were observed three life stages: a coccoid cell, a cyst, and a flagellate. The vegetative coccoid cell is spherical and has 5–7 µm in diameter. Vegetative cells split to create two cells enclosed by a thin wall. Another division can lead to a cyst stage which is about 4 µm bigger than a vegetative cell and it contains four daughter cells. Cells in the flagellate state are slightly curved and prolonged into eggplant-shaped cells roughly about 5 to 3 µm that have a short anterior and long posterior flagellum. There is a reciprocal transition between the two stages of flagellate and coccoid form. The permutation of these different life cycles happens accordingly to changes in physical and chemical conditions in cultures. There wasn't observed sexual reproduction and it's only thought that it occurs in the natural habitat when *C. velia* is hidden inside of stony corals (Oborník et al., 2011). However, the question concerning the trophic mode of *C. velia* (epiphyte, commensal,

host beneficial mutualist or parasite) is quite important but yet not sufficiently solved. In spite of *C. velia* can live as free-living phototrophs, and it was speculated, due to the way how they were isolated (Moore et al., 2008), that the association with corals is most likely symbiotic (Cumbo et al., 2013). However, a recently published transcriptomic study on larvae of *Acropora digitifera* infected by *Chromera velia* showed that at least *C. velia* is rather (facultative) parasite than a mutualist (Mohamed et al., 2018). This finding is not so surprising when the phylogenic position of chromerids is in the the root of apicomplexan parasites.

2.1.1 Affiliation to Alveolates

The Alveolata clade belongs under the new super assemblage of eukaryotes called "SAR", which stands for three major clades Stramenopiles, Alveolates and Rhizaria. Alveolates belong to a monophyletic group of primarily single-celled eukaryotes that have adopted extremely diverse modes of nutrition, such as predation, photoautotrophy and intracellular parasitism (Burki et al., 2007).

C. velia cells have three ultrastructural characteristics typical to alveolates. There is a layer of flattened vesicles called the cortical alveoli and together with underlying microtubules support the cell membrane. Alveolates also contain micropores through the cell surface that are important for pinocytosis (Moore et al., 2008).

2.1.2 Distinctive characteristics of *C. velia*

The special feature which makes *C. velia* different from alveolates is a large, golden-brown chloroplast bound by four membranes. The focus on the plastid is due to its origin. There is a suggestion that the plastid was obtained through secondary endosymbiosis. Another unusual feature of *C. velia* is the chromerosome described as an extrusome-like structure, which is resembled out of fibres (Oborník et al., 2011). The chromerosome structure can extend into a rod-like projection that protrudes out of the cell. In comparison to apicomplexans and dinoflagellates, there are no similar features found. The extrusomes of dinoflagellates are morphologically diverse and significantly different. The purpose of the chromerosome is still unknown and there is only assumption that it is important for coral interaction or hunting (Leander et al., 2008). There was performed an experiment on the mixotrophic ability of *C. velia*. A better growth was

observed in a culture supplemented with specific small organic compounds (glycine and galactose) than in strictly photoautotrophic cultures (Foster et al., 2014).Another interesting discovery was that the phylogenetic analyses displayed that the tetrapyrrole biosynthetic pathway of *C. velia* is a mosaic composed of genes of different origins and that is homologous to the unusual pathway of apicomplexan parasites. Furthermore, it was proved *C. velia* is unique being able to synthesize α aminolevulinic acid, the first step of tetrapyrrole pathway, from glycine and succinyl-CoA by the mitochondrial C4 pathway while other known autotrophs employed C5 pathway where α aminolevulinic acid is synthesized from glutamic acid (Kořený et al., 2011). It appears that *C. velia* has an even smaller mitochondrial genome than Apicomplexan parasites as well as dinoflagellates which are already known for a tiny mitochondrial genome. The other peculiarity of *C. velia* is a divergent respiratory chain which lacks complexes I and III. This is also unprecedented among living creatures (Flegontov et al., 2015).

2.2 Other Chromerids

Vitrella brassicaformis is the second from the two known autotrophic algal relatives to apicomplexans and dinoflagellates which was isolated from the Australian stony coral *Leptastrea purpurea* at One Tree Island, Great Barrier Reef by R. A. Andersen and R. B. Moore. *V. brassicaformis* underwent extensive molecular studies and it was confirmed that it is also closely related to the apicomplexans. *V. brassicafromis* established a novel branch on the evolutionary tree collateral with *C. velia*. Both algae are classified as chromerids according to common metabolic features, photosynthetic ability and molecular phylogeny. In spite of their relatively close relationship, *C. velia* and *V. brassicaformis* do not form sister groups (Gile and Slamovits, 2014, Janouškovec et al., 2015, Oborník and Lukeš, 2015) and they differ significantly in morphology (Oborník et al., 2011, Oborník et al., 2012), life cycle (Oborník and Lukeš, 2015, Füssy et al., 2017), nuclear (Woo et al., 2015), plastid (Janouškovec et al., 2010) and mitochondrial genomes (Flegontov et al., 2015, Oborník and Lukeš, 2015). *V. brassicaformis* own a highly conserved and compact circular plastid genome and uses exclusively the canonical code for all tryptophans in its plastid-encoded genes (Janouškovec et al. 2010).

3 ALGAE CULTIVATION

There are so many media and modifications which are designed for all kinds of algae cultivation. Every laboratory and every phycologist have specific manuals and technique which work best for their purposes. The first algal culture was described by Ferdinand Cohn in 1850 and he called the procedure as "cultivation". The founder of bacteriology managed to keep alive the unicellular flagellate *Haematococcus* (Chlorophyceae) for some time in his laboratory. Many of the methods and basic culture medium concepts that are used today were developed in the late 1800s and early 1900s. Algal culture methods have been described in several books and articles. Nowadays, technologies are still advancing for the best cultivating performance, not only for experimental and biotechnological research but also for a commercial production (Andersen, 2011).

At first, it is important to find out the ecology of the alga. The basic division is to freshwater and marine algae. It is essential to adjust the right balance and composition of macro/micronutrients. Also, it is important to take into consideration the targeted volume of the algae and the purpose of the culture. If it is enough to keep the culture in small flasks for a laboratory experiment or to grow algae in big pools for a commercial production. The work with cultures is very neat. It is performed in a sterile environment with great precision. Chemical constituents necessary for the preparation of media should generally be of the highest quality (Andersen, 2011).

It's common to cultivate algae in different environments to discover the fitness and the durability of algae. The aim is to stress algae to find out how they react. It's usually overproduction of nutrients and secondary metabolites. Afterwards, the chemical compounds and processes are analysed and processed by many laboratory techniques. Biotechnology research projects especially perform this experiments to find a suitable species for desired applications e.g. biofuels (Minhas et al., 2016).

4 LIPIDS

Lipids have the crucial importance in living organisms as they represent a major form of energy storage, they are essential for the membrane structure, they regulate and modify properties of many proteins and as components of certain lipid signalling molecules, they perform an important part in metabolic regulation (Vance and Vance, 2004).

Lipids can be divided into three basic classes according to their functional properties. There are storage lipids, structural lipids and lipids with specific biological functions. The major characteristic of alga species important for biotechnological application is its ability to accumulate high amounts of Triacylglycerols. TGs belong to the group of storage lipids and are used for metabolic fuel deposing. The essential advantages are that the molecule of TG contains fatty acids which are more reduced than sugars and the lipid oxidation can generate twice as much energy (Briggs and Chandler, 1995).

Several research studies have presented that it is possible to control cell metabolism in algae to produce a high content of starch, lipids or both storage compounds at the same time. It depends on alga species which storage compound prefers. As the pathways of lipid accumulation can be various from starch accumulation, there are several approaches to raise starch or lipid overproduction (Brányiková et al., 2011; Dragone et al., 2011; Chen et al., 2011; Lee, 2011). Lipid content can be increased by phosphate or nitrogen depletion (Hsieh et al., 2009; Rodolfi et al., 2009), high salinity (Takagi et al., 2006), high iron concentrations (Liu et al., 2008) or growth under combination of heterotrophic and mixotrophic conditions in cultures (Heredia-Arroyo et al., 2010; Shen et al., 2010).

4.1 Lipidomic analysis

There is growing scientific interest in lipids thanks to technical advances and the better understanding how lipids are involved in crucial biological mechanisms in all eukaryotic and prokaryotic organisms. Lipidomics is useful in various scientific areas such as environmental sciences, pharmacology, nutrition, biophysics, cell biology, physiology, pathology, and disease diagnostics (Astarita et al., 2009).

Basic techniques used in the lipidomic analysis are an extraction of lipids from the biological matrix, lipid localization by microscopy, a separation by chromatography, and a characterization by spectrometry. The liquid-liquid extraction is very popular and is able to separate lipophilic from hydrophilic compounds. Basically, two extraction approaches are used: older one is based on chloroform: methanol solution (Folch et al., 1957) and the newer on methyl-tert-butyl ether: methanol solution. The yield of both methods is comparable, however, the newer one could be performed by automatic extraction (Matyash et al., 2008). The localization of nonpolar lipids like TGs could be achieved by BODIPY® marker staying and confocal microscopy (Tomčala, 2017). The recent fast development of lipidomic methodswas caused due to improved lipid extraction and analysis with the combination of bioinformatics technology (Li et al., 2013).

The main strategies of lipid analysis are qualitative and quantitative methods. The qualitative analysis is focused on known lipids and develops a specific method with a high sensitivity for the targeted lipids. The quantitative methods aim to detect every lipid species simultaneously. In order to successfully realize the qualitative and quantitative analysis of lipids, many analytical methods have been developed, including thin-layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), enzyme-linked immunosorbent assays (ELISA), nuclear magnetic resonance (NMR) and mass spectrometry (MS)(Li et al., 2014).

Among the technologies, MS is one of the most important for lipid analysis due to high sensitivity and specificity. There is a great challenge for MS in identification a large number of categories and the extremely complex structures of lipids to detect and identify the components of a complex mixture (Li et al., 2014). The MS is coupled together with LC to create a very efficient system because liquid chromatography can separate delicate and complex natural mixtures, which chemical composition needs to be well established (Niessen, 2007). The separation and characterization of algal lipids were successfully performed by HPLC MS and detailed described by Tomčala et al., (2017).

Furthermore, the lipidomic studies generate overwhelming amounts of data, which need bioinformatics to help in data processing for obtaining meaningful information (Li et al., 2014).

4.2 Biotechnology

In the last few years, there has been an intense interest in using microalgal lipids in food, cosmetics, chemical and pharmaceutical industries. Research projects are focusing on microalgal lipid production and its aspects of photosynthetic pathways, lipid biosynthesis and catabolism, and applied research dealing with the various biological and technical limitations of the lipid production process (Bellou et al., 2014).

Microalgae belong to primary producers in the aquatic environment, providing a supply of omega-3 polyunsaturated fatty acids, the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that are primary structural components in animal and plant cell membranes. The impact of constantly growing population is increasing stress on fish and seafood production. Nowadays natural production of these essential fatty acids is insufficient to fulfil demands of the whole human population (Sprague et al., 2017).

Furthermore, microalgae are one of the convenient alternative sources of lipids for biofuel production. The main goal of biofuels is a reduction of gas emissions which lead to the greenhouse, climatic changes, and global warming effects. Main advantages to use microalgae are fast growth rate, enable the use of non-arable land and non-potable water, use far less water and do not displace food crops cultures. The production of microalgae is not seasonal and they can be harvested continuously (Gouveia and Oliviera, 2009). High lipid productivity is a required key characteristic of a species for biofuel production (Griffiths and Harrison, 2009). There have been in focus *Chlorella vulgaris, Spirulina maxima, Nannochloropsis sp., Neochloris oleabundans, Scenedesmus obliquus* and *Dunaliella tertiolecta* (Milano at al., 2016).

5 AIM OF THE STUDY

- Algae cultivation
- Growth observation
- Analysis of triacylglycerols accumulation
- Comparison of *C. velia* strains

6 MATERIALS AND METHODS

6.1 Algae cultivation

The start of the experiment was to prepare cultivations of the three strains of *C. velia*. CV - *Chromera velia* original strain, CT - *C. tropica* from the *V. brassicaformis* culture, OTI - *C. velia* strain from the One Tree Island location. Grown cultures were picked from the laboratory growbox. With the help of my supervisor, I prepared basic f/2 enriched seawater medium (Guillard and Ryther, 1962) according to Figure 3. Cultures were inoculated inside of the laboratory flow box. One ml of each *C. velia* strain stationary culture was added to 75 cm^2 flasks with 160 ml of f/2 medium. Cultures were grown under artificial light (fluorescent lamps) with a photoperiod 12h/12h and light exposure 30–50 µmol/m^2/s and at a temperature of 26°C. The cultures were grown for 30 days.

f/2 medium stocks [g/l]	
$NaNO_3$	75
$NaH_2PO_4.2H_2O$	5,65
Trace elements (chelated)	
NA_2 EDTA	4,16
$FeCl_3.6H_2O$	3,15
$CuSO_4.5H_2O$	0,01
$ZnSO_4.7H_2O$	0,022
$CoCl_2.6H_2O$	0,01
$MnCl_2.4H_2O$	0,18
$Na_2MoO_4.2H_2O$	0,006
Vitamin mix	
Cyanocobalamin (Vitamin B_{12})	0,0005
Thiamine HCl (Vitamin B_1)	0,1
Biotin	0,0005

f/2 medium [ml/l]	
$NaNO_3$	1,0
$NaH_2PO_4.2H_2O$	1,0
Trace elements stock solution	1,0
Vitamin mix stock solution	1,0

1 l of distilled water
1 M HCl to adjust pH up to 8,00
33,3 g of sera® marine reef salt

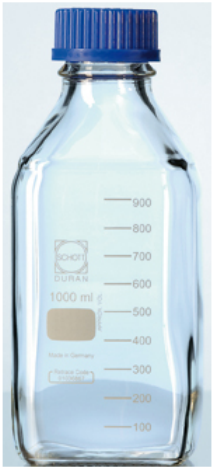

Figure 3 Basic f/2 enriched seawater medium (Guillard and Ryther, 1962). An autoclaved storage glass bottle was filled with distilled water, sea salt and f/2 medium stocks except for the vitamin mix solution. The medium was stirred in the process of adding the components as vitamin mix solution and pH was set to 8,00 by 1 M HCl. After the bottle was once more time autoclaved, the medium was filtered to prevent fungi contamination.

Prepared cultures in the stationary stage were used for the first part of the experiment to grow *C. velia* strains in f/2 mediums in which I dosed different amounts of the artificial sera® marine reef salt (Fig. 4).

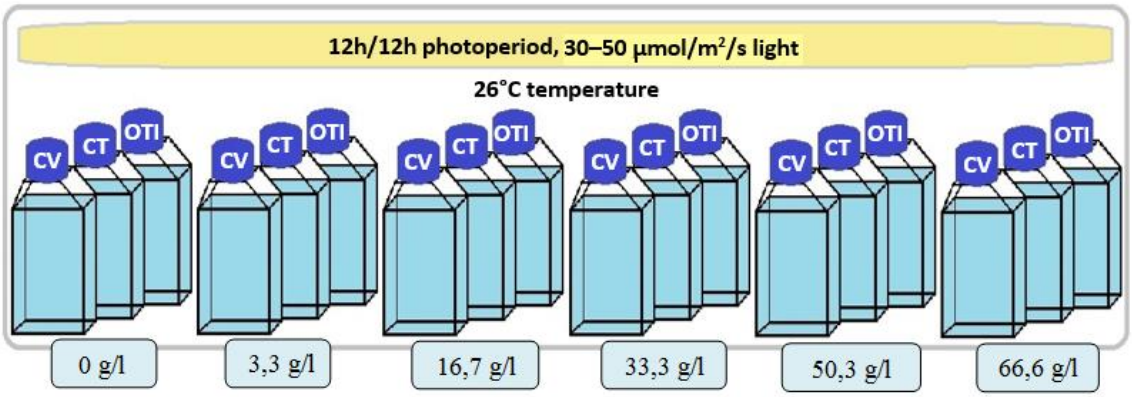

Figure 4 Experiment setup for culturing *C. velia* strains in different salinities. There was prepared f/2 medium in 6 glass storage bottles just as in Figure 3. Bottles were autoclaved, vitamins added and pH adjusted to 8. The amount of artificial sea salt was added (g/1) as it is displayed in the picture. Inside of the laboratory flow box, the media were poured into 75 cm^2 flasks with 160 ml volume inside of the sterile flow box and 1 ml of each culture was inoculated from the start-up cultures. Every strain had 3 repetitions to grow the higher volume of algae for another experiments and analysis.

The second part was to culture *C. velia* strains to grow *C. velia* strains in f/2 mediums with a pH range from 6 to 10 (Fig. 5).

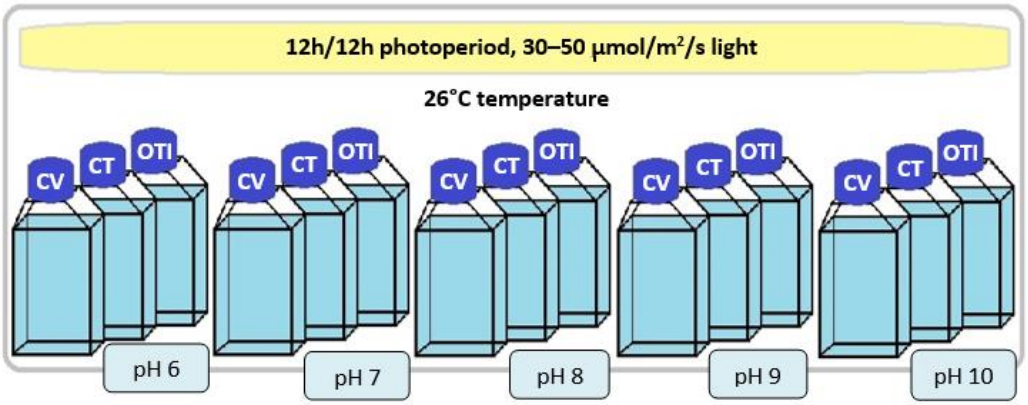

Figure 5 Experiment setup for culturing *C. velia* strains in different pH. There was prepared f/2 medium in 5 glass storage bottles just as in Figure 3. The final step was to adjust pH in every storage bottle as it was demanded by 1 M HCl and 1 M NaOH. Prepared f/2 mediums were poured in 25 cm^2 flasks with 40 ml volume inside of the sterile flow box and 1 ml of each culture was inoculated. Every strain in certain pH had 3 repetitions.

6.2 Growth observation

Culture densities were visually counted under a microscope using a Bürker's counting chamber and also determined by the spectrophotometric instrument TECAN Infinite® 200Pro.The densities were observed once a week in the period of time 35 days (salinity experiment) and 41 days (pH experiment).

Bürker's counting chamber serves to determine the number of particles per volume unit of a liquid. It's usually used for counting blood cells in hospital laboratories. I used the chamber for counting algae cells inside of growing cultures. I took the flasks out of growing box into the sterile flow box. I chose one repetition from each *C. velia* strain and shook the flasks vigorously before taking 1 ml of the culture into Eppendorf tubes. I placed the tube rack with Eppendorf tubes next to the light microscope and prepared Bürker's counting chamber. I applied 10 µl of the culture to the chamber and followed the instructions at the Brand.de "Counting chambers" manual [3].

The preparation for the spectrophotometric method started inside of the flow box. I took 100 µl of the sample from each algae culture and put it into the TECAN microplate. The microplate underwent an analysis inside of the TECAN Infinite® 200 Pro. The analysis provided an absorbance of each sample. Absorbance is a parameter which refers to the density of cells. As there are more cells in the sample they cause a brown colouration. Darker the sample becomes there is a higher density of cells. The spectrophotometer TECAN is sensitive enough to detect slight differences in the absorbance of the light (600 nm) in the sample.

We chose data from spectrophotometric analysis due to its accuracy. This method doesn't have many steps in the preparation so it means there are fewer human errors.

6.3 Lipid analysis

The lipidomic analysis was performed with cultured algae in different salinities and pH in focus how the environmental stress affects lipid accumulation in algae cells. It was performed by my supervisor who is specialized in the chemistry of lipids, but I was present during the procedure and helped with the extraction. Grown cultures were collected by centrifugation and pellets were stored in a temperature –20°C. For the procedure of lipid extraction was used general solvent mixed with chloroform and methanol (Folch et al., 1957). The extracted lipids were analysed followed the procedure proposed by Tomčala et al., (2017). Briefly: the liquid chromatograph and autosampler Accela (Thermo Fisher Scientific,San Jose, CA, USA) was used for injection. The samples (5 μL) were injected and separated on the Gemini column 250 × 2 mm; id 3 μm (Phenomenex, Torrance, CA, USA). The mobile phase comprises of (A) 5 mM ammonium acetate in methanol, (B) water, and (C) 2-propanol. The analysis was completed within 85 min with a flow rate of 250 μl/min by the described gradient. The column temperature was set at 30°C. The linear ion trap LTQ-XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used in both positive and negative ion ESI mode detection. The full scan lipid spectra were collected in the range 140–1400 Da with spectra rate2 Hz. For the dependent MS/MS experiments, the collision-induced dissociation energy was 35 eV for both polarities. Obtained data was recorded and processed by the software Xcalibur 2.2 (Thermo).

6.4 Statistics

I performed a series of statistical methods in the StatSoft® STATISTICA 13.2 software to analyse obtained data. Data from the pH experiment were processed as logarithms for a linear transformation. The first measurements were tested for variability and then I used one-tailed Student's paired t-test to compare the first measurements with the final ones. The ANCOVA method was used to find the covariation between *C. velia* strains and the pH, and one-way ANOVA to see the variance between the specific pH. Data obtained from the salinity experiment were tested identically except for the linear transformation. The level of significant differences was considered at $p<0,05$.

The data obtained from the HPLC MS lipid analyses provide the peak areas of particular lipid, these were statistically evaluated using ordination methods as follows: for linear data - principal component analysis (PCA), redundancy analysis (RDA), Monte-Carlo permutation test (unrestricted permutations, n=999), The data was transformed by using internal standard peak area of the particular sample. For each, the transformed peak areas were calculated in the convoluted total cell and peak area. In the canonical analysis (RDA) the strain, pH and salinity levels stood as a categorical predictor. Monte-Carlo permutation tests were used to statistical significance determination. Statistic software CANOCO 4.5 (Biometrics, Plant Research International, Wageningen UR, Netherlands) was used for the PCA, RDA, and Monte-Carlo permutation test analyses.

7 RESULTS

7.1 Algae growth curves

Data were obtained by Spectrophotometer TECAN Infinite® 200 Pro. Graphs were constructed for visualisation of growth curves of algae. Graphs show the effect of different chemical conditions and they also show variation between the three *C. velia* strains.

7.1.1 Salinity Experiment

Three *C. velia* strains were subjected to cultivation in medium with different salinities ranging from 0 to 66.6 g of salt to 1 litre of distilled water (Fig. 6). The cultivation takes 35 days and samples were collected every 7 days with one exception. Unsurprisingly, the best growth was recorded in medium with natural salinity – 33,3 g/l in all three strains (marked by diamond). The growth of cultures seems to be linear with an exception for cultures grown in 0 g/l and 3,3 g/l salinity where almost no growth was recorded. Surprisingly, in these low salinities the algae of all three strains could survive. Besides the lowest salinities, the worst growth was measured in the highest salinity also for all three strains of *C. velia*. The growing curves in 16,7 g/l and 50,3 g/l media are comparable only in *C. velia* strain. The other two algal strains growth more intensive in higher salinities. On the end of the experiment, the samples were collected via centrifugation and stored for further research as analysis of lipids, saccharides, amino acids and RNA isolation for quantitative PCR investigation.

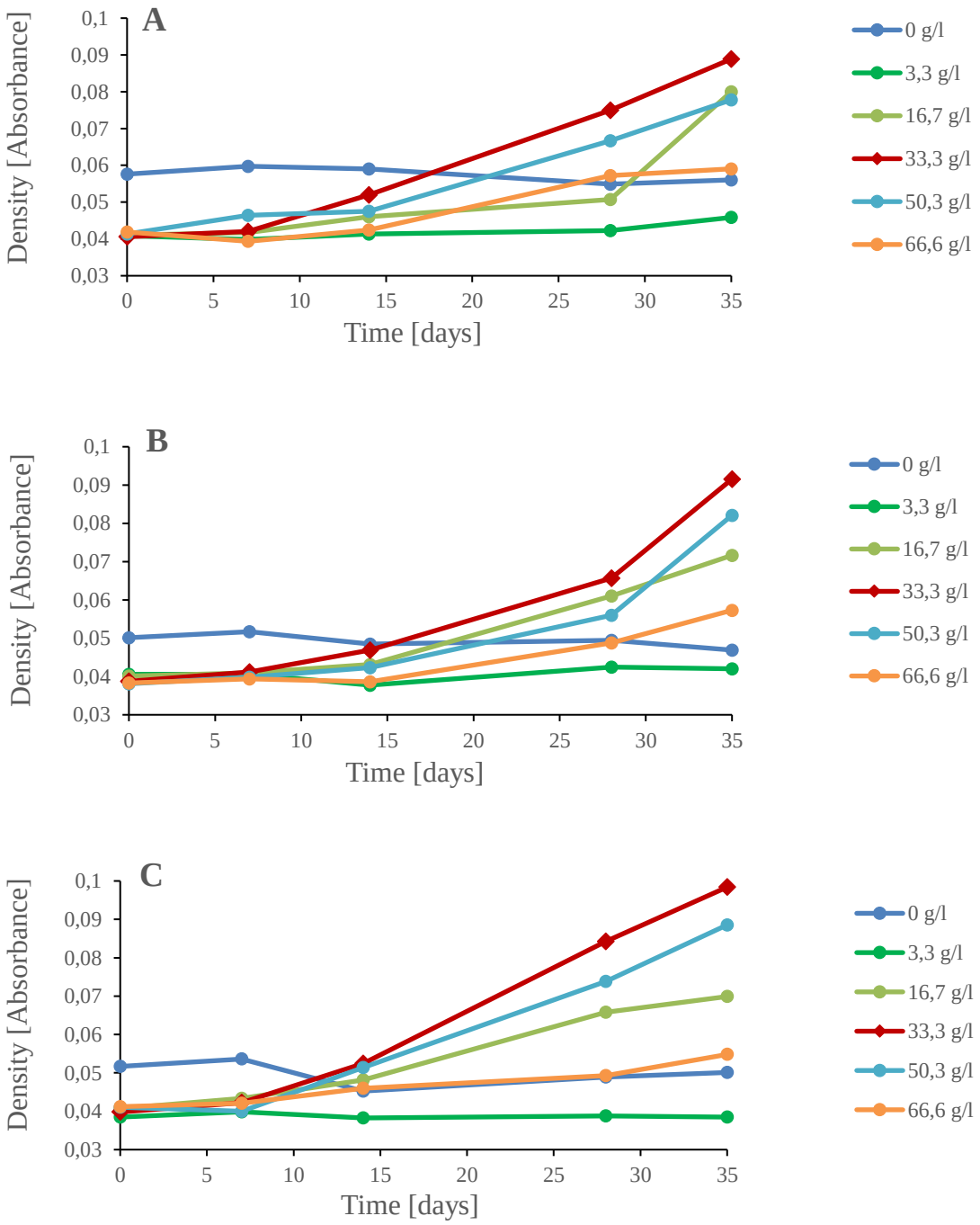

Figure 6 Growth curves of three *C. velia* strains (A – *C. velia*, B – *"C. tropica"*, C – OTI strain) in different salinities. Graphs are displayed as a culture density (absorbance) in different salinity, dependent on time.

7.1.2 pH Experiment

The aim of this experiment was to observe the ability of algae to cope with different pH. There are displayed three graphs in Fig. 7. Each graph represents *C. velia* strains separately. Surprisingly, the best growth curves were not recorded in classical f/2 medium pH optimum which is set between pH 8 and 8.2 (marked by diamond). The worst growth was recorded for all three strains in pH 10 medium. It seems that *C. velia* has the best tolerance to pH among the tested strains, however, the pH 10 seems to be almost lethal. "*C. tropica*" perform the best growth in pH 6 medium, on the other hand, the OTI strain grows best in pH 7 medium. The last graph also shows extraordinary tolerance of OTI strain to pH 10 medium. The growth of the cultures is linear except for the *C. velia*in 10 pH (no density increase was recorded) and the OTI strain in 7 pH where is an abnormal deflection between days 20 and 41.

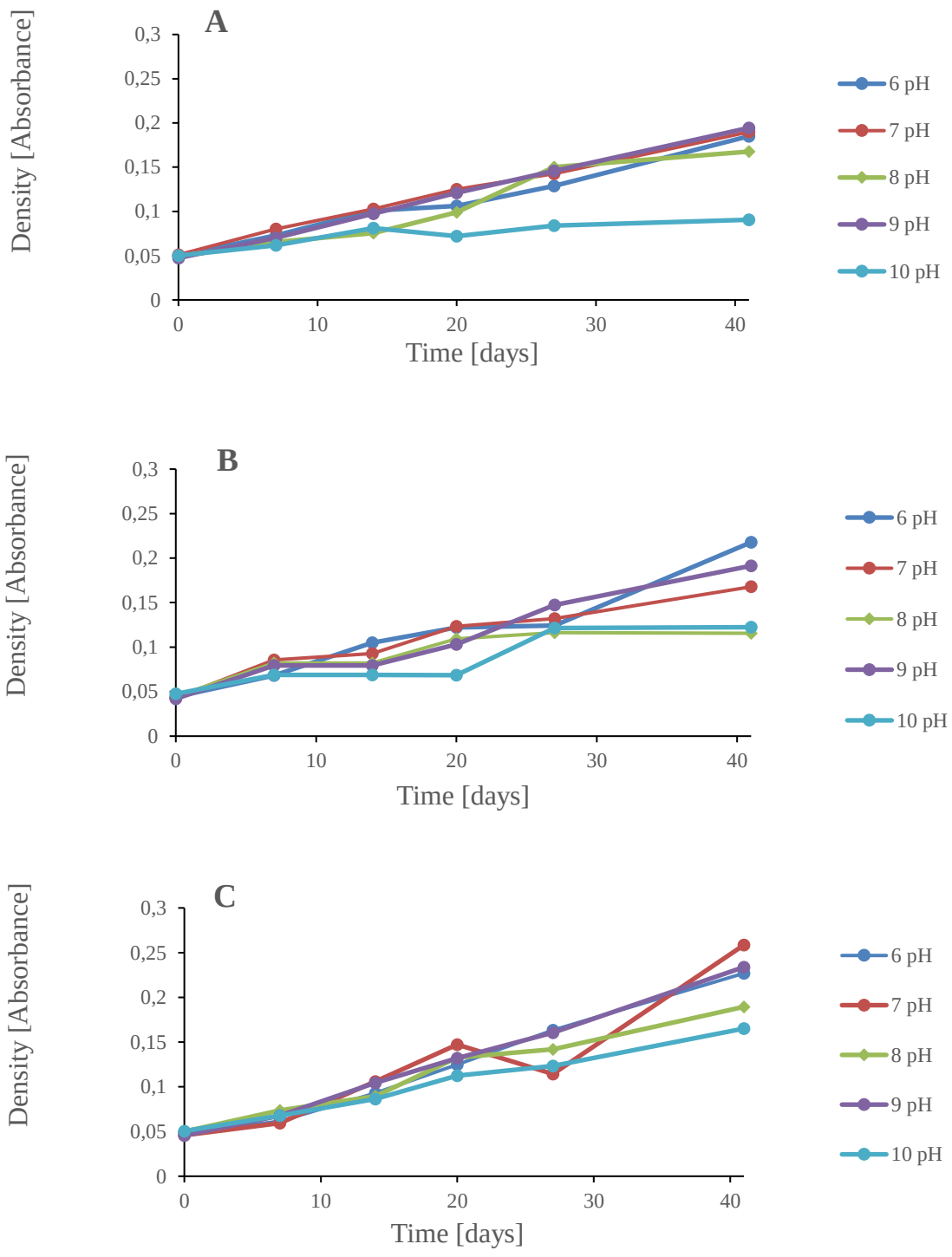

Figure 7 Growth curves of three *C. velia* strains (A – *C. velia*, B – *"C. tropica"*, C – OTI strain) in different pH. Graphs are displayed as a culture density in different pH dependent on time.

7.1.3 Comparison of *C. velia* strains based on culture densities

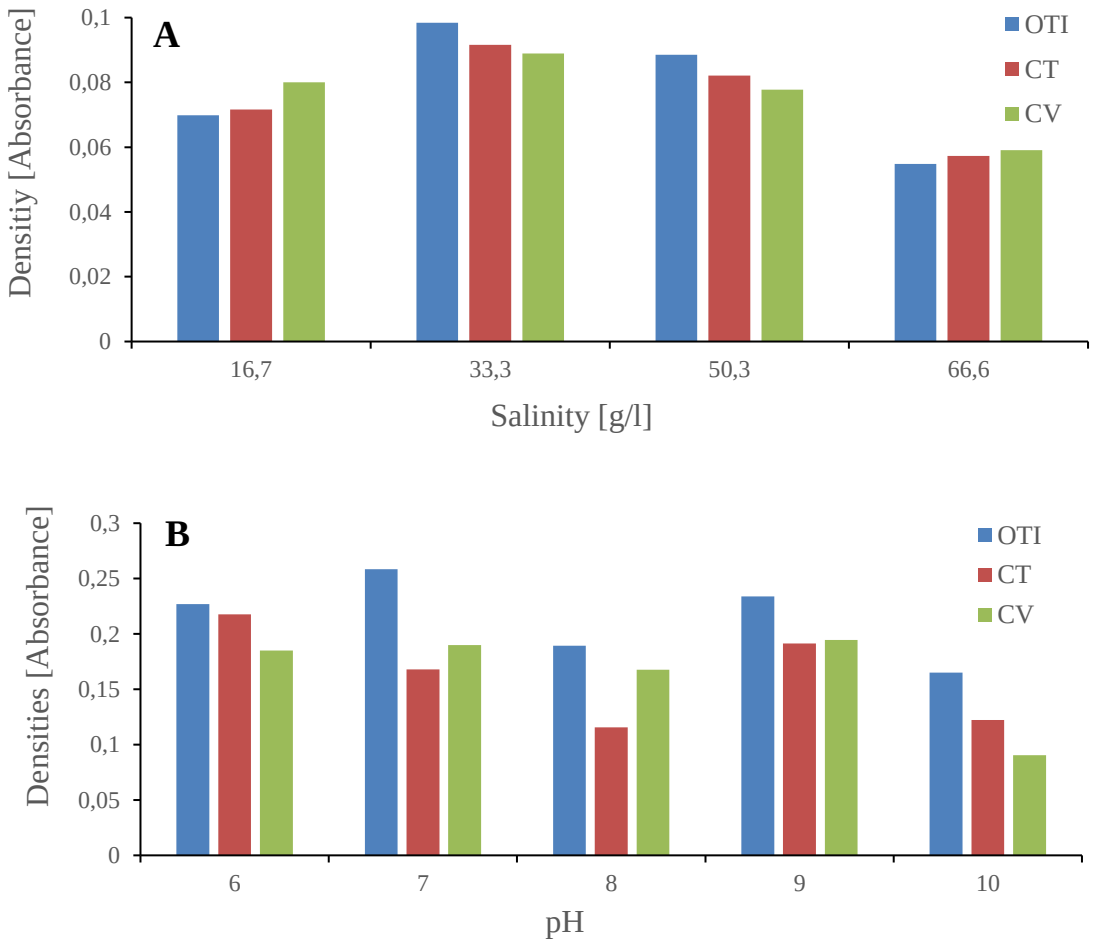

Figure 8 A) The comparison of *C. velia* strains culture densities at the end of the "salinity experiment". Cultures have grown in 0 g/l and 3,3 g/l were excluded due to negligible growth. B) The comparison of *C. velia* strains culture densities at the end of the "pH experiment".

The final densities of both experiments were compared by a statistical approach. In Figure 8 A is visualized salinity experiment. The ANCOVA didn't prove any significant covariance between strains and salinity. The highest values were obtained in media with the 33,3 g/l salt concentration as it is the natural habitat. ANOVA proved significant variation ($p<0,05$) between the 33,3 g/l and all the other concentrations. There wasn't found any significant variance ($p>0,05$) between the strains even though the best growing ability had OTI strain in 33,3 g/l and 50,3 g/l, but it wasn't proved by Tukey's

test. Figure 8 B represents the results from “pH experiment”. ANCOVA show that the cultivation of algae in different pH has a significant influence on the growth, but there weren’t any variations between the strains. Even though ANOVA proved a significant difference between pH, Tukey test didn’t prove any significant differences which one pH is exactly the most significant among the others.

7.2 Amount of accumulated lipids

7.2.1 Salinity experiment

The chemical-analytical approach to determination and semi-quantification of glycerolipids introduced by Tomčala et al. (2017) was used to obtain lipidomic profiles of experimental algal strains. Data were processed by the multivariate statistical analysis. The PCA visualize the samples datasets in to the 2D scatterplot (Fig. 9).The distribution of particular samples shows separation of all samples of OTI strain. It seems that *C. velia* and "*C. tropica*" lipids profiles are more similar then OTI strain. The low levels of salinity 0 and 3,3 g/l were excluded due to an insufficient amount of the sample.

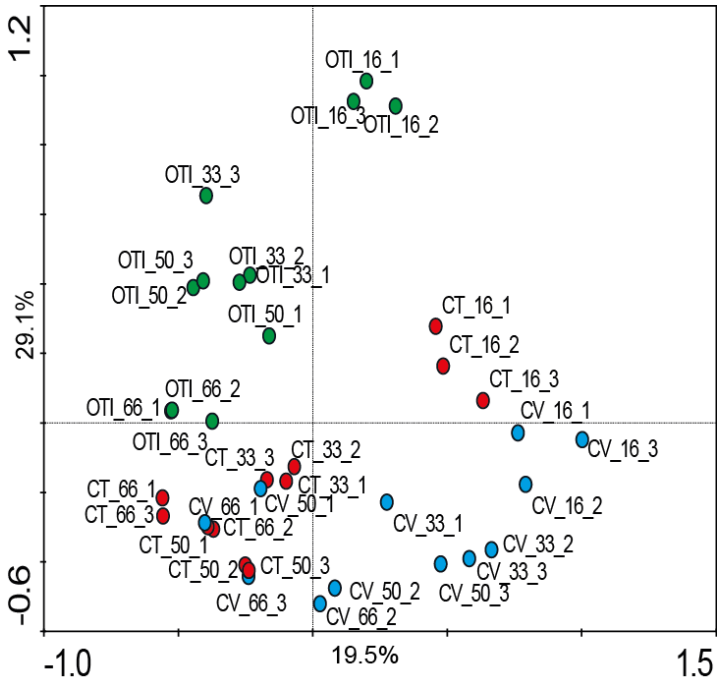

Figure 9 PCA diagram of lipidomic profiles of *C. velia* strains and their distribution in the 2D graphwith relation to salinity. Strains are coloured *C. velia* is blue, "*C. tropica*" is red and green colour is for OTI strain.

The RDA rendered also a 2D scatterplot graph (Fig. 10) to visualise the specific lipid variability dependent on salinity changes and the *C. velia* strains character. The OTI strain has a negative correlation to the CV and CT. That mean CV a CT lipidomic profiles are closer than the lipidomic profile of OTI strain. The main responsible glycerolipids for the data distribution are triacylglycerols, the storage lipids. The strongest changes of the lipidomic profile were recorded in the lowest salinity concentration (16,7 g/l). In the 33,3 g/l salinity algae don't differ from each other in the TG content as significant as in other salinities. The high concentrations (50,3 and 66,6 g/l) have the opposite effect on TG production in comparison to the 16.7 g/l salinity. It seems that high salinities support the production of TGs with short-chain saturated fatty acids. On the other hand, TGs possess long-chain polyunsaturated fatty acids are preferably produced in low salinities.

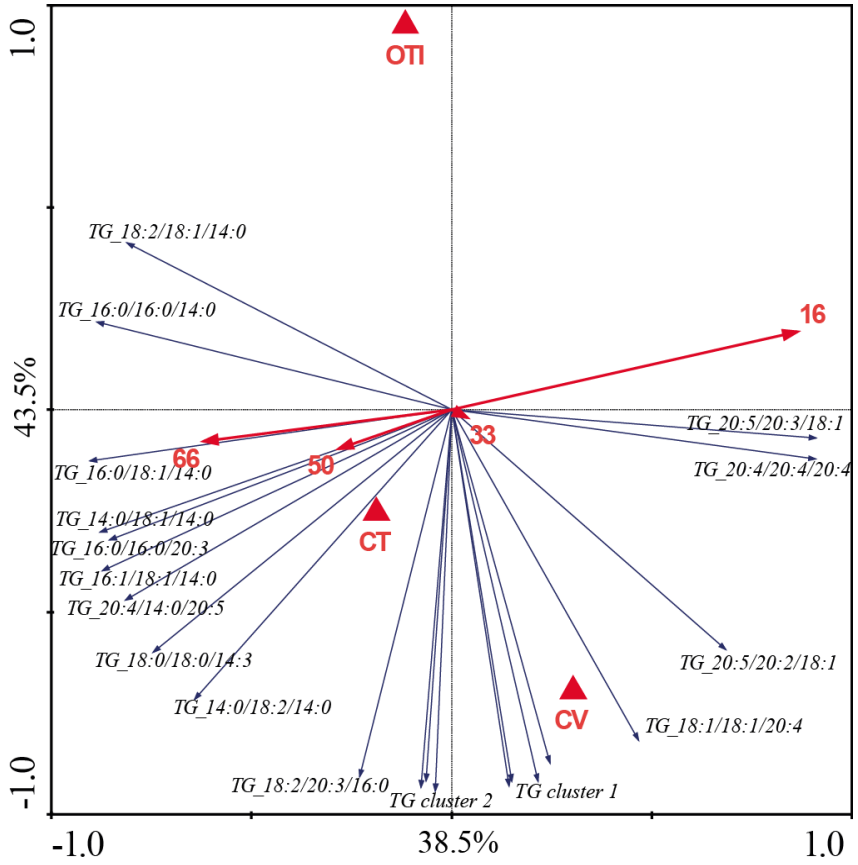

Figure 10 RDA diagram of lipidomic profiles of *C. velia* strains and their distribution in the 2D graph with relation to salinity. In diagram are depicted lipids responsible for the data segregation. For clarity the TG clusters were depicted – TG cluster 1 comprise with TG_18:0/20:2/18:1, TG_18:2/18:2/18:1, TG_18:1/20:2/16:0, TG_20:4/18:2/16:0, TG 2 cluster comprise with TG_20:3/18:1/16:0, TG_20:3/18:1/18:0, TG_16:0/16:0/20:4. Monte Carlo test revealed p=0.001.

7.2.2 pH Experiment

Lipidomic profiles of samples were obtained as mentioned above. Data from lipidomics were displayed in the PCA and RDA 2D scatterplot (Fig. 11, Fig. 12).

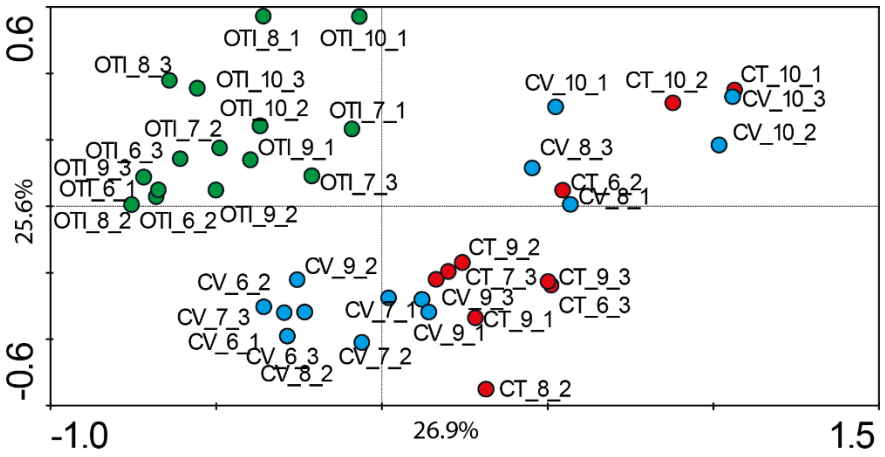

Figure 11 PCA diagram of lipidomic profiles of *C. velia* strains and their distribution in the 2Dgraph with relation to pH level. Strains are coloured *C. velia* is blue, "*C. tropica*" is red and green colour is for OTI strain.

Nevertheless, the experiment is based on another physical parameter, the distribution of the data is very similar to the previous PCA graph (Fig. 9). The OTI strain lipidomic profiles constitute a single group and profiles from *C. velia* and "*C. tropica"* are partly mixed. The distribution of particular samples directly support the findings from the first experiment that OTI strain lipidomic profile differs the most.

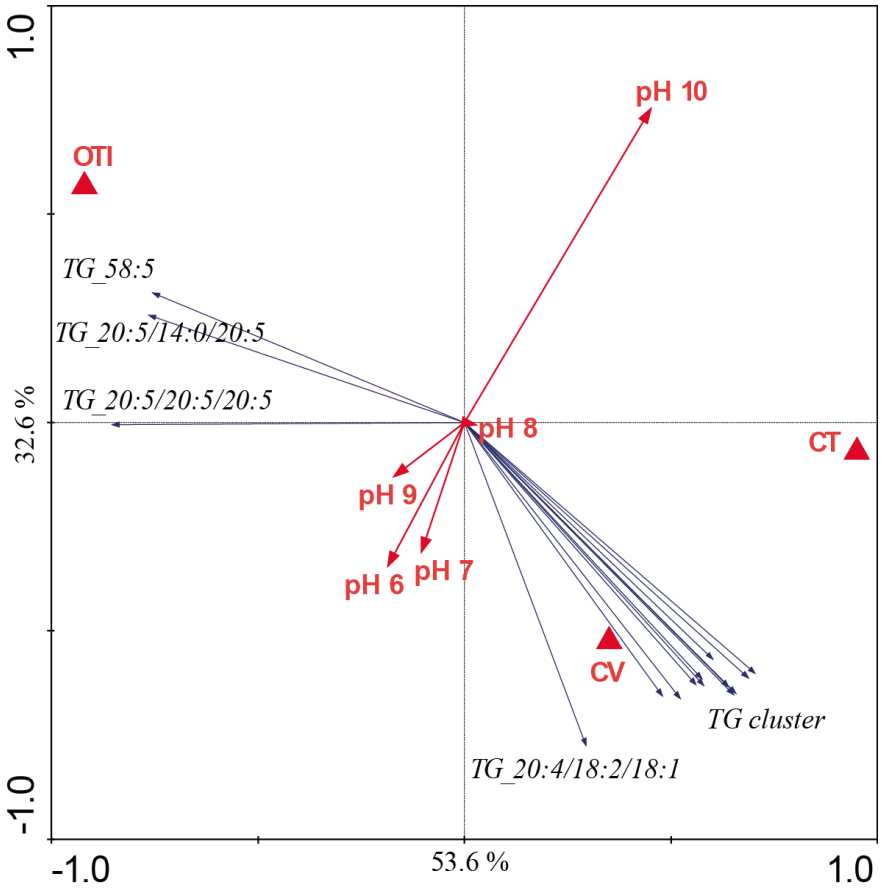

Figure 12 RDA diagram of lipidomic profiles of *C. velia* strains and their distribution in the 2D graph with relation to pH level. In diagram are depicted lipids responsible for the data segregation. For clarity the TG cluster was depicted – TG cluster comprise with TG_18:1/20:2/16:0, TG_18:2/18:2/14:0, TG_18:1/20:3/18:1, TG_20:4/18:1/18:0, TG_18:2/20:3/16:0, TG_18:1/18:1/20:4, TG_18:2/18:2/18:1, TG_18:2/18:1/16:0, TG_16:0/16:0/20:4, TG_20:4/18:2/16:0, TG_51:4. Monte Carlo test revealed p=0.001.

RDA diagram revealed that pH level (Fig. 12) has no such significant effect as salinity (Fig. 10). However, the OTI strain has also a negative correlation between the CV and CT. The main responsible compounds are TGs but no trend in fatty acid occurrence as in salinity experiment is visible. Even the growth curves are not optimal in pH 8 level the lipidomic profile of all algal strains is the most similar in this specific condition. The strongest effect to lipidomic profile has the cultivation in highly alkaline environment - pH 10. Since lipid biosynthesis is a primary metabolic pathway and the lipidomic profile has not been influenced too much as well as growth rate one can hypothesize that *C. velia* strains are very resilient to pH levels.

7.2.3 Comparison of *C. velia* strains based on lipidomic profiles

The growing curves did not reveal any statistically significant differences among tested *C. velia* strains. The highest lipidome profile similarity was recorded in salinity 33.3g/l and 8 pH. RDA of these experimental setups directly shows significant differences among tested strains in lipidome profile view. In both cases has OTI strain negative correlation to *C. velia* and "*C. tropica*". The distribution of the data is not influenced only by TGs as in previous cases but also structural lipids as monogalactosyl diacylglycerol (MGDG), phosphatidyl glycerol (PG), sulfoquinovosyldiacylglycerol (SQDG), and diacylglyceryltrimethylhomoserine (DGTS) and metabolic intermediates (diacylglycerol - DG) are involved.

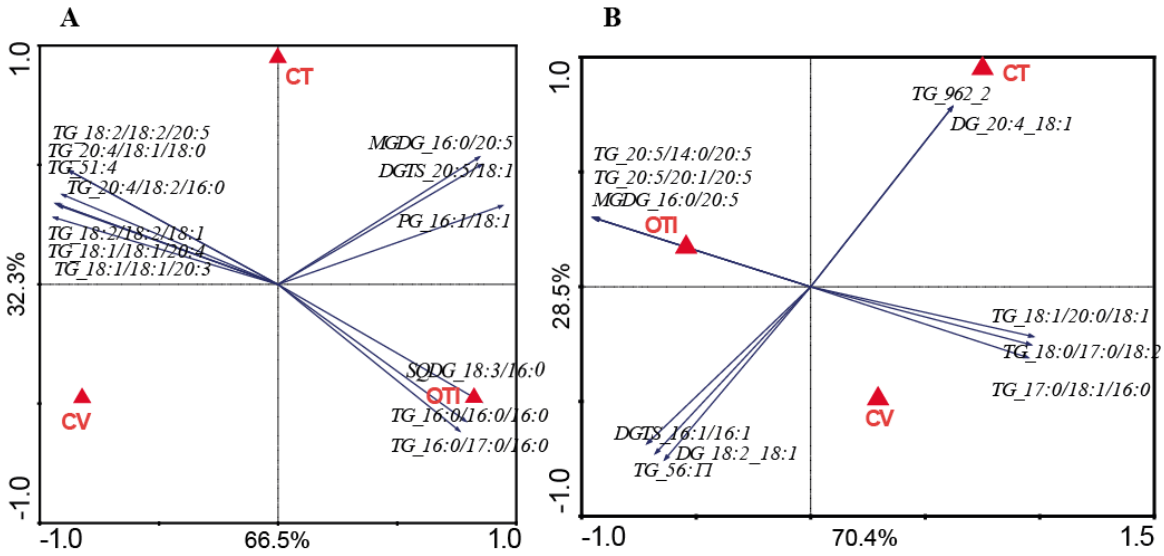

Figure 13 RDA diagrams of lipidomic profiles of *C. velia* strains and their distributions in the 2D graph for salinity 33.3 g/l (A) and for pH level 8 (B). Monte Carlo test revealed p=0.002 and p=0.001 respectively. Monogalactosyl diacylglycerol (MGDG), phosphatidyl glycerol (PG), sulfoquinovosyldiacylglycerol (SQDG), diacylglyceryltrimethylhomoserine (DGTS), diacylglycerol (DG).

8 DISCUSSION

Unicellular algae are great model organisms to experiment with. They are organisms easy to cultivate in a laboratory, they don't have high demands for a maintenance and nutrition, and most of them are fast growing. Microalgae have the potential to bring an understanding of photosynthesis, algae life cycle and metabolism, and revolutionize biotechnology in many areas such as nutrition, aquaculture, pharmaceuticals and biofuels (Rosenberg et al., 2008). Exploring biodiversity and selecting prospective strains that produce the desired products with maximum efficiency can lead the way to the sustainable development (Minhas et al., 2016).

The main area of interest in the alga *C. velia* so far was a phylogenetic and evolutionary research in relation to the Apicomplexan phylum and the rest of SAR supergroup. The high interest of this issue is supported by several research projects which have examined the ultrastructure, morphology, life cycle (Oborník et al., 2011, 2012), plastid, mitochondrial and nuclear genome (Janouškovec et al., 2010; Flegontov et al., 2015; Woo et al., 2015). Though, there are many types of research mapping the special evolutionary position and characteristic features of *C. velia*, the focus could be also directed into the biotechnological application (Tomčala et al., 2018; Lukeš et al., 2017).

Lukeš et al., (2017) have found out that *Chromera velia* grew well in a salinity range between 0.2 M (11,7 g/l) and 1 M (58,4 g/l) with no significant differences in growth rate. My experiments with all three strains of *C. velia* were very similar but with broader spectrum of salinities. The results shows as in Lukeš experiment that algae grew well in a range between 16,7 g/l (0,3 M) and 66,6 g/l (1,1 M). However we found that all strains of *C. velia* even they do not grow they are able to survive almost in distilled water. The mechanism of osmoregulation in *C. velia* is still unknown. Effects of salinity stress on marine algae have not been explored by many researchers and the reports are so far limited on freshwater microalgae (Minhas et al., 2016). The osmoregulation has been extensively studied in *Dunaliella tertiolecta* (Chlorophyceae), in which the compatible solute glycerol is synthesised and metabolised through the glycerol cycle (Wegmann, 1986). The response to variability in external salinity was observed also in *Fucus vesiculosus* and *Laminaria saccharina* (Phaeophyceae). It was found that the responsible osmolyte is a polyol mannitol, synthesised and metabolised through the mannitol cycle

(Reed et al., 1980). The mannitol cycle has been verified in a unicellular red alga *Dixoniella grisea* (Rhodellophyceae). Moreover, the demonstration of the mannitol cycle within the Rhodellophyceae provides evidence that this metabolic pathway is of ancient origin in the red algal lineage (Eggert et al. 2006). The coccolithophorid alga *Emiliania huxleyi* (Haptophyta) underwent profiling of metabolites which revealed mannitol and suggesting its role in carbon storage of the alga (Obata et al. 2013). Results from Michalski et al., (1992) indicate that mannitol plays an important role in the metabolism and development of the intracellular stages of the parasite *Eimeria tenella*. Unsporulated oocysts of this apicomplexan contain large quantities of carbohydrates, namely amylopectin, mannitol and glucose (Michalski et al., 1992). Mannitol was found as a major saccharide as well in *C. velia* (Tomčala, *unpublished*) which leads to the hypothesis that the osmoregulation is similar to brown, red algae, apicomplexans, and haptophytes as they are also closely related to SAR clade.

However, the salinity is a complex stress factor affecting net lipid productivity in the microalgal cell (Minhas et al., 2016). The extreme halophilic alga *D. Tertiolecta (Chlorophyceae)* under high salinity concentration, increased lipid content up to 70% (Takagi et al., 2006). The green freshwater alga *Chlorella sp*. showed the highest growth and lipid content at 24 h photoperiod, pH 8.0 and 0.5 M NaCl (29.2 g/l) concentration. There was approximately two times increase in lipid content of *Chlorella sp.* after keeping all the optimized condition with salt supplemented medium (Rai et al., 2015). Experiments with *C. velia* strains however did not show any significant accumulation of storage lipids which is with accordance with Lukeš's results dealing with fatty acids (Lukeš et al., 2017). He also did not record any differences in fatty acid composition in different salinities. On the other hand, in the contrast to his work the results in my bachelor thesis point out that the lipidomic analysis tracking intact lipids proved significant differences in the TG production. The differences were caused rather by quality than quantity of TGs. High salinities endorsed short-chained saturated fatty acids accumulation suitable for oil production and biofuels. The 16,7 g/l salinity endorsed long-chained polyunsaturated fatty acids suitable for dietary supplements. Especially, the CT strain was the most successful to endorse short-chained saturated fatty acids under the high salinity stress.

There are not many articles about the pH influence and the ability of algae to sustain life in a wide range of pH. One report from Guckert and Cooksey (1990) on *Chlorella sp*. investigates alkaline pH effect which induced lipid accumulation. It was further supported by Gardner et al. (2011) and by Shah et al. (2013). However, my study did not provide any result which points to lipid accumulation in all three *C. velia* strains by different pH level. The main reason for cultivating *C. velia* in different pH was to follow the linkage with Apicomplexa phylum. The assumption was that *C. velia* is going to survive in a wide range of pH due to the affinity with protozoan parasites which must sustain life across the change of environments as they change locations inside of their hosts (Francia et al., 2011). My data showing almost no grown changes in different pH with exception of highly alkaline environment and therefore supports mentioned assumption. The mechanism how *C. velia* strains could grow and survive in so broad spectrum of pH is still unknown. There were found evidence that aquaporins (AQP) located in the plasmatic membraneof apicomplexan parasitescontribute to adaptation in changing environments (Von Bülow and Beitz, 2015). Nevertheless, the mechanism of AQPs wouldn't work without the acidocalcisomes in conjunction with the contractile vacuole complex (Rohloff et al. 2004). Acidocalcisome is an acidic organelle with the main function of storage cations and phosphorus. The organelle participates in the metabolism of phosphorus, calcium homeostasis, maintenance of intracellular pH homeostasis, and osmoregulation. The fact that acidocalcisome-like organelles occur both in bacteria and humans suggests that this organellar compartment was established before prokaryotic and eukaryotic lineages diverged and that these organelles have been conserved over evolutionary time (Moreno and Docampo, 2009). The preliminary studies employed transmission electron microscopy did not revealed any significant changes in cell ultra-structure of experimental algae.

9 CONCLUSION

C. velia strains maintained to grow in media with concentrations 16,7 g/l; 33,3 g/l; 50,3 g/l and 66,6 g/l. There wasn't any significant difference among growing rates of *C. velia* strains. The RDA analysis of lipidomic profiles of all three *C. velia* strains has shown that salinities 50,3 g/l and 66,6 g/l endorse short-chain unsaturated fatty acids and low on the other side, the low salinity 16,7 g/l endorse long-chain polyunsaturated fatty acid. The CT strain proved the best correlation between high salinity stress and short-chain unsaturated fatty acids production. Growth curves show that in the period of 41 days the cultures managed to grow in all media from pH 6 to pH 10, except for *C. velia* strain in 10 pH. There was a deflection of OTI strain in 6 pH. The RDA analysis of lipidomic profiles from the pH experiment has shown no correlation between the pH and specific lipid production. Furthermore, RDA analysis of lipidomic profiles of both experiments based on different physical parameters points to significant differences among all three *C. velia* strains. RDA also revealed that lipidomic profiles of *C. velia* and "*C. tropica"* are more similar to each other rather than to OTI strain.

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